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AMANDA PIRES DE SOUZA

ANGIOGÊNESE EM FERIDAS CUTÂNEAS DE RATOS DIABÉTICOS – EFEITO DA FOTOBIOMODULAÇÃO NA EXPRESSÃO DE FATOR DE CRESCIMENTO DO ENDOTÉLIO VASCULAR E DE ACTINA DE MÚSCULO LISO

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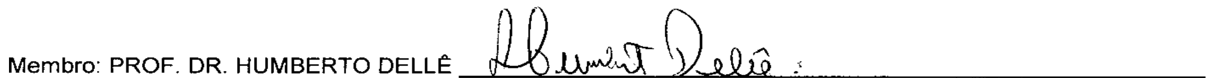
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RESUMO

O diabetes *mellitus* (DM) é uma doença crônica causada tanto pela produção insuficiente de insulina quanto pela resistência periférica à mesma. Essa condição gera hiperglicemia com graves consequências, especialmente na cicatrização tecidual. Nesse contexto, o processo de formação de novos vasos, ou angiogênese, está bastante afetado. A fotobiomodulação (FBM) com laser em baixa intensidade tem sido relacionada a melhorias, tanto na angiogênese, como no reparo tecidual. Para entender parte desses mecanismos de FBM em animais diabéticos, esse trabalho avaliou se a FBM causa diferença na expressão de fator de crescimento do endotélio vascular (VEGF) e vasos maduros que expressam actina de músculo liso (SMA) em feridas cutâneas. Foram utilizados 10 ratos Wistar provenientes do biotério da UNINOVE. O diabetes foi induzido com estreptozotocina (50 mg/kg) e quinze dias após a indução os animais convertidos foram anestesiados e foram feitas duas úlceras dorsais com *punch* de 8 mm. Os animais foram aleatoriamente divididos em 2 grupos: Controle (úlcera sem tratamento), Laser (laserterapia logo após a realização da úlcera usando laser de diodo, λ= 660 ± 2 nm, com potência de saída igual a 100 mW e área do feixe de 0,04 cm^2–Therapy XT, DMC, São Carlos, Brasil), com irradiância de 32,7 mW/cm^2 durante 122 s, entregando uma dose de 4 J/cm^2. A eutanásia foi feita 15 dias após a lesão. As amostras foram processadas rotineiramente para inclusão em parafina e coloração com hematoxilina e eosina, imunohistoquímica para SMA e VEGF. Os vasos foram contados com auxílio do software ImageJ e os dados analisados estatisticamente. Não houve diferença estatística entre a quantidade de vasos sanguíneos marcados com VEGF entre os grupos controle e laser (p=0,22 - Mann-Whitney). A quantidade de vasos maduros marcados com SMA também foi semelhante entre os grupos (p=0,36). Nas condições do estudo, aos 15 dias de reparo tecidual de ratos diabéticos, a fotobiomodulação não causou diferença na angiogênese.

Palavras-chave: Diabetes mellitus, angiogênese, fototerapia, lasers, cicatrização

ABSTRACT

Diabetes mellitus (DM) is a chronic disease caused by both insufficient insulin production and peripheral resistance to insulin. This condition generates hyperglycemia with serious consequences, especially in tissue healing. In this context, the process of formation of new vessels, or angiogenesis is greatly affected. Low-intensity laser photobiomodulation (PBM) has been associated to improvements in both angiogenesis and tissue repair. To understand part of these mechanisms of PBM in diabetic animals, this study evaluated whether PBM causes difference in the expression of vascular endothelial growth factor (VEGF) and mature vessels that express smooth muscle actin (SMA) in cutaneous wounds. Ten Wistar rats were used from the UNINOVE breeding stock. Diabetes was induced with streptozotocin (50 mg/kg) and fifteen days after induction the diabetic animals were anesthetized and two dorsal ulcers were made with 8 mm punch. The animals were randomly divided into 2 groups: Control (untreated ulcer), Laser (laser therapy shortly after performing ulcer using diode laser, λ = 660 ± 2 nm, with output power equal to 100 mW and beam area of 0,04 cm^2– Therapy XT, DMC, São Carlos, Brazil), with irradiance of 32.7 mW/cm^2 for 122 s, delivering a dose of 4 J/cm^2. Euthanasia was done 15 days after the injury. Samples were routinely processed for paraffin inclusion and staining with hematoxylin and eosin, immunohistochemistry for SMA and VEGF. The vessels were counted using ImageJ software and the data analyzed statistically. There was no statistical difference between the number of VEGF-labeled blood vessels between the control and laser groups ($p = 0.22$ - Mann-Whitney test). The amount of mature pots labeled with SMA was also similar between the groups ($p = 0.36$ - Mann-Whitney Test). Under the study conditions, at 15 days of tissue repair of diabetic rats, photobiomodulation did not cause any difference in angiogenesis.

Key words : Diabetes mellitus, angiogenesis, fototherapy, lasers, wound healing

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LISTA DE ABREVIAÇÕES

ATP: adenosina trifosfato;
COBEA: Colégio Brasileiro de Experimentação Animal;
DM: diabetes mellitus;
FAK: quinase de adesão focal;
FBM: fotobiomodulação;
FGF: fator de crescimento de firoblastos;
HFI: fator induzido por hipóxia;
ICLAS: International Council of Laboratory Animal Science;
IL: interleucina;
LASER: Amplificação da Luz por Emissão Estimulada de Radiação;
MEC: matriz extracelular;
PDGF: fator de crescimento derivado de plaquetas;
RTK: receptor de tirosina quinase;
SBD: Sociedade Brasileira de Diabetes;
SMA: actina de músculo liso;
SVCAL: Sociedade Brasileira de Ciência em Animais de Laboratório;
TGF- beta: fator de crescimento transformante beta;
TLR: receptores toll- like;
TNF: fator de necrose tumoral;
UNINOVE: Universidade Nove de Julho;
VEGF: fator de crescimento do endotélio vascular.

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1. CONTEXTUALIZAÇÃO

1.1. Diabetes mellitus

A palavra diabetes vem do grego e quer dizer sifão (tubo para aspirar água), nome dado devida a grande quantidade de urina, *mellitus*, do latim, significa mel. Logo, diabetes *mellitus* significa urina doce. [1,2]

O hormônio que regula a concentração de glicose sanguínea, é a insulina. O diabetes *mellitus* (DM) é uma doença crônica causada quando o pâncreas não produz insulina suficiente ou o corpo não utiliza com eficácia a insulina que produz. A insulina quando não regulada corretamente pode ocasionar a hiperglicemia, um efeito comum do diabetes que se não controlada pode levar a micro e macroangiopatias, principalmente neuropatias e amputações de membros. [3,4,5]

Segundo a Organização Mundial de Saúde (OMS), em 2011, cerca de 9% dos adultos (maiores de 18 anos) apresentaram diabetes. Em 2012, 1,5 milhões de mortes foram causadas pelo DM, mais de 80% dessas mortes são nos países em desenvolvimento nos indivíduos de média e baixa renda. [6,7,8]

Em 2015, foi avaliado pela OMS, por meio de um formulário, que a capacidade de prevenir e controlar o diabetes varia de acordo com a região e nível econômico do país. Grande parte dos países afirma ter políticas para prevenção e controle do diabetes, porém dependendo da região nos países de baixa renda, os profissionais da saúde carecem de tecnologias básicas para os diabéticos gerenciarem corretamente a doença. Neste mesmo formulário também foi constatado que 1 em cada 3 países com renda média ou baixa possui as tecnologias básicas para o controle e prevenção do diabetes nos postos de cuidados básicos de saúde. [9]

O DM é atualmente classificado segundo sua etiologia em: DM tipo 1, DM tipo 2, diabetes gestacional e outros. O diabetes tipo 1 é observado principalmente na infância, causado pela destruição auto-imune das células do pâncreas, ocasionando uma falta acentuada de insulina. Os sintomas são poliúria (excesso de urina), polidipsia (sede), fome constante, perda de peso, alterações na visão e fadiga. [10,11,12]

O DM tipo 2, presente em adultos, corresponde a 90% dos casos no mundo, sendo resultado de herança poligênica, excesso de peso, resistência à insulina, sedentarismo e posterior falência das células beta-pancreáticas. Os sintomas são semelhantes ao diabetes do tipo 1, apresentando-se de forma atenuada a princípio, ocasionando muitas vezes um diagnóstico tardio, quando já existem complicações. [10,11,13]

O diabetes gestacional é caracterizado por valores acima do normal de glicose no sangue, porém inferiores aos de diagnóstico de diabetes. O diabetes gestacional pode acarretar riscos maiores durante a gravidez e no parto e apresentam maior risco de diabetes tipo 2 no futuro. [10,11]

Devido à hiperglicemia, os diabéticos possuem alterações micro e macrovasculares que dificultam o processo inflamatório, dentre essas alterações podemos citar a proliferação de células endoteliais nos capilares da retina, degeneração dos neurônios, das células de Schwann e alterações na membrana basal dos vasos periféricos. [6,14,15,16,17.]

Pacientes diabéticos apresentam um retardo da cicatrização de feridas agudas que muitas vezes acabam tornando-se úlceras crônicas. A predominância dessas úlceras é em pés e membros inferiores. Para que a cicatrização ocorra de maneira correta, vários tipos celulares estão envolvidos. Em diabéticos, ocorre uma diminuição na proliferação e migração dos queratinócitos epidérmicos que são responsáveis por manter a estrutura e a função da epiderme. [18,19]

Em lesões cutâneas, a expressão de citocinas inflamatórias ocorre através da ativação dos receptores de Toll-like (TLRs), porém, a produção de citocinas pró-inflamatórias, fator de necrose tumoral (TNF) e a interleucina (IL) - 6 é aumentada em diabéticos, induzindo a infiltração leucocitária prolongada. [20]

Outro fator que contribui para o retardo da cicatrização em diabéticos são os altos índices de VEGF no plasma sanguíneo, o que pode produzir um estado de pseudo-hipóxia e ter um efeito direto no endotélio. Estudos indicam que o VEGF desempenha um papel importante nas retinopatias isquêmicas, sendo a molécula alvo nesta doença. [21, 22]

O fator de crescimento do endotélio vascular (VEGF) é das famílias das citocinas, está diretamente relacionado a neovascularização. O VEGF pertence

ao grupo de glicoproteínas diméricas da qual compreende sua família de múltiplos membros (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E). [21,23]

O VEGF-A é importante no reparo tecidual porque promove os primeiros estímulos para a angiogênese, particularmente a migração, proliferação, sobrevivência e diferenciação das células endoteliais, queratinócitos, fibroblatos, células musculares lisas, plaquetas, neutrófilos e macrófagos, também responsável pelo aumento da permeabilidade vascular.[23, 24, 25]

Em estudos com animais diabéticos, a administração de VEGF-A mostrou-se capaz de restaurar a angiogênese e promover a reepitelização associada à formação de vasos.[22]

O modelo utilizado para a indução do Diabetes mellitus tipo 2 foi descrito por Correia- Santos e*t. al.,* em 2012, e adaptado para as necessidades propostas na dissertação. O método de indução consiste em uma injeção intraperitonial de streptozotocina na dose de 50 mg/kg dissolvida em citrato de sódio (0,01 M, pH 4,5) com intuito de mimetizar o metabolismo da doença em humanos (disfunção da células β-pancreáticas). [26]

1.2. Reparo Tecidual e Angiogênese

A cicatrização de feridas cutâneas está didaticamente dividida em três etapas que se sobrepõem: inflamação, proliferação e remodelação. A primeira fase, a inflamação, acontece imediatamente após o dano tecidual e os componentes da cascata da coagulação, cascata inflamatória e ativação do sistema imune, são necessários para realizar a hemostasia, exsudação plasmática e celular. [27,28,29]

A segunda fase do reparo, a proliferativa, é caracterizada pela migração de diferentes tipos celulares: como queratinócitos, células angioprogenitoras, fibroblastos e macrófagos, a fim de substituir a matriz de fibrina por tecido de granulação. [30,31,32]

Entre 24 e 72 horas do processo de reparação, fibroblastos e células endoteliais vasculares começam a proliferação para a formação do tecido de granulação, possui esta nomenclatura devido à sua aparência rósea, mole, granular na superfície das feridas. Histologicamente, está ocorrendo a formação de novos vasos (angiogênese) e fibroplasia. [30,33,34,35]

A angiogênese é a formação de novos vasos, sendo assim, responsável pelo aporte sanguíneo do local em reparo tecidual. Esse processo faz com que as células, fatores de crescimento e citocinas consigam chegar ao local lesionado de modo que o processo como um todo se torne mais eficaz e eficiente. [33, 34]

A angiogênese é um processo complexo e orquestrado que envolve a proliferação e migração de células endoteliais, interações com outros tipos celulares e modificações da matriz extracelular (MEC). Durante esse processo as células endoteliais migram por meio da MEC, rica em colágeno, laminina, fibronectina e proteoglicanas. [35,36]

A regulação da angiogênese envolve vários mecanismos de controle em multiescala, revisados por Briquez et al., 2016 (Fig. 1).

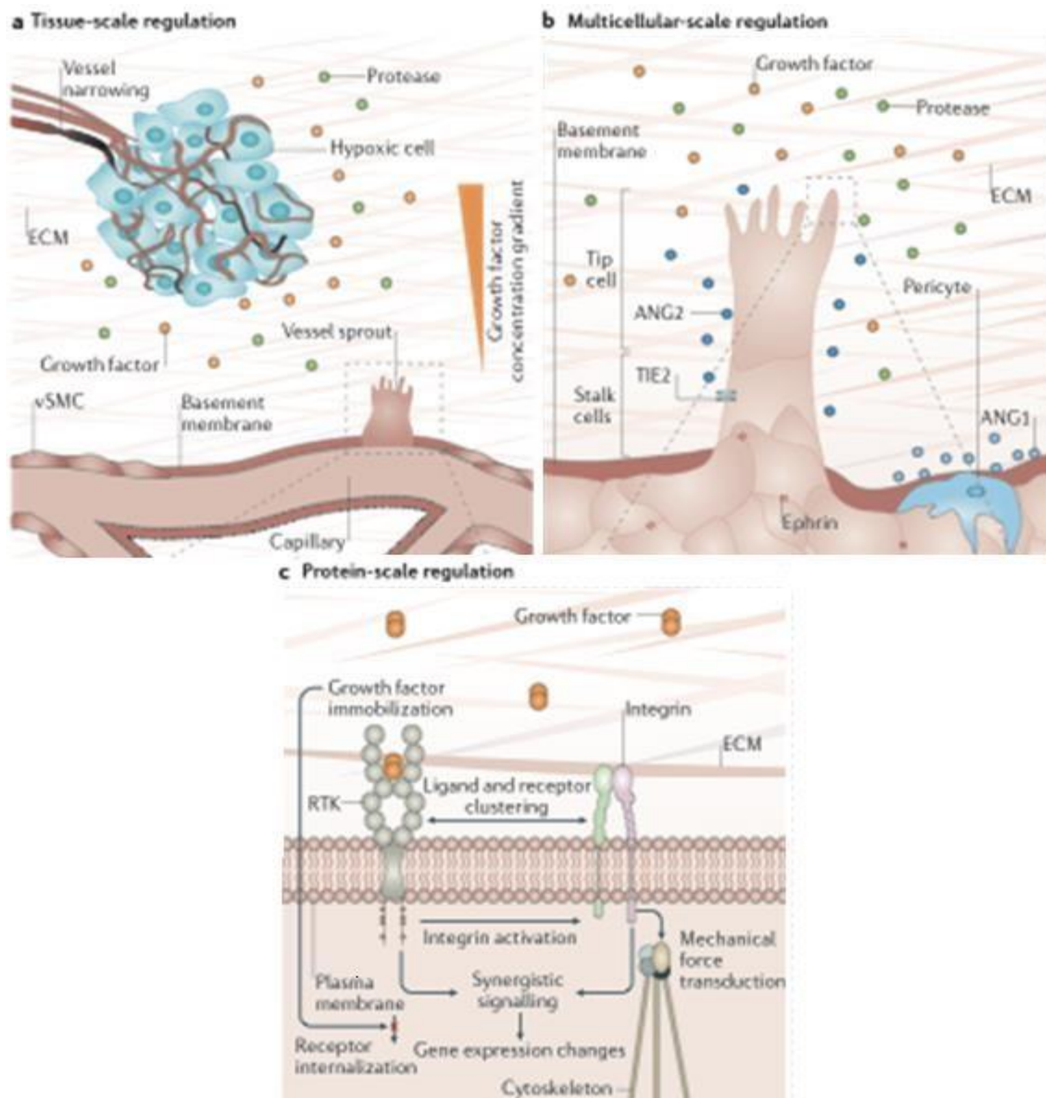

Fig 1: Regulação em multiescala da angiogênese. A) Comunicação entre as células e a matriz extracelular (MEC). B) Detalhe do brotamento do vaso e dos principais fatores envolvidos. C) Em uma escala molecular, os receptores de superfície endotelial que são ativados nessa fase e as vias de sinalização intracelular. [Adaptado de Briquez PS, Clegg LE, Martino MM, Gabhann FM, Hubbell. Design principles for therapeutic angiogenic material. Nat Rev Materials. 2016; 1:1-15.]

Brevemente, em uma situação de hipóxia, há a liberação de fatores de crescimento, como o VEGF, fator de crescimento derivado de plaquetas – (PDGF), fator de crescimento de fibroblastos (FGF1, FGF2, FGF4), eritropoietina, fator induzido por hipóxia - HFI-1 e fator de crescimento transformante (TGF beta), que formam gradientes de concentração dentro do tecido e se ligam aos receptores de superfície do endotélio, desencadeando o brotamento de novos vasos a partir dos vasos adjacentes. Nesse processo, a comunicação célula-célula é crítica para o brotamento apropriado do vaso e seu crescimento. [36]

Para abertura de caminhos de migração dentro da MEC (especialmente através do colágeno IV e da laminina na membrana basal do vaso), o broto vascular e as células adjacentes liberam proteases ou as expressam na sua superfície. As proteínas da MEC promovem a formação de complexos de ligação entre os fatores de crescimento, seus receptores e as integrinas na membrana celular, induzindo uma sinalização celular sinergística que resulta em mudanças na expressão gênica do endotélio. [37]

Concomitantente, as integrinas traduzem os sinais mecânicos para o citoesqueleto celular deixando à mostra sítios de ligação que anteriormente estavam escondidos, levando a mudanças na sinalização protéica e regulação de outras integrinas. Com as alterações mecânicas, as caudas das intreginas citoplasmáticas também interagem diretamente com moléculas sinalizadoras que são consideradas críticas para a sinalização reciproca entre as integrinas e os fatores de crescimento. Por último, os brotos formam pontes intercelulares e canalizam, formando o lúmen, adquirindo a forma achatada, tornando-se vasos maduros.[36]

Artéria e arteríolas maduras expressam o filamento de actina de músculo liso (SMA). A manutenção ou interrupção da estabilidade desses vasos maduros ocorre pela interação entre as células endoteliais e entre endotélio e pericito. Os pericitos são células oriundas de células mesenquimais, localizadas ao redor dos

capilares, arteríolas, vênulas pós-capilares e completamente imersas na MEC do endotélio adjacente. Em sua maioria, eles expressam SMA e são responsáveis pela regulação da morfogênese vascular, função e estabilização dos vasos. [37]

Numa reposta inflamatória normal, parte da função do VEGF é aumentar a permeabilidade, afetando as proteínas das células endoteliais, que poderiam aumentar a formação do tecido de granulação. Na angiogênese, o VEGF desempenha o papel de vasculogênese através do recrutamento de células progenitoras endoteliais a partir da medula óssea, por fim estimulam os pericitos para revestir e estabilizar a vasculatura. [2,38,39,40]

O estágio de remodelamento começa de 2 a 3 semanas após o dano celular e pode durar até um ano. Durante este estágio, todos os processos ativados durante as outras fases diminuem e cessam. A maioria das células endoteliais, macrófagos e miofibroblastos sofrem apoptose, ou saem da ferida, deixando no local tecido fibrótico, que é gradualmente remodelado pelos fibroblastos. [41,42,43]

1.3. Laser em baixa intensidade

O LASER (Light Amplification by Stimulated Emisson of Radiation), ou seja, Amplificação da Luz por Emissão Estimulada de Radiação) é um dispositivo que produz uma radiação eletromagnética com 3 características muito próprias: ela é coerente (os fótons que compõe o feixe estão na mesma fase), monocromática (comprimento de onda bem definido) e é colimada (propaga- se com um feixe de ondas paralelas). Desde o final da década de 60 o laser vem sendo usado como terapia alternativa para auxiliar o processo de cicatrização. [44]

A literatura fundamenta os mecanismos de ação da terapia com laser de baixa potência em células e tecidos: aumento do aporte de adenosina trifosfato (ATP), aumento da permeabilidade da membrana celular, viabilizando o influxo de cálcio, regulação de fatores de crescimento e citocinas inflamatórias, estimulação da diferenciação e proliferação celular, indução da síntese e remodelamento de colágeno, aumento da resistência tênsil e angiogênese. [45,46]

Todavia, a literatura ainda e controversa sobre a questão se o laser em baixa intensidade influencia ou não a síntese de VEGF. [22] Brassolati et. al., 2016 mostraram que a FBM favoreceu o aumento do VEGF em queimaduras de 3° grau em ratos. Tim et. al., também observaram aumento da expressão de VEGF após aplicações de laser em feridas de calvárias de ratos. [46]

No entanto, Marques et. al., 2015, estudando o mesmo modelo de calvária de rato com doses de 16 J/cm^2 de laser em baixa intensidade não encontraram diferenças na marcação de VEGF. [47]

Em modelo experimental de lesões de tendão de Aquiles, tampouco foram encontradas diferenças na expressão de VEGF nos grupos que receberam laser na dose de 17,5 cm^2. [48]

Silva et. al., 2010, mostraram aumento na expressão de RNA mensageiro do VEGF durante o processo de reparo tecidual após duas irradiações com um comprimento de onda de 780 nm, 70 mW de potência e doe de 35 J/cm^2. [49]

Um estudo *in vitro* com células endoteliais humanas mostrou que o laser em baixa intensidade diminuiu a quantidade de VEGF no meio sobrenadante e aumentou a proliferação celular. [50]

Na tabela 1 temos um comparativo entre os parâmetros do Laser utilizados por Brassolati et. al., Tim et. al., Silva et. al., Marques et. al. e Góralczyk et. al.:

Tabela 1 – Avaliação da literatura recente comparando dosimetria, fontes de luz e reparo tecidual (P= potência, DE= densidade de energia)

Autor	**Descrição**	**Modelo experimental**	**Resultados**
Peplow et. al., 2011 [16]	Lasotronic GmbH (λ= 660 nm, P= 50 mw ou 25 mw, 40 s ou 80 s, DE= 4,7 J/cm^2 ou 6,3 J/cm^2)	Ferida circular no flanco esquerdo	Ambos tratamentos estimularam a cicatrização em feridas de diabéticos
De Loura Santana et. al.,2015 [31]	Diodo (λ = 660 ± 2 nm, P = 30 mW, DE= 4 J/cm^2) em dose única e dose fracionada irradiando 1 J/cm^2 nos dias 1, 3, 8, e 10	Ferida dorsal com punch em rato diabético	Aceleração no fechamento 40% nos 3 primeiros dias em relação ao controle

De Loura Santana et. al. , 2016 [52]	GaAlAs (λ = 660 ± 2 nm, P= 30mW, DE= 4 J/cm^2) em dose única e dose fracionada irradiando 1 J/cm^2 nos dias 1, 3, 8, e 10	Ferida dorsal com punch em rato diabético	Leva a uma resposta inflamatória mais equilibrada em diabéticos, transitando de uma rápida infiltração de neutrófilos através da polarização de macrófagos M2.
Tim et. al, 2015 [47]	Thera Laser, DMC® (λ= 80 nm, P= 30 mW, por 94 s)	Cirurgia na tíbia a fim de produzir defeitos ósseos	Aumento de osso recém-formado e aumento significativo na expressão de genes relacionados com a inflamação e angiogênese
Brassolati et. al, 2016 [46]	Therapy XT, DMC® (λ= 660 nm, P= 50 mw ou 100 mw, 10 s, DE= 12.5 J/cm^2 ou 25 J/cm^2	Queimadura de 3° grau na região dorsal	O tratamento com maior densidade de energia foi mais efetivo no estimulo do processo de cicatrização
Marques et. al., 2015 [48]	P= 50mW, 9 s ou 3 s, DE= 16 J/cm^2 ou 3.7 J/cm^2	Defeito ósseo no crânio de 8 mm produzidos cirurgicamente	Funciona nos estágios iniciais da regeneração óssea
Silva et.al. 2010 [49]	GaAlAs (λ= 780 nm, P= 70 mW; 40 s, DE= 35 J/cm^2) AlGaInP (λ= 660 nm, P= 40 mW, 10 s, DE= 5 J/cm^2)	Ferida de excisão de 12,4 cm^2 na língua dos ratos	O tratamento com o laser GaAlAs inibiu a expressão do mRNA de VEGF-A165 durante a cicatrização de feridas 1 dia após um procedimento cirúrgico na língua de ratos Wistar
Góralczyk et. al., 2015 [50]	GaAlAs (λ= 660 nm, DE= 2 J/cm^2, 4 J/cm^2, 8 J/cm^2) Infravermelho (λ=808 nm, DE= 2 J/cm^2, 4 J/cm^2, 8 J/cm^2)	Linhagem celular de HUVEC armazenadas em incubadora de umidificada com 5% CO^2	O laser GaAlAs se mostrou eficaz na produção de VEGF-A em células endoteliais da linha HUVEC
Mathur et.al., 2016 [54]	Diodo (λ=660±20 nm, 60s DE= 3 J/cm^2)	Úlcera em pele de humanos diabéticos	As feridas em indivíduos tratados com laser contraíram (37,2%) significativamente mais do que as feridas no grupo não tratado (15,12%).

Tatmatsu-Rocha et.al., 2016 [55]	GaAS diodo (λ=904 nm, P= 39,9 mW, 60 s DE= 18, 28 J/cm²)	Lesão dorsal com lâmina cirúrgica de aço carbono	Baixa nos níveis de nitrito, aumento na proteção contra danos oxidativos e melhor organização do colágeno.
Poriran R et.al., 2016 [56]	Laser infravermelho de diodo (λ= 890 nm, P=1,08 mW, 180 ns, DE=0,2 J/cm²)	Duas incisões longitudinais de 12 mm de espessura total foram feitas na região dorsal com lâmina e bisturi	Acelerou o processo de cicatrização
Fahimipour et. al. 2016 [57]	He-Ne (λ=632,8 nm , P=5 mW, 16 s, DE=4 J/cm²) GaAlAs (λ=830 nm, P=25 mW, 16 s, DE=4 J/cm²)	Incisão na rafe mediana, 2 mm posterior à superfície lingual dos dentes incisivos, utilizando um bisturi.	Laserterapia de baixo nível com laser He-Ne em comparação com o laser GaAlAs na cicatrização em feridas gengivais do palato duro em camundongos 20diabéticos.
Lau P et.al. 2015 [58]	Diodo (λ= 808 nm, P=100mW, 200mW, 300mW, 50 s, 25 s e 17 s, DE=5 J/cm²	Punch cirúrgico de 6mm	Aumentou contração da ferida, melhorou o processo de cicatrização e síntese de colágeno.
Sharifian et. al. 2014 [59]	Laser infravermelho de diodo (λ=890 nm, P=1,08 mW, 200s, DE= 0,2 J/cm²)	Duas incisões dorsais de 1, 5 cm.	Acelerou o processso de cicatrização tanto no grupo tratado quanto no grupo não tratado
Dancáková et.al. 2014 [60]	Diodo (λ=810 nm, P= 30mW, 60 s durante 7 dias, DE= 1,8 J/cm²	Incisão dorsal de 40 mm de comprimento e 4 mm de diâmetro	Estimulou a formação do tecido de granulação
Kilík et. al. 2014 [61]	GaAlAs (λ=635 nm, 5min 33s; 16min 40s; 83min 20)	Feridas circulares realizadas com alicate de punção com 4 mm de diâmetro, que foram suturadas	Todos os tratamentos mostraram um efeito positivo na cicatrização de feridas em ratos diabéticos e não-diabeticos
Dadpay et. al. 2012 [62]	Laser infravermelho de diodo (λ=890 nm, P=1,08 mW, 30 s ou 200s, DE= 0,03 J/cm² 0,2 J/cm²)	Duas incisões dorsais com 1,5 cm de comprimento	O tratamento com 0,2 J/cm² se mostrou mais eficaz o processo de cicatrização

Hegde et.al. 2011 [63]	He-Ne (λ=632,8nm, P=7mW, 4min 15sec-^{4} J/cm^{-2}, 8 min 32 sec^{-2} J/cm^{-2}, 12 min 46sec^{-3} J/cm^{-2}, 17 min 3sec^{-4} J/cm^{-2} e 21min 17sec^{-5} J/cm^{2}	Ferida de excisão de espessura total de 15 mm	Ocorreu um efeito benéfico na progressão da cicatrização da ferida diabética, possivelmente através da ativação de fibroblastos da ferida e elevação da síntese de colágeno

Em um estudo anterior do nosso grupo, foi observado que o laser em baixa intensidade induziu a angiogênese e o reparo tecidual em queimaduras de 3° grau em ratos, tanto quanto o laser era aplicado uma vez ou em 4 dias alternados[51]. Em seguida, comparamos esses dois regimes de entrega de luz na cicatrização de feridas em ratos diabéticos, mostrando que nas feridas que receberam dose única de laser, totalizando 4 J/cm^2 (comprimento de onda de 660 nm, potência de 30 mW), houve maior quimiotaxia para macrófagos e neutrófilos do que o grupo que recebeu os 4 J/cm^2 divididos em doses de 1J/cm^2 em dias alternados. Ao final, a análise do colágeno demonstrou que uma dose única de 4 J/cm^2 favoreceu o reparo tecidual nos animais diabéticos. [31,53,54]

Com bases nesses resultados anteriores, o presente trabalho foi realizado com objetivo de esclarecer se o laser em baixa intensidade promove a angiogênese no reparo tecidual via expressão de VEGF ou pela diferenciação de vasos maduros, SMA positivos.

2. OBJETIVOS

2.1. Objetivos Gerais

Avaliar se fotobiomodulação com laser em baixa intensidade causa diferença na expressão de VEGF e vasos maduros que expressam SMA em feridas cutâneas de ratos diabéticos.

2.2. Objetivos Específicos

- Avaliar de modo quantitativo se há diferença na expressão de VEGF em ratos diabéticos tratados com LASER e ratos não tratados;
- Quantificar arteríolas maduras caracterizadas pela presença de actina de músculo liso em ratos diabéticos tratados com LASER e ratos não tratados.

3. MATERIAIS E MÉTODOS

O protocolo experimental utilizado neste estudo seguiu os princípios de ética e experimentação animal, elaborados pelo COBEA (Colégio Brasileiro de Experimentação Animal), entidade filiada ao InternationalCouncilofLaboratory Animal Science (ICLAS), com base nas normas internacionais, que visam o aprimoramento de condutas na experimentação animal baseando-se em três princípios básicos: sensibilidade, bom senso e boa ciência.

A metodologia utilizada neste estudo foi submetida à avaliação pelo Comitê de Ética em Pesquisa Animal da UNINOVE e aprovada com o número de protocolo 006.2014 (anexo 1).

3.1. Animais

Foram utilizadas 10 ratas Wistar oriundas e mantidas no biotério da Universidade Nove de Julho (UNINOVE) – unidade Vergueiro. Os animais foram mantidos em caixas plásticas apropriadas, num máximo de 2animais por caixa, a temperatura ambiente (22°C) e luminosidade controlados com ciclo de 12 horas sendo que os animais possuiram comida e água *ad libitum*.

3.2. Indução do DM

Para a indução do diabetes, 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 (SVCAL) e receberam injeção intraperitoneal de streptozocina (cat S0130, Sigma-Aldrich, St. Lois, MO, USA), que apresenta toxicidade para células β- pancreáticas na dose de 50 mg/ kg de massa corpórea dissolvida em tampão de citrato estéril, pH 4,5 [26].

A indução aconteceu em até sete dias e foi confirmada por exame de glicemia por meio do Accu-Chek(Roche Diagnostics, Indianapolis, Indiana, USA).

A glicemia foi aferida semanalmente, retirando- se uma gota de sangue da cauda do animal e fazendo a leitura com um aparelho Accu-Chek (Roche). Foram incluídos no estudo os animais com glicemia superior a 220 mg/ dL o que

caracteriza um diabetes bem estabelecido [55]. O consumo de água e ração foi monitorado diariamente.

3.3. Grupos Experimentais

Cada grupo teve 5 animais diabéticos distribuídos aleatoriamente. O número de animais foi estabelecido para dar poder aos testes estatísticos, fundamentar os resultados obtidos e viabilizar as comparações entre os grupos.

Grupo controle (GC) – as úlceras dorsais não foram submetidas a tratamento;

1- Grupo TLBI (GTLBI) – a úlcera dorsal dos animais recebeu laserterapia (4 J/cm²) imediatamente após a realização da ferida (no dia 1).

Esquematicamente os grupos foram divididos desta forma:

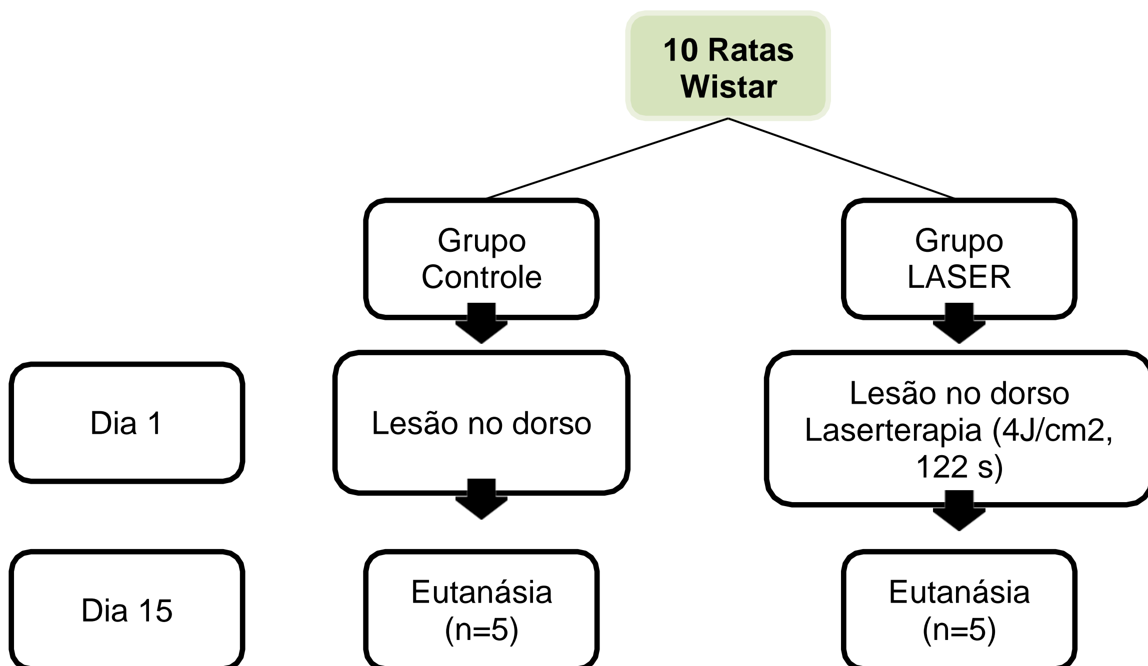

Fig. 2: Fluxograma dos grupos em função dos dias experimental.

3.4. Procedimentos cirúrgicos – indução da úlcera

Após a confirmação do diabetes, os animais foram anestesiados com injeção intraperitonial de uma mistura de Ketamina (Dopalen, Vetbrands, Jacareí, SP) na dose de 100 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 biseltrifacetado.

Depois de anestesiados, o dorso dos animais foi tricotomizado e houve a realização de úlcera, lateral à linha média do animal, com *punch* cirúrgico de 8 mm.

Posteriormente os animais foram mantidos em gaiolas com aquecimento para prevenir a hipotermia.

3.5. Laserterapia

Um laser vermelho, λ= (660 ± 2) nm, com potência de saída igual a 100 mW e área do feixe de 0,04 cm^2 (Therapy XT, DMC, São Carlos, Brasil), com irradiância de 32,7 mW/cm^2durante 122 s, entregando uma dose de 4 J/cm^2.

O dia 15 foi escolhido para se avaliar a angiogênese porque representa o pico de formação de vasos durante a fase proliferativa do reparo.

3.6. Eutanásia

A eutanásia foi realizada no dia 15 nos cinco animais de cada grupo com sobredose de anestésico (quatro vezes a dose necessária para anestesia).

A úlcera foi removida, armazenada em formaldeído 10% para inclusão em parafina e coloração de rotina com HE (hematoxilina e eosina) e imunohistoquímica.

3.7. Imunohistoquímica para actina de músculo liso e VEGF

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, foram desparafinizados, por meio da imersão das lâminas em xilol aquecido (60- 65° C), e colocados em estufa histológica durante 5 minutos, sendo posteriormente passadas em 3 banhos de xilol frio.

Para a hidratação dos cortes, as lâminas foram colocadas em dois banhos de álcool absoluto, um banho de álcool 95° e um banho de 70°. Em seguida, foram 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).

O próximo passo foi a recuperação dos sítios antigênicos realizada em alta temperatura com solução tampão de ácido cítrico 10 mM pH 6 (95-98 °C) por 15 minutos. Após esta etapa, ocorre o esfriamento do tampão por mais 20 minutos.

Em seguida, foi feito o bloqueio da peroxidase endógena presente nas hemácias com 3 banhos de 5 minutos de água oxigenada 10v (3%). 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 anti-actina de músculo liso (Abcam # 5694) e anti VEGF-A (Abcam #1316), ambos na diluição de 1:1000 por 16 horas a 4°C. O anticorpo primário foi diluído em diluente especifico (Spring BioscienceCorp – Pleasanton, EUA) que contém em sua fórmula 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 – NichireiBiosciences Inc., Tokio, Japão), por 30 minutos em estufa 37°C.

Após esta incubação, os cortes foram lavados em TBS e incubados com cromógeno DAB- Diaminobenzidina (Dako – Dinamarca) por 5 a 10 minutos. Posteriormente ocorreu a lavagem em água corrente por 10 minutos e a contra-coloração com Hematoxilina de Harris (Merk, Darmstadt, Alemanha) por 1 minuto.

3.8. Contagem celular

Após a marcação de imunohistoquímica, 5 campos consecutivos da área da ferida, sempre da borda esquerda para o centro, percorrendo toda a área da lesão, foram fotografados em um aumento de 400 x (Leica Microsystems, Wetzlar, Germany) e as imagens das células marcadas foram contadas com auxílio do *software*ImageJ 1.45 (*software* livre, NIH, Bethesda, Maryland,USA) usando- se o pluggin “cell counter”.

3.9. Analise Estatística

Os dados das contagens dos vasos marcados foram submetidos a testes estatísticos apropriados para avaliação da diferença entre o grupo controle e o Laser em baixa intensidade. A distribuição estatística dos dados foi avaliada por meio de normalidade de Shapiro-Wilk. Os dados eram não paramétricos e o teste de Mann-Whitney foi para comparar os diferentes grupos (nível de significância $p < 0,05$).

Toda análise dos dados foi realizada com o software SPSS® (SPSS statistics v23, USA).

4. RESULTADOS

Durante o mestrado, a aluna participou ativamente dos trabalhos dessa linha de pesquisa, comprovado, tanto pelo artigo como pelos resultados da tese submetido para revista Lasers in Medical Science, quanto pelos artigos já publicados listados a seguir:

4.1. Artigo 1

Os resultados desse trabalho foram enviados para publicação na revista Laser in Medical Science (anexo 2).
Souza AP, Silva DFT, Bussadori SK, Mesquita-Ferrari RA, Fernandes KP, França CM. Efeito da fotobiomodulação na expressão de VEGF e actina de músculo liso em feridas cutâneas de ratos diabéticos. Laser in Medical Science.

4.2. Artigo 2 (anexo 3)

de Loura Santana C, Sliva 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. Tisssue responses to postoperative laser therapy in diabetic rats submitted to excisional wounds. PLoS One. 2015 Apr 24;10(4):e0122042. Doi: 10.1371/journal.pone0122042.eCollection 2015.

Abstract: In a previous study low-level laser therapy biomodulation on a full-thickness burn model we showed that single and fractionated dose regimens 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 ηm; P= 30 mW; energy density: 4 J/cm^2) and

fractionated-dosse 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 processes for histological and immunohistochemitry 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 myofibroblat 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 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.

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

4.3. Artigo 3 (anexo 4)

Calisto FC, Calisto SL, Souza AP, França CM, Ferreira AP, Moreira MB. Use of low-power laser to assist the healing of traumatic wounds in rats.Acta Cir Bras. 2015 Mar; 30(3): 204-8. doi: 10.1590/s0102-865020150030000007.

Abstract: Purpose: To investigate the morphological aspects of the healing of traumatic wound in rats using low-power laser. Methods: Twenty four non isogenic, young adult male Wistar rats (Rattus norvegicus) weighing between 200 and 300g was used. The animals were randomly distributed into two group: Control (GC) and Laser (GL), with 12 animals each. After having, anesthesia was performed in the dorsal region and then a surgical procedure using a scalpel was carried out to make the traumatic wound. GL received five sessions of laser therapy in consecutive days using the following laser parameters: wavelength 660 nm, power 100mW, dose 10 J/cm^2. The wound were evaluated through measurement of the area and depth of the wound (MW) and histological analysis (HA). Results: When comparing the GC with the GL in MW there was difference

in area (p<0.001) and depth p=0.003) measurement of the wounds in GL. The laser group presented more epithelization than GC (p=0.03). The other histological parameters were similar. Conclusion: The healing of wounds in rat was impoved with the use of the laser.

4.4. Artigo 4 (anexo 5)

De Loura Santana C, de Fátima Teixeira Silva D, de Souza AP, Jacinto MV, Bussadori SK, Mesquita-Ferrari RA, Fernande KP, França CM. Effect of laser therapy on immune cells infiltrate after excisional wounds in diabetic rat. 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 wound in those individuals, the aim of this tudy was to evaluate the reponse of the main effector cell of immunity (neutrophils, macrophages, and T lymphocytes) and to compare the effects of two laser therapy regimens in the postoperative treatment of excision wounds. Methods: 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 660nm, output power of 30mW, 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/lm.22445/abstract;jsessionid=0DD9770EE5CFA1BF692753554D5E565.f02t02.

5. DISCUSSÃO

Este trabalho demonstrou que em ratas diabéticas, no dia 15 do reparo cutâneo, não existem diferenças significantes na quantificação de VEGF e no número de arteríolas maduras quando comparamos o grupo tratado com laser e o grupo não tratado.

A literatura ainda é controversa quando se fala na FBM na expressão do fator de crescimento do endotélio vascular. Existe uma série de estudos que exibem resultados conflitantes e nenhum deles coincide os parâmetros. Foi demonstrado que a FBM pode tanto aumentar a angiogênese quanto não fazer efeito sobre a mesma[45,9].Isso depende do tecido, do momento da aplicação da luz e da dose entregue[54].

Brassolati et al., 2016, Tim et al., 2016 e Silva et al., 2010 encontraram resultados positivos na expressão do VEGF usando o laser em baixa intensidade[45,46,47], porém Marques et. al. não encontrou diferenças na marcação de desse fator de crescimento após aplicação do laser[48]. Outro estudo in vitro com células endoteliais humanas mostrou que o laser em baixa intensidade diminuiu a quantidade de VEGF no meio sobrenadante, porém aumentou a proliferação celular[50].

Como podemos observar na tabela 1, os parâmetros utilizados pelos autores foram muito diferentes dos utilizados no nosso estudo (Therapy XT, λ= 660 nm, com potência de 100 mW, irradiância de 32,7 mW/cm^2, durante 122 s, entregando uma dose de 4 J/cm^2), dificultando assim, a comparação entre os resultados de VEGF mediante a tratamentos diferentes.

O fato de não haver diferença estatística na quantificação de vasos marcados com VEGF e SMA indica que ambos anticorpos foram muito sensíveis na detecção de vasos sanguíneos. Além disso, as marcações em outros tecidos foram todas dentro do descrito na literatura, sendo o VEGF presente em epitélio e músculo, enquanto a SMA estava presente em endoteliócitos, células musculares lisas, pericitos e miofibroblastos.

Prováveis explicações dos achados do presente estudo podem ser a dose do laser, que deveria ser maior, ou com maior frequência. Outra possibilidade seria a de que há uma antecipação do pico da angiogênese no grupo laser para

o dia 8, e isso não foi detectado no dia 15. Ou que realmente o laser interfira com outros fatores, como a produção de TGF-beta, e não com a via do VEGF. Todas essas hipóteses precisam ser testadas em experimentos futuros.

Uma limitação desse estudo é a análise de somente um dia experimental. O ideal teria sido a análise do dia 8 e 22 para observar se há um adiantamento do início da angiogênese nos grupos laser ou se há um retardo na apoptose dos vasos sanguíneos ao final do reparo, com 3 semanas. Outra proposta interessante seria avaliar a angiogênese gerada pelo LED, como a fez Corazza et al., 2007 que comparam a angiogênese em feridas circulares tratadas ou com Laser ou com LED e verificaram que a angiogênese foi superior em todos os grupos que receberam luz, mostrando que a coerência da luz não é essencial para formação de novos vasos sanguíneos.[27]

6. CONCLUSÃO

Sob essas condições de estudo, no 15° dia do reparo tecidual de feridas cutâneas de ratos diabéticos, a fotobiomodulação não apresentou diferenças na angiogênese quando comparado o grupo controle com o grupo laser.

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8. ANEXOS

8.1. Comitê de Ética

Protocolo de Pesquisa referente ao Projeto nº AN006.2014

Título do Projeto: Influência dos parâmetros do laser de baixa potência na quimiotaxia de macrófagos e produção de CCL2 durante o reparo cutâneo

Orientador: Dra Cristiane Miranda França

Aluno: Amanda Pires de Souza

Objetivos: Avaliar se a mesma dose de energia do laser de baixa potência aplicada com diferentes tempos e potências interferem na produção da quimiocina CCL-2 e na quantificação de macrófagos em feridas cutâneas de ratos.

Método: Serão utilizadas 60 ratas Wistar (*Rattus novergicus*). A úlcera será realizada com punch de 8 mm no dorso do animal após tricotomia. Grupos experimentais: grupo controle (GC) (n=20)- a úlcera dorsal será submetida a laserterapia sham; grupo laser 50 (GL 50) (n=20)- - a úlcera dorsal receberá laserterapia no dia 1, duas horas após a realização da ferida (D1) e nos dias 3, 8 e 15 usando potência de 50 mW; grupo laser 100 (GL 100) (n=20)- - a úlcera dorsal receberá laserterapia no dia 1, duas horas após a realização da ferida (D1) e nos dias 3, 8 e 15 usando potência de 100 mW. Os parâmetros da aplicação do laser serão: $\lambda = (660 \pm 2)$ nm, P= 1 J, spot= 0,04 cm2, e tempo de 20 s (GL 50) e 10 s (GL 100). Um dia (24 horas) após a laserterapia, (nos dias 2, 4, 9, e 16) cinco animais de cada grupo sofrerão eutanásia, as úlceras serão removidas, processadas rotineiramente para coloração de hematoxilina e eosina, imunohistoquímica para macrófagos e Western blot para CCL2. As células imunomarcadas serão fotografadas e quantificadas com auxílio do software ImageJ. Os dados serão analisados estatisticamente quanto à normalidade e aqueles com distribuição paramétrica serão submetidos ao teste ANOVA seguido pelo teste de Tukey para comparação entre os grupos. Os níveis de confiança serão ajustados para 95% ($p<0.05$).

Animais (procedência, raça, linhagem, número de animais, peso, sexo): Biotério (UNINOVE), Serão utilizadas 60 ratos (ratas?) Wistar (Rattus novergicus), machos (150/ 250g).

Condições de alojamento e nutrição: Alimento e água: a vontade; lotação: 5 animais por caixa;
exaustão de ar: sim.

Medidas para evitar estresse e/ou dor nos animais: Anestésicos e analgésicos.

Procedimento Anestésico e/ou Analgésico (incluir dose e vias de administração): Os animais serão anestesiados com injeção intraperitoneal de uma mistura de ketamina (Dopalen, Vetbrands, Jacareí, SP) na dose de 100mg/kg do animal e de xilazina

(Anasedan, Vetbrands, Jacarei, SP) na dose de 10mg/kg do animal. Posteriormente analgesia no pós operatório com Tramal (IP – dose 5mg/kg) 12/12 h por 3 dias.

Eutanásia: Eutanásia com sobredose de anestésico (4x o volume usado para anestesia). 400 mg/ kg de Ketamina e 40 mg/kg de Xilazina, via intraperitoneal.

Pertinência e valor científico do estudo proposto: A partir dos resultados será conhecido como o macrófago reage a diferentes parâmetros da laserterapia. Sendo o macrófago o pivô do processo de reparo celular, este estudo permitirá se conhecer o quanto o processo de cicatrização pode ser beneficiado com o uso do laser. Existe uma grande prevalência de acidentes na população, produzindo feridas que afastam as pessoas das suas atividades diárias e do trabalho, com a laserterapia o processo de cicatrização pode ser beneficiado, por diminuir o tempo da cicatrização e aumentar a qualidade do reparo tecidual.

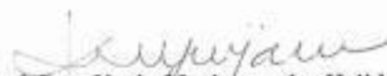
Apresentado a este Comitê para análise ética, foi considerado:

(X) Aprovado, sendo que este projeto deverá permanecer arquivado por 05 (cinco) anos nesta Secretaria.
( ) Com pendência, devendo o Pesquisador encaminhar as modificações sugeridas, e iniciar a coleta de dados somente após a aprovação do projeto por este Comitê.
( ) Não-Aprovado

Comentário:

- Cabe ao pesquisador elaborar e apresentar ao CEUA-UNINOVE, o relatório final sobre a pesquisa (Lei Procedimentos para o Uso Científico de Animais - Lei Nº 11.794 -8 de outubro de 2008).

São Paulo, 29 de abril de 2014



Prof. Dra. Karin Marie van der Heijden
Vice-Coordenadora do Comitê de Ética no Uso de Animais
Universidade Nove de Julho

8.2. Anexo 2

Effect of the photobiomodulation in the expression of VEGF and smooth muscle actin in cutaneous wounds of diabetic rats.

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Abstract

To understand part of these mechanisms of PBM in diabetic animals, this study evaluated whether PBM causes difference in the expression of vascular endothelial growth factor (VEGF) and mature vessels that express smooth muscle actin (SMA) in cutaneous wounds. Ten Wistar rats were used from the UNINOVE breeding stock. Diabetes was induced with streptozotocin (50 mg / kg) and fifteen days after induction the diabetic animals were anesthetized and two dorsal ulcers were made with 8 mm punch. The animals were randomly divided into 2 groups: Control (untreated ulcer), Laser (laser therapy shortly after performing ulcer using diode laser, $\lambda = 660 \pm 2$ nm, with output power equal to 100 mW and beam area of 0,04 cm^2 (TwinLaser, MMoptics, São Carlos, Brazil), with irradiance of 32.7 mW/cm^2 for 122 s, delivering a dose of 4 J/cm^2). Euthanasia was done 15 days after the injury. Samples were routinely processed for paraffin inclusion and staining with hematoxylin and eosin, immunohistochemistry for SMA and VEGF. The vessels were counted using ImageJ software and the data analyzed statistically. There was no statistical difference between the number of VEGF-labeled blood vessels between the control and laser groups ($p = 0.22$ - Mann-Whitney test). The amount of mature pots labeled with SMA was also similar between the groups ($p = 0.36$ - Mann-Whitney Test). Under the study conditions, at 15 days of tissue repair of diabetic rats, photobiomodulation did not cause any difference in angiogenesis.

Key words: Diabetes mellitus, angiogenesis, phototerapy, lasers, cicatrization

Introduction

LASER (Light Amplification by Stimulated Emission of Radiation, or Amplification of Light by Stimulated Emission of Radiation) is advice that produces an electromagnetic radiation with three characteristics: it is coherent (the photons that make up the beam are in the same phase), monochromatic (well defined wavelength) and is collimated (propagates with a beam of parallel waves. Since the late 1960s the laser has been used as an alternative therapy to aid the healing process[1].

The literature supports the mechanisms of action of low-power laser therapy in cells and tissues: increase of adenosine triphosphate (ATP), increase of cell membrane permeability, viability of calcium influx, regulation of growth factors and inflammatory cytokines , Stimulation of cell differentiation and proliferation, induction of collagen synthesis and remodeling, increase of tensile strength and angiogenesis[2][3]

However, the literature is still controversial as to whether or not the low-intensity laser influences VEGF synthesis. Brassolati et al., 2016 showed that FBM at a dose of 25 J / cm 2 and potency of 1 J favored the increase of VEGF in 3rd degree burns in rats{Brassolatti[4], 2016 #27}. Tim et al., also observed an increase in VEGF expression after laser applications with an intensity of 2.8 J for 94 seconds in rat calvarial wounds[4]. However, Marques et al., 2015, studying the same model of rat calvaria, and using doses of 16 J/cm^2 of low intensity laser found no differences in VEGF labeling[5].Silva et al., 2010, showed an increase in VEGF messenger RNA expression during the tissue repair process after two irradiations with a wavelength of 780 nm, 70 mW of power and a dose of 35 J/cm^2[6].

In an experimental model of Achilles tendon lesions, no differences were found in the expression of VEGF in the groups that received laser at the dose of 17.5 cm^2.[7]

An in vitro study with human endothelial cells showed that the laser at low intensity decreased the amount of VEGF in the supernatant medium and increased cell proliferation [8]

In a previous study of our group, it was observed that low intensity laser induced angiogenesis and tissue repair in 3rd degree burns in rats, as much as the laser was applied once or in 4 alternate days[9]. Then, we compared these two light delivery regimes in wound healing in diabetic rats, showing that in the wounds that received the single dose of laser, totaling 4 J (wavelength 660 nm, 30 mW power), there was greater chemotaxis for macrophages And neutrophils than the group receiving the 4 J/cm^2 divided into doses of 1 J/cm^2 daily on alternate days. At the end, collagen analysis demonstrated that a single dose of 4 J favored tissue repair in diabetic animals[10, 11]

The present work was carried out with the objective of clarifying if the laser in low intensity promotes the aniogenesis in the tissue repair by VEGF expression or by the differentiation of mature, SMA positive vessels.

Materials and Methods

Animals

This study was approved by the Animal Research Ethics Committee of University Nove de Julho (Brazil, process number: ANS 006/2014) and was performed in compliance with Brazilian ethical principles for animal experimentation. Ten female adult Wistar rats (body mass: 250 ± 50 g) were

kept in plastic cages with five animals each, free access to water and ration and a 12-h light/dark cycle.

Chemical induction of diabetes

Diabetes was induced according to the following protocol: fasting for 12 h with free access to water, then perform 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). On a weekly basis blood glucose levels were measured. Animals with fasting blood glucose greater than 220 mg/dL and stable body mass after one week were selected for the experiment.

Injury model

Anesthesia was performed with80 mg/kg of ketamine HCl (Dopalen, Vetbrands, SP, Brazil and 10 mg/kg of xylazine (Anasedan, Vetbrands, SP, Brazil). Fur from the back of each animal was removed with an electric shaver, then the skin was then cleaned with a 0.12% chlorhexidine solution. An 8 mm surgical punch (Richter, SP, Brazil) was used to produce two parallel round wounds in the dorsum. Until complete recovery from the anesthesia the animals were maintained on a warm plate (37° C) to prevent hypothermia [10].

Experimental groups

The animals were divided into two groups with five animals each: the control group, where the ulcers did not receive treatment and the laser group,

where the dorsal ulcers received laser therapy immediately after the wound was performed.

Laser system

A diode laser was used with the following parameters, λ = (660 ± 2) nm, with output power equal to 100 mW and a beam area of 0.04 cm^2 (TwinLaser, MMoptics, São Carlos, Brazil), with irradiance of 32.7 mW/cm^2, applied for 122 sec, delivering a dose of 4 J/cm^2. To avoid double exposure, a box with a circle of 8 mm diameter was used, thus allowing the irradiation of one wound at a time.

The choice of the experimental day occurred because it represents a crucial moment of the tissue repair, being day 15 representative of the proliferative phase.

Immunohistochemistry and inflammatory cell counts

Five animals from each group were euthanized on Day15with an overdose of anesthesia. The injured tissue was removed, fixed in 10% buffered formalin (pH7.4) and embedded in paraffin.

Serial sections of paraffin-embedded tissues (3 µm) were placed on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma-Aldrich, St. Louis, MO) and de-paraffinized 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-SMA (Abcam # 5694) and with anti-VEGF-A (Abcam # 1316).

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).

After the immunohistochemical marking, 5 consecutive fields of the wound area, always from the left border to the center, traversing the entire area of the lesion, were photographed in an increase of 400 x (Leica Microsystems, Wetzlar, Germany) and the images of the cells (Free software, NIH, Bethesda, Maryland, USA) using the pluggin "cell counter". 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.

Statistical analysis

The data of marked vessel counts were submitted to appropriate statistical tests to evaluate the difference between the control group and the low intensity laser. The statistical distribution of the data was assessed using normality of Shapiro-Wilk. The data were non-parametric and the Mann-

Whitney test was used to compare the different groups. The level of significance was set at $p < 0.05$. All data analysis was performed with SPSS® software (SPSS Statistics v23, IBM, USA)

Results

On Day 15, the wounds of both groups were in the proliferative phase of tissue repair, characterized by the presence of angiogenesis and fibroblasts (Figure 1).

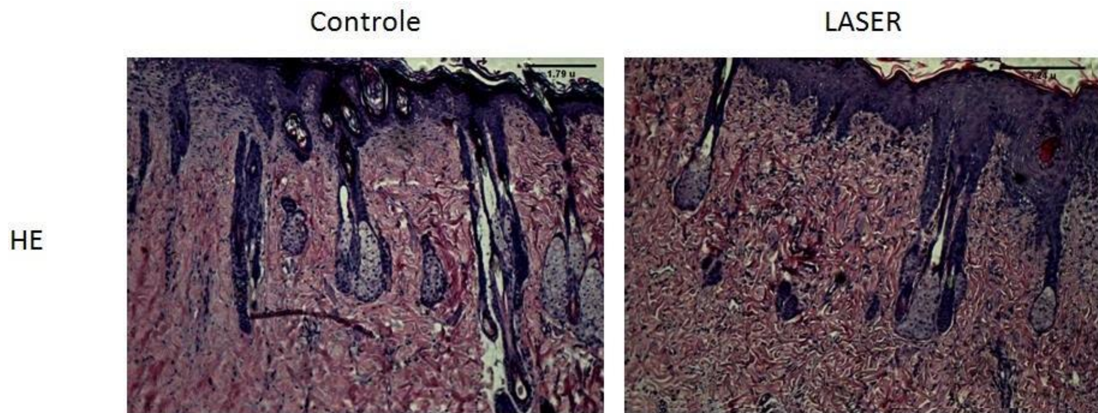


Figure 1: Photomicrographs of the wounds showing granulation tissue, characterized by angiogenesis and fibroplasia. (Hematoxylin & Eosin, original magnification 100 x)

The VEGF positive cells were observed both in the epithelial and connective tissue. The control group and laser group revealed abundant VEGF positive blood vessels (Figure 2) with no statistical difference between the groups ($p = 0.22$ - Mann-Whitney test) (Figure 3).

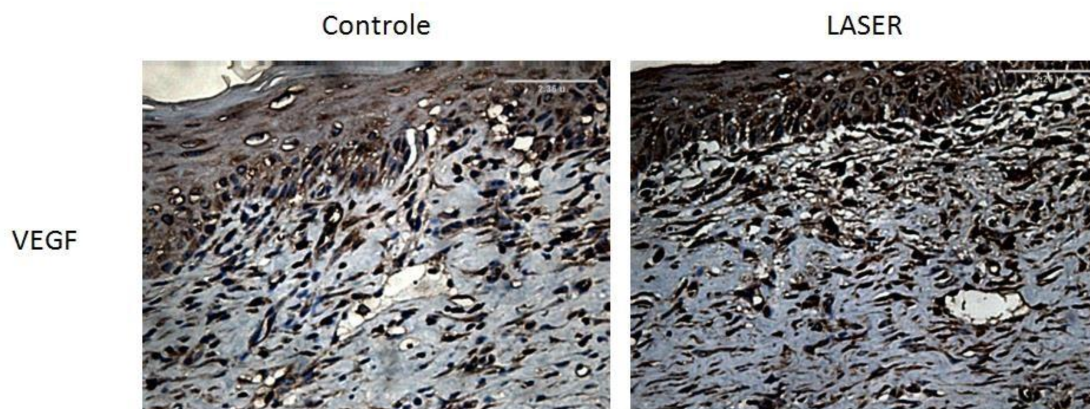
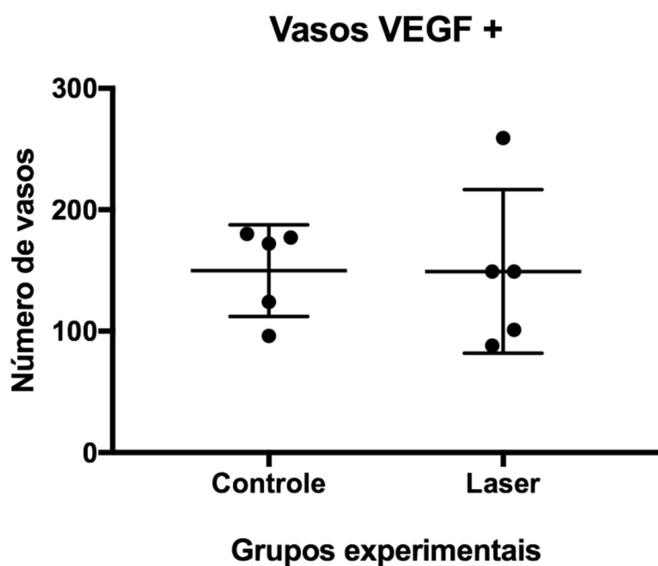


Figure 2: Photomicrographs of the VEGF positive blood vessels, showing similarity between the groups control and laser on day 15 ($p = 0.22$, Mann-Whitney test). (Original magnification 400 x)



The SMA positive cells and cells were observed the connective tissue. The control group and laser group revealed abundant SMA positive blood vessels (Figure 4) with no statistical difference between the groups (p = 0.36 - Mann-Whitney test) (Figure 5).

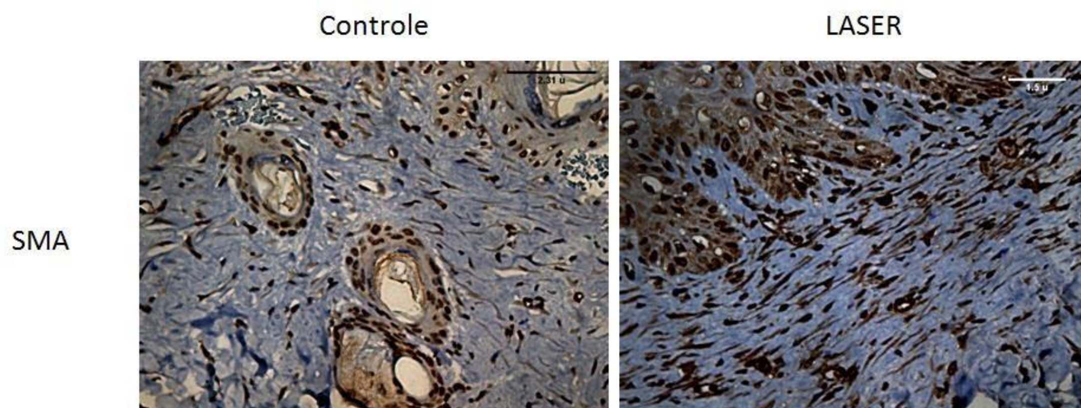
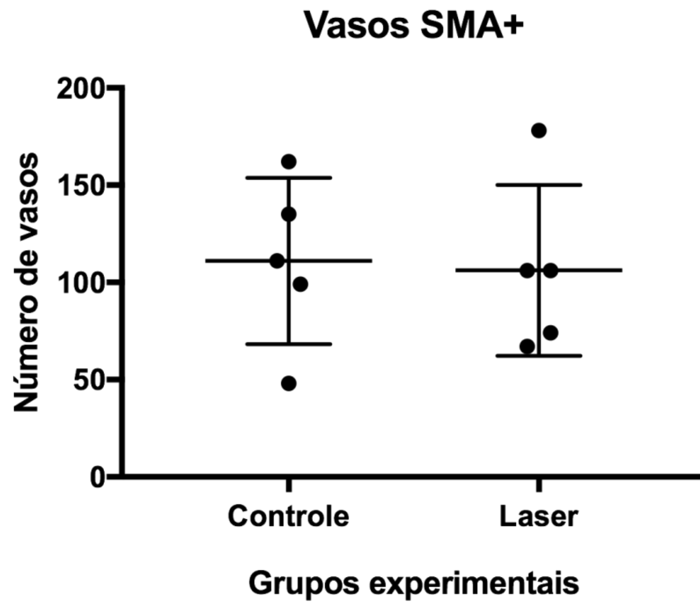
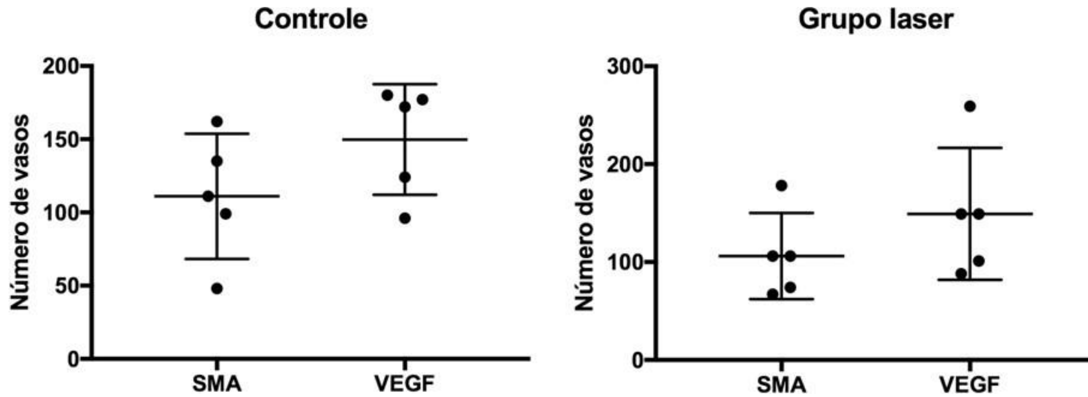


Figure 4: Photomicrographs of the SMA positive blood vessels, showing similarity between the groups control and laser on day 15 (p = 0.36, Mann-Whitney test). (Original magnification 400 x)



There was no difference between the number of vessels detected with VEGF and SMA showing that both antibodies are sensitive to label vessels (p = 0.65 - Mann-Whitney test) (Figure 6).



Discussion

This study demonstrated that in diabetic rats at day 15 of cutaneous repair there are no significant differences in the quantification of VEGF and in the number of mature arterioles when comparing the laser treated group and the untreated group.

The literature is still controversial when we speak of photobiomodulation in the expression of vascular endothelial growth factor. There are a number of studies that exhibit conflicting results and none of them match the parameters. It has been shown that FBM can both increase angiogenesis and have no effect on angiogenesis. 45-9 This depends on the tissue, the moment of application of the light and the delivered dose54.

Brassolati et al. (2016), Tim et al., 2016 and Silva et al., 2010 found positive results in the expression of VEGF using the laser at low intensity45,46,47, but Marques et. Al. Did not find differences in the marking of this growth factor after laser application48. Another in vitro study with human endothelial cells showed that the laser at low intensity decreased the amount of VEGF in the supernatant medium, but increased the cell proliferation50.

The fact that there is no statistical difference in the quantification of vessels labeled with VEGF and SMA indicates that both antibodies were very sensitive in the detection of blood vessels. In addition, labeling in other tissues was all within the literature described, with VEGF being present in epithelium and muscle, while SMA was present in endotheliocytes, smooth muscle cells, pericytes and myofiboblasts.

Likely explanations of the findings of the present study may be the dose, which should be higher, or more frequently. Another possibility would be that there is an anticipation of the angiogenesis peak in the laser group for day 8, and this was not detected on day 15. Or that the laser actually interferes with other factors, such as the production of TGF-beta, and not With the VEGF pathway. All these hypotheses need to be tested in future experiments.

One limitation of this study is the analysis of only one experimental day. The ideal would have been the analysis of day 8 and 22 to observe if there is an advance of the onset of angiogenesis in the laser groups or if there is a delay in the apoptosis of the blood vessels at the end of the repair, with 3 weeks. Another interesting proposal would be to evaluate the angiogenesis generated by the LED, as did Corazza et al., 2007 that compared the angiogenesis in treated circular wounds or with Laser or LED and verified that angiogenesis was superior in all groups that received light, showing That the coherence of light is not essential for the formation of new blood vessels.[27]

Diabetic animals have some peculiarities and because of their nature suffer from severe dehydration, we realized throughout the work that it would be interesting to make a replacement of mineral salts and water by means of subcutaneous injection of saline solution. Due to the polyuria it is necessary to change one or more times a day of the shaving and also the reduction of animals per box from 5 to 2 or at most 3. For the next work we suggest that the mesh or the granulate is used.

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8.3. Anexo 3

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RESEARCH ARTICLE

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

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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 50th 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/cm²	4 J/cm²
Laser exposition time		26 s	104 s
Treatment frequency		Four times	Once

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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., 22nd 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 (Δn) of the samples in nm, the angle (α) read in the microscope is multiplied by 3.03 nm [32]. Five α 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

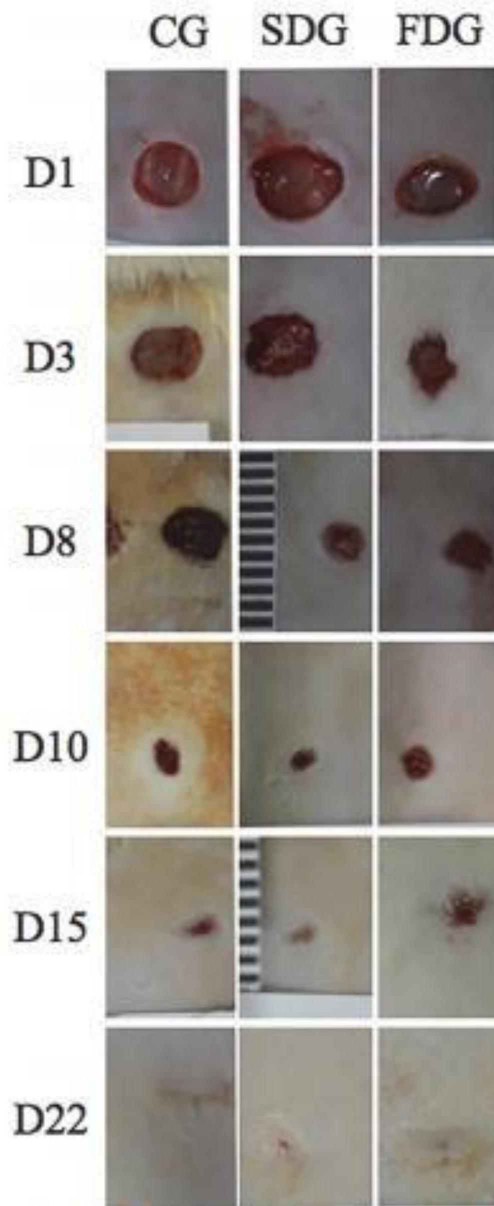

Fig 1. Wound healing in different groups throughout experiment (D = Day, CG = Control Group, SDG = Single Dose Group, FDG = Fractionated Dose Group). Original magnification 10x)

doi:10.1371/journal.pone.0122042.g001

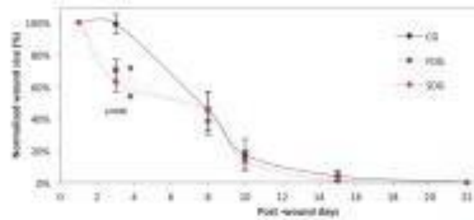

Fig 2. Percentage of normalized wound closure throughout experiment demonstrating the effect of laser therapy in the early tissue repair process. (mean ± SEM, $p<0.05$)

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

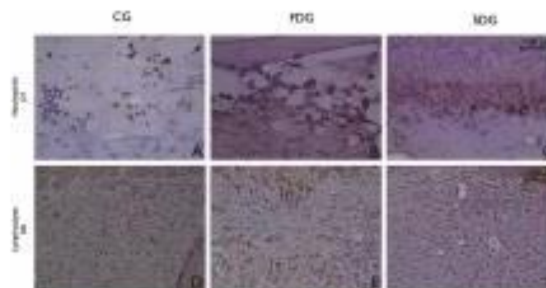

Fig 3. Histological examination of healing tissue—Day 3: all groups in inflammatory phase of tissue repair (A, B, C), with a crust over the ulcer and an intense inflammatory infiltrate (*); Day 8: wounds in proliferative phase with granulation tissue (D, E, F); Some samples in FDG exhibited acute inflammatory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

doi:10.1371/journal.pone.0122042.g003

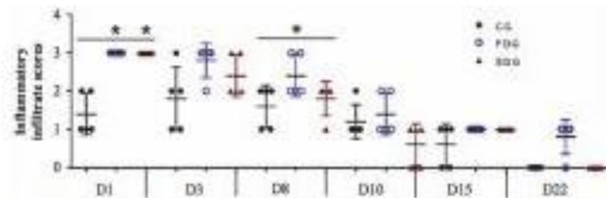

Fig 4. Inflammatory infiltrate score—SDG exhibited more leukocytes than other groups through to Day 10.

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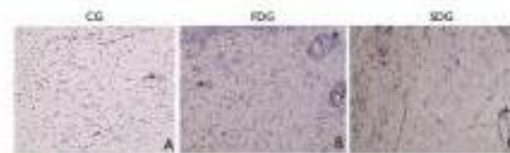

Fig 5. Immunohistochemical analysis with anti-α smooth muscle actin; myofibroblasts within granulation tissue stained brown; arrow heads point to smooth muscle in hair follicles; asterisks indicate smooth muscle in arterioles used as internal positive control of reaction; immunohistochemistry with DAB;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

doi:10.1371/journal.pone.0122042.g005

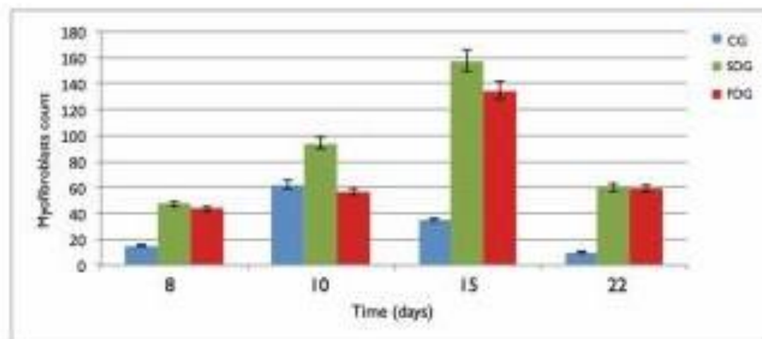

Fig 6. Myofibroblast count showing more cells in both laser groups on Days 8, 15 and 22 in comparison to control; SDG exhibited more myofibroblasts than other groups on Days 10 and 15. Bars represent mean counts with respective standard deviation values.

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.

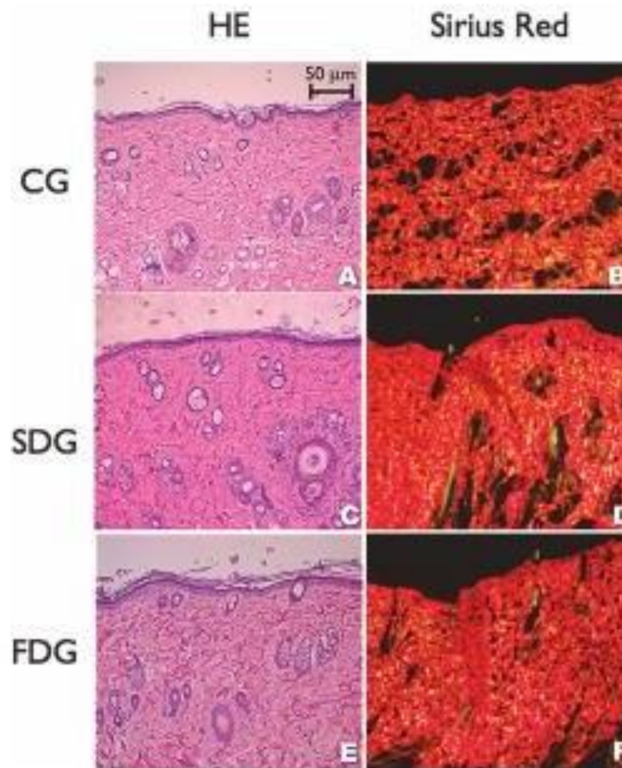

Fig 7. Morphology of wound healing on Day 22, showing similarity among experimental groups (hematoxilyn and eosin—A, C, E; Picrosirius Red—B D, F)

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 studies in the literature report an increase in collagen synthesis and deposition following laser therapy [20]. In the present investigation, however, collagen organization was significantly affected by laser therapy as well as the energy density. A single laser application of 4 J/cm² after surgery led to better collagen organization in the scar tissue, demonstrating that the initial inflammatory events following an injury are crucial to the final modulation of the repair process. The fractionated dose (four applications of 1 J/cm²) also led to improved final scar quality, but the energy density in the inflammatory phase may not have been sufficient to accelerate this phase of the tissue repair process in rats with diabetes. However, cells and tissues under stress are more prone to be influenced by laser biomodulation and low fractionated doses are believed to be better than a single dose for "normally responding" tissue [16].

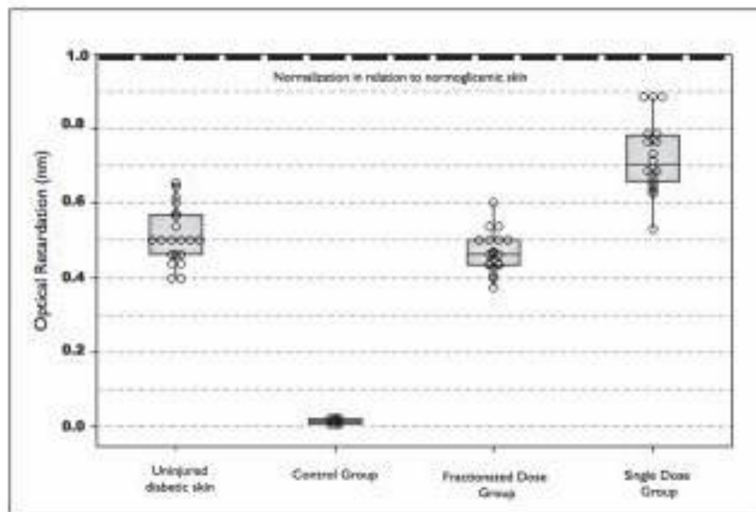

Fig 8. Optical retardation analysis of collagen on Day 22, showing most organized collagen in SDG, followed by uninjured skin, FDG and CG

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.

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8.4. Anexo 4

7 - ORIGINAL ARTICLE
WOUND HEALING

Use of low-power laser to assist the healing of traumatic wounds in rats[1]

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ABSTRACT

PURPOSE: To investigate the morphological aspects of the healing of traumatic wounds in rats using low-power laser.

METHODS: Twenty four non isogenic, young adult male Wistar rats (*Rattus norvegicus*) weighing between 200 and 300g was used. The animals were randomly distributed into two groups: Control (GC) and Laser (GL), with 12 animals each. After shaving, anesthesia was performed in the dorsal region and then a surgical procedure using a scapel was carried out to make the traumatic wound. GL received five sessions of laser therapy in consecutive days using the following laser parameters: wavelength 660 nm, power 100 mW, dose 10 J/cm ^{2}. The wounds were evaluated through measurement of the area and depth of the wound (MW) and histological analysis (HA).

RESULTS: When comparing the GC with the GL in MW there was a difference in area ($p<0.001$) and depth ($p=0.003$) measurement of the wounds in GL. The laser group presented more epithelization than GC ($p=0.03$). The other histological parameters were similar.

CONCLUSION: The healing of wounds in rats was improved with the use of the laser.

Key words: Laser Therapy. Wound Healing. Rats.

Introduction

A wound is the loss of continuity of a body tissue with varied etiology, which can reach from the epidermis to deeper structures such as muscles, tendons and bones. Wound treatment is probably one of the oldest areas of medicine[1].

Classification of wounds includes the evolution (acute or chronic); the presence of infection (uncontaminated clean, clean contaminated, contaminated or dirty and infected); the depth (stage I, II, III and IV) and/or the cause (intentional or unintentional and surgical or traumatic)[2,3].

Acute traumatic wounds can result in injuries with extensive skin loss and damage to tissue viability or exposure of important organs. Wounds comprise a major cause for the use of hospital emergency services: 11 million patients treated annually in emergency departments in the United States are for this cause[2].

The population most affected by traumatic wounds is economically active, aged 20 to 59 years, resulting in a significant social and economic impact, because during the treatment period these individuals are restricted from their work activities, and even after recovery, chronic sequelae, as aesthetic and functional limitation, may be present[2,3].

The treatment and management of traumatic wounds depends on their characteristics, extent, the general conditions of the patient and the conditions of care. Daily cleaning, debridement and even surgical reconstruction are common practices and there may be an association with other adjuvant treatments such as hyperbaric oxygen therapy, negative pressure and the use of hydrocolloids and gels[4-8].

Irradiation with low-power laser has been used in clinical practice as a complementary tool to enhance healing with positive cosmetic and anti-inflammatory effects in various types of injuries, such as epithelial, muscle and bone repair.

The biomodulator effect of the laser is based on the theory that photon energy is absorbed by a photoreceptor cell, for example oxyhemoglobin, hemoglobin, cytochrome c oxidase, and melanin cells, since the energy of photons is absorbed by these cells. The molecule assumes a electronically excited state and that energy is converted into chemical energy within the cell. These events are biomodulated in the different types of cells involved in tissue repair and include increased neoangiogenesis, changes in cytokine synthesis and aid in the conversion of fibroblasts in myofibroblasts[10-14].

The objective of this study of the macro and microscopic effects of laser therapy was to investigate the closure of traumatic wounds in rats.

Methods

This study was approved by the Ethics Committee and Ethics Studies and Research (CEDEP)/Ethics Committee on Animal Use (CEUA) of the Federal University of San Francisco Valley - UNIVASF, under protocol number 0001/170913.

The sample consisted of 24 male Wistar rats (*Rattus norvegicus albinus*), weighing between 200 and 300 grams. The animals came from the central vivarium of the University and remained in adaptation for six days in the Nucleus for Experimental Surgery laboratory, of the Federal University of San Francisco Valley (UNIVASF).

Surgery and experimental groups

The animals were anesthetized by intraperitoneal injection of ketamine and xylazine (80 mg/kg and 10 mg/kg body weight), and tramadol hydrochloride (4 mg/kg body weight). Once anesthetized, the dorsal region of each animal was sterilized with alcohol-iodine prior to trichotomy. The tip of a scalpel was used to make a longitudinal incision (5 x 3 cm) at a depth of 6 mm along the right portion of the back of the animal and the fragment removed to expose the muscle and laceration of the dorsal muscle fibers to similate a traumatic wound. A pilot study provided the standard for the operations and all operations were carried out by the same person.

After completion of surgery, animals were housed individually in polypropylene cages with standard food and water *ad libitum*, under controlled temperature and moisture with the light cycle automatically adjusted every 12 hours. And after recovery from anesthesia, the rats were randomly distributed into two groups, with 12 animals each:

- CG: control group, untreated, only subjected to handling and physical restraint of five sessions on consecutive days, simulating laser application;

- GL: Laser Group, treated with five applications of low level laser on consecutive days, using the following parameters: laser diode (Photon Laser III, DMC, São Carlos, Brazil), wavelength 660 nm, visible red, power 100 mW power density per point of 10 J/cm^2 and irradiation time of 20 seconds per spot with a total dose of 2 J/dot.

Macro and microscopic evaluation of the wound

After inflicting the wound, all animals were subjected to an assessment on the first day (D1) and were reassessed on the sixth day (D6) after the end of each respective group procedures.

The measurements of the wound were performed using a digital caliper with an accuracy of 0.01 mm, from which the largest transverse (CT) and longitudinal lengths (CL) were collected from each lesion, where the product (CL x CT) of these measures resulted in the area expressed in mm^2. The depth values were expressed in absolute display obtained using the lower shaft of the caliper, given in millimeters.

At the end of the sixth day the animals were euthanized with an overdose of anesthetic (4 times the dose required for analgesia) and then the fragments of skin were removed and fixed in 10% paraformaldehyde and embedded in paraffin and hematoxilia and eosin staining for performing routing histology. A pathologist blinded to the groups did two evaluations in different weeks. In cases of disagreement scores, a third evaluation was made.

After making the slides, the following histological parameters were analyzed: reepithelialization, intensity of inflammatory infiltrate, presence of granulation tissue and neoangiogenesis. The scores were established according to the methodology of Melo *et al.*[19], namely: (+1) = less than 10%; (+ 2) = between 10 and 50%; (+3) = More than 50%.

Statistical analysis

The original data were transferred to a database in Microsoft Excel (version 2010), where statistical tests were performed. Part of the statistical analysis was performed using the software program Statistical Package for Social Science version 10 (SPSS 10) for Windows.The Shapiro-Wilk test was used first to assess the normal distribution (Gauss) variables; then the Student t-test was applied to the values obtained from the measurements; scores were analyzed with the Wilcoxon test, and all tests were considered to be statistically significant at $p<0.05$.

Results

During the protocols of the two groups, surgical procedures were carried out without complexities, with no deaths. Anesthetic recovery was satisfactory with maintenance of general health and appetite.

The macroscopic evaluation consisted of measurement of the wound area performed with calipers and expressed in graphics, as shown in Figure 1, which is the average of the averages of these areas assessed by Student t test unpaired. It was observed that the areas were reduced in sixth day compared with the first day, and also that there was a difference between the groups ($p<0.001$).

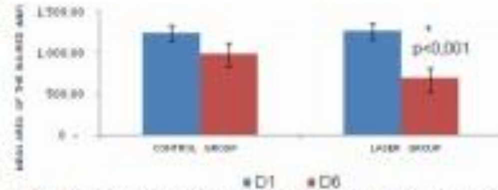

FIGURE 1 - Graph comparing the values of mean wound areas in the two periods studied showing the same decrease between days and a statistical difference between groups ($p<0.001$).

Regarding the depth of the wound, the laser group showed a decrease of the same in relation to the control group ($p = 0.003$, t Student test), as shown in Figure 2.

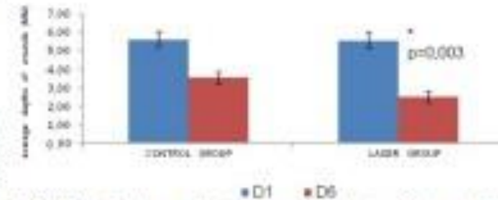

FIGURE 2 - Graph comparing the values of the depths of the wounds in the two periods studied showing that both groups underwent a decrease in the average depth of the wound on day 6, and the laser group showed a statistically greater decrease ($p = 0.003$, t Student test).

Regarding the descriptive microscopic analysis, histological sections revealed that on day 1, the traumatic wounds of both groups were in the inflammatory phase of repair; inflammation of moderate to intense. On day 6, the wounds were in the proliferative phase of repair, showing granulation tissue with angiogenesis and proliferation of fibroblasts, with complete or almost complete reepithelialization, especially in the laser group of animals (Wilcoxon test - $p = 0.03$) wounds (Figure 3).

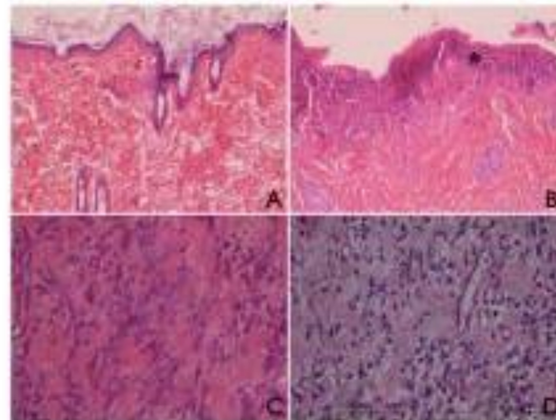

FIGURE 3 - Photomicrograph of histological section of the wounds on day 6 of GL (A) showing complete epithelialization, with a thin epithelial layer, keratin plates and skin appendages. In the same period, the wounds of the GC (B) showed acute inflammatory infiltrate (*). Granulation tissue was similar in both the laser group (C) and control group (D).

The parameters of inflammatory infiltrate, vascular proliferation and fibroblasts were similar between groups, with a statistical difference in the reepithelialization (Table 1) variable.

TABLE 1 - Table showing the correspondence of histological scores and the amount of wounds The CG and GL, on day 6 (after the protocol in each group):

Score	Reepithelialization		Inflammatory infiltrate		Angiogenesis		proliferation of fibroblasts	
	Control	Laser (p=0,03)	Control	Laser	Control	Laser	Control	Laser
+1	4	3	0	2	10	11	10	11
+2	5	7	8	8	0	1	0	0
+3	1	2	2	2	0	0	0	1

Discussion

The present study demonstrated the use of laser in the healing of traumatic wounds with secondary intention healing. This finding was confirmed by both clinical measures and by histological evaluation.

These findings corroborate with the literature, where the use of the laser increases wound contraction with apparent acceleration of the repair process and delineation of visible scar scabbing and raised edges and reddish center, due to the presence of blood flow in the treated area[13], due to the granulation tissue[14].

The histological analysis found increased reepithelialization in the animals that received laser, indicating progression of almost-full to full and regular healing and the normal presence of skin appendages. This finding is important because the epithelial barrier prevents the installation of infectious processes, which constitute one of the common complications of wounds[22].

In the study by Carvalho *et al.*[11] the wounds were sutured and healed by first intention, therefore the number of inflammatory cells in the group irradiated for seven days was less evident. In the present work, the wounds were larger in size and healed by secondary intention and reduction of the inflammatory infiltrate was similar between GL and GC groups, indicating that laser therapy is effective in both healing processes, either by modulating the inflammatory process in repair by first intention, and increasing epithelialization repair by secondary intention[11].

The laser parameters were chosen based on literature, which indicates that doses of between 1 and 10 J/cm^2 are the most favorable for repair biomodulation[17]. Lower doses would not take effect and might induce larger doses inhibiting cell activity, delaying the closing of the wound[15,16,17].

From the results it can be stated that the laser is an adjunct in the treatment of complex wounds, accelerating the closure of the same and favoring the restoration of morphofunctional tissue.

Conclusion

Laser therapy can be an adjunct to the healing of traumatic wounds, especially by accelerating reepithelialization of traumatic wounds.

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8.5. Anexo 5

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

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

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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.

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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.

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During the normal repair process, neutrophils undergo apoptosis, and are recognized and ingested by macrophages. As the number of neutrophils decreases, the number of T lymphocytes and macrophages increases, which become the predominant cell type in the inflammatory 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 inflammatory infiltrate was the same across groups); by Day 8: the FD has a higher inflammatory infiltrate score but not difference in neutrophils. On day 15, the number of myofibroblasts began to decrease in the control group, but continued to increase in the laser groups. PBM induced a 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

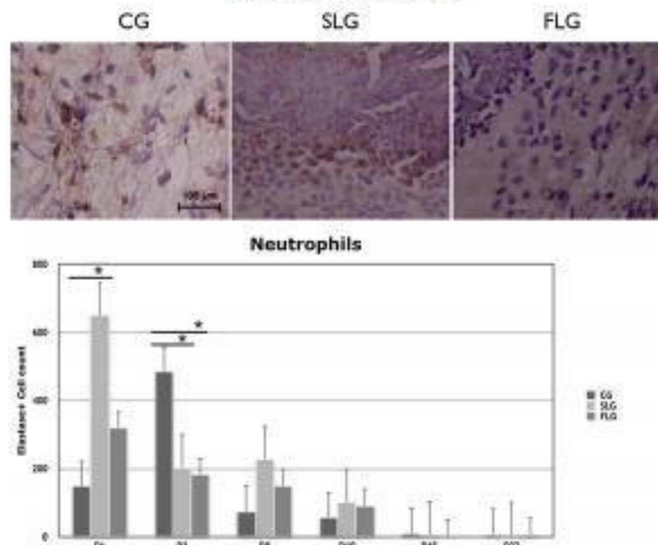

Fig. 1. Photomicrographs of neutrophil count; results expressed as mean ± SD; Predominance of neutrophils in SLG on Day 1; Significantly more neutrophils in CG on Day 3; Progressive decrease in neutrophil count similar in all groups beginning at Day 8 (Kruskal–Wallis test with Dunn's *post hoc* test);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

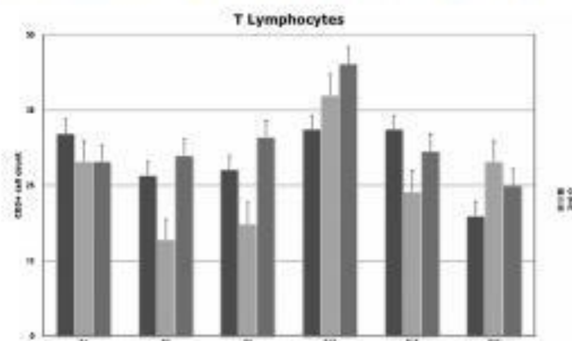

Fig. 2. T lymphocyte (CD3 + cells) count; Results expressed as mean ± SD; similarity among all groups throughout experiment (Kruskal–Wallis test with Dunn's *post hoc* test); CG, control group; SLG, single-dose laser group; FLG, fractionated-dose laser group.

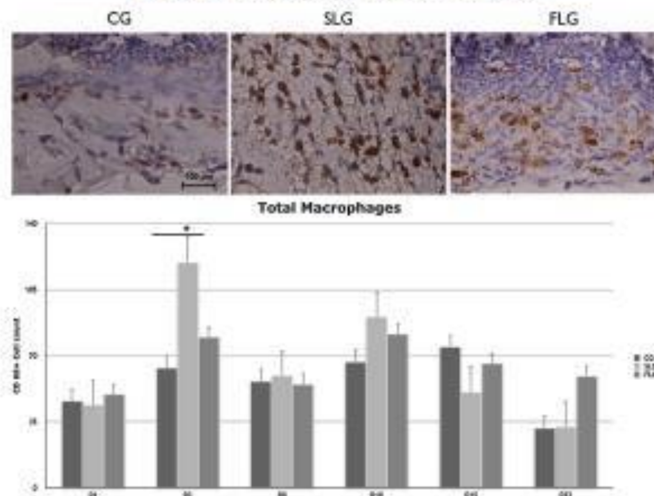

Fig. 3. Photomicrograph of CD68 + cells count (pan macrophage marker); Results expressed as mean ± SD; SLG exhibited higher total macrophage count ($P < 0.05$) on Day 3. Similar macrophage count in all groups at other evaluation times (Kruskal–Wallis test with Dunn's *post hoc* test); CG, control group;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

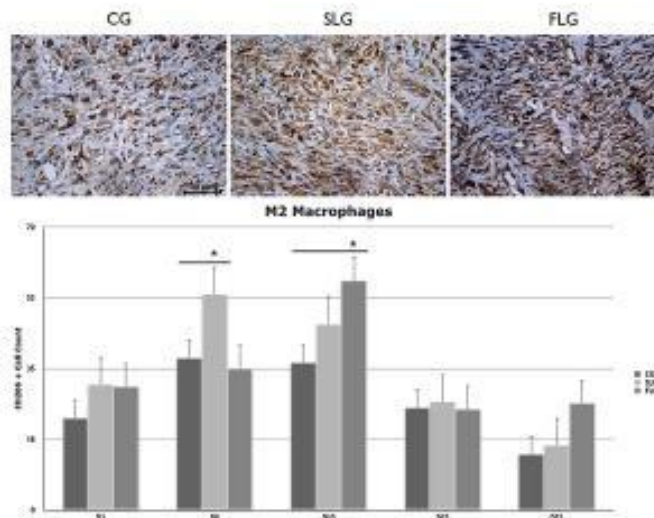

Fig. 4. CD206 + cell count (M2 macrophage marker); Results expressed as mean ± SD; SLG exhibited more antiinflammatory macrophages than CG on Day 8 ($P < 0.05$). FLG exhibited more anti-inflammatory macrophages than CG on Day 10 ($P < 0.05$). No significant differences in M2 macrophage counts among groups 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 (T regs—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 4 J/cm^2 was able to recruit more macrophages on Day 3, which is in agreement with data described by Sahrifian et al. (2014), who found that low doses of an infrared pulsed laser were able to increase the macrophages count on Day 4 following surgery in diabetic animals [19].

Macrophages in the wounds of diabetic mice exhibit an impaired transition from M1 pro-inflammatory to M2 pro-healing phenotypes, which may contribute to the 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.

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