**UNIVERSIDADE NOVE DE JULHO**
**PROGRAMA DE PÓS-GRADUAÇÃO EM BIOFOTÔNICA**
**APLICADA ÀS CIÊNCIAS DA SAÚDE**

**CRISTIANO DE LOURA SANTANA**

# Efeito da fotobiomodulação no infiltrado inflamatório em feridas de pele de ratos diabéticos em dois regimes de entrega de energia

**São Paulo – SP**

**2016**

UNIVERSIDADE NOVE DE JULHO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOFOTÔNICA
APLICADA ÀS CIÊNCIAS DA SAÚDE

# Efeito da fotobiomodulação no infiltrado inflamatório em feridas de pele de ratos diabéticos em dois regimes de entrega de energia

**Tese apresentada à Universidade Nove de Julho, para obtenção do título de Doutor em Biofotônica Aplicada às Ciências da Saúde**

**Aluno:** Cristiano de Loura Santana
**Orientadora:** Profa. Dra. Cristiane Miranda França

São Paulo
Julho / 2016

# FICHA CATALOGRÁFICA

*Santana, Cristiano de Loura.*

*Efeito da fotobiomodulação no infiltrado inflamatório em feridas de pele de ratos diabéticos em dois regimes de entrega de energia. / Cristiano de Loura Santana. 2016.*

*67 f.*

*Tese (doutorado) – Universidade Nove de Julho - UNINOVE, São Paulo. 2016.*

*Orientador (a): Profª Dra. Cristiane Miranda França.*

Sao Paulo, 14 de junho de 2016.

TERMO DE APROVA/AO

Aluno (a): Cristiano de Loura Santana

Titulo da Tese: “efeito da fotobiomodula§âo no infiltrado inflamatério em feridas de pele de ratos diabéticos"

Presidente: PROF^. DR^. CRISTIANE MIRANDA FRANg/

Membro: PROF^. DR". KRISTIANNE PORTA SANTOS FERNANDES

Membro: PROF^. DRª. RAQUEL AGNELLI MESQUITA FERRARI

Membro: PROF^. DR^. MARTHA SIMDES RIBEIRO

Membro: PROF. DR. FABIO DAUMAS NUNES

## DEDICATÓRIA

Dedico esta dissertação à minha família que me deu muito apoio em todos os momentos da minha vida, à minha querida professora orientadora, pela confiança, amizade e segurança, aos meus amigos do laboratório que sempre me deram suporte, à UNINOVE, pela oportunidade de crescer profissionalmente, aos professores que me ensinaram com toda a dedicação e, sobretudo aos mais que queridos alunos de iniciação científica, um obrigado mais do que especial, vocês são a diferença!

Obrigado por tudo!

**RESUMO**

O processo de cicatrização dos diabéticos é alterado e, muitas vezes, ineficiente levando a complicações pós-cirúrgicas importantes, como retardo no reparo, deiscência e infecção. A laserterapia é uma ferramenta promissora para auxiliar o fechamento destas feridas, porém a falta de padronização da dosimetria, regimes de entrega de energia e falta de entendimento dos seus mecanismos celulares e moleculares de ação limitam a disseminação do seu uso. Estudos apontam que a modulação de macrófagos e outras células inflamatórias em determinadas fases do reparo tecidual induzem a maior eficácia nesse processo. O objetivo deste trabalho foi comparar dois regimes diferentes de entrega de luz laser analisando o influxo de neutrófilos, linfócitos T, fibroblastos e polarização de macrófagos. Foram utilizadas 90 fêmeas Wistar, com massa corpórea entre 140 e 250 g com diabetes induzido por injeção intraperitoneal de estreptozotocina (50 mg/kg) e com lesões dorsais feitas com *punch* cirúrgico. Foram divididas em Grupo Controle (GC - sem tratamento), Grupo Dose Única (GDU – 1 aplicação de 4 J/cm²) e Grupo Dose Fracionada (GDF – 4 aplicações de 1 J/cm² em 1, 3, 8 e 10 dias) e submetidos à laserterapia com 660 nm e 30 mW. Após o tratamento, as lesões foram removidas e acondicionadas em solução tamponada de formol 10% e processadas rotineiramente para emblocamento em parafina, coloração histológica com hematoxilina e eosina, marcação imunohistoquímica para linfócitos T, macrófagos totais, macrófagos M2, neutrófilos e miofibroblastos. As lâminas foram fotografadas, quantificadas e submetidas aos testes estatísticos apropriados. A aplicação do laser em ambos grupos acelerou o fechamento da ferida em 40% nos três primeiros dias. O grupo dose única apresentou maior número de neutrófilos no primeiro dia, os macrófagos totais no dia 3 e macrófagos M2 no dia 8. A contagem de linfócitos T foi semelhante em todos os grupos no durante todo o experimento. Comparados aos controle, os grupos tratados com laser apresentaram um maior número de miofibroblastos no dia 15 e maior organização do colágeno no dia 22. Os resultados demonstram que

a fotobiomodulação pode alterar a composição do infiltrado inflamatório nas feridas em diabéticos, especialmente com uma única aplicação de 4 J/cm² indicando que a laserterapia no período pós-operatório imediato pode melhorar o processo de reparação tecidual em um modelo de diabetes.

**Palavras-chave:** cicatrização, diabetes mellitus, terapia a laser, macrófagos

**ABSTRACT**

The healing process of diabetic individuals is altered and is often ineficiente, leading to significant post-surgical complications such as delayed repair, dehiscence and infection. Laser therapy is a promising tool to assist the closure of these wounds, but the lack of standardization of dosimetry, of the power delivery systems and a lack of understanding of the cellular and molecular mechanisms of action limit the spread of its use. Studies indicate that modulation of macrophages and other inflammatory cells in certain stages of tissue repair induce more effective healing of the injury. The aim of this study was to compare two different regimes of application laser analyzing the influx of neutrophils, T lymphocytes, fibroblasts and total macrophages and M2 macrophages. 90 female Wistar rats were used, with body weight between 140 g and 250 g with diabetes induced by intraperitoneal injection of streptozotocin (50mg/kg) and back injuries made with surgical punch. The animals were divided into control group (CG - no treatment), Single Dose Group (SDG - 1 Application 4 J/cm²) and Fractionated Dose Group (GDF - 4 applications of 1 J/cm² at 1, 3, 8 and 10 days and undergoing laser therapy with 660 nm and 30 mW. After treatment, the lesions were removed and placed in buffered formalin 10% solution and routinely processed for paraffin inclusion, and then histologic staining with hematoxylin & eosin, immunohistochemical staining for T lymphocytes, total macrophages, M2 macrophages, neutrophils and myofibroblasts. The samples were photographed, the cells quantified, and subjected to appropriate statistical tests. The laser application in both groups accelerated the wound closure in 40% during the first 3 days. SDG presented a higher number of neutrophils in the first day, more macrophages on Day 3, more M2 macrophages on Day 8. The T lymphocyte count was similar in all groups during the experimental. Compared to controls, the laser treated groups presented more myofibroblasts on Day 15 and more collagen organization on Day 22. The results showed that photobiomodulation can alter the inflammatory

infiltrate composition of the diabetic wounds, especcially with a single dose application of the 4 J/cm².

**Key words:** healing, diabetes mellitus, laser therapy, macrophage

**SUMÁRIO**

**LISTA DE TABELAS**

**LISTA DE FIGURAS**

## LISTA DE ABREVIATURAS

| | |
|---|---|
| AlGaInP | Alumínio gálio fosfeto de índio |
| ATP | Trifosfato de adenosina |
| DM | *Diabetes mellitus* |
| Ga-As | Arsenieto de gálio |
| He-Ne | Helio-Neônio |
| IFN-γ | Interferon gama |
| IGF | Fator de crescimento semelhante à insulina |
| IL | Interleucina |
| LBI | Laser em baixa intensidade |
| LED | Diodo emissor de luz |
| LPS | Lipopolissacarídeo |
| ROS | Espécies reativas de oxigênio |
| TGF- β | Fator de transformação do crescimento beta |
| TNFα | Fator de necrose tumoral alfa |
| VEGF | Fator de crescimento do endotélio vascular |

# 1. CONTEXTUALIZAÇÃO

## 1.1 Diabetes mellitus

O *diabetes mellitus* (DM) é um grupo de desordens metabólicas que têm em comum a hiperglicemia. Essa pode resultar de defeitos na secreção da insulina, na ação da insulina ou, mais comumente, em ambos. A classificação atual do DM baseia-se na sua etiologia e inclui quatro classes clínicas: DM tipo 1 (DM1), DM tipo 2 (DM2), outros tipos de DM e DM gestacional. O DM1 ocorre quando o diabetes se estabelece pela destruição autoimune das células beta pancreáticas. O diabetes é classificado como DM2 no caso da hiperglicemia ser resultante de uma combinação entre a resistência periférica à ação da insulina e uma resposta secretória inadequada das células beta pancreáticas. [1, 2]

São 380 milhões de diabéticos no mundo e, seguindo essa tendência, poderá chegar a 592 milhões até 2035 ou 1 em cada 10 adultos. No Brasil, o número é aproximadamente de 12 milhões de pessoas, uma média de 7% da população podendo chegar a 25% na faixa etária acima de 65 anos. [3]

O diabetes gera alterações micro e macrovasculares e dentre elas, deficiência no reparo tecidual, o que pode resultar em infecções, deiscências, feridas crônicas, amputações e em morte de 5% dos diabéticos por feridas infectadas. Essa condição é preocupante uma vez que cerca de 50% dos pacientes diabéticos têm chance de necessitar de alguma cirurgia (por exemplo: transplantes, cesáreas, cirurgias torácicas, bariátricas) ao longo da vida.[4,5 6]

## 1.2 Reparo tecidual e principais células efetoras

As fases do reparo são didaticamente divididas em três etapas que se sobrepõem: inflamatória, proliferativa e remodelamento. Muitos eventos

biológicos estão envolvidos nesses processos, tais como coagulação do sangue, inflamação, formação de tecido de granulação, contração da ferida e remodelação tecidual.[7]

A fase inflamatória é caracterizada pelas alterações vasculares (vasodilatação e aumento da permeabilidade) e exsudativas. Dentre as células que migram do interior do vaso para os tecidos, os neutrófilos são os primeiros leucócitos a serem recrutados para o local de inflamação e são capazes de eliminar os patógenos através de diferentes mecanismos. A vida média de um neutrófilo é de oito a doze horas em camundongos e pode chegar a vários dias em humanos, mas durante a inflamação, eles são ativados e aumentam sua longevidade consideravelmente, o que garante sua presença em atividades mais complexas, como contribuição para a resolução da inflamação ou a resposta imune adaptativa. Possuem papel crucial nas fases iniciais do processo inflamatório, sendo bastante responsivos a agentes quimiotáxicos como: produtos de clivagem de frações do complemento (C3a e C5a) e substâncias liberadas por mastócitos e basófilos.[8]

Em condições de normalidade, os neutrófilos entram em apoptose horas após sua diapedese, sendo que a falha no mecanismo pode levar doenças inflamatórias e imunológicas. [17]

Durante o processo de reparo normal, essas células sofrem apoptose e são reconhecidos e ingeridos por macrófagos. Na medida em que diminui o número de neutrófilos, aumenta o número de linfócitos T e macrófagos, que se tornam os tipos celulares predominantes no infiltrado inflamatório.[4]

Os linfócitos são leucócitos uniformes na aparência, mas variados em função e incluem os linfócitos T com função reguladora, linfócitos B responsáveis pelo efeito de memória imunológica e produção de anticorpos e linfócitos natural killer (NK), responsáveis também pela destruição de células infectadas por vírus e células tumorais.

São ativadas após a interação de moléculas co-estimulatórias e a identificação do antígeno apresentado pelas células apresentadoras de

antígenos (APC), via complexo principal de histocompatibilidade (MHC) classe I (que apresenta antígenos de patógenos intracelulares) e II (que apresenta antígenos de patógenos extracelulares), as células da imunidade adaptativa tornam-se aptas a reconhecer uma multiplicidade de moléculas diversas e ao mesmo tempo, “lembrar-se” de um encontro anterior com mesmo patógeno.[29]

Os linfócitos T respondem pela imunidade celular, reconhecem os antígenos de microrganismos intracelulares, destruindo-os ou destruindo as células infectadas. Elas não produzem anticorpos, apresentam especificidade restrita para antígenos, ou seja, atacam diretamente os invasores estranhos. Apesar de também se originarem na medula óssea, amadurecem no timo, onde passam a apresentar em sua membrana celular, receptores denominados receptores de células T, também específicos para um único antígeno. [30, 31]

As células T incluem as células T efetoras, que se dividem em células T auxiliares/helper (Th), células T citotóxicas/citolíticas (Tc), as células T supressoras e as células T de memória. [32]

Ainda na fase inflamatória do reparo, os monócitos derivados do sangue, ao adentrar no tecido se diferenciam em macrófagos. O macrófago ativado é a principal célula efetora do processo de reparo tecidual, degradando e removendo componentes do tecido conjuntivo danificado, secreta fatores quimiotáticos e a permeabilidade dos microvasos.[9,10]

Os macrófagos são células cruciais na regulação do reparo, com diferentes funções em cada fase do mesmo. Em experimentos realizados por Lucas et al. (2010)[19], foi verificado que se a depleção de macrófagos ocorrer na fase inflamatória (quatro dias após a lesão), ocorre uma grave alteração no processo de reparação, com redução no tecido de granulação, epitelização e na deposição de colágeno. Caso a diminuição de macrófagos ocorra na fase proliferativa (8 a 14 dias após a lesão), há uma associação com hemorragia na ferida, aumento da apoptose de células endoteliais e o colapso dos vasos sanguíneos recém-formados.

Macrófagos são classicamente ativados como M1 em resposta ao interferon gama (IFN-γ) e aos lipopolissacarídeos de membrana bacteriana (LPS) ou em tipo M2 após estímulo com IL-4 e IL-13. Os macrófagos M1 possuem ação microbicida e nas funções de defesa pela liberação de citotóxicos por altas concentrações de citocinas pró-inflamatórias (TNFα, IL-1b, IL-6 e IL-12) e espécies reativas de oxigênio (ROS).[20, 21]

A resposta inflamatória inicial é mediada por macrófagos M1, que produzem grandes quantidades de espécies reativas de oxigênio (ROS) e citocinas pró-inflamatórias. A fase de cura está relacionada com macrófagos M2, que têm uma maior capacidade fagocítica produzindo principalmente citocinas anti-inflamatórias e são caracterizados pelo aumento da expressão do receptor de manose de CD206.[5,6]

Ao contrário do M1, os macrófagos M2 produzem citocinas anti-inflamatórias (IL-10), fatores de crescimento (TGF-b1, VEGF e PDGF), matriz extracelular e expressam marcadores associados à reparação tecidual.[22,23,24]

A classificação da ativação foi expandida para incluir novos fenótipos de M2, como M2a/b/c. O fenótipo M2a é pela exposição à IL-4 ou IL-13, que agem através do receptor comum IL-4R para aumentar a expressão de CD206, arginase e TGF-β.[25]

O fenótipo M2b é produzido pela exposição a uma combinação de complexos imunes como IgG e LPS, que aumenta a produção de IL-10 e diminui a produção de IL-12, com potentes propriedades anti-inflamatórias. [26, 27]

Exposição de IL-10 ou glicocorticóides produz o fenótipo M2c, que da mesma forma é caracterizado pela elevação de IL-10 e baixa produção de IL-12 bem como aumento expressão do receptor de CD163. O fenótipo de macrófagos também pode ser alterado por fagocitose de corpos apoptóticos ou células necróticas.[28]

Em paralelo à ativação de macrófagos M2, os fibroblastos também secretam fatores quimiotáticos de células progenitoras endoteliais e promovem

a neoangiogênese. Novos vasos, miofibroblastos e macrófagos constroem o tecido de granulação com uma liberação adicional de fatores de crescimento que estimulam a proliferação de queratinócitos e reepitelização.[20]

A fase de proliferação é a responsável pelo fechamento da lesão propriamente dito. Compreende: a reepitelização que tem início horas após a lesão, com a movimentação das células epiteliais oriundas tanto da margem como de apêndices epidérmicos localizados no centro da lesão; a fibroplasia e angiogênese, compondo o tecido de granulação responsável pela ocupação do tecido lesionado cerca de quatro dias após a lesão.[11]

Os fibroblastos produzem a nova matriz extracelular necessária ao crescimento celular enquanto os novos vasos sanguíneos carreiam oxigênio e nutrientes necessários ao metabolismo celular local. A fibroplasia é um mecanismo que envolve a proliferação de fibroblastos e sua migração para o interior da ferida por estímulo de fatores de crescimento como o fator de crescimento derivado das plaquetas (PDGF), fator básico de crescimento de fibroblasto (FGF) e fator de crescimento transformante beta (TGF-β) a qual, semelhantes às células epiteliais, migram para a ferida valendo-se das alterações morfológicas que possibilitam a expansão de segmentos da membrana plasmática.[11,12]

A angiogênese é uma etapa fundamental do processo de cicatrização, onde novos vasos sanguíneos são formados a partir de vasos pré-existentes e participam da formação do tecido de granulação provisório e suprem de nutrientes e de oxigênio o tecido em crescimento. De forma diferencial, a vasculogênese refere-se aos primeiros estádios do desenvolvimento vascular, durante o qual as células precursoras do endotélio vascular sofrem diferenciação, expansão e junção para formar a rede de túbulos primitivos do organismo.[11]

Vários fatores de crescimento e citocinas parecem estar envolvidos com a angiogênese como o FGF (fator de crescimento de fibroblastos), fator de crescimento epidérmico (EGF), fator de crescimento transformante alfa (TGFα),

PDGF (fator de crescimento de plaquetas), TGF-β, fator de crescimento endotelial vascular (VEGF), dentre outros.[12, 13, 14]

A fase de remodelação é uma tentativa de retorno à estrutura tecidual normal onde há equilíbrio entre a formação do colágeno novo e degradação do colágeno velho através da ação das colagenases, há o desaparecimento dos macrófagos junto com a redução da angiogênese e proliferação dos fibroblastos e sua conversão em miofibroblastos. Ocorre a remodelação da matriz extracelular pelas metaloproteinases que incluem colagenases intersticiais, colagenase tipo IV e gelatinases tendo como principais citocinas envolvidas a interleucina 1 (IL-1), TNFα (fator de crescimento transformante alfa), TGFβ, PDGF produzidas pelos fibroblastos e EGF e TGFβ produzidas pelas células epiteliais.[15]

No primeiro mês, há uma proporcionalidade entre a resistência tênsil e a quantidade e qualidade do colágeno no ferimento.[7]

### 1.3 Reparo tecidual e diabetes

O DM tem sido associado clinicamente e experimentalmente a um processo cicatricial mais demorado. Uma das explicações para esse fato é a disfunção dos leucócitos polimorfonucleares, macrófagos e fibroblastos, com uma fase inflamatória prolongada, decréscimo na biossíntese de colágeno e glicosaminoglicanas, resultando no retardo da formação do tecido de granulação. As falhas mais importantes do reparo ocorrem em estágios iniciais de cicatrização, levando à diminuição dos elementos celulares e alterações na síntese de colágeno.[41]

Nas feridas crônicas associadas com o DM, uma subpopulação de macrófagos exibe características de M1 persistentes nas fases finais de cicatrização de feridas. Esse fato está associado com prejuízo da formação de tecido de granulação, angiogênese, deposição de colágeno e cicatrização por causa dos níveis menores de citocinas anti-inflamatórias (por exemplo: IL-10) e

fatores de crescimento (fator de crescimento semelhante à insulina [IGF]-1, TGF-β1 e VEGF).[42]

Também foi descrita a incapacidade de macrófagos para alternar para a ativação de M2 e promover a reparação de tecido é atribuída à sua fagocitose deficiente para as células apoptóticas no microambiente do diabético.[43]

### 1.4 Fotobiomodulação com laser em baixa intensidade na ferida

A fototerapia é a aplicação de luz em aspecto geral no intervalo de 1 mW - 500 mW para promover a regeneração dos tecidos, reduzir a inflamação e aliviar a dor. A luz é tipicamente de largura espectral na faixa do vermelho visível ao infravermelho próximo (600 nm - 1000 nm), com uma densidade de potência (irradiância) entre 1 mW-500 mW/cm$^2$.,[44]

Os efeitos iniciais da luz ocorrem nas mitocôndrias, levando a maior produção de ATP, modulação de espécies reativas de oxigênio e indução de fatores de transcrição. Estes efeitos, por sua vez, modulam a proliferação celular e migração (particularmente por fibroblastos), modulação dos níveis de citocinas, fatores de crescimento e mediadores inflamatórios e aumento da oxigenação dos tecidos.[44]

Os lasers terapêuticos apresentam uma série de indicações, podendo ser usados isoladamente ou como coadjuvante de outros tratamentos, sempre que se necessite de efeito biológico local, uma vez que, dentre as suas funções, modulam as células do sistema imune, estimulam a microcirculação, ativam a liberação de endorfinas e estimulam também a proliferação e a migração celulares, desempenhando ação analgésica, anti-inflamatória e bioestimulante ou cicatrizante.[45,46]

A fotobiomodulação promovida pelo laser em baixa intensidade tem sido empregada de maneira bastante eficaz, no pós-operatório de feridas cirúrgicas e no tratamento de lesões ulceradas, resultando em uma reparação tecidual mais rápida e com padrão de qualidade morfológica superior.[47] Em uma meta-análise publicada por FULOP et al., (2009)[45], cujo objetivo era avaliar o efeito

da fototerapia no reparo tecidual, foi observado que a fototerapia foi considerada auxiliar do reparo de lesões.

No DM, observou-se que a fotobiomodulação com LBI em fibroblastos cultivados em meio com alta concentração de glicose causa a diminuição da apoptose.[48]

Nos pacientes diabéticos a laserterapia acelera o fechamento das úlceras crônicas, independente dos níveis de glicemia do paciente.[49]

Estudos realizados por Kajagar et al (2012)[50] utilizando 2–4J/cm$^2$ e 60 mW verificaram uma maior contração nas lesões de pacientes com pé diabético onde o LBI atuou significativamente no melhor fechamento das lesões comparando-se ao grupo não tratado (40,24 % vs 11,87 % , $p < 0,001$ ) , indicando que o LBI é uma modalidade eficaz para facilitar a contração de feridas em e pode ser utilizada como um adjuvante para o modo convencional de tratamento.

Santos et al (2013)[51] utilizando comprimento de 660nm e 40mW observaram diminuição na área das lesões tratadas com LBI entre o oitavo e décimo quinto dia, comparado com o grupo controle sem tratamento.

Alguns estudos demonstraram aceleração no fechamento de lesões agudas com maturação no tecido de granulação em menor tempo, organização do colágeno, aumento na angiogênese além da modulação no infiltrado inflamatório e fibroblastos em grupos irradiados com densidades de potência de 1J/cm² a 9J/cm² e diferentes regimes de entrega conforme Tabela 1.

Tabela 1 – Avaliação da literatura recente comparando dosimetria, fontes de luz e reparo tecidual

| **Autor** | **Descrição** | **Modelo experimental** | **Resultados** |
|---|---|---|---|
| Lau, 2015 [52] | Diodo λ= 808 nm e 5 J/cm$^2$ com densidades: 0.1 W/cm2, 0.2 W/cm² e 0.3 W/cm$^2$ exposições de 50 s, 25 s e 17 s | Punch, diabético | Cicatrização otimizada e aumento colágeno |
| Santana et al, 2015 [53] | Diodo (λ = 660 ± 2 nm; P = 30 mW; 4 J/cm$^2$) em dose única e dose fracionada irradiando 1 J/cm$^2$ nos dias 1, 3, 8, e 10 | Punch, diabético | Aceleração no fechamento 40% nos 3 primeiros dias em relação ao controle |
| Kilík, 2014 [54] | AlGaInP (λ=635nm, 5J/cm$^2$, dose diária) densidades de 1, 5, e 15mW/cm² | Punch, diabético | Aumento na angiogênese nos grupos irradiados (5, 15mW/cm²) |
| Danca´kova´, 2013 [55] | Diodo λ=810 nm , 30 mW, densidade 30mW/cm$^2$ por 30s (dose diária de 0.9J/cm$^2$/ferida/dia) | Punch, diabético | Aumento de maturação de tecido de granulação |
| Ferreira, 2013 [41] | LED (λ=945 ± 20 nm) 6 J/cm² (30 mW, 157s, 4 pontos (total 24 J/cm$^2$ ) | Incisão suturada, diabético | Aumento fibroblastos |
| Carvalho, 2010 [7] | InGaAlP 100 mW λ= 660 nm (4 J/cm$^2$ ) 24 s | Punch, diabético | Aumento de fibras colágenas e macrófagos |
| Busnardo, Simões, 2010 [56] | He-Ne com energia de 4J/cm$^2$ aplicado 12 segundos por ponto da ferida no modo contínuo, 5 mW, comprimento de onda de 632, 8 nm e área de raio do laser de 0,015 cm$^2$. | Punch, normoglicêmico | Aumento de colágeno tipo III, diminuição do infiltrado inflamatório e resolução precoce da fase inflamatória das feridas |
| Silva et al., 2010 [57] | Laser aplicado em 15 ratos Wistar divididos em três grupos: G1(controle), G2 (2 J/cm$^2$) e G3 (4 J/cm$^2$), com comprimento de670nm e | Punch, normoglicêmico | A dose de 4J/cm$^2$ diferiu significativamente das demais quanto ao |

| | | | |
|---|---|---|---|
| | irradiados durante 10 dias consecutivos sobre lesão cutânea. | | processo de reepitelização. |
| Felice et al., 2009 [58] | Aplicação de laser AlGaInP (658 nm, 4J/cm$^2$, de forma pontual e em varredura sobre úlceras de decúbito e venosas em humanos. | Traumáticas pós AVE e tetraplegia | Redução da área das feridas. |
| Maiya et al., 2009 [59] | Laser He-Ne sobre feridas cutâneas de ratos diabéticos, com comprimento de onda de 632,8 nm e doses de 3-9 J/cm$^2$, durante cinco dias/semana até a completa cicatrização. | Punch, diabético | Maior produção de tecido de granulação entre os animais que receberam doses entre 4-5 J/cm$^2$, especialmente no quinto dia de tratamento. |
| Inoe et al., 2008 [60] | Laser de He-Ne utilizados em doses de 3 e 6 J/cm$^2$,45 W de potência e comprimento de onda de 632 nm e grupo controle, para feridas cirúrgicas de coelhos hígidos. Os animais foram avaliados nos 7º, 14º e 21º dias. | Punch, normoglicêmico | No 14º dia observou-se presença de tecido de granulação maduro e no 21º dia ausência de hemorragia e exsudato |
| Channual et al., 2008 [61] | Laser de baixa potência com comprimento de onda de 585 nm e dose de 7J/cm$^2$ sobre feridas cutâneas em ratos. | Lesão tipo janela, normoglicêmco | Proliferação vascular permanente após o quinto dia de aplicação |
| Pinto et al., 2007 [62] | Primeira semana duas vezes com intervalo de 48 h, nas semanas seguintes 1x/semana, de forma pontual e sem uso de medicação adicional | Deiscência, normoglicêmico | A lesão respondeu com tecido de granulação, diminuição do processo inflamatório e analgesia desde a primeira aplicação |
| Rocha Júnior et al., 2006 [63] | 12 animais foram divididos em dois grupos: experimental e controle. A ferida foi tratada durante sete dias com laser de AsGa, pulsátil, dose 3,8 | Punch, normoglicêmico | O reparo tecidual foi significativamente maior e mais organizado no grupo |

| | | | |
|---|---|---|---|
| | J/cm2, potencia de 15 mW e tempo de 15 segundos. | | experimental |
| Hopkins et al., 2004 [64] | Induziram a ocorrência de lesão por abrasão em membro superior não dominante em dois grupos de pessoas saudáveis. Um grupo foi tratado com dose de 8 J/cm$^2$, comprimento de onda de 820 nm e tempo de dois minutos. O outro grupo foi tratado sob os mesmos parâmetros e tempo de cinco segundos. E um terceiro grupo não foi tratado. | Abrasão, normoglicêmico | Os grupos tratados com laser de baixa potência apresentaram redução estatisticamente significativa da ferida quando comparados ao grupo controle para o 6º, 8º e10º dias de tratamento. |
| Reddy, 2003 | Laser 904 nm 7 mW e 1 J/cm$^2$, He-Ne e infravermelho Ga-As 5 dias três vezes por semana | Punch, diabético | O laser He-Ne é superior ao Ga-As avaliando-se a força tensional da ferida e quantidade de colágeno |
| Envemeka, 2001 [65] | Utilizou laser He-Ne (632.8 nm, modo contínuo) e laser AsGa (904 nm, modo contínuo) em lesões cutâneas de ratos. | Incisão, normoglicêmico | Houve melhora da cicatrização para ambos os comprimentos de onda adotados, embora o último tenha apresentado resultados mais evidentes |

Observa-se que a maioria dos estudos utilizou o laser vermelho que por possuir uma menor penetração tecidual é ideal para tratamentos na pele e doses de 4 a 6 J/cm² foram eficazes mesmo com única aplicação.·

Tendo como base a necessidade do desenvolvimento de novas terapias para o tratamento de lesões cutâneas pós-cirúrgicas relacionadas ao DM, e tentando obter-se o melhor resultado com o menor número de aplicações, o objetivo deste trabalho é avaliar o mecanismo de ação da laserterapia em regimes diferentes de entrega de energia traçando um perfil do infiltrado inflamatório e diferenciação fenotípica dos macrófagos.

## 2. OBJETIVOS

### 2.1 Objetivo geral

Avaliar se aplicar uma dose de 4J/cm² de laser vermelho em feridas cutâneas de ratos diabéticos equivale a quatro doses de 1J/cm² em dias alternados.

### 2.2 Objetivos específicos

**Artigo 1**

I- Avaliar macroscopicamente o fechamento das feridas dos animais diabéticos comparando os dois grupos experimentais;

II- Analisar o tipo e quantidade do infiltrado inflamatório, presença de tecido de granulação e organização do colágeno comparando os dois grupos experimentais;

III- Quantificar os miofibroblastos entre os grupos.

**Artigo 2**

IV- Avaliar e quantificar a presença de neutrófilos durante o reparo tecidual;

V- Verificar a quantidade de macrófagos totais e M2 nos diferentes dias experimentais;

VI- Avaliar e quantificar linfócitos T durante o reparo tecidual.

## 3. MATERIAL E MÉTODOS

### 3.1. Animais

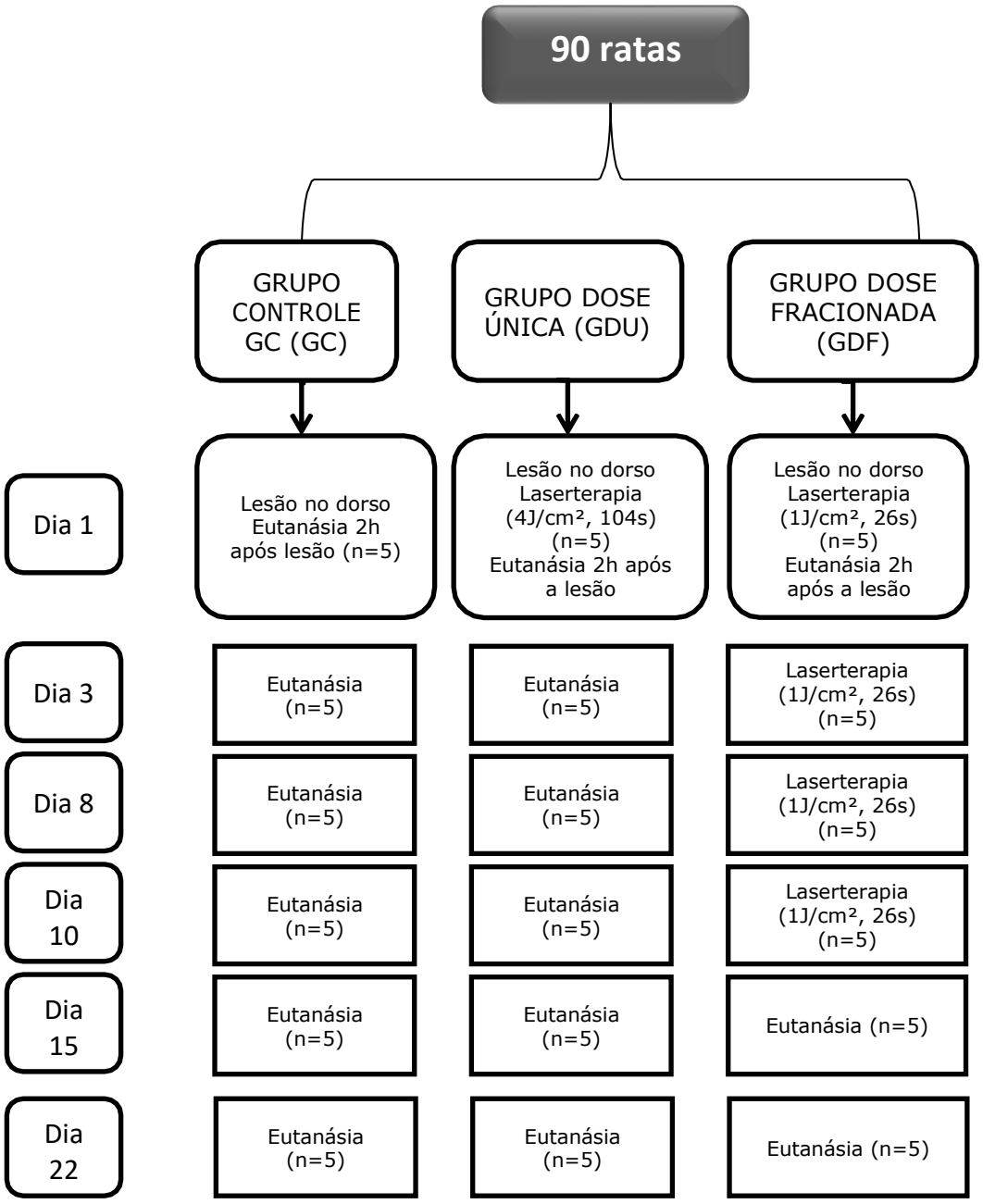
Foram utilizadas 90 ratas fêmeas da linhagem Wistar (*Rattus novergicus albinus*) pesando entre 140 e 250 g, mantidas no biotério da UNINOVE – unidade Vergueiro. Os animais foram mantidos em caixas plásticas apropriadas, num máximo de 5 animais por caixa, a temperatura ambiente (22°C) e luminosidade controlados com ciclo de 12 horas sendo que os animais tiveram comida e água *ad libitum*.

Protocolo de aprovação no Comitê de Ética em Pesquisa Animal (AN 03/2013) (Anexo 1). Um estudo piloto foi conduzido comparando-se as contagens em animais machos e fêmeas e os resultados não demonstraram diferencas significativas.

### 3.2 Animais e grupos experimentais

Os animais foram divididos em grupos:

Figura 1: Fluxograma dos procedimentos experimentais diabéticos



### 3.3. Indução do diabetes

A indução do diabetes foi realizada de acordo com o protocolo descrito por Reddy (2003)[66]. Os animais ficaram 12 horas em jejum alimentar de acordo com o protocolo e as recomendações da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL) e receberam injeção intraperitoneal da toxina para células beta pancreáticas, estreptozocina (cat S0130, Sigma-Aldrich, St. Louis, MO, USA) na dose de 50 mg/kg peso dissolvida em tampão citrato estéril, pH 6,0. A indução ocorreu em média de sete dias sendo confirmada por exame de glicemia por meio do Accu-Chek (Boehringer Mannheim Cor. Indianapolis, IN, USA). O monitoramento do nível de glicemia foi realizado semanalmente pelo mesmo método. O consumo de água e ração foi monitorado diariamente.

### 3.4. Procedimentos cirúrgicos - indução da úlcera

As duas úlceras foram realizadas lateralmente em todos os grupos após 07 dias de indução do diabetes. Para a anestesia os animais receberam injeção intraperitoneal de uma mistura de ketamina (Dopalen, Vetbrands, Jacareí, SP) na dose de 80 mg/kg do animal e de xilazina (Anasedan, Vetbrands, Jacareí, SP) na dose de 10 mg/kg do animal. Foram utilizadas seringas da marca BD 100 Unidades com Agulha BD Ultra-Fine®, modelo insulina com a agulha Ultra-Fine® (regular), comprimento: 12,7 mm, calibre: 0,33 mm e bisel trifacetado.

Depois de anestesiados, o dorso dos animais foi tricotomizado com máquina elétrica e a finalização com uso de um creme depilatório a base de tioglicolato (Veet, Reckitt Benckiser, São Paulo, Brasil). Em seguida, realizada uma úlcera com *punch* cirúrgico de 5 mm (modelo R-806-9-5**,** Richter, São Paulo, Brasil) até a exposição da fáscia muscular dorsal.

Posteriormente aos procedimentos cirúrgicos para a recuperação da anestesia, os animais foram colocados separadamente em gaiolas que foram expostas a pouca luz, evitando-se a manipulação e o estresse do animal. Após a recuperação da anestesia, nos dois primeiros dias os animais receberam uma injeção subcutânea de cloridrato de tramadol (5 mg/kg) associado a 50 mg/kg de dipirona, intramuscular a cada 8 horas para o controle da dor.[52, 53]

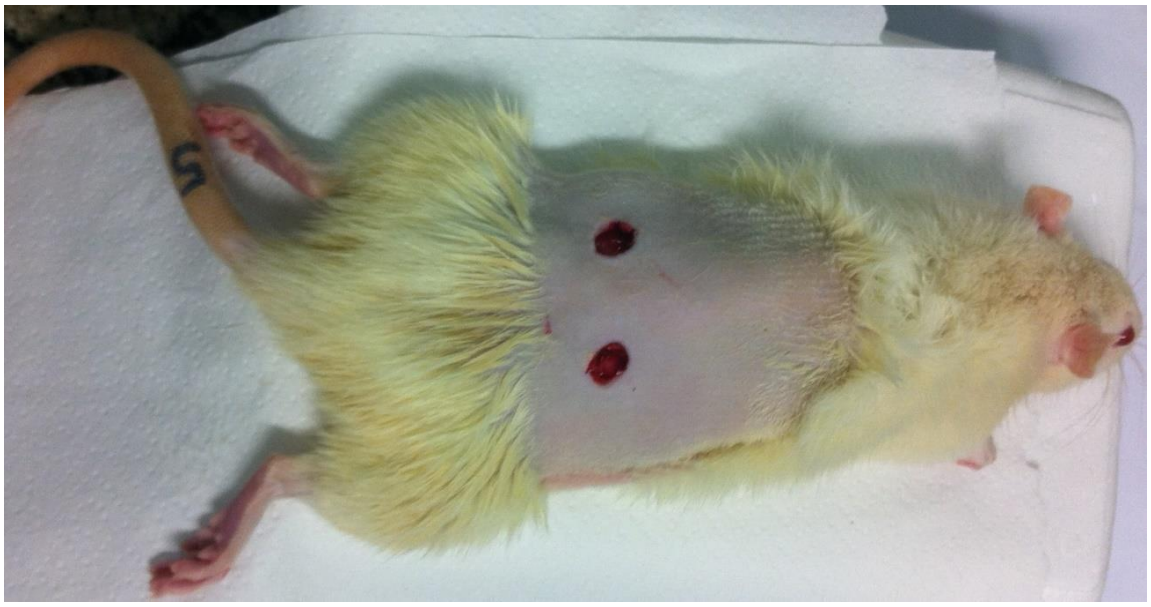


Fig.2 – Aspecto das lesões dorsais

### 3.5. Laserterapia

Um laser vermelho (MMOptics, São Carlos, SP, Brazil) (λ 660 ± 2 nm) com saída de 0,03 $cm^2$, foi acoplado a um sistema óptico composto por uma lente divergente fixada num suporte e a ponteira era afastada ou aproximada de modo a obter-se um feixe expandido de 5 mm de diâmetro que cubrisse as bordas das feridas, garantindo a irradiação de maneira uniforme com 4 $J/cm^2$ de fluência. Os animais do grupo dose única receberam uma aplicação de 4 $J/cm^2$ logo após a realização da lesão, enquanto o grupo de dose fracionada receberam 4 aplicações de 1 $J/cm^2$ nos dias 1, 3, 8 e 10. Para evitar exposição dupla, a porção da ferida já irradiada foi protegida com uma máscara.

Esses dias experimentais foram escolhidos porque representam momentos cruciais do reparo tecidual, sendo os dias 1 e 3 a fase inflamatória, os dias 8, 10 e 15 a fase proliferativa.

### 3.6. Eutanásia

Para a eutanásia, os animais receberam injeção intraperitoneal duas horas após a irradiação de uma mistura de ketamina (Dopalen, Vetbrands, Jacareí, SP) na dose de 320 mg/kg do animal e de xilazina (Anasedan, Vetbrands, Jacareí, SP) na dose de 40 mg/kg do animal. Foram utilizadas seringas da marca BD 100 Unidades com Agulha BD Ultra-Fine®, modelo insulina com a agulha Ultra-Fine® (regular), comprimento: 12,7 mm, calibre: 0,33 mm e bisel trifacetado.

### 3.7 Análise histológica

Nos dias experimentais (1, 3, 8, 10, 15 e 22), três animais de cada grupo sofreram eutanásia em câmara de $CO_2$. As peles foram cuidadosamente removidas, fixadas com formaldeído a 10% tamponado e processadas rotineiramente para inclusão em parafina e posterior coloração com hematoxilina e eosina seguindo o roteiro abaixo:

1- Desparafinização em estufa 45°C - 40 min;
2 – Desparafinização Xilol I - 10 min;
3 - Desparafinização Xilol II - 10 min;
4 - Hidratação Álcool Etílico 100% - 5 min;
5 - Hidratação Álcool Etílico 80% - 5 min;
6 - Hidratação Álcool Etílico 70% - 5 min;
7 - Lavagem Água - 7 min;
8 - Coloração com Hematoxilina - 3 min;
9 - Lavagem Água corrente - 3 min;
10- Coloração Eosina - 7 min;

11- Lavagem Água corrente - 2 min;
12 - Desidratação Álcool Etílico 70% - 5 min;
13 - Desidratação Álcool Etílico 80% - 5 min;
14 - Desidratação Álcool Etílico 100% I - 3 min;
15 - Desidratação Álcool Etílico 100% II - 5 min;
16 - Fixação com Xilol I - 5 min;
17 - Fixação com Xilol II - 10 min.

O reparo foi avaliado por dois avaliadores calibrados, sem conhecimento prévio dos grupos. Os seguintes parâmetros foram avaliados de modo qualitativo: intensidade do infiltrado inflamatório (ausente, discreto, moderado ou intenso), presença de tecido de granulacão e remodelamento do colágeno. Para tal foi utilizado microscópio (NIKON ECLIPSE 50i, Japão) e os dados foram submetidos a análise estatística apropriada.

### 3.8. Imunohistoquímica

Cortes de tecido com 5 µm foram dispensados em lâminas silanizadas (Sigma Chemical Co.; St. Louis, Missouri, EUA) e posteriormente agrupados em suporte adequado. Em seguida foi realizada a desparanifinização, por meio da imersão das lâminas em xilol (60 – 65º C), e colocados em estufa histológica durante 5 minutos e posteriormente passadas em 3 banhos de xilol frio.

Para hidratação dos cortes, as lâminas foram colocadas em dois banhos de álcool absoluto, um banho de álcool 95°ou 80° e um banho de álcool 70°. Em seguida, lavadas em água corrente, água deionizada e deixadas em tampão fosfato salino pH 7,4 (PBS) ou tampão tris-base salino pH 7,4 (TBS) com concentração final de 1M. O próximo passo foi a recuperação dos sítios antigênicos realizada de acordo com o protocolo de cada anticorpo descrito em tabela abaixo:

**Tabela 2: Anticorpos, diluição e recuperação antigênica**

| Antígeno | Anticorpo | Diluição | Recuperação de sítio |
|---|---|---|---|
| **Neutrófilo (elastase)** | Anti-elastase (Abcam cat. 68672) | 1:3500 | Pepsina, temperatura ambiente,15 min |
| **Macrófagos totais (M1 e M2)** | Anti-CD68 (Abcam cat. 31630) | 1:1000 | Tampão citrato, pH 6.0, 100° C, 15 min |
| **Macrófagos M2** | Anti-CD206 (Abcam cat 64693) | 1:250 | Tampão citrato, pH 6.0, 100° C, 15 min |
| **Linfócitos T** | Anti-CD3 (Abcam cat.5690) | 1:300 | Tampão citrato, pH 6.0, 100° C, 15 min |
| **Miofibroblastos** | Anti- actina alfa de músculo liso (ABCAM, ab5694) | 1:1000 | Tampão citrato, pH 6.0, 100° C, 15 min |

A diluição foi otimizada utilizando diluente específico (Spring Bioscience Corp – Pleasanton - EUA) que contém em sua fórmula albumina sérica bovina (BSA) e tampão fosfato, pH 7,4.

Em seguida, foi feito o bloqueio da peroxidase endógena presente nas hemácias com 3 banhos 5 min. de água oxigenada 3% (m/v), após esta etapa as lâminas foram lavadas em água corrente, água destilada e deixadas em tampão salino (PBS/TBS).

A próxima etapa foi a incubação do anticorpo primário por 16 horas a 4ºC. Todos os anticorpos listados em tabela anexa foram diluídos de maneira apropriada (Spring Bioscience Corp – Pleasanton -EUA) em soluções que contém albumina sérica bovina (BSA) e tampão fosfato, pH 7,4. No dia seguinte os cortes foram lavados por 2 vezes durante 5 minutos em TBS e posteriormente incubados com o anticorpo secundário não diluído (Histofine – Nichirei Biosciences Inc., Tokio, Japão) por 30 minutos em estufa a 37 ºC.

Após a marcação de imunohistoquímica, 5 campos consecutivos da área da ferida foram fotografados em um aumento de 400 x (Leica Microsystems,

Wetzlar, Germany) e as imagens digitalizadas das células marcadas foram contadas por um avaliador calibrado sem conhecimento dos grupos usando o *software* ImageJ 1.45 (*software* livre, NIH, Bethesda, Maryland, USA) usando-se o *plug-in* “cell counter”.

## 4. ANÁLISE DOS RESULTADOS

O reparo foi avaliado por dois avaliadores calibrados, sem conhecimento prévio dos grupos nas contagens imunohistoquímicas e submetidas a testes estatísticos apropriados para avaliação da diferença entre os dois regimes de entrega da radiação, dose única e dose fracionada.

O teste de Shapiro-Wilk revelou que as variáveis não seguiram distribuição Gaussiana (normal), exceto para a contagem de miofibroblastos. Assim, foi utilizado o teste Kruskal-Wallis para detectar diferenças possíveis para todas as variáveis, exceto a contagem miofibroblastos, para o qual foi utilizado o teste t de Student. O *software* Minitab 16 (Minitab Inc., EUA) foi utilizado para todas as análises estatísticas, com o nível de significância de 95% ($\alpha = 0,05$).

## 5. RESULTADOS

### 5.1 ARTIGO 1

de Loura Santana C, Silva D de F, Deana AM, Prates RA, Souza AP, Gomes MT, de Azevedo Sampaio BP, Shibuya JF, Bussadori SK, Mesquita-Ferrari RA, Fernandes KP, França CM. **Tissue responses to postoperative laser therapy in diabetic rats submitted to excisional wounds.** *PLoS One*. 2015 Apr 24;10(4):e0122042. doi: 10.1371/journal.pone.0122042. eCollection 2015.

**Abstract**: In a previous study about low-level laser therapy biomodulation on a full-thickness burn model we showed that single and fractionated dose regimens increased wound healing and leukocyte influx similarly when compared with untreated control. In order to verify if this finding would be similar in an impaired wound model, we investigated the effect of single and multiple irradiations on wound closure rate, type of inflammatory infiltrate, myofibroblasts, collagen deposition, and optical retardation of collagen in diabetic rats. Female Wistar rats in the same estrous cycle had diabetes induced with streptozotocin and an 8-mm excisional wound performed with a punch. The experimental groups were: control group--untreated ulcer; single-dose group--ulcer submitted to single dose of diode laser therapy (λ = 660 ± 2 nm; P = 30 mW; energy density: 4 J/cm2) and fractionated-dose group--ulcer submitted to 1 J/cm2 laser therapy on Days 1, 3, 8, and 10. The ulcers were photographed on the experimental days and after euthanasia tissue samples were routinely processed for histological and immunohistochemistry analyses. Independently of the energy density, laser therapy accelerated wound closure by approximately 40% in the first three days in comparison to the control group. Laser therapy increased acute inflammatory infiltrate until Day 3. Both laser groups exhibited more myofibroblasts and better collagen organization than the control group. The findings demonstrate that low-level laser therapy in the

immediate postoperative period can enhance the tissue repair process in a diabetes model. Similar effects were achieved with laser therapy applied a single time with an energy density of 4 J/cm2 and applied four times with an energy density of 1 J/cm2. The application of laser therapy in the inflammatory phase was the most important factor to the enhancement of the tissue repair process.

Link: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4409316/

### 5.2 ARTIGO 2

de Loura Santana C, de Fátima Teixeira Silva D, de Souza AP, Jacinto MV, Bussadori SK, Mesquita-Ferrari RA, Fernandes KP, França CM. **Effect of laser therapy on immune cells infiltrate after excisional wounds in diabetic rats.** *Lasers Surg Med*. 2016 Jan;48(1):45-51. doi: 10.1002/lsm.22445.

**Abstract**: BACKGROUND AND OBJECTIVE: Diabetes alters innate and specific immunity, causing an imbalanced tissue repair process. Very active neutrophils and macrophages are found for a long time in chronic wounds in those individuals. The aim of this study was to evaluate the response of the main effector cells of immunity (neutrophils, macrophages, and T lymphocytes) and to compare the effects of two laser therapy regimens in the postoperative treatment of excision wounds. METHOD: Diabetes was induced in female Wistar rats and a punch was used to cause wounds in the dorsum of each individual. The animals were randomly allocated to a control group (CG), in which the wound was untreated, a single-dose laser group (SLG), in which the wound was submitted to single dose of laser therapy at wavelength of 660 nm, output power of 30 mW, energy density of 4 J/cm(2) , and 26-second exposure time, and a fractionated-dose laser group (FLG), submitted to 1 J/cm(2) of laser therapy on Days 1, 3, 8, and 10. Euthanasia was performed on five animals

from each group Days 1, 3, 8, 10, 15, and 22. The wound was removed and routinely processed for immunohistochemistry against elastase, CD3, CD68, and CD206 antibodies. The samples were photographed and labeled cells were counted by a blinded observer. The Kruskal-Wallis test was used for the statistical analysis. RESULTS: Neutrophils were predominant in the SLG on Day 1, whereas these cells were mostly found in the CG on Day 3 ($P < 0.05$). The T lymphocyte count was similar in all groups in the throughout the experiment. On Day 3, the SLG exhibited a greater number of total macrophages (CD68+) ($P < 0.05$), whereas the macrophage count was similar among the different groups on the other evaluation days. The CD206 + cell counts revealed that the SLG had more M2 macrophages than the CG on Day 8 ($P < 0.05$), whereas the FLG exhibited more M2 macrophages than the CG on Day 10 ($P < 0.05$). CONCLUSION: The present findings demonstrate that laser therapy can alter the composition of inflammatory infiltrate in diabetic wounds, leading to a more balanced response transiting from a rapid neutrophil infiltration through to M2 macrophage polarization, especially with a single application of 4 J/cm(2) in the immediate postoperative period.

Link:http://onlinelibrary.wiley.com/doi/10.1002/lsm.22445/abstract;jsessionid=0DDD9770EE5CFA1BF692753554D5E565.f02t02

## 6. DISCUSSÃO

A laserterapia mostrou ser capaz de interferir no recrutamento de neutrófilos, polarização de macrófagos, diferenciação de fibroblastos em miofibroblastos e redução da área das feridas, sobretudo quando aplicada em dose única de 4 J/cm$^2$ logo após a realização da lesão.

Sabol et al, (2012) realizaram experimentos com lesões em pele de ratos analisando a fisiologia do tecido e concluíram ser um modelo dinâmico para este tipo de estudo.

Independentemente da densidade de energia, a laserterapia acelerou o fechamento das feridas em cerca de 40% nos primeiros três dias em comparação com o grupo de controle, após esse período o fechamento ocorreu de maneira semelhante em todos os grupos do dia 8 em diante.

Em pacientes diabéticos, a resposta inflamatória após lesão é frequentemente prolongada e excessiva devido à desregulação da coagulação e resposta inflamatória bem como glicação de proteínas, que leva ao espessamento da membrana basal capilar e alteração na permeabilidade.[48,67]

Uma das complicações do DM é a lentidão na migração de células inflamatórias para o local da lesão, resultando em inflamação crônica. Estudos relatam que a laserterapia aumenta a quimiotaxia de neutrófilos.[68,69] No presente trabalho, a laserterapia promoveu o aumento o número de leucócitos (principalmente neutrófilos) em duas horas após a lesão, com um pico no dia 3, independentemente da densidade de energia (1 ou 4 J/cm²). Importante observar que o grupo submetido a uma dose única da irradiação teve a melhor resposta inflamatória, como demonstrado pelos valores significativamente mais elevados do dia 3 a dia 10 em comparação com os outros grupos.

Núñez et al., (2013)[70] utilizaram ratos normoglicêmicos com queimaduras com a mesma dosimetria deste estudo e encontraram uma

quimiotaxia de neutrófilos superior, bem como a formação de novos vasos sanguíneos acelerando a fase inflamatória e promovendo a cicatrização.

Estudos com um modelo de artrite e o modelo de doença fúngica, constataram que a laserterapia pode melhorar a quimiotaxia de neutrófilos.[68,74] No entanto, os resultados na literatura em modelos diabéticos são conflitantes. Kilík et al. (2014)[54] realizaram uma análise morfológica semi-quantitativa da contagem de neutrófilos em feridas diabéticas tratadas com laserterapia e encontraram uma significativa menor infiltração nos grupos irradiados. Considerando que Sharifian et al (2014)[75] demonstraram que a laserterapia foi incapaz de melhorar a quimiotaxia de neutrófilos no dia 4. Ambos os estudos não levaram em consideração que o período mais importante, onde ocorre a diapedese de neutrofilos, é de quatro horas após a lesão. Além disso, as análises foram apenas morfológicas e não utilizaram técnicas de imunomarcação.

Os neutrófilos responderam prontamente na laserterapia na fluência de 4J/cm$^2$, imediatamente após a lesão, com uma diminuição significativa no número no dia 3. No entanto, o grupo controle demonstrou um pico de neutrófilos no dia 3, e ainda assim, com menos células que as encontradas no grupo dose única. Este é um importante achado, como a maior quantidade de neutrófilos é encontrada no tecido de indivíduos normoglicêmicos, cerca de quatro horas após a lesão[72]. No entanto, sob condições diabéticas, estas células demoram mais tempo para chegar ao local da lesão e permanecem por um periodo maior, liberando proteases que contribuem para a cronicidade da ferida[8,73]. Assim, a fotobiomodulação para neutrófilos no dia 3 em diante é importante para evitar estímulo pro-inflamatório desnecessário.

A laserterapia mostrou modulação na presença de neutrófilos e macrófagos nas feridas e aumento na diferenciação de macrófagos. Com base nas condições deste estudo, uma única dose de 4 J/cm² mostrou-se mais eficaz no aumento do número de neutrófilos e macrófagos que quatro doses

de 1 J/cm², indicando que o método de entrega de luz no início da cascata inflamatória é crucial para desencadear uma resposta celular efetiva e que 1 J/cm² é uma dose menos efetiva para cicatrização de feridas diabéticas.

Souza et al (2014)[71] irradiaram macrófagos em cultura e encontraram maior atividade mitocondrial e portanto, alteração no estado de ativação dessa célula *in vitro*, o que se mostrou evidente no tecido lesionado onde a laserterapia foi capaz de recrutar os macrófagos e estimular sua polarização, bem como promover a liberação de fatores de crescimento e diferenciação de fibroblastos em miofibroblastos promovendo a contração da ferida .

Embora os macrófagos sejam cruciais para a cicatrização de feridas normais, a desregulação da sua função poderia contribuir para reparar o tecido deficiente em pacientes com diabetes.[4,19] Nosso grupo constatou que a laserterapia numa fluência de 4J/cm$^2$ foi capaz de recrutar mais macrófagos no dia 3, que está de acordo com os dados descritos por Sharifian et al. (2014)[75], que descobriu que doses baixas de um laser pulsado de infravermelho foram capazes de aumentar a contagem de macrófagos no dia 4 após a cirurgia em animais diabéticos. Os macrófagos nas feridas de ratos diabéticos exibem uma transição prejudicada de M1 pró-inflamatória para o fenótipo M2 pró-reparo, o que pode dificultar o fechamento da ferida[4,5]. No presente estudo, fotobiomodulação inicial usando 4 J/cm² exerceu um impacto sobre a polarização de macrófagos M2 no dia 8 e as doses sub-ótimas fracionadas de 1J/cm², apesar de não afetar o número total de macrófagos, também levou a um aumento na contagem de macrófagos M2 no dia 10. Estes são resultados importantes que podem ajudar a esclarecer os mecanismos pelos quais a laserterapia melhora a cicatrização de feridas, especialmente em diabéticos. Macrófagos M2 produzem TGF-β, que é responsável pela conversão dos fibroblastos em miofibroblastos aumentando a contração da ferida, que é deficiente em indivíduos com diabetes.[76]

Embora muitos fatores possam contribuir para a disfunção de macrófagos encontrada em indivíduos com diabetes, o aumento do montante de citocinas pro-inflamatórias interferon gama e IL-1β, junto com a diminuição da IL-10 que é anti-inflamatória, podem desempenhar um papel significativo.[77] Como macrófagos são responsivos à luz, estas células podem ser moduladas tanto com a luz vermelha e próximo ao infravermelho, a laserterapia pode restaurar parcialmente o balanço deste microambiente devido à redução de interferon[78], IL-1[80] e marcadores de estresse oxidativo[73] na cicatrização de tecidos.

Nas feridas diabéticas ocorre falha na formação do tecido de granulação adequado, a angiogênese é pobre e a cicatriz não se contrai corretamente, muitas vezes resultando em deiscência ou feridas crônicas.

A fase proliferativa do reparo do tecido é caracterizada pela formação de tecido fibroso e angiogênese e é fortemente modulada por transformar o fator de crescimento beta (TGF-β), que induz a proliferação de fibroblastos e sua diferenciação em miofibroblastos. Laserterapia induz o aparecimento de miofibroblastos em tecido de granulação durante a proliferação e fases de remodela do tecido reparar o processo, provavelmente através da modulação da síntese de TGF-β, o que é de grande valor para o paciente com feridas. [81, 82,

Araújo et al. (2005)[83], utilizando feridas cirúrgicas no dorso de ratos, analisaram o padrão histológico das feridas ao 8º, 15º e 22º dias e observaram que o número de miofibroblastos aumentou gradativamente no grupo controle ao longo do período analisado, enquanto na derme irradiada a população de miofibroblastos foi significativamente maior no início e muito inferior no 22º dia, corroborando com nosso estudo, onde a laserterapia promoveu aumento na quantidade de miofibroblastos nos grupos tratados em relação ao controle na fase de remodelamento – dias 15 ao 22 - que pode ser útil para contração das feridas e remodelação em pacientes diabéticos.

No presente trabalho, a laserterapia não estimulou o recrutamento d estas células. Células T são uma importante parte da resposta imune inata em feridas da pele. Mais estudos devem ser realizados para investigar outras células T com marcação diferenciada (T regs – CD4 + FOXP3 +) e a relação CD4/CD8.[84]

## 7. CONCLUSÕES

Aplicar uma dose de 4J/cm² de laser vermelho em feridas cutâneas de ratos diabéticos gera uma cicatrização de qualidade superior do que a a quatro doses de 1J/cm² em dias alternados. E independentemente do regime de aplicação, fazer a fotobiomodulação é melhor do que não realizá-la.

A fotobiomoduação mostrou-se efetiva na aceleração do fechamento da ferida nos três primeiros dias, aumento do número de neutrófilos, polarização de macrófagos, diferenciação de miofibroblastos e organização do colágeno, sobretudo quando aplicado em dose única.

Não houve diferença significativa na contagem de linfócitos T.

## 8. REFERÊNCIAS BIBLIOGRÁFICAS


1 World Health Organization. Disponível em: http://www.who.int/countries/bra/es/ acessado em 28 February 2015

2 American Diabetes Association - Diabetes Statistics. Data from the 2011 National Diabetes Fact Sheet. Available: http://www.diabetes.org/diabetes-basics/statistics/. Acessed 28 February 2015

3 Brasil. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. Diabetes Mellitus / Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Atenção Básica. – Brasília : Ministério da Saúde, 2006. 64 p. il. – (Cadernos de Atenção Básica, n. 16) (Série A. Normas e Manuais Técnicos)

4 Brancato, SK, Albina, JE. Wound Macrophages as Key Regulators of Repair Origin, Phenotype, and Function. The American Journal of Pathology, Vol. 178, No. 1, January 2011 DOI: 10.1016/j.ajpath.2010.08.00

5 Kittan NA, Allen RM, Dhaliwal A, Cavassani KA, Schaller M, Gallagher KA, et al. Cytokine induced phenotypic and epigenetic signatures are key to establishing specific macrophage phenotypes. PLoS One. 2013; 8: e78045

6 Mirza R, DiPietro LA, Koh TJ: Selective and specific macrophage ablation is detrimental to wound healing in mice. Am J Pathol 2009, 175:2454–2462

7 Carvalho, PTC, Schettert Silva, IS, Reis, FA, Perreira, DM, Aydos, RD.

Influence of ingaalp laser (660nm) on the healing of skin wounds in diabetic rats. Acta Cirúrgica Brasileira - Vol. 25 (1) 2010

8 Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013; 13: 159-75

9 Clark RA. Molecular and Cellular Biology of Wound Repair, (Second Edition). Plenum Press, New York, 1995.

10 Eming SA, Krieg T, Davidson JM. Gene therapy and wound healing. Clin Dermatol. 2007;25:79-92

11 Mendonca RJ, Coutinho-Netto J. Aspectos celulares da cicatrização. An. Bras. Dermatol. [online]. 2009, vol.84, n.3 [cited 2011-12-06], pp. 257-262

12 FRADE, M. **Úlcera de perna: caracterização clínica e perfil imunohistológco da cicatrização na presença da biomembrana de látex natural da seringueira *Hevea brasiliensis***. 2003. 164 p. Tese (Doutorado em Clínica Médica) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto.

13 Arnold F, West DC. Angiogenesis in wound healing. Pharmacol Ther. 1991;52:407-22.

14 BELPERIO, J. A.; KEANE, M. P.; ARENBERG, D. A.; ADDISON, C. L.; EHLERT, J. E.; BURDICK, M. D.; STRIETER, R. M. CXC chemokines in angiogenesis. J. Leukoc. Biol., v. 68, p. 1-8, 2000.

15 Karukonda SR, Flynn TC, Boh EE, McBurney EI, Russo GG, Millikan LE. The effects of drugs on wound healing--part II. Specific classes of drugs

and their effect on healing wounds International journal of dermatology.2000;39:321-33.

16 Cruvinel, WM, Mesquita Júnior, D, Araújo, JAP, Catelan, TTT, Souza, AWS, Silva, NP, Andrade, LEC, Fundamentos da imunidade inata com ênfase nos mecanismos moleculares e celulares da resposta inflamatória. Rev. Bras Reumatol 2010;50(4):434-61

17 Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS et al. Neutrophil extracellular traps kill bacteria. Science 2004; 303:1477-8.

18 Clark RA. Basics of cutaneous wound repair. J Dermatol Surg Oncol. 1993 Aug;19(8):693-706

19 Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Müller W, et al. Differential roles of macrophages in diverse phases of skin repair. J Immunol.2010; 184: 3964-77

20 Sindrilaru, A.; Scharffetter-Kochanek, K.. Disclosure of the Culprits: Macrophages—Versatile Regulators of Wound Healing ADVANCES IN WOUND CARE, VOLUME 2, NUMBER 7. DOI: 10.1089/wound.2012.0407

21 HUPPES, D. Padronização da diferenciação in vitro e a ativação clássica ou alternativa da linhagem de células humanas U937 em macrófagos. Dissertação de Mestrado. UFRS 2013

22 Willenborg, S., Lucas, T., van Loo, G., Knipper JA, Krieg, T., Haase, I., Brachvogel, B., Hammerschmidt, M., Nagy, A., Ferrara, N., Pasparakis,

M., Eming, S.A. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. Blood 2012; 120:61

23 Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, Hainzl A, Schatz S, Qi Y, Schlecht A, Weiss JM, Wlaschek M, Sunderkotter C, and Scharffetter-Kochanek K: An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. J Clin Invest 2011; 121: 985

24 Sica A and Mantovani A: Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012;122:787.

25 Novak, LM; Timothy J. Koh, T. Macrophage phenotypes during tissue repair. Journal of Leukocyte Biology. Volume 93, June 2013

26 Buechler, C., Ritter, M., Orsó, E., Langmann, T., Klucken, J., Schmitz, G. (2000) Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli.J. Leukoc. Biol.67,97–103

27 Ambarus, C. A., Krausz, S., van Eijk, M., Hamann, J., Radstake, T. R., Reedquist, K. A., Tak, P. P., Baeten, D. L. (2012) Systematic validation of specific phenotypic markers for in vitro polarized human macrophages.J. Immunol. Methods 375,196 –206.

28 Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K., Suttles, J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J. Immunol.175,342–349 - 2005

29 Malagutti, W, Imunização, Imunologia e Vacinas. Rio de Janeiro: Rubio, 2011

30 Manual dos Centros de Referência de Imunobiológicos Especiais / elaborado pelo Comitê Técnico Assessor de Imunizações do Ministério da Saúde. - Brasília: Ministério da Saúde: Fundação Nacional de Saúde, 2001 142 p.

31 Robbins SL, Cotran RS, Kumar V, Abbas AK, Fausto N. Tecido de renovação e reparação: regeneração, cicatrização e fibrose. In: Robbins e Cotran. Patologia: bases patológicas das doenças. 7ª ed. Rio de Janeiro: Elsevier;2005

32 Brunner & Suddarth - Manual de Enfermagem Médico e Cirúrgica - 13ed - 2015 - Guanabara Koogan

33 Broughton G 2nd, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg. 2006;117(7 Suppl):12S-34S

34 Isaac C, Ladeira PRS, Rego FMP, Aldunate JCB, Ferreira MC. Processo de cura das feridas: cicatrização fisiológica. Rev Med (São Paulo). 2010 jul.-dez.;89(3/4):125-31.

35 Feghali, C. A., Wright, T. M. . Cytokines in acute and chronic inflammation.Front. Biosci.2, d12–d26. – 1997

36 Gharaee-Kermani M, McCullumsmith RE, Charo IF, Kunkel SL, Phan SH: CC-chemokine receptor 2 required for bleomycin-induced pulmonary fibrosis. Cytokine 2003, 24:266 –276

37 Gharaee-Kermani, M., Kasina, S., Moore, B. B., Thomas, D., Mehra, R., Macoska, J. A. CXC-type chemokines promote myofibroblast phenoconversion and prostatic fibrosis.PLoS ONE7:e49278. doi: 10.1371/journal.pone.004927 - 2012

38 Scotton, C. J., and Chambers, R. C. Molecular targets in pulmonar fibrosis: the myofibroblast in focus.Chest132, 1311–1321. doi: 10.1378/chest. 06-256 – 2007

39 Amara, N., Goven, D., Prost, F., Muloway, R., Crestani, B., and Boczkowski, J. (2010). NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta1-induced fibroblast differentiation into myofibroblasts.Thorax65, 733–738. doi:10.1136/thx.2009.113456 Arcasoy, S. M., Christie, J.

40 Bondi, C. D., Manickam, N., Lee, D. Y., Block, K., Gorin, Y., Abboud, H. E., et al. (2010). NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts.J. Am. Soc. Nephrol. 21, 93–102. doi: 10.1681/ASN.2009020146

41 Ferreira, CLR; Nicolau, RA; Oliveira, MA; Davidson Ribeiro Costa, DR; Prianti Júnior, AGC.. Efeito da terapia LED (λ = 945 ± 20 nm) de baixa intensidade sobre tecido epitelial de ratos diabéticos em processo de reparo. Rev. Bras. Eng. Bioméd., v. 29, n. 4, p. 404-413, dez. 2013 Braz. J. Biom. Eng., 29(4), 404-413, Dez. 2013

42 Mirza R and Koh TJ: Dysregulation of monocyte/macrophage phenotype in wounds of diabetic mice. Cytokine 2011;56:256.

43 Khanna S, Biswas S, Shang Y, Collard E, Azad A, Kauh C, Bhasker V, Gordillo GM, Sen CK, and Roy S: Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice. PLoS One 2010;5:e9539

44 Hamblin MR, Huang Y-Y, Chen ACH, Carroll JD. Biphasic dose response in low level light therapy. International Dose-Response Society 2009; 7:358-383

45 Fulop AM, Dhimmer S, Deluca JR, Johanson DD, Lenz RV, Patel KB, Douris PC, Enwemeka CS (2009) A meta-analysis of the efficacy of phototherapy in tissue repair. Photomed Laser Surg 27:695–702

46 Lins RDA, Dantas EU, Lucena KCR, Granville-Garcia AF, da Silva JSP. Aplicação do laser de baixa potência na cicatrização de feridas. Odontol. Clín.-Cient., Recife, Suplemento 511-516, out./dez., 2011

47 Franciscon LMGS. Avaliação do laser de baixa potência e medicação tópica na reparaçãode ulceras cutânras em diabéticos. Dissertação de Mestrado. Unicastelo, 2014

48 Houreld NN, Sekhejane PR, Abrahamse H. Irradiation at 830 nm stimulates nitric oxide production and inhibits pro-inflammatory cytokines in diabetic wounded fibroblast cells. Lasers Surg Med. 2010 Aug;42(6):494-502.

48 Houreld, N; Abrahamse, H. Irradiation with a 632.8 nm helium-neon laser with 5 J/cm² stimulates proliferation and expressiono of Interleukin-6 in diabetic wounded fibroblast cells. Diabetes Technol Ther 9:451-459, 2007

49 Kaviani A, Djavid GE, Ataie-Fashtami L, Fateh M, Ghodsi M, Salami M, Zand N, Kashef N, Larijani B. Photomedicine and Laser Surgery. February 2011, 29(2): 109-114. doi:10.1089/pho.2009.2680

50 Kajagar, BM, Godhi, AS, Pandit, A, Khatri, Efficacy of Low Level Laser Therapy on Wound Healing in Patients with Chronic Diabetic Foot Ulcers—A Randomised Control Trial Indian J Surg (September–October 2012) 74(5):359–363

51 Santos, CFF, Santos, AP, Machado, TGP, Avelar, NCP, Oliveira, MX, Cicatrização de feridas cutâneas em ratos após terapia laser de baixa intensidade (660nm). Revista Vozes dos Vales da UFVJM: Publicações Acadêmicas – MG – Brasil – Nº 03 – Ano II – 05/2013 Reg.: 120.2.095–2011 – PROEXC/UFVJM – ISSN: 2238-6424

52 Lau, PS, Bidin, N, Krishnan, G, Anaybbaleg, SM, Marsin, FM, Sum, MBM, Baktiar, H, Nassir, Z, Chong, PL & Hamid, A2015, 'Wound treatment on a diabetic rat model by a 808 nm diode laser' Laser Physics, vol 25, no. 7, 075601.,10.1088/1054-660X/25/7/075601

53 Santana, CL, Silva, DFT, Deana, AM, Prates, RA, Souza, AP, Gomes, MT, Sampaio, BPA, Shibuya, JF, Bussadori, SK, Mesquita-Ferrari, RA, Fernandes, KPS, França, CM. Tissue Responses to Postoperative Laser Therapy in Diabetic Rats Submitted to Excisional Wounds. PLoS ONE 10(4): e0122042. doi:10.1371/journal.pone.0122042

54 Kilík R, Lakyová L, Sabo J, Kruzliak P, Lacjaková K, Vasilenko T, Vidová M, Longauer F, Rado J. Effect of Equal Daily Doses Achieved by Different Power Densities of Low-Level Laser Therapy at 635nm on Open Skin Wound Healing in Normal and Diabetic Rats. BioMed Research

International .Volume 2014, Article ID 269253, 9 pages http://dx.doi.org/10.1155/2014/269253

55 Danca´kova´ L, Vasilenko T, MD, Kova´ I, Jakub_cova´ K, Holly´ M, Revajova´ V , Sabol F, Tomori Z, Iversen M, Ga´ l P, M. Bjordal JM. Low-Level Laser Therapy with 810nm Wavelength Improves Skin Wound Healing in Rats with Streptozotocin-Induced Diabetes. Photomedicine and Laser Surgery . Volume 32, Number 4, 2014

56 Busnardo VL, Biondo-Simões MLP. Os efeitos do laser hélio-neônio de baixa intensidade na cicatrização de lesões cutâneas induzidas em ratos. Rev bras fisioter. 2010;14(1):45-51

57 Silva TS, Mendes F, Alves AMP, Alves EPB, Bertolini GRF. Estudo microscópio da lesão tecidual em pele de ratos Wistar tratados com laser de baixa potência. Rev Bras Bioci. 2010;8(3);264-7

58 Felice TD, Pinheiro AR, Menchik EDS, Silva ACD, Souza LS, Caires CSA, et al. Utilização do laser de baixa potência na cicatrização de feridas. Interbio. 2009;3(2);42-52

59 Maiya AG, Kumar P, Nayak S. Photo-stimulatory effect of low energy helium-neon laser irradiation on excisional diabetic wound Healing dynamics in wistar rats. Indian J Dermatol. 2009;54(4):323-9

60 Inoe AP, Zafanelli CCG, Rossato RM, Leme MC, Sanches AWD, Araújo CV, et al. Avaliação morfológica do efeito do laser de baixa potência He-Ne em feridas cutâneas de coelhos. Arq ciênc vet zool Unipar. 2008;11(1):27-30.

61 Channual J, Choi B, Osann K, Pattanachinda D, Lotfi J, Kelly KM. Vascular effects of photodynamic and pulsed dye laser therapy protocols. Lasers Surg Med. 2008;40(9);644-50

62 Pinto NC, Pereira, HC, Stolf NAG, Chavantes MC. Laser de baixa intensidade em deiscência aguda safenectomia: proposta terapêutica. Rev Bras Cir Cardiovasc. 2009;24(1);88-91.

63 Rocha Júnior AM, Oliveira RG, Farias RE, Andrade LCF. Aarestrup FM. Modulação da proliferação fibroblástica e da resposta inflamatória pela terapia a laser de baixa intensidade no processo de reparo tecidual. An Bras Dermatol. 2006;81(2):150-6.

64 Hopkins JT, McLoda TA, Seegmiller JG, Baxter GD. Low-level laser therapy facilitates superficial wound healing in humans: a tripleblind, sham-controlled study. J Athl Train. 2004;39(3):223-9.

65 Envemeka CS. Attenuation and penetration of visible 632.8nm and invisible infra-red 904nm light in soft tissues. Laser Therapy. 2001;13:95-101

66 Reddy GK. Comparison of the photostimulatory effects of visible HeNe and infrared GaAs lasers on healing impaired diabetic rat wounds. Lasers Surg Med. 2003;33:344-351.

67 Chen J, Kasper M, Heck T, Nakagawa K, Humpert PM, Bai L, et al. Tissue factor as a link between wounding and tissue repair. Diabetes. 2005; 54: 2143–2154. PMID: 15983216

68 Alves AC, Vieira R, Leal-Junior E, dos Santos S, Ligeiro AP, Albertini R, et al. Effect of low-level laser therapy on the expression of inflammatory mediators and on neutrophils and macrophages in acute joint inflammation. Arthritis Res Ther. 2013; 15: R116. PMID: 24028507

69 Firat ET, Dag A, Gunay A, Kaya B, Karadede MI, Kanay BE, et al. The effects of low-level laser therapy on palatal mucoperiosteal wound healing and oxidative stress status in experimental diabetic rats. Photomed Laser Surg. 2013; 31: 315–321. doi: 10.1089/pho.2012.3406 PMID: 23789588

70 Núñez SC, França CM, Silva DFT , Nogueira GEC , Prates RA, Ribeiro, MS. The influence of red laser irradiation timeline on burn healing in rats. Lasers Med Sci (2013) 28:633–641 DOI 10.1007/s10103-012-1105-4

71 Souza NHC, Ferrari RAM, Silva DFT, Nunes FD, Bussadori SK, Fernandes KPS. Effect of low-level laser therapy on the modulation of the mitochondrial activity of macrophages. Braz J Phys Ther. 2014 July-Aug; 18(4):308-314. http://dx.doi.org/10.1590/ bjpt-rbf.2014.0046

72 Oehmichen M. Vitality and time course of wounds. Forensic Sci Int. 2004; 144: 221-31

73 Pillay J, den Braber I, Vrisekoop N, Kwast LM, de Boer RJ, Borghans JA, Tesselaar K, Koenderman L. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood. 2010 Jul 29;116(4):625-7

74 Burger E, Mendes AC, Bani GM, Brigagão MR, Santos GB, Malaquias LC, et al. Low-level laser therapy to the mouse femur enhances the fungicidal response of neutrophils against Paracoccidioides brasiliensis. PLoS Negl Trop Dis. 2015 Feb 12;9(2):e0003541

75 Sharifian Z, Bayat M, Alidoust M, Farahani RM, Bayat M, Rezaie F, et al. Histological and gene expression analysis of the effects of pulsed low-level laser therapy on wound healing of streptozotocin-induced diabetic rats. Lasers Med Sci. 2014; 29: 1227-35

75 Fukuda TY, Tanji MM, Silva SR, Sato MN, Plapler H. Infrared low-level diode laser on inflammatory process modulation in mice: pro- and anti-inflammatory cytokines. Lasers Med Sci. 2013; 28: 1305-13.

76 Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest. 2007; 117: 1219-22

77 Venneri MA, Giannetta E, Panio G, De Gaetano R, Gianfrilli D, Pofi R, et al. Inhibition of PDE5 Limits Pro-Inflammatory Monocyte-Macrophage Polarization in Streptozotocin - Induced Diabetic Mice. PLoS One. 2015 May 11;10(5):e0126580

78 Oliveira RG, Ferreira AP, Côrtes AJ, Aarestrup BJ, Andrade LC, Aarestrup FM. Low-level laser reduces the production of TNF-α, IFN-γ, and IL-10 induced by OVA. Lasers Med Sci. 2013; 28: 1519-25.

79 Fernandes KP, Alves AN, Nunes FD, Souza NH, Silva JA Jr, Bussadori SK, Ferrari RA. Effect of photobiomodulation on expression of IL-1β in skeletal muscle following acute injury. Lasers Med Sci. 2013; 28: 1043-6

80 Souza NH, Marcondes PT, Albertini R, Mesquita-Ferrari RA, Fernandes KP, Aimbire F. Low-level laser therapy suppresses the oxidative stress-induced glucocorticoids resistance in U937 cells: relevance to cytokine secretion and histone deacetylase in alveolar macrophages. J Photochem Photobiol B. 2014; 130: 327-36.

81 Hawkins D, Houreld N, Abrahamse H. Low level laser therapy (LLLT) as an effective therapeutic modality for delayed wound healing. Ann N Y Acad Sci. 2005;1056:486-93.

82 Posten W, Wrone DA, Dover JS, Arndt KA, Silapunt S, Alam M. Low level laser therapy for wound healing: mechanism and efficacy. Dermatol Surg. 2005;31(3):334-40.

83 Araújo CEN, Ribeiro MS, Favaro R, Zezell DM, Zorn TMT. Ultrastructural and autoradiographical analysis show a faster skin repair in HeNe lasertreated wounds. J Photochem Photobiol B. 2007;86:8796

84 Szymanska J, Goralczyk K, Klawe JJ, Lukowicz M, Michalska M, Goralczyk B, et al. Phototherapy with low-level laser influences the proliferation of endothelial cells and vascular endothelial growth fator and transforming growth factor-beta secretion. J Physiol Pharmacol. 2013; 64: 387–391.

## 9. Anexos

### *9.1* Artigo 1: Tissue responses to postoperative laser therapy in diabetic rats submitted to excisional wounds. *PLoS One*

PLOS | ONE

RESEARCH ARTICLE

# Tissue Responses to Postoperative Laser Therapy in Diabetic Rats Submitted to Excisional Wounds

**Cristiano de Loura Santana, Daniela de Fátima Teixeira Silva, Alessandro Melo Deana, Renato Araujo Prates, Amanda Pires Souza, Mariana Teixeira Gomes, Brunna Pileggi de Azevedo Sampaio, Josiane Ferraretto Shibuya, Sandra Kalil Bussadori, Raquel Agnelli Mesquita-Ferrari, Kristianne Porta Santos Fernandes, Cristiane Miranda França***

Postgraduate Program in Biophotonics Applied to Health Sciences, University Nove de Julho (UNINOVE), São Paulo, São Paulo, Brazil

* cristiane321@gmail.com



OPEN ACCESS

**Citation:** de Loura Santana C, de Fátima Teixeira Silva D, Deana AM, Prates RA, Souza AP, Gomes MT, et al. (2015) Tissue Responses to Postoperative Laser Therapy in Diabetic Rats Submitted to Excisional Wounds. PLoS ONE 10(4): e0122042. doi:10.1371/journal.pone.0122042

**Academic Editor:** Alexander V. Ljubimov, Cedars-Sinai Medical Center; UCLA School of Medicine, UNITED STATES

**Received:** September 24, 2014

**Accepted:** February 5, 2015

**Published:** April 24, 2015



**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** This study was supported by Research Grants # 2012/01944-0 (CMF), 2012/02801-8 (BPAS) and 2012/03334-4 (JAFS) from the State of São Paulo Research Foundation (FAPESP), Brazil. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

In a previous study about low-level laser therapy biomodulation on a full-thickness burn model we showed that single and fractionated dose regimens increased wound healing and leukocyte influx similarly when compared with untreated control. In order to verify if this finding would be similar in an impaired wound model, we investigated the effect of single and multiple irradiations on wound closure rate, type of inflammatory infiltrate, myofibroblasts, collagen deposition, and optical retardation of collagen in diabetic rats. Female Wistar rats in the same estrous cycle had diabetes induced with streptozotocin and an 8-mm excisional wound performed with a punch. The experimental groups were: control group – untreated ulcer; single-dose group – ulcer submitted to single dose of diode laser therapy (λ = 660 ± 2 nm; P = 30 mW; energy density: 4 J/cm$^2$) and fractionated-dose group – ulcer submitted to 1 J/cm$^2$ laser therapy on Days 1, 3, 8, and 10. The ulcers were photographed on the experimental days and after euthanasia tissue samples were routinely processed for histological and immunohistochemistry analyses. Independently of the energy density, laser therapy accelerated wound closure by approximately 40% in the first three days in comparison to the control group. Laser therapy increased acute inflammatory infiltrate until Day 3. Both laser groups exhibited more myofibroblasts and better collagen organization than the control group. The findings demonstrate that low-level laser therapy in the immediate postoperative period can enhance the tissue repair process in a diabetes model. Similar effects were achieved with laser therapy applied a single time with an energy density of 4 J/cm$^2$ and applied four times with an energy density of 1 J/cm$^2$. The application of laser therapy in the inflammatory phase was the most important factor to the enhancement of the tissue repair process.

Competing Interests: The authors have declared that no competing interests exist.

## Introduction

Diabetes mellitus (DM) is a common disease with the estimated prevalence of more than 371 million people worldwide and an increasing incidence in every country. Patients with diabetes often have surgical needs due to health disorders caused mostly by chronic hyperglycemia and are at risk for postoperative complications related to the non-healing of surgical wounds [1]. DM impairs wound healing due to an imbalance in the inflammatory response, the altered production of cytokines, altered collagen synthesis, reduced angiogenesis, and reduced tensile strength [2–4]. This leads to a decrease in wound strength, poor wound contraction, an increased incidence of infection, and dehiscence, which prolong hospitalization and increase the mortality rate [5]. The World Health Organization (WHO) estimates that there will be 366 million individuals with diabetes in 191 countries by the year of 2030 [6, 7].

Low-level laser therapy has been used in the clinical setting as a complementary tool for pain relief as well as due to its anti-inflammatory effects and has also been employed to accelerate the healing process in cases of muscle injury [8, 9], burns [10], surgical wounds [11, 12] and chronic ulcers [13, 14]. The biomodulatory effects of laser therapy are based on the theory that photon energy is absorbed by cellular photoacceptor molecules, such as oxyhemoglobin, hemoglobin, cytochrome c oxidase and melanin. Once the photon energy is absorbed, the photoacceptor assumes an electronically excited state and this energy is converted into chemical energy within the cell [15]. Cytochrome c oxidase receives photons and promotes a change in the mitochondrial redox state and/or pumping of ions across the inner mitochondrial membrane as well as an increase in ATP synthesis [15]. There is also an increase in intracellular calcium ($Ca^{2+}$), which stimulates DNA and RNA synthesis, thereby activating a cascade of intracellular signals [16]. This ultimately stimulates DNA duplication, increases protein synthesis, regulates oxidative stress, and modulates the production of different cytokines [17, 18]. These events lead to the biomodulation of different cell types involved in tissue regeneration [19], including an increase in fibroblast mitosis [20], greater angiogenesis [21, 22], changes in the synthesis of cytokines [23–25], and assistance in the conversion of fibroblasts into myofibroblasts [26].

These effects have been demonstrated in both pre-clinical studies and clinical trials [8–14, 27]. However, the lack of standardization regarding dosimetry and light delivery regimens as well as the incomplete understanding of the associated cellular and molecular mechanisms of action limit the use of this treatment modality [28]. The importance of dose versus irradiation moment is still a matter of investigation. If a single laser exposure would be enough to produce the same effect as three or four exposures, regarding the compliance of the therapy, and also the costs involved, a single application would be better [10].

The hypothesis of this study was whether lasertherapy delivery regimen would impact on the final repair tissue under hyperglycemic conditions. Considering the diabetes epidemics and that these individuals suffer traumatic injuries and surgeries, to apply lasertherapy on alternate days for more than two weeks is not a reliable therapy due to the general lack of patients' compliance. We searched for a photobiomodulation alternative regimen that could trigger the expected tissue responses of improved healing in less time. Thus, we compared the effect of two laser delivery regimens (single dose and fractionated dose) on the postoperative repair of diabetic wounds using objective parameters of tissue healing: wound closure rate, inflammatory infiltrate type, myofibroblasts count, collagen deposition, and optical retardation of collagen.

## Materials and Methods

### Animals

Ninety female adult Wistar rats (body mass: 250 ± 50 g) were kept in vivarium in plastic cages with five animals each, with free access to water and chow, 12-h light/dark cycle, 22°C, and 70% umidity). The animals were monitored daily. This study received approval from the Animal Research Ethics Committee of University Nove de Julho (Brazil, process number: ANS 026/12) and was carried out in compliance with Brazilian ethical principles for animal experimentation.

### Chemical induction of diabetes

After fasting for 12 h with free access to water, diabetes was induced in all animals with an intraperitoneal injection of streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.05 M of citrate buffer (dose: 60 mg/kg of body mass). Blood glucose levels were measured on a weekly basis. Animals with fasting blood glucose greater than 220 mg/dL and stable body mass after one week were selected for the experiment.

We used a 60 mg/kg dose to avoid unnecessary suffering and death due a 100mg/kg dose, which is highly toxic to the animals. Considering that blood glucose peak is before the 50$^{th}$ day [29], and that we wanted to study the wound healing in the maximum hyperglycemia, we calculate the experiment to start 15 days after the diagnosis of diabetes and the last group wound end on day 21. Our experiment reproduced how lasertherapy could aid a post surgical wound in a diabetes type 1 individual, not a chronic wound in an old person.

### Injury model

Anesthesia was performed with 80 mg/kg of ketamine HCl (Dopalen, Vetbrands, SP, Brazil) and 10 mg/kg of xylazine (Anasedan, Vetbrands, SP, Brazil). Fur was removed from the back of each animal with an electric shaver and a hair removal cream (Veet Cream, SP, Brazil). The skin was then cleaned with a 0.12% chlorhexidine solution. An 8-mm surgical punch (Richter, SP, Brazil) was used to produce round wounds in the central portion of the dorsum. The animals were maintained on a warm plate (37°C) to prevent hypothermia until complete recovery from the anesthesia. Then, to prevent pain the animals received an intramuscular injection of tramadol hydrochloride (5 mg/kg) twice a day for two days.

### Experimental groups

The animals were divided into three groups with thirty animals each (Table 1).

### Laser system

A gallium-aluminum-arsenide diode laser (MMOptics, São Carlos, SP, Brazil) (wavelength [λ]: 660 ± 2 nm) was employed with a beam spot of 0.04 cm$^2$, which was enlarged to 10 mm in diameter using a diverging lens to ensure complete coverage of the ulcer. The output power was 30 mW. The energy density and exposure time in the single-dose and fractionated dose groups (SDG and FDG, respectively) are displayed in Table 1. The output power was measured before and after irradiation to guarantee the parameters used (LaserCheck, Coherent, Santa Clara, CA, USA).

**Table 1. Experimental groups and treatment parameters.**

| Number of animals | 30 | 30 | 30 |
|---|---|---|---|
| Group | Control group (CG) | Fractionated-dose group (FDG) | Single-dose group (SDG) |
| Condition | Untreated | Laser therapy | Laser therapy |
| Laser energy density | | 1 J/cm$^2$ | 4 J/cm$^2$ |
| Laser exposition time | | 26 s | 104 s |
| Treatment frequency | | Four times | Once |

doi:10.1371/journal.pone.0122042.t001

## Wound closure rate

The animals were anesthetized as described above and placed in the prone position. Pictures were taken of the ulcers using a Canon T1i with a 100-mm Canon macro lens (Kunisaki, Oita Prefecture, Japan). The ulcers were photographed daily until the closing of the wound (i.e., 22$^{nd}$ day of the study). The ulcerated area was measured manually with the aid of the ImageJ 1.45 program (free software, NIH, Bethesda, Maryland, USA). Measurements were compared with a fully automated numerical method for the validation of the results. A complete description of the method can be found in a paper by Deana (2013) [30]. Photos from Day 1 and the time of euthanasia were compared to determine the wound closure rate.

## Analysis of the healing morphogy, inflammatory infiltrate and myofibroblasts count

Five animals from each group were euthanized on Days 1 (2 hours after injury and laser irradiation), 3, 8, 10, 15 and 22 with an overdose of anesthesia. The ulcerated tissue was removed, fixed in 10% buffered formalin (pH 7.4) and embedded in paraffin. Three 5-μm section from each animal sample was stained with hematoxylin and eosin for morphological analysis. An experienced pathologist blinded to the allocation of the samples to the different groups performed the analysis, searched the complete extension of each sample with an light microscope (Leica Microsystems, Wetzlar, Germany) and recorded the presence/absence of ulcer, epithelization, granulation tissue, and fibrosis.

The inflammatory cells neutrophil and T lymphocyte identification was made with immunohistochemistry (described below) and it was scored as: 0 = absent, 1 = low (up to 25% of cells), 2 = moderate (25 to 50% of cells), and 3 = high (50% to 100% of cells). To do this score, the complete area of the injured tissue of all animals was analyzed in triplicate. The highest scores were recorded.

Immunohistochemical analysis was performed for the myofibroblast count and inflammatory cells identification. Serial sections of paraffin-embedded tissues (3 μm) were placed on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma-Aldrich, St. Louis, MO) and deparaffinized in xylene, followed by immersion in alcohol and incubation with 3% hydrogen peroxide diluted in Tris-buffered saline (TBS) (pH 7.4). The sections were blocked by incubation with 3% normal goat serum for 20 minutes and immersed in citrate buffer (pH 6.0) at 95°C for 20 minutes for antigen retrieval. The slides were then incubated with anti-alpha smooth muscle actin (ABCAM, ab5694), anti-neutrophil elastase (ABCAM cat 68672, 1:3500), and anti-CD3 T lymphocyte marker (- ABCAM 5690, 1:300). The samples were kept overnight at 4°C in a humidified chamber, followed by washing of the sections with TBS, incubation with N-Histofine Simple Stain (Nichirei Biosciences Inc., Tokyo, Japan) for 30 minutes and incubation in 3,3'-diaminobenzidine in a chromogen solution (Dako) at room temperature for two to five minutes. The sections were then stained with Mayer's hematoxylin and covered. For the

negative controls, the primary antibodies were replaced with 1% PBS/bovine serum albumin and non-immune mouse serum (X501-1, Dako).

To count the myofibroblasts, five consecutive microscope fields (magnification: 400 x) with the most myofibroblasts (hot spot) were photographed (Leica Microsystems, Wetzlar, Germany). An experienced pathologist blinded to the allocation of the samples to the different groups performed the analysis of the images with the aid of the ImageJ 1.45 program (free software, NIH, Bethesda, Maryland, USA), using the "cell counter" plug-in. Analysis were made in triplicate.

### Collagen deposition

The analysis of collagen deposition was performed using interference colors, which are directly proportional to the thickness and packing state of the fibers. The constant thickness of the cuts allowed the study of the packing state: wider, packed fibers appeared as orange to red and thin, less-packed fibers appeared as green [31]. For this analysis, histological cuts measuring 8 μm were obtained, stained with Picrosirius Red, and examined under a polarizing microscope (Pol-Interferencial Photomicroscope, Model 61282, Carl Zeiss, Germany). The photographs were digitalized and examined using the ImageJ 1.45 program for the quantification of each color (green, orange and red) and determination of the proportion of the different packing states.

### Optical retardation of collagen

For the quantification of the optical retardation of collagen, birefringence was measured in deparaffinized, unstained histological cuts measuring 8 μm. Readings were performed with the samples soaked in distilled water using a polarizing microscope (Pol-Interferencial Photomicroscope, Model 61282, Carl Zeiss, Germany) with a high-pressure mercury bulb (HBO 200W) for illumination and an interference filter (PIL 546) for the determination of monochromatic light at $\lambda = 546$ nm. The readings were conducted with a compensator, which introduces optical retardation of $\lambda/4$. When the difference in the optical path of the sample is equal to the retardation of the compensator, destructive interference occurs between the ordinary and extraordinary rays emanating from the sample, characterized by a dark background seen in the ocular of the microscope. The field varies in position (degrees) in relation to the light beam. Upon encountering this dark field, the angle in the microscope is read by the accessory that complements the equipment. Thus, to determine optical retardation ($\Delta n$) of the samples in nm, the angle ($\alpha$) read in the microscope is multiplied by 3.03 nm [32]. Five $\alpha$ readings were performed for each histological section.

### Statistical analysis

Shapiro-Wilk test revealed that the variables did not follow Gaussian (normal) distribution, except for the myofibroblast count. Thus, the Mann-Whitney test was used to detect differences between all possible pairs in the within-day analyses for all variables except the myofibroblast count, for which the Student's t-test was used. The Minitab 16 software program (Minitab Inc, USA) was used for all statistical analyses, with the level of significance set to 95% ($\alpha = 0.05$).

## Results and Discussion

The present findings demonstrate that laser therapy delivered either once or four times enhances the early phase of tissue repair by accelerating initial wound closure and leukocyte chemotaxis, with more myofibroblasts and more organized fibrous tissue in the wound.

Independently of the energy density, laser therapy accelerated wound closure by approximately 40% in the first three days in comparison to the control group, after which the closure rate decreased in a similar rate in all groups from Day 8 onward (Figs 1 and 2).

On Day 3, the laser groups had a significantly smaller injury area in comparison to the control group. Beginning with Day 8, no statistically significant differences in injury area were found among the groups, and the ulcer was no longer apparent in any group by Day 22 (Fig 2).

Thus, it cannot be stated that laser therapy accelerated the healing process, as wound closure did not occur earlier in any group in comparison to the other groups, which is in agreement with data reported in previous studies [33, 34]. However, the biomodulation caused by laser therapy was sufficient to achieve perceptible effects in the inflammatory phase of the healing process (Day 3), which can be of considerable assistance in major surgeries to which patients with diabetes are submitted, exposing such patients to a lower risk of infection.

Laser therapy increased acute inflammatory infiltrate measured by neutrophils count two hours after the induction of the wound, which remained high through to Day 3. From Day 8 onward, acute inflammatory infiltrate was gradually replaced with chronic infiltrate, measured by the T lymphocyte count, with significant differences among the groups (Figs 3 and 4).

In patients with diabetes, the inflammatory response following injury is often prolonged and excessive [15] due to dysregulated coagulation and inflammatory response [35] as well as protein glycation, which leads to thicker capillary basal membranes with altered permeability. The migration of inflammatory cells to and from the injury site is delayed, resulting in chronic inflammation. Studies report that low-level laser therapy increases neutrophil chemotaxis [18, 36]. In the present investigation, laser therapy triggered leukocyte chemotaxis (especially neutrophils) beginning at two hours after injury, with a peak on Day 3, independently of the energy density (1 or 4 J/cm$^2$) (Fig 4). It should be stressed that the group submitted to a single-dose of low-level laser irradiation had the best inflammatory response, as demonstrated by the significantly higher scores from Day 3 to Day 10 in comparison to the other groups.

Laser application altered the inflammatory infiltrate trend line (Fig 5). Independently of the energy density (1 or 4 J/cm$^2$), laser therapy induced leukocyte chemotaxis in the early stages of tissue repair. The leukocytes scores were similar among the different groups from Day 8 onward.

As expected, myofibroblasts appeared on Day 8 and remained through to Day 22 in all groups (Fig 5).

On Day 15, the number of myofibroblasts began to decrease in the control group, but continued to increase in the laser groups. On Day 22, all groups had fewer myofibroblasts, demonstrating that the scars were in the remodeling phase, but both laser groups had more of these cells than the control group.

The proliferative phase of tissue repair is characterized by the formation of fibrous tissue and angiogenesis and is strongly modulated by transforming growth factor beta (TGF-β), which induces the proliferation of fibroblasts and their differentiation into myofibroblasts. Laser induces the appearance of myofibroblasts in granulation tissue during the proliferation and remodeling phases of the tissue repair process, likely through the modulation of TGF-β synthesis. Szymanska et al. (2013) demonstrated that LLLT at a wavelength of 635 nm increases endothelial cell proliferation, with a corresponding decrease in the concentration of vascular endothelial growth factor, suggesting the role of this growth factor in this process; in contrast, the 830 nm wavelength was associated with a decrease in TGF-β secretion [22]. Visible red laser (660 nm) was employed in the present study and led to a significant increase in the appearance of myofibroblasts in the healing process, especially on Days 15 and 22 (Fig 6), which may be helpful to wound contraction and remodeling in patients with diabetes.

Interference colors with the use of Picrosirius Red and polarized light revealed that laser therapy did not affect the proportion of larger, more packed collagen fibers in relation to

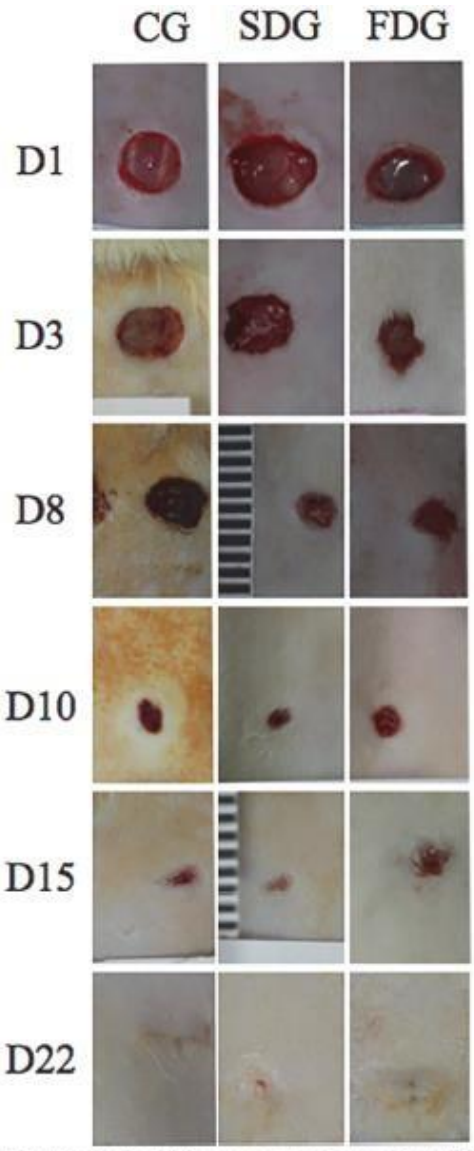



**Fig 1. Wound healing in different groups throughout experiment (D = Day, CG = Control Group, SDG = Single Dose Group, FDG = Fractionated Dose Group).** Original magnification 10x)

doi:10.1371/journal.pone.0122042.g001

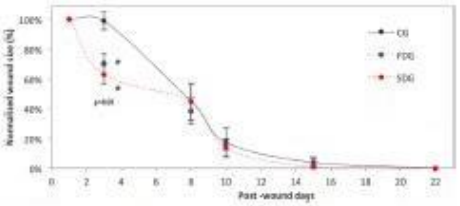



**Fig 2. Percentage of normalized wound closure throughout experiment demonstrating the effect of laser therapy in the early tissue repair process.** (mean ± SEM, $p<0.05$)

doi:10.1371/journal.pone.0122042.g002

thinner, less packed fibers on Day 22 (Fig 7). However, the optical retardation analysis demonstrated that the collagen fibers were significantly more organized in the SDG. All wounds in the three groups were closed by Day 22, with well-formed epithelium showing a mature stratum corneum and epidermal appendages (sebaceous glands and hair follicles). The skin barrier function is mainly assigned to the stratum corneum layer of the epidermis, which prevents exogenous substances from entering the body while also minimizing transepidermal water loss.

On Day 22, the collagen fibers were more organized in the SDG in comparison to intact, uninjured diabetic skin. The FDG had slightly less organized tissue than uninjured skin and the control group exhibited the worst degree of collagen organization (Fig 8).

Using second harmonic generation (SHG), which is sensitive to the molecular orientation of collagen fibers, Kim et al. found that changes in protein structure caused by glycation lead to a reduction in the signal measured by SHG, indicating less organization of the fibers [37]. In the present study, optical retardation was employed for the inference of the molecular orientation of the collagen fibers, as this method is well established, more accessible and correlated with the signal obtained through SHG [32]. Since tissue glycation is a biochemical characteristic of diabetes, a low degree of molecular organization was expected and, consequently, low optical retardation. However, the SDG exhibited significantly different values in comparison to the other groups, including in comparison to uninjured diabetic tissue. The buildup of glycation products can lead to the loss of organization in the fibrillar arrangement, which is reflected in the fibers, bundles and extracellular matrix [38]. As both laser groups had significantly

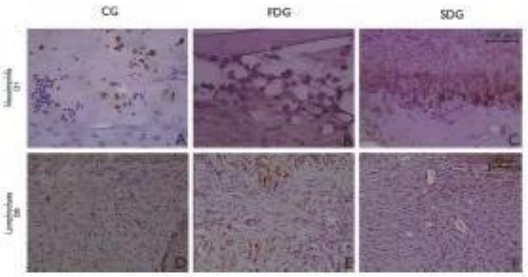



**Fig 3. Histological examination of healing tissue—Day 3: all groups in inflammatory phase of tissue repair (A, B, C), with a crust over the ulcer and an intense inflammatory infiltrate (*); Day 8: wounds in proliferative phase with granulation tissue (D, E, F); Some samples in FDG exhibited acute inflammatory infiltrate at this time (*).** hematoxilyn & eosin staining; original magnification: 200 x

doi:10.1371/journal.pone.0122042.g003

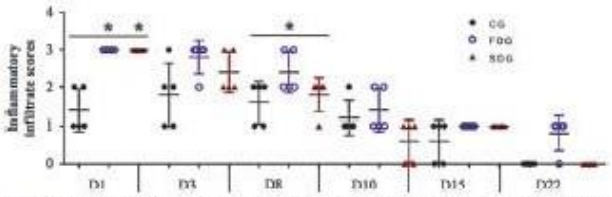



**Fig 4. Inflammatory infiltrate score—SDG exhibited more leukocytes than other groups through to Day 10.**

doi:10.1371/journal.pone.0122042.g004

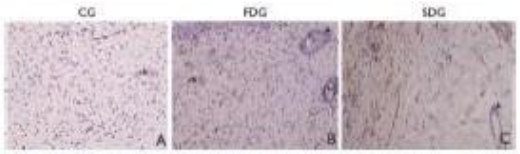



**Fig 5. Immunohistochemical analysis with anti-α smooth muscle actin; myofibroblasts within granulation tissue stained brown; arrow heads point to smooth muscle in hair follicles; asterisks indicate smooth muscle in arterioles used as internal positive control of reaction; immunohistochemistry with DAB; original magnification: 400 x**

doi:10.1371/journal.pone.0122042.g005

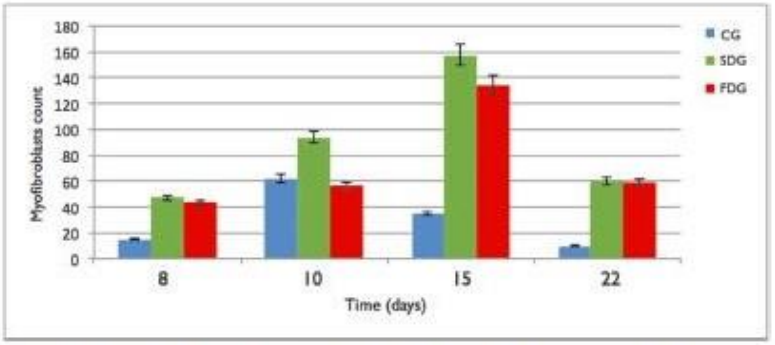



**Fig 6. Myofibroblast count showing more cells in both laser groups on Days 8, 15 and 22 in comparison to control; SDG exhibited more myofibroblasts than other groups on Days 10 and 15. Bars represent mean counts with respective standard deviation values.**

doi:10.1371/journal.pone.0122042.g006

greater optical retardation in comparison to the control group, one may infer that the laser-glycated tissue interaction is an important factor in the organization of collagen fibers in individuals with diabetes. Moreover, the energy density employed should be considered in this interaction, as greater collagen organization was found in the SDG than the FDG.

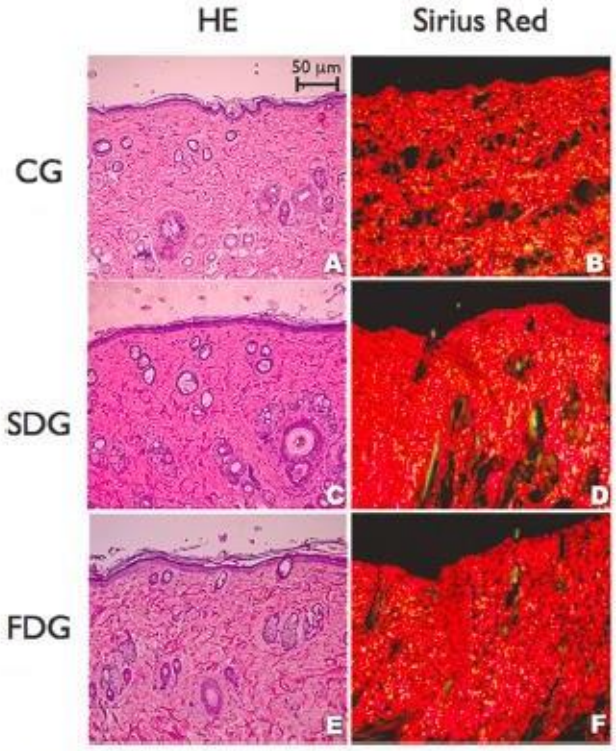



**Fig 7. Morphology of wound healing on Day 22, showing similarity among experimental groups (hematoxilyn and eosin—A, C, E; Picrosirius Red—B D, F)**

doi:10.1371/journal.pone.0122042.g007

Diabetic wounds fail to form adequate granulation tissue; angiogenesis is poor and the scar does not contract properly, often resulting in dehiscence or chronic wounds. No previous stud-

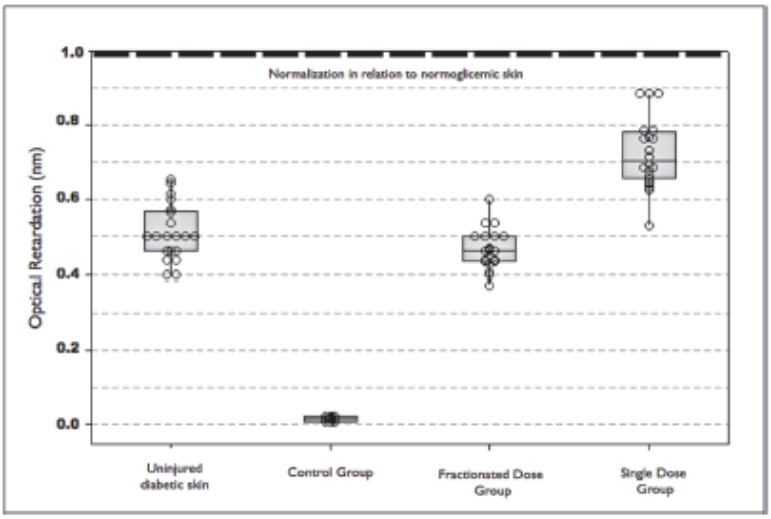



**Fig 8. Optical retardation analysis of collagen on Day 22, showing most organized collagen in SDG, followed by uninjured skin, FDG and CG**

doi:10.1371/journal.pone.0122042.g008

## Conclusion

The present findings demonstrate that low-level laser therapy in the immediate postoperative period can enhance the tissue repair process in patients with diabetes by modulating the inflammatory process, increasing the synthesis of myofibroblasts and enhancing collagen organization. Similar effects were achieved with laser therapy applied a single time with an energy density of 4 J/cm$^2$ and applied four times with an energy density of 1 J/cm$^2$. Moreover, the application of laser therapy in the inflammatory phase was the most important factor to the enhancement of the tissue repair process. Further studies should be conducted to determine the role of the red wavelength on the modulation of glycation through biochemical analysis and compare the advantages of different energy delivery regimens (single higher dose or multiple lower doses).

## Author Contributions

Conceived and designed the experiments: SKB RAMF KPSF CMF. Performed the experiments: CLS DFTS APS MTG BPAS JAFS. Analyzed the data: AMD RAP CMF. Wrote the paper: DFTS CMF.

## References

1. Buchleitner AM, Martinez-Alonso M, Hernandez M, Sola I, Mauricio D. Perioperative glycaemic control for diabetic patients undergoing surgery. Cochrane Database Syst Rev. 2012; 9: CD007315. doi: 10.1002/14651858.CD007315.pub2 PMID: 22972106
2. Oviedo-Socarras T, Vasconcelos AC, Barbosa IX, Pereira NB, Campos PP, Andrade SP. Diabetes alters inflammation, angiogenesis, and fibrogenesis in intraperitoneal implants in rats. Microvasc Res. 2014; 93: 23–29. doi: 10.1016/j.mvr.2014.02.011 PMID: 24594441

3. Ferraro F, Lymperi S, Mendez-Ferrer S, Saez B, Spencer JA, Yeap BY, et al. Diabetes impairs hematopoietic stem cell mobilization by altering niche function. Sci Transl Med. 2011; 3: 104ra101. doi: 10.1126/scitranslmed.3002191 PMID: 21998408
4. Rosado P, Cheng HT, Wu CM, Wei FC. Influence of diabetes mellitus on postoperative complications and failure in head and neck free flap reconstruction: A systematic review and meta-analysis. Head Neck. 2014.
5. Peppa M, Stavroulakis P, Raptis SA. Advanced glycoxidation products and impaired diabetic wound healing. Wound Repair Regen. 2009; 17: 461–472. doi: 10.1111/j.1524-475X.2009.00518.x PMID: 19614910
6. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004; 27: 1047–1053. PMID: 15111519
7. *American Diabetes Association—Diabetes Statistics. Data from the 2011 National Diabetes Fact Sheet.* Available: http://www.diabetes.org/diabetes-basics/statistics/. Acessed 2015 Feb 28.
8. Leal Junior EC, de Godoi V, Mancalossi JL, Rossi RP, De Marchi T, Parente M, et al. Comparison between cold water immersion therapy (CWIT) and light emitting diode therapy (LEDT) in short-term skeletal muscle recovery after high-intensity exercise in athletes—preliminary results. Lasers Med Sci. 2011; 26: 493–501. doi: 10.1007/s10103-010-0866-x PMID: 21088862
9. Franca CM, de Loura Santana C, Takahashi CB, Alves AN, De Souza Mernick AP, Fernandes KP, et al. Effect of laser therapy on skeletal muscle repair process in diabetic rats. Lasers Med Sci. 2013; 28: 1331–1338. doi: 10.1007/s10103-012-1249-2 PMID: 23262549
10. Nunez SC, Franca CM, Silva DFT, Nogueira GE, Prates RA, Ribeiro MS. The influence of red laser irradiation timeline on burn healing in rats. Lasers Med Sci. 2013; 28: 633–641. doi: 10.1007/s10103-012-1105-4 PMID: 22618156
11. Pinto FC, Chavantes MC, Pinto NC, Alho EJ, Yoshimura EM, Matushita H, et al. Novel treatment immediately after myelomeningocele repair applying low-level laser therapy in newborns: a pilot study. Pediatr Neurosurg. 2010; 46: 249–254. doi: 10.1159/000319363 PMID: 21160232
12. Dixit S, Maiya A, Rao L, Rao MA, Shastry BA, Ramachandra L. Photobiomodulation by helium neon and diode lasers in an excisional wound model: A single blinded trial. Adv Biomed Res. 2012; 1: 38. doi: 10.4103/2277-9175.100132 PMID: 23326769
13. Kajagar BM, Godhi AS, Pandit A, Khatri S. Efficacy of low level laser therapy on wound healing in patients with chronic diabetic foot ulcers-a randomised control trial. Indian J Surg. 2012; 74: 359–363. doi: 10.1007/s12262-011-0393-4 PMID: 24082586
14. Kaviani A, Djavid GE, Ataie-Fashtami L, Fateh M, Ghodsi M, Salami M, et al. A randomized clinical trial on the effect of low-level laser therapy on chronic diabetic foot wound healing: a preliminary report. Photomed Laser Surg. 2011; 29: 109–114. doi: 10.1089/pho.2009.2680 PMID: 21214368
15. Houreld NN. Shedding light on a new treatment for diabetic wound healing: a review on phototherapy. ScientificWorldJournal. 2014; 2014: 398412. doi: 10.1155/2014/398412 PMID: 24511283
16. Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol B. 1999; 49: 1–17. PMID: 10365442
17. Silveira PC, Silva LA, Fraga DB, Freitas TP, Streck EL, Pinho R. Evaluation of mitochondrial respiratory chain activity in muscle healing by low-level laser therapy. J Photochem Photobiol B. 2009; 95: 89–92. doi: 10.1016/j.jphotobiol.2009.01.004 PMID: 19232497
18. Alves AC, Vieira R, Leal-Junior E, dos Santos S, Ligeiro AP, Albertini R, et al. Effect of low-level laser therapy on the expression of inflammatory mediators and on neutrophils and macrophages in acute joint inflammation. Arthritis Res Ther. 2013; 15: R116. PMID: 24028507
19. Wang CZ, Chen YJ, Wang YH, Yeh ML, Huang MH, Ho ML, et al. Low-level laser irradiation improves functional recovery and nerve regeneration in sciatic nerve crush rat injury model. PLoS One. 2014; 9: e103348. doi: 10.1371/journal.pone.0103348 PMID: 25119457
20. Pereira AN, Eduardo Cde P, Matson E, Marques MM. Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers Surg Med. 2002; 31: 263–267. PMID: 12355572
21. Cury V, Moretti AI, Assis L, Bossini P, Crusca Jde S, Neto CB, et al. Low level laser therapy increases angiogenesis in a model of ischemic skin flap in rats mediated by VEGF, HIF-1 alpha and MMP-2. J Photochem Photobiol B. 2013; 125: 164–170. doi: 10.1016/j.jphotobiol.2013.06.004 PMID: 23831843
22. Szymanska J, Goralczyk K, Klawe JJ, Lukowicz M, Michalska M, Goralczyk B, et al. Phototherapy with low-level laser influences the proliferation of endothelial cells and vascular endothelial growth factor and transforming growth factor-beta secretion. J Physiol Pharmacol. 2013; 64: 387–391. PMID: 23959736

23. Fukuda TY, Tanji MM, Silva SR, Sato MN, Plapler H. Infrared low-level diode laser on inflammatory process modulation in mice: pro- and anti-inflammatory cytokines. Lasers Med Sci. 2013; 28: 1305–1313. doi: 10.1007/s10103-012-1231-z PMID: 23179306

24. Giannopoulou C, Cappuyns I, Cancela J, Cionca N, Mombelli A. Effect of photodynamic therapy, diode laser, and deep scaling on cytokine and acute-phase protein levels in gingival crevicular fluid of residual periodontal pockets. J Periodontol. 2012; 83: 1018–1027. doi: 10.1902/jop.2011.110281 PMID: 22181685

25. Fukuda TY, Tanji MM, Jesus JF, Sato MN, Duarte AJ, Plapler H. Single session to infrared low level diode laser on TNF-alpha and IL-6 cytokines release by mononuclear spleen cells in mice: a pilot study. Lasers Surg Med. 2010; 42: 584–588. doi: 10.1002/lsm.20949 PMID: 20662036

26. Ribeiro MA, Albuquerque RL Jr., Ramalho LM, Pinheiro AL, Bonjardim LR, Da Cunha SS. Immunohistochemical assessment of myofibroblasts and lymphoid cells during wound healing in rats subjected to laser photobiomodulation at 660 nm. Photomed Laser Surg. 2009; 27: 49–55. doi: 10.1089/pho.2007.2215 PMID: 19250051

27. Oberoi S, Zamperini-Netto G, Beyene J, Treister NS, Sung L. Effect of prophylactic low level laser therapy on oral mucositis: a systematic review and meta-analysis. PLoS One. 2014; 9: e107418. doi: 10.1371/journal.pone.0107418 PMID: 25198431

28. Junker JP, Philip J, Kiwanuka E, Hackl F, Caterson EJ, Eriksson E. Assessing quality of healing in skin: review of available methods and devices. Wound Repair Regen. 2014; 22 Suppl 1: 2–10. doi: 10.1111/wrr.12162 PMID: 24813357

29. Kataoka M, Kawamuro Y, Shiraki N, Miki R, Sakano D, Yoshida T, et al. Recovery from diabetes in neonatal mice after a low-dose streptozotocin treatment. Biochem Biophys Res Commun. 2013; 430: 1103–1108. doi: 10.1016/j.bbrc.2012.12.030 PMID: 23257160

30. Deana AM, de Jesus SH, Sampaio BP, Oliveira MT, Silva DFT, Franca CM. Fully automated algorithm for wound surface area assessment. Wound Repair Regen. 2013; 21: 755–761. doi: 10.1111/wrr.12085 PMID: 23937087

31. Vidal BC, Mello ML, Pimental ER. Polarization microscopy and microspectrophotometry of Sirius Red, Picrosirius and Chlorantine Fast Red aggregates and of their complexes with collagen. Histochem J. 1982; 14: 857–878. PMID: 6184330

32. Silva DFT, Gomes AS, de Campos Vidal B, Ribeiro MS. Birefringence and second harmonic generation on tendon collagen following red linearly polarized laser irradiation. Ann Biomed Eng. 2013; 41: 752–762. doi: 10.1007/s10439-012-0720-3 PMID: 23247985

33. Ribeiro MS, Silva DF, Maldonado EP, de Rossi W, Zezell DM. Effects of 1047-nm neodymium laser radiation on skin wound healing. J Clin Laser Med Surg. 2002; 20: 37–40. PMID: 11902353

34. Silva DFT, Vidal BC, Zezell DM, Zorn TMT, Nunez SC, Ribeiro MS. Collagen birefringence in skin repair in response to red polarized-laser therapy. J Biomed Opt. 2006; 11: 024002.024001–024002.024006. PMID: 16674192

35. Chen J, Kasper M, Heck T, Nakagawa K, Humpert PM, Bai L, et al. Tissue factor as a link between wounding and tissue repair. Diabetes. 2005; 54: 2143–2154. PMID: 15983216

36. Firat ET, Dag A, Gunay A, Kaya B, Karadede MI, Kanay BE, et al. The effects of low-level laser therapy on palatal mucoperiosteal wound healing and oxidative stress status in experimental diabetic rats. Photomed Laser Surg. 2013; 31: 315–321. doi: 10.1089/pho.2012.3406 PMID: 23789588

37. Kim B-M, Eichler J, Reiser KM, Rubenchik AM, Silva LBD. Collagen structure and nonlinear susceptibility: effects of heat, glycation and enzymatic cleavage on second harmonic signal intensity. Lasers Surg Med. 2000; 27: 329–335. PMID: 11074509

38. Tanaka S, Avigad G, Brodsky B, Eikenberry EF. Glycation induces expansion of the molecular packing of collagen. J Mol Biol. 1988; 203: 495–505. PMID: 3143838

## *9.2* Artigo 2: Effect of Laser Therapy on Immune Cells Infiltrate After Excisional Wounds in Diabetic Rats – *Lasers in Surgery and Medicine*

Lasers in Surgery and Medicine 48:45–51 (2016)

# Effect of Laser Therapy on Immune Cells Infiltrate After Excisional Wounds in Diabetic Rats

**Cristiano de Loura Santana, Daniela de Fátima Teixeira Silva, Amanda Pires de Souza, Marcos Vinicius Jacinto, Sandra Kalil Bussadori, Raquel Agnelli Mesquita-Ferrari, Kristianne Porta Santos Fernandes, and Cristiane Miranda França***

*Postgraduate Program in Biophotonics Applied to Health Sciences, University Nove de Julho, São Paulo, SP, Brazil*

**Background and Objective:** Diabetes alters innate and specific immunity, causing an imbalanced tissue repair process. Very active neutrophils and macrophages are found for a long time in chronic wounds in those individuals. The aim of this study was to evaluate the response of the main effector cells of immunity (neutrophils, macrophages, and T lymphocytes) and to compare the effects of two laser therapy regimens in the postoperative treatment of excision wounds.

**Method:** Diabetes was induced in female Wistar rats and a punch was used to cause wounds in the dorsum of each individual. The animals were randomly allocated to a control group (CG), in which the wound was untreated, a single-dose laser group (SLG), in which the wound was submitted to single dose of laser therapy at wavelength of 660 nm, output power of 30 mW, energy density of 4 J/cm$^2$, and 26-second exposure time, and a fractionated-dose laser group (FLG), submitted to 1 J/cm$^2$ of laser therapy on Days 1, 3, 8, and 10. Euthanasia was performed on five animals from each group Days 1, 3, 8, 10, 15, and 22. The wound was removed and routinely processed for immunohistochemistry against elastase, CD3, CD68, and CD206 antibodies. The samples were photographed and labeled cells were counted by a blinded observer. The Kruskal–Wallis test was used for the statistical analysis.

**Results:** Neutrophils were predominant in the SLG on Day 1, whereas these cells were mostly found in the CG on Day 3 ($P < 0.05$). The T lymphocyte count was similar in all groups in the throughout the experiment. On Day 3, the SLG exhibited a greater number of total macrophages (CD68+) ($P < 0.05$), whereas the macrophage count was similar among the different groups on the other evaluation days. The CD206+ cell counts revealed that the SLG had more M2 macrophages than the CG on Day 8 ($P < 0.05$), whereas the FLG exhibited more M2 macrophages than the CG on Day 10 ($P < 0.05$).

**Conclusion:** The present findings demonstrate that laser therapy can alter the composition of inflammatory infiltrate in diabetic wounds, leading to a more balanced response transiting from a rapid neutrophil infiltration through to M2 macrophage polarization, especially with a single application of 4 J/cm$^2$ in the immediate postoperative period. Lasers Surg. Med. 48:45–51, 2016.


**Key words:** laser therapy; neutrophil infiltration; diabetes; macrophages; wound healing

## INTRODUCTION

Diabetes is a serious public health problem affecting more than 250 million individuals around the world that can lead to neurological, macrovascular, and microvascular alterations, resulting in difficulties regarding tissue repair, infection, dehiscence, chronic wounds, and amputation of an affected organ [1].

With diabetes, alterations occur in innate, specific immunity, leading to an imbalanced tissue repair process. Active macrophages and neutrophils last a long time in the wounds of diabetic patients. Inflammation is persistent, with a reduction in angiogenesis and the formation of granulation tissue, which exerts a negative impact on the closure of the wound [2,3].

Efficient wound repair requires the coordinated effort of many different cell types. Neutrophils are the first leukocytes to be recruited to the inflammation site and are capable of eliminating pathogens through different mechanisms. The mean life of a neutrophil is 1.5–12 hours in mice and can reach several days in humans; during inflammation, however, these cells are activated, increasing their longevity considerably, which ensures their presence in more complex activities, such as the resolution of inflammation or the adaptive immune response [4,5].

Cristiano de Loura Santana, Amanda Pires de Souza, and Marcos Vinicius Jacinto performed the experiments.

Sandra Kalil Bussadori, Raquel Agnelli Mesquita-Ferrari, Kristianne Porta Santos Fernandes, and Cristiane Miranda França conceived and designed the experiments.

Daniela de Fátima Teixeira Silva and Cristiane Miranda França analyzed the data.

Conflicts of interest: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported.

Contract grant sponsor: São Paulo Research Foundation (FAPESP); Contract grant number: # 2012/01944-0.

*Correspondence to: Dr. Cristiane Miranda França, PhD, Postgraduate Program in Biophotonics Applied to Health Sciences, University Nove de Julho, SP, Brazil.
E-mail: cristiane321@gmail.com

Accepted 4 November 2015
Published online 2 December 2015 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/lsm.22445

© 2015 Wiley Periodicals, Inc.

tory infiltrate [6].

Monocytes/macrophages are involved in both the initiation and resolution of the inflammatory response. Mirroring the Th1 and Th2 classification of T helper cells, two distinct macrophage subsets have been recognized: M1 and M2. The initial inflammatory response is mediated by M1 macrophages, which produce large amounts of reactive oxygen species (ROS) and pro-inflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin [IL]-1 and IL-6). The healing phase is related to M2 macrophages, which have greater phagocytic capacity, mainly produce anti-inflammatory cytokines (IL-4, IL-10, and IL-13) and are characterized by the increased expression of the mannose receptor CD206. Thus, macrophages participate in all phases of the tissue repair process, controlling cellularity, and the remodeling of the wound [7,8].

Experiments have been conducted in which macrophages are depleted in different phases of the repair process, such as the study conducted by [9]. With the occurrence of depletion in the inflammatory phase (up to 4 days following injury), the repair process is severely altered, leading to reductions in granulation tissue, the formation of new epithelial tissue, and the depositing of collagen. Macrophages ablated in the proliferation phase (8–14 days following injury) are associated with wound hemorrhaging, increased endothelial cell apoptosis, and the failure of newly formed blood vessels. When macrophages are ablated in the remodeling phase (15 days following injury), no effects in the wounds are found [9].

Diabetes is largely considered a pro-inflammatory condition. Data from a study by [10] confirm that the infiltration of M1 macrophages is associated with a reduction in the survival rate and more extensive tissue damage. This lends support to the theory that tissue injury in individuals with diabetes is aggravated by a reduction in the control of the inflammatory response, with impairment seen in remodeling and repair after an injury, and subsequent inflammation [10].

The biomodulatory effects of photobiomodulation (PBM) have been widely described both in animal models and clinical studies [11–13]. PBM is believed to promote angiogenesis, enhance neovascularization, increase collagen synthesis, and accelerate the healing of skin wounds [14]. Recent studies have found that PBM at different wavelengths can modulate neutrophil chemotaxis [15,16] and the cellular activation status of macrophages in inflammation processes [17–19].

In a previous study by our research group on tissue repair in the wounds of diabetic rats, laser therapy, either in a single dose (SL), or in fractionated doses (FD), was found to modulate the inflammatory process and end quality of the wound. Briefly, on Day 1: SL induced the presence of more neutrophils and inflammatory infiltrate; on Day 3: control group had more neutrophils (though 40% of wound closure in the initial days after injury, with an increase in the number of leukocytes, a larger number of myofibroblasts, and greater collagen organization [20].

In an attempt to clarify the mechanisms by which laser therapy exhibits this property of photobiomodulation, a hypothesis was put forth stating that laser therapy leads to differences in the composition of the inflammatory infiltrate in diabetic wounds, which was implied by the end quality of the wound.

The aim of the present study was to evaluate the response of the main immunity effector cells (neutrophils, T lymphocytes, and macrophages) and compare the effects of two laser therapy regimens in the postoperative treatment of wounds in diabetic rats.

## MATERIALS AND METHODS

### Animals

Ninety female adult Wistar rats (body mass: 250 ± 50 g) were kept in plastic cages of five animals each with free access to water and ration and a 12-hours light/dark cycle. This study was approved by the Animal Research Ethics Committee of University Nove de Julho (Brazil, process number: ANS 026/12) and was conducted in compliance with Brazilian ethical principles for animal experimentation.

### Chemical Induction of Diabetes

After fasting for 12 hours with free access to water, diabetes was induced in all animals with an intraperitoneal injection of streptozotocin (Sigma–Aldrich, St. Louis, MO) dissolved in 0.05 M of citrate buffer (dose: 60 mg/kg of body mass). Blood glucose levels were measured weekly. Animals with fasting blood glucose greater than 220 mg/dL and stable body mass after 1 week were selected for the experiment.

### Injury Model

Xylazine (10 mg/kg; Anasedan, Vetbrands, SP, Brazil) and ketamine (80 mg/kg; Dopalen, Vetbrands, SP, Brazil) were administered for the anesthetic effect. Fur was removed from the dorsum of each animal with an electric shaver and hair removal cream (Veet Cream, SP, Brazil). The skin was cleaned with 0.12% chlorhexidine. A surgical punch (Richter, SP, Brazil) was employed to cause two round wounds measuring 8 mm in diameter in the dorsum.

The punch was inserted until the complete thickness of the skin. When the punch reaches the fascia overlying the panniculus carnosus the operator feels the reduction of tissue resistance that offers to the cutting blade and this defines the cutting depth and allows complete removal of the skin without injuring the adjacent muscle tissue.

The animals were then kept on a warm plate at 37°C for the prevention of hypothermia [20].

**TABLE 1. Experimental Groups and Treatment Parameters**

| Number of animals | 30 | 30 | 30 |
|---|---|---|---|
| Group | Control group (CG) | Single-dose laser group (SLG) | Fractionated-dose laser group (FLG) |
| Condition | No treatment | Laser therapy 4 J/cm$^2$ | Laser therapy 1 J/cm$^2$ |
| Wavelength | | 660 ± 2 nm | 660 ± 2 nm |
| Output power | | 30 mW | 30 mW |
| Power density | | 38 mW/cm$^2$ | 38 mW/cm$^2$ |
| Laser exposition time | | 104 s | 26 s |
| Energy per application | | 3.12 J | 0.78 J |
| Treatment frequency | | Day 1 (after excisional wound) | Four times (after excisional wound and on Days 3, 8, 10) |

### Experimental Groups

The animals were divided into three groups of thirty animals each (Table 1).

### Administration of Laser Therapy

A gallium-aluminum-arsenide diode laser (MMOptics, São Carlos, SP, Brazil) operating at a wavelength of 660 ± 2 nm was used. The 0.04 cm$^2$ beam spot was enlarged (10 mm in diameter) with the aid of a diverging lens to cover the wound completely. Table 1 displays the laser parameters in the SLG and FLG. Output power (30 mW) was determined before and after PBM to ensure the proper parameters with the aid of LaserCheck (Coherent, Santa Clara, CA).

### Immunohistochemistry and Inflammatory Cell Counts

Five animals from each group were euthanized on Days 1 (2 hours after injury and laser irradiation), 3, 8, 10, 15, and 22 with an overdose of anesthesia. The injured tissue was removed, fixed in 10% buffered formalin (pH 7.4), and embedded in paraffin.

The tissue samples were imbedded in paraffin and cut in serial sections measuring 3 μm. The samples were placed on slides with a 2% solution of 3-aminopropyltriethylsilane (Sigma–Aldrich, St. Louis, MO). Deparaffinization was performed with xylene and the samples were immersed in alcohol, followed by incubation in a 3% hydrogen peroxide solution diluted in Tris-buffered saline (TBS) (pH 7.4). Incubation was then performed with a 3% solution of normal goat serum (20 minutes) and the blocked samples were immersed in citrate buffer (pH 6.0) for 20 minutes at 95° C for the retrieval of the antigen. The slides were then incubated with the following antibodies listed in Table 2.

The samples were stored in a humidified chamber at 4°C overnight. Next, the tissue sections were washed in TBS and incubated with N-Histofine Simple Stain (Nichirei Biosciences Inc., Tokyo, Japan) for 30 minutes, followed by 3,3′-diaminobenzidine incubation at room temperature at room temperature in a chromogen solution (Dako) for 2–5–minutes. The samples were stained with Mayer's hematoxylin and covered. The primary antibodies were replaced with 1% PBS/bovine serum albumin and non-immune mouse serum (X501-1, Dako) for the negative controls.

In the microscopic analysis, five fields with the largest amount of labeled cells were photographed (Leica Microsystems, Wetzlar, Germany) at a magnification of 400 fold. The analysis of the images was performed by an experienced pathologist who was blinded to the different study groups using the "cell counter" plug-in of the ImageJ 1.45 program (free software, NIH, Bethesda, MD).

### Statistical Analysis

The BioEstat 5.3 software program (free software, Mamirauá Institute, AM, Brazil) was used for all statistical analyses, with the level of significance set to 95% ($\alpha = 0.05$). As the data were non parametric, the Kruskal–Wallis test was used, followed by Dunn's *post hoc* test.

## RESULTS

On Day 1, 2 hours after the injury and PBM, the SLG exhibited more neutrophils than the other groups (Kruskal–Wallis with Dunn *post hoc* test, $P < 0.05$) (Fig. 1). On Day 3, the control group had the highest neutrophil count ($P-< 0.05$). From Day 8 onward, the neutrophil count progressively decreased similarly in all groups.

**TABLE 2. Antibodies and Antigen Retrieval**

| Antigen | Antibody | Dilution | Retrieval |
|---|---|---|---|
| Neutrophil (neutrophil elastase) | Anti-elastase (abcam cat 68672) | 1:3500 | Pepsin, room temperature, 15 minutes |
| Pan macrophages (M1 and M2) | Anti-CD68 (abcam cat 31630) | 1:1000 | Citrate buffer, pH 6.0, 100°C, 15 minutes |
| M2 macrophages | Anti-CD206 (abcam cat 64693) | 1:250 | Citrate buffer, pH 6.0, 100°C, 15 minutes |
| T lymphocytes | Anti-CD3 (abcam cat5690) | 1:300 | Citrate buffer, pH 6.0, 100°C, 15 minutes |

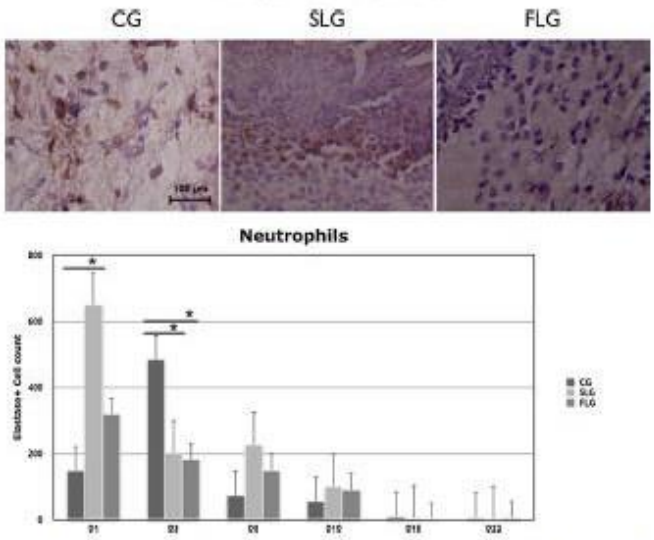



Fig. 1. Photomicrographs of neutrophil count; results expressed as mean ± SD; Predominance of neutrophils in SLG on Day 1; Significantly more neutrophils in CG on Day 3; Progressive decrease in neutrophil count similar in all groups beginning at Day 8 (Kruskal–Wallis test with Dunn's *post hoc* test); CG, control group; SLG, single-dose laser group; FLG, fractionated-dose laser group.

The T lymphocyte (CD3+ cells) count was similar in all groups throughout the experiment, with no statistically significant differences (Fig. 2).

The total macrophage count, which was identified by the pan-macrophage marker CD68 antibody, demonstrated that all groups exhibited a similar quantity of macrophages on Day 1. The SLG exhibited more macrophages than the other groups on Day 3 (Kruskal–Wallis test with Dunn's *post hoc* test, $P < 0.05$). From Day 8 onward, all groups exhibited similar macrophage counts (Fig. 3).

M2 macrophages were identified using the CD206 mannose receptor antibody. The SLG exhibited more M2 macrophages than the CG on Day 8 ($P < 0.05$). Interestingly, the FLG exhibited more macrophages than CG on Day 10 ($P < 0.05$). Similar counts among the different groups were found on all other evaluation days (Fig. 4).

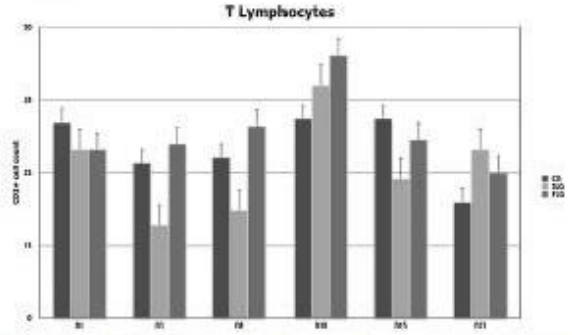



Fig. 2. T lymphocyte (CD3 + cells) count; Results expressed as mean ± SD; similarity among all groups throughout experiment (Kruskal–Wallis test with Dunn's *post hoc* test); CG, control group; SLG, single-dose laser group; FLG, fractionated-dose laser group.

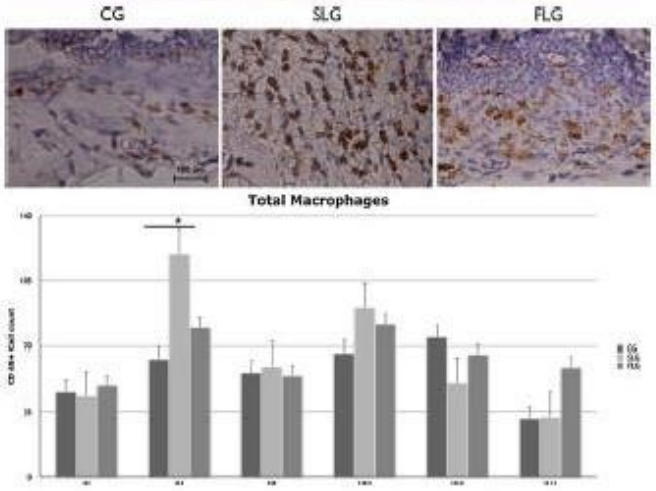



Fig. 3. Photomicrograph of CD68+ cells count (pan macrophage marker); Results expressed as mean ± SD; SLG exhibited higher total macrophage count ($P < 0.05$) on Day 3. Similar macrophage count in all groups at other evaluation times (Kruskal–Wallis test with Dunn's *post hoc* test); CG, control group; SLG, single-dose laser group; FLG, fractionated-dose laser group.

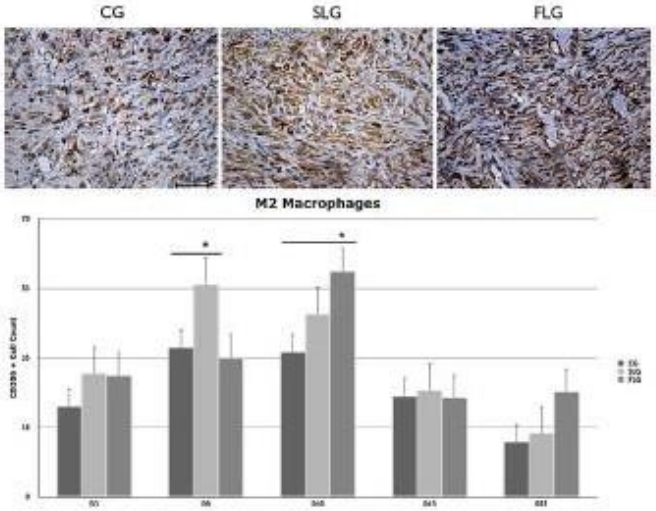



Fig. 4. CD206+ cell count (M2 macrophage marker); Results expressed as mean ± SD; SLG exhibited more anti-inflammatory macrophages than CG on Day 8 ($P < 0.05$). FLG exhibited more anti-inflammatory macrophages than CG on Day 10 ($P < 0.05$). No significant differences in M2 macrophage counts among groups on other evaluation days (Kruskal–Wallis test with Dunn's *post hoc* test); CG, control group; SLG, single-dose laser group; FLG, fractionated-dose laser group.

## DISCUSSION

The present study shows that PBM modulates the chemotaxis of neutrophils and macrophages in the diabetic excisional wounds and enhances M2 macrophage differentiation. Based on the conditions of this study, a single dose of 4 J/cm$^2$ proved to be a more effective manner to attract neutrophils and macrophages than four doses of 1 J/cm$^2$, indicating that the light delivery method at the beginning of the inflammatory cascade is crucial to triggering an enhanced cell response and that 1 J/cm$^2$ is a sub-optimal dose for diabetic wound healing.

Neutrophils responded promptly to the laser therapy at a fluency of 4 J/cm$^2$ immediately after injury, with a significant decrease in number on Day 3. In contrast, the CG demonstrated a peak in neutrophils on Day 3, but with fewer cells than those found in the SLG. This is an important finding, as the largest amount of neutrophils is found in the tissue of normoglycemic individuals about 4 hours after injury [21]. However, under diabetic conditions, these cells take more time to arrive at the injury site and stay for a longer period, releasing proteases that contribute to the chronicity of the wound [4,5]. Thus, the fact that PBM enhanced the neutrophil count but these cells left the injury site from Day 3 onward is important in order to avoid unnecessary pro-inflammatory stimulus from neutrophil products.

Studies with an arthritis model [15], and fungal disease model [16] have found that laser therapy can improve the chemotaxis of neutrophils. However, the results in the literature on diabetic models are conflicting. Kilik et al. (2014) performed a semi-quantitative morphological analysis of the neutrophil count in diabetic wounds treated with PBM and found significantly lower infiltration in the irradiated group [22], whereas [19] found that PBM was unable to improve neutrophil chemotaxis on Day 4. Both studies failed to investigate the initial 4 hours after injury, which is the most important time for studying the diapedesis of neutrophils [19]. In addition, cell analysis was purely morphological with no use of sensitive techniques, such as immunohistochemistry.

T cells are an important part of the innate immune response in injured skin. In the present study, laser therapy did not stimulate the recruitment of these cells. Further studies should be conducted to investigate other T cells present as regulatory T cells (Tregs–CD4 + FOXP3 +) and the CD4/CD8 ratio.

Although, macrophages are crucial to normal wound healing, the dysregulation of their function could contribute to impaired tissue repair in patients with diabetes [6–9]. The present investigation shows that PBM at a fluency of pro-inflammatory wound environment and poor healing response [6,7]. In the present study, initial photobiomodulation using 4 J/cm$^2$ exerted an impact on M2 macrophage polarization on Day 8 and the fractionated sub-optimal doses of 1 J/cm$^2$, despite not affecting the total number of macrophages, also led to an increase in the M2 macrophage count on Day 10. These are important findings that can help clarify the mechanisms by which laser therapy improves wound healing, especially diabetic wounds. M2 macrophages produce TGF-β, which is responsible for the conversion of fibroblasts into myofibroblasts and increasing the contractility of the wound, which is impaired in individuals with diabetes [23].

Although, many factors likely contribute to the macrophage dysfunction found in individuals with diabetes, the increase in the amount of the pro- inflammatory cytokines interferon gamma and IL-1β, along with a decrease in the amount the anti-inflammatory IL-10, may play a significant role [10]. As macrophages are light-sensitive cells that can be modulated both in the red light and near infrared range, PBM may partially restore the balance of this microenvironment due to the reduction in interferon [24,25], IL-1 [26] and oxidative stress markers [27] in healing tissues.

Fulop et al., 2009 performed a literature review with meta-analysis in order to investigate the effect of PBM on tissue repair. Both normoglicemic and diabetes conditions were analyzed and it was found that phototherapy is a very effective form of treatment for wound healing, with stronger supporting evidence resulting from animal studies than human studies [13]. However, lacks in the literature studies using the differential count of inflammatory cell (neutrophils, M1 and M2 macrophages, lymphocytes) in normoglycemic animals to compare the results with diabetic individuals.

In conclusion, PBM can alter the composition of the inflammatory infiltrate in diabetic wounds, leading to a more balanced response, transiting from a rapid neutrophil infiltration through M2 macrophage polarization.

## ACKNOWLEDGMENT

The authors thank the São Paulo Research Foundation (FAPESP) for the grants # 2012/01944-0.

## REFERENCES

1. American diabetes association-diabetes statistics. Data from the 2011 National diabetes fact sheet. Available: http://www.diabetes.org/diabetes-basics/statistics/ Accessed 28 february 2015.
2. Wilgus TA, Roy S, McDaniel JC. Neutrophils and wound Repair: Positive actions and negative reactions. Adv Wound

5. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013;13:159–175.
6. Brancato SK, Albina JE. Wound macrophages as key regulators of repair: Origin, phenotype, and function. Am J Pathol 2011;178:19–25.
7. Kittan NA, Allen RM, Dhaliwal A, Cavassani KA, Schaller M, Gallagher KA, Carson WF 4th, Mukherjee S, Grembecka J, Cierpicki T, Jarai G, Westwick J, Kunkel SL, Hogaboam CM. Cytokine induced phenotypic and epigenetic signatures are key to establishing specific macrophage phenotypes. PLoS ONE 2013;8:e78045.
8. Mirza RE, Koh TJ. Contributions of cell subsets to cytokine production during normal and impaired wound healing. Cytokine 2015;71:409–412.
9. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Müller W, Roers A, Eming SA. Differential roles of macrophages in diverse phases of skin repair. J Immunol 2010;184: 3964–3977.
10. Venneri MA, Giannetta E, Panio G, De Gaetano R, Gianfrilli D, Pofi R, Masciarelli S, Fazi F, Pellegrini M, Lenzi A, Naro F, Isidori AM. Inhibition of PDE5 limits pro-inflammatory monocyte-macrophage polarization in streptozotocin-induced diabetic mice. PLoS ONE 2015;10(5):e0126580.
11. Huang YY, Sharma SK, Carroll J, Hamblin MR. Biphasic dose response in low level light therapy—An update. Dose Response 2011;9:602–618.
12. Chung H, Dai T, Sharma SK, Huang YY, Carroll JD, Hamblin MR. The nuts and bolts of low-level laser (light) therapy. Ann Biomed Eng 2012;40:516–533.
13. Fulop AM, Dhimmer S, Deluca JR, Johanson DD, Lenz RV, Patel KB, Douris PC, Enwemeka CS. A meta-analysis of the efficacy of phototherapy in tissue repair. Photomed Laser Surg 2009;27:695–702.
14. Corazza AV, Jorge J, Kurachi C, Bagnato VS. Photobiomodulation on the angiogenesis of skin wounds in rats using different light sources. Photomed Laser Surg 2007;25: 102–106.
15. Alves AC, Vieira R, Leal-Junior E, dos Santos S, Ligeiro AP, Albertini R, Junior J, de Carvalho P. Effect of low-level laser therapy on the expression of inflammatory mediators and on neutrophils and macrophages in acute joint inflammation. Arthritis Res Ther 2013;15:R116.
16. Burger E, Mendes AC, Bani GM, Brignão MR, Santos GB, Malaquias LC, Chavasco JK, Verinaud LM, de Camargo ZP, Hamblin MR, Sperandio FF. Low-level laser therapy to the mouse femur enhances the fungicidal response of neutrophils against Paracoccidioides brasiliensis. PLoS Negl Trop Dis 2015;9(2):e003541.
17. Souza NH, Ferrari RA, Silva DF, Nunes FD, Bussadori SK, Fernandes KP. Effect of low-level laser therapy on the modulation of the mitochondrial activity of macrophages. Braz J Phys Ther 2014;18:308–314.
18. Chen CH, Wang CZ, Wang YH, Liao WT, Chen YJ, Kuo CH, Kuo HF, Hung CH. Effects of low-level laser therapy on M1-related cytokine expression in monocytes via histone modification. Mediators Inflamm 2014;2014:625048.
19. Sharifian Z, Bayat M, Alidoust M, Farahani RM, Bayat M, Rezaie F, Bayat H. Histological and gene expression analysis of the effects of pulsed low-level laser therapy on wound healing of streptozotocin-induced diabetic rats. Lasers Med Sci 2014;29:1227–1235.
20. de Loura Santana C, Silva Dde F, Deana AM, Prates RA, Souza AP, Gomes MT, de Azevedo Sampaio BP, Shibuya JF, Bussadori SK, Mesquita-Ferrari RA, Fernandes KP, França CM. Tissue responses to postoperative laser therapy in diabetic rats submitted to excisional wounds. PLoS ONE 2015;10:e0122042.
21. Oehmichen M. Vitality and time course of wounds. Forensic Sci Int 2004;144:221–231.
22. Kilík R, Lakyová L, Sabo J, Kruzliak P, Lacjaková K, Vasilenko T, Vidová M, Longauer F, Radoňak J. Effect of equal daily doses achieved by different power densities of low-level laser therapy at 635 nm on open skin wound healing in normal and diabetic rats. Biomed Res Int 2014;2014:269253.
23. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest 2007;117:1219–1222.
24. Oliveira RG, Ferreira AP, Cortes AJ, Aarestrup BJ, Andrade LC, Aarestrup FM. Low-level laser reduces the production of TNF-α, IFN-γ, and IL-10 induced by OVA. Lasers Med Sci 2013;28:1519–1525.
25. Fukuda TY, Tanji MM, Silva SR, Sato MN, Plapler H. Infrared low-level diode laser on inflammatory process modulation in mice: Pro- and anti-inflammatory cytokines. Lasers Med Sci 2013;28:1305–1313.
26. Fernandes KP, Alves AN, Nunes FD, Souza NH, Silva JA, Jr., Bussadori SK, Ferrari RA. Effect of photobiomodulation on expression of IL-1β in skeletal muscle following acute injury. Lasers Med Sci 2013;28:1043–1046.
27. Souza NH, Marcondes PT, Albertini R, Mesquita-Ferrari RA, Fernandes KP, Aimbire F. Low-level laser therapy suppresses the oxidative stress-induced glucocorticoids resistance in U 937 cells: Relevance to cytokine secretion and histone deacetylase in alveolar macrophages. J Photochem Photobiol B 2014;130:327–336