UNIVERSIDADE FEDERAL DE SÃO CARLOS-UFSCAr

PROGRAMA DE PÓS-GRADUAÇÃO EM FISIOTERAPIA

DEPARTAMENTO DE FISIOTERAPIA

José Carlos Tatmatsu Rocha

Efeitos da Fototerapia em Marcadores Inflamatórios, na Função Mitocondrial e no Estresse Oxidativo em Roedores com Diabetes Induzida

São Carlos - SP

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Tese apresentada ao Programa de Pós-Graduação em Fisioterapia da Universidade Federal de São Carlos, para obtenção do Título de Doutor em Fisioterapia. Área de Concentração: Processos de avaliação e intervenção em Fisioterapia do sistema músculo-esquelético.
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São Carlos - SP

2016

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária UFSCar
Processamento Técnico
com os dados fornecidos pelo(a) autor(a)

R672e

Rocha, José Carlos Tatmatsu
Efeitos da fototerapia em marcadores inflamatórios, na função mitocondrial e no estresse oxidativo em roedores com diabetes induzida / José Carlos Tatmatsu Rocha. -- São Carlos : UFSCar, 2016.
115 p.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2016.

1. Catalase. 2. Diabetes mellitus. 3. Espécies reativas de oxigênio. 4. Colágeno. 5. Óxido nítrico. I. Título.

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Fisioterapia

Folha de Aprovação

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Prof. Dr. Paulo Sérgio Bossini
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Apoio Financeiro

Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP)

Coordenação e Aperfeiçoamento de Pessoal de Nível Superior - CAPES

A Deus, pela oportunidade de viver e de compreender nossas falhas e limitações, buscando compreender também de modo fraterno as limitações do meu próximo.

À minha família, em especial minha esposa, agradeço por conseguir me suportar ao longo de 15 anos de casamento, e a meus filhos, por terem pais tão próximos mas às vezes tão longe ...

Aos amigos que conseguem nos compreender e dar o alento necessário em tempo oportuno.

AGRADECIMENTOS

A realização desta Tese só foi possível graças ao apoio e colaboração direta ou indireta de muitas pessoas, em especial:

Ao Professor Nivaldo Parizotto, por acreditar em meu potencial e pelo seu trabalho que tornou a área de Fototerapia no Brasil respeitável mundialmente. Meus agradecimentos e afeto.

Às Professoras Patrícia Druso e Lucimar Avo e ao professor Rubens Bernardes Filho, pela paciência e pela disponibilidade em nos receber sempre que precisamos.

Aos membros da Banca por estarem dispostos a contribuir com suas considerações e sugestões que são de grande relevância para o enriquecimento deste trabalho.

Aos colegas e amigos do laboratório de Recursos Fisioterapêuticos, pelos momentos bons e ruins que passamos juntos e pela grande troca de experiências que me proporcionaram ao longo do doutorado, em especial a Carla Roberta Tim, Hueliton Kido, Patrícia Brassolatti, Lia Mara Neves, Cintia Martignago, Ana Laura Martins e Cynthia Castro.

Aos meu amigos e colegas da Harvard Medical School/Massachussetts General Hospital Cleber Ferraresi e Fernanda Freire pela troca de experiências enriquecedoras e pelos momentos de apoio mútuo, meu muito obrigado!

Aos amigos de Boston, Margarida Rue, Maria Cida Champlin e Calvin James Champlin, cuja amizade, apoio e generosidade foram fundamentais para que minha família pudéssemos seguir nossa jornada nos EUA. Com vocês aprendemos o significado da palavra Ser Humano.

A família Fernandes, aos amigos do grupo de voluntários do Grupo Posto de Rua de São Carlos, Cauê e Taty Ribeiro, Anália Rosa Gussen, Solange e Luiz Hortenci, cuja amizade, carinho e atenção nos fizeram sentir parte de suas famílias.

A Iolanda da Silva Vilela, funcionária do Departamento de Fisioterapia, cujo trabalho dedicado faz do Departamento de Fisioterapia da UFSCAR um ambiente mais humanizado.

Aos meus pais que sempre me apoiaram nos estudos, a Fernando Brito meu cunhado que deu o suporte familiar necessário no período de ausência da família

À minha esposa Daniely Tatmatsu, por sempre estar ao meu lado me incentivando e apoiando nos momentos mais difíceis, para mim um exemplo de profissional, mãe e mulher. Muito obrigado por me escolher como seu marido e pai dos nossos amados filhos.

Aos meus filhos, Nathália Brito Tatmatsu e Benjamin Brito Tatmatsu, minhas paixões e orgulho. Obrigado por serem filhos maravilhosos que muito me orgulham e pela paciência (embora nem sempre consciente) que tiveram comigo ao longo desta jornada.

Á Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico – FUNCAP e à Universidade Federal do Ceará-UFC que apoiaram e financiaram minha permanência na cidade de São Carlos, o que foi essencial e decisivo para a conclusão desta pesquisa. Espero que os resultados possam contribuir para o crescimento científico e social de nosso País.

A CAPES e ao professor Michael R. Hamblin, que apoiaram a pesquisa de doutorado sanduiche na Harvard Medical School/Massachussetts General Hospital, cuja experiência me mostrou novos horizontes a percorrer. A todos, meus mais sinceros agradecimentos.

Ninguém cruza nosso caminho por acaso e nós não entramos na vida de alguém sem nenhuma razão.

Chico Xavier

ABREVIATURAS E SÍMBOLOS

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

ANOVA: Analysis of variance

AsGa- Arsenite Galium

ATP: Adenosine triphosphate

Ca^{2+}: Calcium ion

CCO- citochrome C oxidase

COX2 – cyclooxygenase-2

DLASER - diabetic Laser treated

DLASER- diabetic Laser treated

DLED-diabetic Light-emitting diode treated

DMT1: DM mellitus Type 1

DMT2: DM mellitus Type 1

DNA: Deoxyribonucleic Acid

DRP1- Dynamin-1-like protein

ECM: extracellular matrix

FIS1 - Mitochondrial fission 1 protein

GLP-1 : Glucagon-like peptide- 1

GLUT: glucose transporter

GLUT4:glucose transporter 4

GTT: Glucose tolerance test

IDF- international Diabetes Federation

IGF: Insulin-like growth factor

IL: Interleukin

iNOS- nitric oxide isozyme

ITT: Insulin tolerance test

Kg: kilogram

KITT: glucose decrease constant

L: liter

LED – Light-emitting diode

LLLT - Low level laser therapy

MFN1- Mitofusin 1

MFN2- Mitofusin 2

mg: milligram

mL: milliliter

MMPs: Matrix metalloproteinases

NAD- Nicotinamide adenine dinucleotide

NFkappa: factor nuclear kappa B

NID – no irradiated diabetic

nm: nanometer

NO – nitric oxide

NOS - nitric oxide species

OPA1- Dynamin-like 120 kDa protein

RNAm: Messenger Ribonucleic acid

ROS – reactive oxygen species

rpm: Revolutions per minute

SHAM – placebo / no treated

STZ - streptozotocin

TBARS - thiobarbituric acid

TNF- α: Tumor necrosis factor

VEGF- Vascular endothelial growth factor

WHO– world health organization

α: alfa

β: beta

μ: micro

RESUMO

O reparo tecidual em pacientes diabéticos é prejudicado por vários fatores, dentre eles a deficiência da secreção de insulina que conduz à hiperglicemia e consequente produção de radicais livres. A disfunção mitocondrial tem sido apontada como um dos prováveis fatores etiopatogênicos do DM mellitus. Sabe-se que a Fototerapia tem efeitos proliferativos em tecidos com algumas patologias. O presente estudo teve como objetivo avaliar os efeitos da Fototerapia utilizando-se Laser e LED de baixa Potência sobre a regeneração tecidual, estresse oxidativo e marcadores mitocondriais em animais diabéticos. Metodologia: 20 camundongos Swiss, pesando entre 30g , 6 semanas, para o estudo relacionado ao estresse oxidativo e 20 ratos Wistar pesando 250 g, 8 semanas, foram submetidos a indução do DM (injeção de streptozotocina 70mg/kg) e após 120 dias de seguimento, sofreram lesão cutânea e foram tratados com Terapia Laser de Baixa Intensidade e LED durante 5 dias consecutivos e no 6º dia os animais foram mortos e retirada a pele. Um outro grupo de animais (20 ratos Wistar pesando 250 g, 8 semanas) foi submetido à irradiação Laser e LED na região do pâncreas durante 5 dias consecutivos e no 6º dia os animais foram mortos e retirado o pâncreas, fígado e músculos gastrocnêmio. Foram divididos em quatro grupos: Sham (controle saudável), NID (diabético não irradiado), DLED(diabético irradiado pelo LED) e DLASER (diabético irradiado pelo LED). Mensurou-se níveis de catalase, àcido tiobarbitúrico (TBARS), nitrito, conteúdo de colágeno, fator de crescimento endotelial vascular (VEGF), Ciclo-oxigenase-2 (COX2) Mitofusin 2 (MFN2), Mitochondrial fission 1 protein (FIS1), glicogênio hepático e muscular bem como testes funcionais de glicose e insulina. Resultados: Observou-se na pele desses animais, aumento no conteúdo de colágeno e angiogênese nos animais diabéticos irradiados pela Terapia Laser de Baixa Intensidade

904nm bem como níveis de TBARS e nitrito menores nos diabéticos irradiados. Além disso, a expressão da COX-2 foi maior entre NID quando comparados aos grupos SHAM e DLED. DLED apresentou os maiores scores em relação a MFN2 e a expressão da FIS1 foi maior no grupo DLASER. As Terapias Laser e LED alteraram a expressão de VEGF e COX2 e estimularam a proliferação das fibras colágenas maduras em feridas de animais diabéticos. Os resultados relacionados à irradiação sobre o pâncreas demonstraram que no grupo NID, o conteúdo de glicogênio hepático e muscular foram estatisticamente menores que os grupos DLASER e DLED. Somando-se a esses dados, a densidade dos ductos pancreáticos e ilhotas pancreáticos diferiram significativamente quando comparados ao grupo diabético controle.Constatamos com esses resultados que tanto o Laser de Baixa Intensidade quanto o LED obtiveram potenciais efeitos terapêuticos sobre a pele e o pâncreas sob condições hiperglicêmicas e que ambas as terapias atuaram modulando parâmetros histológicos, angiogênicos e mitocondriais no pâncreas e pele dos animais diabéticos.

Palavras chave: Catalase. Diabetes mellitus. Espécies reativas de oxigênio. Colágeno. Óxido nítrico. TBARS. Fototerapia. FIS1. MFN2.

ABSTRACT

Tissue repair in diabetic patients is hampered by several factors, including secretion of insulin deficiency that leads to hyperglycemia and production of free radicals. Mitochondrial dysfunction has been identified as one of the probable factors of DM. It is known that phototherapy has proliferative effects in tissues with some pathologies. This study aimed to evaluate the effects of phototherapy using laser and low power LED on tissue regeneration, oxidative stress and mitochondrial markers in diabetic animals. Methodology: 20 Swiss mice (30g, 6 weeks) for the study relating to oxidative stress and 20 Wistar rats (250 g, 8 weeks) were subjected to induction of DM (streptozotocin injection 70 mg / kg) and after 120 days follow up, suffered skin lesions and were treated with Low Level Laser Therapy and LED for 5 consecutive days and on day 6 the animals were killed and the skin removed. Another group of animals (20 male Wistar rats weighing 250 g, 8 weeks) was subjected to laser irradiation and in the LED region of the pancreas for 5 consecutive days and on day 6 animals were killed and the pancreas, liver, and gastrocnemius muscle were removed. Animals were divided into four groups: Sham (healthy control), NID (non-irradiated diabetic), DLED (diabetic irradiated by LED) and DLASER (diabetic irradiated by LED). Levels of catalase, thiobarbituric acid (TBARS), nitrite, collagen content, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX2) Mitofusin 2 (MFN2), Mitochondrial fission 1 protein (FIS1) liver and muscle glycogen and functional insulin and glucose tests were measured. Results: It was observed in the skin of these animals, increase in collagen content and angiogenesis in irradiated diabetic animals, TBARS levels and lower nitrite in irradiated diabetic. Furthermore, COX-2 expression was higher among NID compared to SHAM and DLED groups. DLED showed the highest scores against

MFN2 and FIS1 expression was higher in DLASER group. Laser and LED Therapy altered the expression of VEGF and COX2 and stimulated the proliferation of mature collagen fibers in diabetic animal wounds. About irradiation over pancreas results showed that the NID group, hepatic and muscle glycogen content were statistically lower than DLASER and DLED groups. Adding to these data, the density of the pancreatic duct and pancreatic islets were significantly different when compared to NID. These results suggest that both the low-level laser LED obtained as potential therapeutic effects on the skin and pancreas under conditions hyperglycemic and that both therapies acted modulating histological, angiogenic and mitochondrial parameters on pancreas and skin of diabetic animals.

Keywords: Catalase. Diabetes mellitus. Reactive oxygen species. Collagen. Nitric oxide. TBARS. Phototherapy. FIS1. MFN2.

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Sumário

INTRODUÇÃO

O Diabetes Mellitus gera altos custos financeiros ao país. O Brasil aparece como o 8.º país no mundo com maior prevalência da doença(1). Devido ao comprometimento neurovascular, os diabéticos frequentemente sofrem uma perda sensorial e pequenas feridas podem se desenvolver em áreas corporais com proeminências ósseas (calcanhar, no caso do pé diabético, por exemplo) que evoluem para úlceras mais profundas de difícil manejo (2).

Sabe-se que uma das principais patologias que afetam os diabéticos é a cicatrização tardia(3). Deficiências na angiogênese, em particular, podem conduzir ao atraso no reparo tecidual (4) e um grande esforço tem sido dedicado para produção de novas drogas e outros agentes capazes de promover uma revascularização segura e eficaz(5). Em adição à vascularização deficitária encontra-se o estresse oxidativo, que pode ocasionar falhas na geração de ATP induzida pela glicose, a diminuição da secreção de insulina e um aumento da produção de Espécies Reativas de Oxigênio (do inglês:ROS) nas mitocôndrias (6) .

As mitocôndrias são organelas-chave devido ao seu fornecimento de energia a todas as células e participação da maioria dos processos fisiológicos. Essas organelas formam uma rede dinâmica dentro da maioria das células e constantemente sofrem fissão e fusão para sua própria manutenção. O equilíbrio entre a fusão e fissão é importante na manutenção da integridade da mitocôndria e facilita a troca de DNA entre mitocôndrias. As proteínas Mitofusin 1 e 2 (MFN1 e MFN2) mediam a fusão da membrana externa, enquanto a Dynamin-like 120 kDa protein, mitochondrial (OPA1) está envolvido na fusão da membrana interna.(7) Jà a fissão ocorre por divisão binária e, uma vez que requerem o DNA mitocondrial para a sua

função, a fissão é coordenada com a replicação do DNA, através das proteínas FIS1 e Dynamin-1-like protein (DRP1) .(8)

Sabe-se que a Fototerapia atua sobre a função mitocondrial, estimulando a atividade respiratória e aumentando a síntese de ATP(9). Karu sugere que os fótons no espectro da luz vermelha e infravermelha são absorvidos pelos cromóforos da cadeia respiratória mitocondrial e aumentam o potencial de membrana mitocondrial , o transporte de elétrons e consumo de oxigênio (10).

No entanto, poucos trabalhos investigaram a relação entre a Fototerapia e suas conseqüências sobre estresse oxidativo e nitrosativo em pele de ratos diabéticos.

O estresse oxidativo induzido pela hiperglicemia do DM pode causar danos nos tecidos e induzir uma aceleração da produção de radicais hidroxilo relacionados com o nível de ácido tiobarbitúrico (TBARS), que é marcador de peroxidação lipídica (18, 19).

Nossa hipótese é que a Fototerapia Laser e LED de espectro de comprimento de onda infravermelha poderiam influenciar positivamente o processo de regeneração tecidual de animais diabéticos através da modulação de agentes antioxidantes bem como fatores envolvidos na homeostasia mitocondrial.

CONTEXTUALIZAÇÃO

1.1 Epidemiologia do Diabetes Mellitus (DM)

A Organização Mundial da Saúde (do inglês : WHO) estima que cerca de 347 milhões de pessoas no mundo são portadores de DM e a previsão é que em 2030 a DM se tornará a 7ª principal causa de morte no mundo (11, 12). Nos Estados Unidos da América, o número de diabéticos é de 29,1 milhões de pessoas (13). No Brasil, segundo a Sociedade Brasileira de Endocrinologia 12 milhões de brasileiros já foram diagnosticados com a doença(14). Os dados são preocupantes quando a atenção volta-se para os jovens, pois de acordo com a Federação Internacional de DM (IDF) o número de crianças com DM tipo 1 em 2015 chegou a 542.000 e cerca de 86.000 novos casos irã ocorrer a cada ano (1).

Prevê-se que a prevalência de Diabetes Melito Tipo 2 (DM2) em adultos irá aumentar nas próximas duas décadas e grande parte desse aumento ocorrerá nos países em desenvolvimento sendo que a maioria dos pacientes na faixa etária entre 45 e 64 anos (15).

Esses números alarmantes relacionados a prevalência bem como as comorbidades associadas ao DM demonstram que pesquisas abordando novas estratégias terapêuticas para o tratamento do DM devem ser encorajados, bem como uma melhor compreensão acerca dos mecanismos envolvidos na fisiopatologia do DM.

1.2 Fisiopatologia do DM - Disfunção mitocondrial e Estresse oxidativo

O termo DM define um grupo de doenças metabólicas crônicas caracterizadas por hiperglicemia devido à produção insuficiente de insulina e / ou

resistência periférica à insulina (16). Dentre os sintomas característicos do DM estão: fome constante, poliúria (excreção excessiva de urina), alterações na visão, polidipsia (sede excessiva), e fadiga (14). As principais complicações associadas ao DM são retinopatia diabética(14), hipertensão arterial(17) amputações de membros inferiores(18, 19) glaucoma(20), e vasculopatias periféricas(21).

No DM ocorre diminuição da sensibilidade das células à insulina causada pela queda na produção de insulina, resistência insulínica, e/ou eventual incompetência das células beta do pâncreas em produzir insulina (22) alterando o transporte de glucose para músculos, fígado e adipócitos. A insulina reduz a glicemia ao promover o transporte de glicose nas células, sendo essencial para o metabolismo dos carboidratos, sintese proteica e armazenamento lipidico. Níveis inadequados de insulina e aumento da resistência à insulina produzem altos índices glicêmicos, sinal patognômico do DM (23).

Recentemente, a disfunção das células-beta tem sido apontada como uma das causas da fisiopatologia da DM2 (24). Idade e genes (25) são variáveis que influenciam diretamente sobre a homeostase das células beta. Tem sido estudado mais recentemente o polimorfismo rs7903146 no gene TCF7L2, que codifica um fator de transcrição importante no desenvolvimento das ilhotas pancreáticos e a adipogénese e que hipoteticamente poderia estar relacionado à etiologia do DM (26).Outros fatores, como a hipersecreção de polipeptideo amilóide das ilhotas, que é co-segregado com a insulina pode levar a progressiva falha dessas células(27).

A morfologia mitocondrial depende do equilíbrio entre fissão e fusão mitocondrial que atuam mantendo a morfologia e funcionalidade dessa organela, mas o mecanismo envolvido ainda não está claro(28). Nos mamíferos, a fusão mitocondrial é controlada

pelas Mitofusinas 1 e 2 (MFN1 e MFN2) na membrana externa da mitocôndria e OPA1 na membrana interna(29). A fissão mitocondrial é regulada pela proteína citoplasmática e pela proteína Mitochondrial fission 1 protein (FIS1), que funciona presumivelmente como o adaptador de DRP1 na membrana mitocondrial externa(30). Avanços acerca da função mitocondrial e possíveis mecanismos de controle sobre a homeostasia mitocondrial das células beta (28, 31-33) apresentam dados que nos induzem a crer numa forte relação etiológica entre a disfunção mitocondrial e o DM (32). Um estudo utilizando modelo de disfunção mitocondrial experimental resultou na promoção de resistência insulínica em tecidos periféricos, como músculos, fígado e adipócitos (34). Em outra vertente, um estudo com modelo de resistência insulinica experimental estimulou a disfunção mitocondrial (35), o que era esperado, uma vez que a sinalização de insulina é fundamental para a biogênese e metabolismo mitocondrial (34).

O DM e o estresse oxidativo por ele gerado produzem disfunção mitocondrial levando a danos mitocondriais e, portanto, lesão celular (28). Bach et al. (2005) & Hernández-Alvarez (2010) relataram que a expressão de MFN2 se apresentou reduzida em músculos esqueléticos de indivíduos diabéticos tipo 2 (36, 37). Entretanto, não se sabe ao certo qual dos dois mecanismos, fissão ou fusão, seja mais eficaz na manutenção da função mitocondrial, haja visto que a fissão mitocondrial é importante não só para a divisão mitocondrial, mas também como um sistema de eliminação de resíduos mitocondriais, crucial para a manutenção da respiração mitocondrial. Ao longo de sua vida, uma mitocôndria pode acumular uma quantidade excessiva de espécies reativas de oxigênio (do inglês: ROS) e proteínas disfuncionais, muitas vezes levando para cadeias de transporte de eletrons desacoplados. Uma mitocôndria pode dispor de tais detritos moleculares segregando parte da mitocôndria por fissão, englobando-o por autofagia(38).

Além da disfunção das células beta, outros fatores são fundamentais no desenvolvimento do DM, como perturbações no metabolismo lipídico e da glicose no fígado, contribuindo para a resistência insulinica e dislipidemia / hiperlipidemia, levando ao DM e hepatopatias e caracterizando a sindrome metabólica (39). O papel do fígado na resistência insulínica e a superprodução de lipoproteína foram demonstrados em modelos animais diabéticos geneticamente modificados (40) em que a produção de lípidos e de glicose pelo fígado foi elevada e a depuração prejudicada (41). Portanto, não só o fígado desempenha um papel importante nas dislipidemias, como também atua no desenvolvimento de resistência à insulina (42) através de desequilíbrios no estado de energia. Fatores como resistência à insulina, aumento da sensibilidade hepática ao glucagon, hiperglucagonemia, glicotoxicidade e lipotoxicidade aumentam a gluconeogênese hepática. Entretanto, algumas moléculas atuam sobre este defeito metabólico, como a metformina, que atua na redução da neoglicogênese, diminuição da absorção de glicose no trato gastrointestinal e aumento da sensibilidade à insulina (43), e o GLP-1 (Glucagon-like peptide- 1 **)**, incretina derivada do produto da transcrição do gene pró-glucagon, que dentre outras ações aumenta a secreção de insulina do pâncreas dependente de glicose (44).

Um outro sistema fundamental para que o metabolismo glicídico ocorra de maneira equilibrada é o tecido muscular, onde ocorre a maior parte da resistência à insulina e responde por mais de 85-90% da captação de glicose corporal. Entretanto, importantes defeitos intramiocelulares sobre o transporte de glucose, fosforilação glicolitica , glicogênese e oxidação da glicose prejudicam a ação da insulina sobre esse tecido. Park et al. (2006) e Cetinus et al. (2005) observaram que força muscular foi significativamente menor em pacientes com DM2 do que os indivíduos sem a doença (45, 46). Interessantemente, pacientes com DM2 parecem ativar um número limitado de

unidades motoras responsáveis pelas contrações musculares, que podem ser relacionadas com a desnervação das fibras musculares e / ou aumento da gordura intramuscular (46). A indústria farmacêutica tem utilizado esse conhecimento acerca do papel do tecido muscular sobre o DM e dentre os medicamentos para DM, surgiram as glitazonas, que aumentam a sensibilidade à insulina, interferindo na liberação de sinais que agem no tecido muscular e no fígado, como a adiponectina e o fator de necrose tumoral alfa (TNFa)(47).

A partir dos dados supracitados acerca da fisiopatologia do DM, compreender como o DM prejudica o reparo tecidual e a interferência do estresse oxidativo sobre esse processo pode favorecer a assimilação entre os diversos estudos até então realizados.

1.3 DM, Cicatrização Tecidual e estresse oxidativo

O processo de cicatrização tecidual requer uma integração de eventos biológicos e moleculares complexos que envolve migração celular, proliferação celular, e deposição da matriz extracelular (ECM) (48). O reparo tissular perpassa por 3 fases a saber: inflamação, proliferação e remodelação (49).

Na fase inflamatória, ocorre uma modulação celular com aumento da população de células inflamatórias e liberação de fatores de crescimento (50). Na fase proliferativa, ocorre aumento da proliferação de fibroblastos, angiogênese (51) e deposição de colágeno (52) contribuindo para formação de tecido de granulação. Na última fase, a remodelação do tecido ocorre com intensa neovascularização e deposição de colágeno com orientação elevada e fibras maduras (53).

O reparo nos tecidos ativa mediadores inflamatórios, como citocinas e ROS, ambos gerando efeitos deletérios sobre o tecido hígido (54). A resposta inflamatória induzida pela lesão epidérmica provoca a migração de neutrófilos e macrófagos, que realizam um rápido consumo de O_2, formando ROS, que, por sua vez, reduzem os

níveis plasmáticos de óxido nítrico (NO). Todavia, a ação do óxido nítrico sobre o reparo tem um papel dúbio, às vezes benéfico, às vezes prejudicial, pois ao mesmo tempo que está envolvido no relaxamento vascular e protege os vasos sanguíneos, o excesso dessa molécula leva à lesão endotelial(55).

Os radicais livres podem combinar-se rapidamente com o NO, formando peroxinitrito, podendo reduzir a quantidade de NO disponível (56). Além disso, o estresse oxidativo inibe a cadeia respiratória, permitindo a transferência de elétrons para o oxigénio molecular, formando superóxido e bloqueando as enzimas envolvidas no metabolismo de glicose (gliceraldeído-3-fosfato desidrogenase a partir da glicólise), o que conduz a uma redução do ATP / ADP e liberação insulínica deficitária (57).

Adicionalmente, a hiperglicemia desencadeia o estresse oxidativo por meio do aumento da produção de ânion superóxido mitocondrial e do aumento da glicosilação não-enzimática de proteínas, assim como por meio da ativação de vários fatores de transcrição celular (58). Apesar da enorme quantidade de pesquisas sobre cicatrização de feridas no DM, ainda não há resposta clara acerca da sua patogênese, muito embora existam fortes evidências relacionando distúrbios da microvasculatura nos diabéticos com angiogênese inadequada(59).

Atualmente, o mais importante mecanismo pelo qual acredita - se que o estresse oxidativo altera a função endotelial é a inativação do NO pelos ânions superóxidos e lipoproteínas de baixa densidade oxidadas. Essas ROS desativam os receptores endoteliais para acetilcolina, serotonina, trombina, bradicinina e outros mediadores, diminuindo a estimulação da NOS nas células endoteliais e, consequentemente, reduzindo a produção de NO, prejudicando o relaxamento das células musculares lisas e predispondo à formação da placa aterosclerótica(60).

Além disso, o peroxinitrito tem sido implicado na patogênese de muitas doenças crônicas, incluindo o DM. Cuzzocrea et al. (61) ao analisar a catálise da decomposição do peroxinitrito através de um catalisador de decomposição específico, observou a redução da incidência e da gravidade do DM mellitus em ratos submetidos a múltiplas doses baixas de estreptozotocina, fortalecendo as evidências de que o excesso das ROS provocaria alterações fisiológicas importantes no processo de cicatrização tecidual (61).

O mecanismo pelo qual níveis elevados de glicose causam lesões vasculares e resultam em alterações estruturais e funcionais em vários tecidos pode ser multifatorial, e dentre os mais importantes estão o estresse oxidativo, o aumento da síntese/acúmulo de diacilglicerol, a ativação da proteína quinase C, o aumento da ativação da via do sorbitol do metabolismo glicídico, a glicosilação não-enzimática das proteínas e as alterações relativas ou absolutas na produção de substâncias vasoativas, tais como endotelina, prostaglandinas e subprodutos de óxido nítrico (62). Ademais, a susceptibilidade da catalase pelos radicais livres gerados pelo DM (63) contribui para a progressão da doença.

O processo de cicatrização tecidual depende do fornecimento de ATP, que desempenha um papel importante na manutenção da natureza do citoesqueleto das células (64). Isto posto, o incremento da produção de ATP mitocondrial através da Fototerapia já está bem estabelecido (9, 65, 66) e, portanto, analisar os trabalhos que relacionam Fototerapia, reparo tecidual e estresse oxidativo, podem nos elucidar como a Fototerapia poderia contribuir no reparo tecidual em pacientes diabéticos.

1.3 Fototerapia, Cicatrização Tecidual e estresse oxidativo

LLLT é a aplicação de um laser ou diodo emissor de luz no intervalo de 1 mW - 500 mW a determinada condição patológica para promover a regeneração dos tecidos, reduzir a inflamação e aliviar a dor (67).

A LLLT estimula a atividade eletroquímica mitocondrial e aumenta a síntese de ATP (9). Esse tipo de irradiação exerce efeito em cascata sobre a sinalização celular, promovendo uma proliferação celular e citoproteção (65). Chung et al. sugeriram que na Fototerapia vias de sinalização são ativadas, conduzindo a uma cascata de eventos que promovem a sobrevivência celular, proliferação celular, citoproteção, migração celular e a reparação de tecidos (68).

Apesar dos efeitos benéficos já observados pela LLLT, estes dispositivos requerem alta energia e os custos para sua produção ainda são relativamente altos. Atualmente, o uso de Diodos Emissores de Luz (LEDs) ou Light-Emitting Diode Therapy (LEDT) tem sido apontados como uma alternativa nova e barata de Fototerapia (69, 70). LEDs emitem uma luz não-coerente e tem sido apresentados como alternativa terapêutica aos Lasers, já que é o comprimento de onda da luz o mais importante na fototerapia e não a coerência ou a falta da mesma. LEDs são pequenos e robustos dispositivos que emitem radiação eletromagnética que varia em comprimento de onda ultravioleta ao infravermelho, normalmente gerando luz de baixa intensidade na faixa miliwatt (71). Os primeiros estudos com LEDs demonstraram promover o alívio da dor(72), melhorar o desempenho muscular (73) minimizar a fadiga muscular (74) e estimular a cicatrização de feridas (71).

Entretanto, é importante esclarecer que os efeitos da LLLT dependem de alguns parâmetros, como a energia total, a densidade de energia, tempo e frequência da irradiação e do tipo de Laser ou fonte de luz utilizada (67). Segundo Hamblin et al., tal

como com outras formas de medicamentos, a LLLT tem os seus princípios ativos (parâmetros de irradiação ou “remédio”) e uma "dose" (o tempo de irradiação) (67). A Tabela 1 lista as principais variáveis que definem os parâmetros de irradiação:

Tabela 1. Variáveis envolvidas na determinação dos parâmetros da LLLT

(*Adaptado de Huang & Hamblin,2009*)

Irradiação	Unidade de medida	Comentário
Comprimento de onda	nm	Luz é uma energia eletromagnética transportada em “pacotes” que também tem propriedade semelhante a onda e é visível entre 400-700nm
Irradiância	W/cm^2	Muitas vezes chamado de intensidade ou densidade de potência e é calculado: Irradiância = Potência (W) / Área (cm2)
Pulso	Pico de energia(W) Frequencia de pulso (Hz) Largura do pulso(s) ciclo de trabalho (%)	Se o feixe é pulsado, a energia deve ser a média. Potência média (W) = pico de potência (W) × largura de pulso (s) x Frequencia pulso(Hz)
Coerência	Comprimento da banda de coerência depende da largura de banda espectral.	Luz coerente produz salpicos do laser, que tem sido postulados desempenhando um papel na interação com fotobiomodulação das células e organelas
Polarização	Polarização linear ou circular	Luz polarizada pode ter efeitos diferentes do que a luz não polarizada. No entanto, sabe-se que a luz polarizada sofre espalhamento rapidamente, como nos tecidos

Tempo	segundos	o tempo de irradiação poderia ser definido como "dose"
Densidade de Energia	J/cm^2	Possui uma relação de reciprocidade entre irradiância e tempo.
Intervalo de Tratamento	Horas, dias ou semanas	Os efeitos de diferentes intervalos de tratamento são pouco explorados embora haja evidências suficientes para sugerir que este é um parâmetro importante.

No que se refere ao mecanismo de ação da luz sobre os tecidos biológicos, algumas pesquisas sugerem que fótons de baixa energia são absorvidos nos cromóforos da cadeia respiratória da mitocôndria. Esses estudos (75-77) sustentam a hipótese de que a absorção destes fótons aparentemente incrementa o potencial de membrana, podendo estar associada com um aumento da produção de ATP na célula. Esse fato pode representar um mecanismo fundamental a respeito dos efeitos fotomoduladores da LLLT (75). Entretanto, os mecanismos envolvidos nas respostas teciduais à estimulação LLLT ainda permanecem obscuros, principalmente no que se refere aos efeitos na cadeia respiratória mitocondrial e sobre os marcadores de estresse oxidativo (78).

A Fototerapia age sobre o metabolismo do NO através do fenômento de fotodissociação da citocromo c oxidase (CCO) com o NO. O CCO é um fotoaceptor localizado na membrana mitocondrial interna, cuja função é o de catalisar a redução do citocromo c, ao bombear prótons para fora da matriz mitocondrial. O CCO tem dois centros heme (aa e a3) e dois centros Cobre (A e B), dos quais o ferro heme citocromo a3 forma junto com o oxigênio no local de ligação. Assim, o NO pode competir com oxigénio para se ligar ao complexo de ferro-enxofre e de centros de ferro e cobre na

cadeia respiratória e inibem a síntese mitocondrial de ATP (23). Em adição a isto, o NO-CCO pode ser quebrado por energia luminosa visível e infravermelho (24) para restaurar a função mitocondrial para a síntese de ATP (24, 25).

Alguns trabalhos comprovam essa ação da Fototerapia sobre marcadores de estresse oxidativo , como Rubio et al. (79) , que observou diminuições significativas nos níveis plasmáticos de fibrinogênio, NO, L-citrolina e nitrotirosina em animais irradiados pela LLLT (He-Ne 640nm, 8 J/cm2, 3x/dia), demonstrando uma modulação na resposta oxidativa pela LLLT.

Outro possível mecanismo de ação do LLLT sobre o estresse oxidativo é através do aumento dos radicais superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2), que conduzem a regulação do metabolismo celular, em equilíbrio com algumas enzimas antioxidantes (9). Karu demonstrou efeito biomodulador da LLLT sobre o metabolismo oxidativo ao verificar que a produção do radical superóxido e da atividade da enzima catalase foram aumentadas, levando ao incremento da síntese protéica em cultura de células de levedura (9).

Por outro lado, Fillipin (80) ao analisar os efeitos da LLLT sobre o processo de cicatrização em tendões (laser Ga-As, 904 nm, 45 mW de potência média, 5 J / cm^2, 35 s, modo continuo, por 21 dias), observou que a concentração de substâncias reativas do ácido tiobarbitúrico (TBARS) não diferiram significativamente entre os animais irradiados versus controles. Adicionalmente, estudos anteriores indicaram que fluências acima de 4 J/cm^2 apresentaram características de inibição do crescimento celular (81, 82), que poderia estar relacionado com o estresse oxidativo, já que as ROS podem infligir danos diretos aos constituintes celulares vitais, tais como lipídios, proteínas e DNA.(83)

As mitocôndrias não são estáticas e numerosos eventos de fusão e / ou de fissão ocorrem periodicamente. A fissão e a fusão são acompanhadas por variações no tamanho, número e a massa mitocondrial, acionados por uma variedade de estímulos fisiológicos. Karu (10) afirma que o NO• via NO•- sistema citocromo c oxidase é um importante mensageiro-chave para ativar o programa de biogênese mitocondrial em vários tipos de células. Experiências realizadas com células de levedura (84, 85) concluíram que irradiação com LLLT He-Ne (632,8 nm) influenciou de um modo dose-dependente a ultraestrutura mitocondrial ao lado de mudanças na taxa de proliferação celular por sete passagens sucessivas. Além disso, mitocôndrias bipartidas e hipertrofiadas foram observadas após a irradiação de fígado de rato com LLLT He-Ne (86).

Frente ao exposto, existem fortes evidências que nos direcionam a utilizar a Fototerapia como tratamento no reparo tecidual na condição diabética, no entanto, ainda há muitas lacunas acerca não somente dos mecanismos envolvidos nesse processo, mas também dos parâmetros a serem utilizados.

OBJETIVO

Diante do exposto, o presente estudo teve como objetivo avaliar o efeito da Fototerapia Laser e LED sobre a regeneração tecidual e do estresse oxidativo em animais diabéticos e não diabéticos.

Objetivos Especificos

Investigar se a Fototerapia foi capaz de melhorar os parâmetros de estresse oxidativo e nitrosativo durante o processo de cicatrização de feridas em ratos diabéticos mensurando-se a quantidade de colágeno, concentração de catalase, nitrito e TBARS.

Analisar os efeitos da Fototerapia (Laser e LED) sobre a cicatrização da pele em roedores diabéticos e possíveis mecanismos envolvidos. Para tanto, parâmetros histológicos, estudo comparativo do colágeno pela coloração picrosirius e microscopia de força atômica, imunoexpressão de VEGF, COX2, FIS1 e MFN2 foram observados.

Examinar os efeitos da Fototerapia Laser e LED no pâncreas após a injeção de estreptozotocina e suas implicações sobre o metabolismo de carboidratos avaliando-se parâmetros histológicos, glicogênio hepático e muscular, teste intraperitoneal de tolerância à insulina e teste de tolerância à glicose intraperitoneal.

HISTÓRICO DA CONSTRUÇÃO DA TESE

A presente Tese de Doutorado é composta por três artigos originais. Além disso, um quarto artigo será escrito com resultados colhidos a partir da experiência do doutorando no estágio *sandwich* realizado no Massachussetts General Hospital, vinculado a Harvard Medical School na cidade de Boston-EUA. Esses dados não puderam compor o escopo desta Tese devido ao protocolo de sigilo dos dados assinados pelo discente.

O experimento inicial do doutorado, que teve como produto o artigo intitulado "Low-Level Laser Therapy (904nm) Can Increase Collagen and Reduce Oxidative and Nitrosative Stress in Diabetic Wounded Mouse Skin" submetido na Revista Photochemistry and Photobiology B, apresentou resultados que nos fizeram refletir em uma mudança de paradigmas a respeito dos prováveis mecanismos de ação da Fototerapia sobre a mitocôndria de animais diabéticos e, então, iniciamos um segundo experimento, desta vez acrescentando Diodos emissores de luz (LEDs) ou Light-Emitting Diode Therapy (LEDT), com intuito de averiguarmos se, de fato, seria mais importante o comprimento de onda do que coerência da luz para ocorrerem os efeitos metabólicos da Fototerapia e se de alguma forma, haveria diferenças de resultados entre os animais tratados com LEDT ou por LLLT. Este novo artigo, intitulado "Phototherapy Laser and LED acts over Mitochondrial Function and estimulates collagen organization in diabetic animals" que foi submetido para a revista Lasers in Medical Science acrescentou novas informações a respeito dos possiveis efeitos da Fototerapia sobre a homeostasia mitocondrial e nos fez pensar na possibilidade desss efeitos serem benéficos sobre o tecido pancreático, órgao vital no controle da glicemia e em especial, as células beta, fundamentais na fisiopatologia do DM, gerando o terceiro artigo,

intitulado “LLLT and DM: Is there a light of the end tunnel?”, submetido para a Revista Diabetologia.

ESTUDO I

Low-Level Laser Therapy (904nm) Can Increase Collagen and Reduce Oxidative and Nitrosative Stress in Diabetic Wounded Mouse Skin

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ABSTRACT

Background and Objective: Over the last decade we have seen an increased interest in the use of Low-Level Laser Therapy (LLLT) in diseases that involve increased oxidative stress. It is well established that hyperglycemia in diabetes elicits a rise in reactive oxygen species (ROS) production but the effect of LLLT remains unclear. This study aimed to investigate whether LLLT was able to improve oxidative/nitrosative stress parameters in the wound healing process in diabetic mice. **Study Design/Materials and Methods**: Twenty Swiss male mice, 30 weight, 6 months, were divided into four groups: non-irradiated control (NIC), irradiated control (IC), non-irradiated and diabetic (NID), irradiated and diabetic (ID). Diabetes was induced by administration of streptozotocin. Wounds were created 120 days after the induction of diabetes in groups IC and ID and these groups were irradiated daily for 5 days (superpulsed 904 nm laser, average power 40 mW, 60 sec). All animals were sacrificed 1 day after the last irradiation and histology, collagen amount, catalase activity, nitrite and TBARS were measured. **Results**: Histology showed collagen fibers were more organized in IC and ID when compared to NID group and significant differences in collagen content were found in group ID versus NID. Catalase activity was higher in IC group compared to other groups ($p < 0.001$). TBARS levels were higher in IC vs NIC, but were lower in ID vs NID ($p<0.001$). Nitrite was lower in both irradiated groups vs the respective non-irradiated groups ($p < 0.001$). **Conclusions:** Increased production of collagen and photobiomodulation over oxidative and nitrosative stress suggests that LLLT may be a viable therapeutic alternative in diabetic wound healing.

Key words: catalase, diabetes mellitus, oxidative stress, collagen production, nitric oxide, TBARS, photobiomodulation.

1.0 INTRODUCTION

Diabetes mellitus (DM) is characterized by elevated blood glucose levels, an impaired blood supply, and increased production of reactive oxygen species (ROS) (1). Chronic ROS generation is implicated in the pathogenesis of many illnesses, including atherosclerosis and inflammation (2, 3). The hyperglycemia caused by diabetes elicits an increase in ROS production, due to overproduction of superoxide by the mitochondrial electron-transport chain(4). In addition to this oxidative stress, the inflammatory response induced by epidermal injury in wounds provokes the migration and accumulation of neutrophils and macrophages, which also produce ROS via their respiratory burst. When there is increased production of ROS coupled with decreased antioxidant defenses, oxidative stress occurs, and the ROS interacts with cellular molecules and enhances the process of lipid peroxidation (LPO), causing DNA damage and/or inducing protein and nucleic acid turnover (5).

Furthermore, macrophages release nitric oxide (NO) via activated inducible nitric oxide synthase (iNOS) (6). However, the synthesis of large amounts of NO can produce nitrosative stress by reacting with superoxide to form peroxynitrite. Many authors have proposed that low level-laser therapy (LLLT) is able to influence oxidative stress parameters as well as to change the activity of antioxidant enzymes and the production of ROS (1). Over the last decade the literature has been reported an increased interest in the application of photobiomodulation using different light sources in diseases that involving increased oxidative stress, but the mechanisms involved in this response remain unclear, especially in relation to the effects of LLLT on the mitochondrial respiratory chain and on biomarkers of oxidative stress. The basic biological mechanism behind the effects of LLLT is thought to be via absorption of red and infra red light by cytochrome c oxidase (complex IV of the mitochondrial respiratory chain) (7). In addition, Karu (8) and other authors (9) have proposed that one of the possible mechanisms of action of LLLT, is a brief and modest increase in production of ROS such as superoxide (O_2-) and hydrogen peroxide (H_2O_2), leading to restoration of the redox imbalance as a consequence of enhanced production of antioxidant enzymes. LLLT alters the redox state in cells and can induce the activation of intracellular signaling, increase activation of redox-sensitive transcription factors (10), and affect enzyme activation and cell cycle progression (7) which are fundamental mechanisms involved in wound healing. Several parameters are important for optimizing treatment

using photobiomodulation. These parameters include wavelength, power density, energy, time, and frequency of application. Based on these facts, our hypothesis was to test if near-infrared laser (904 nm) could reduce nitrosative/oxidative stress parameters in diabetic mice and thus accelerate the tissue repair process.

2.0 MATERIALS AND METHODS

2.1 Animals

CEPA/UFC (IACUC) approved the study under protocol number 01/2013. All experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The animals used were bred in the Department of Pathology, Faculty of Medicine, Federal University of Ceara and kept in an environment with a constant temperature of 24^{o}C and light/dark cycle of 12 hours. We used 20 male Swiss mice, weighing 25-30 g at baseline. The animals were randomly allocated into four groups: non-irradiated control (NIC), irradiated control (IC), non-irradiated and diabetic (NID) and irradiated diabetic (ID). Ten animals were induced into a diabetic state by administration of streptozotocin and 10 animals remained euglycemic (control).

2.2 Diabetes induction

For induction of diabetes we used a previously described methodology (11). The mice were subjected to fasting for 6 hours and then anesthetized by thiopental (40mg/kg) and kept in a prone position for an intraperitoneal injection of streptozotocin (solution 70mg/kg - STZ) dissolved in citrate buffer solution (pH 4.5). Six hours after administration, the animals had their water supply replaced by an aqueous solution of glucose (10%) for 24 hours. Glucose levels were determined in blood samples taken from the tail vein and measured on a glucose meter (*AccuChek*, Roche Diagnostics, Indianapolis, IN).

2.3 Wound creation

The animals were anesthetized by intraperitoneal injection of thiopental. Each animal was placed in a prone position, without immobilization, in order to shave the dorsal region with a razor and soap. The skin of the animal was cleaned with an antiseptic solution of 2% chlorhexidine and it was cut with a carbon steel surgical blade

until the depth reached the hypodermis. In order to standardize the lesions, we used a surgical field with 2mm width and 2 cm long, with reference to the animal's posterior iliac crest, which was labeled with an alcohol soluble marker (see figure 1).

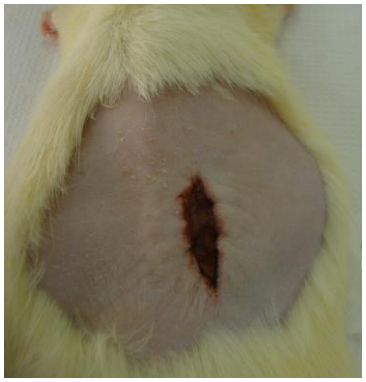

Figure 1.Wound creation: the skin was cut with a carbon steel surgical blade until it reached the hypodermis with 2mm width and 2 cm long.

2.4 Low-level laser therapy (LLLT)

The wounds were irradiated at a single point over the incision with laser device: Laserpulse (IBRAMED, Amparo, Brazil), class 3B, superpulsed 904 nm GaAs diode laser with parameters according to Table 1. The laser treatment was repeated daily for 5 consecutive days. The animals in groups IC and ID received laser irradiation with the probe perpendicular to the wound. Other groups received contact with the probe laser off and the lesions were then treated with saline 0.9% NaCl. Wood chip bedding in the cages were not used due to the risk of infection/contamination, and cages were cleaned daily. One day after the last irradiation, the animals were euthanized by decapitation to remove the skin lesion for homogenization and subsequent analysis.

Table 1: Laser Parameters Parameter [unit]	value
Center wavelength [nm]	904
Operating mode	pulsed
Frequency [Hz]	9500
Pulse duration [ns]	60
Duty cycle [%]	20
Energy per pulse [J]	42 nJ
Peak power [W]	70
Average power [mW]	39.9
Polarization	No
Spot size [cm^2]	0.1309
Beam shape	elliptical
Beam profile	Gaussian
Irradiance at target [mW/cm^2]	304.8
Exposure duration [sec]	60
Radiant exposure [J/cm^2]	18.288
Total Radiant energy [J]	2.394
Number of points irradiated	1
Area irradiated [cm^2]	0.1309
Application technique	Contact
Number and frequency of treatment sessions	1 x day / 5 days

Table 1.Irradiation and Treatment Parameters (AsGa laser, 904nm; model Laserpulse, handheld probe), used in contact to the mice skin

2.5 *Histopathological analysis*

The skin samples were fixed in 10 % buffered formalin (Merck, Darmstadt, Germany) for 24 h. Afterwards, they were dehydrated and embedded in paraffin blocks. Three sections (5µm) from each specimen were sectioned (Microtome Leica Microsystems SP 1600, Nussloch, Germany) and stained with hematoxylin and eosin (H.E. stain, Merck, Darmstadt, Germany). The morphological description of the healing characteristics was performed with optical microscopy (Olympus Optical Co., Tokyo, Japan) and histological analysis was made by a pathologist in a double-blind manner. Five slides from each specimen were analyzed and the following criteria were scored as absent, discrete, moderate or intense: granulation tissue, inflammatory process, area of fibrosis and fibroblast number.

2.6 Collagen Analysis by Masson Staining/

Sections stained with Masson were examined microscopically and the images used for analysis were captured by microscopy using a 20X objective (Carl Zeiss, Germany) and a capture system consisting of a camera (Olympus Optical Co., Tokyo, Japan) equipped with AxioVision 4.7.2.0 software. The processing and image analysis were performed with the public domain software ImageJ 1.36 version (National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/) using plugin *color deconvolution* and percentage of area occupied in relation to the total area was measured as described in previous studies (12-14).

2.7 ***Procedure for determination of the antioxidant activity.***

The skin was immediately dissected and homogenized in 1.15% KCl, volume equivalent to 10 times its weight (1g/10 ml solution). The mixture was centrifuged (5,800 rpm, 10 min at 10°C), and the supernatant was used for biochemical determinations (TBARS, catalase activity and nitrite) according to Figure 2:

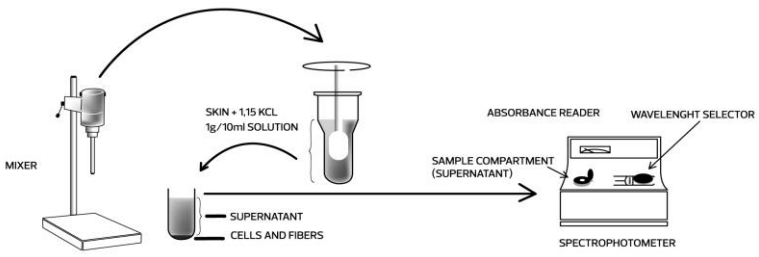

Figure 2.Schematic drawing showing procedure for determination of the antioxidant activity. The skin was dissected and homogenized in 1,15% KCL (1g/10nml solution). The mixture was centrifuged and the supernatant used for biochemical determinations

2.7.1 Concentration of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was assessed by measuring the concentration of thiobarbituric acid reactive substances (TBARS). In this process, two molecules of thiobarbituric acid react with a molecule of malondialdehyde to form a pink pigment with maximum absorbance in acid solution at 532-535 nm. TBARS was determined in the skin homogenates that had been diluted to 10% (w/v). Then, 250 µL of homogenate were incubated in a water bath at 37°C for 1 hour, followed by addition of 400 µL of 35% perchloric acid to precipitate the protein. The mixture was centrifuged at 14,000 rpm at 4 ° C for 10 minutes and then the supernatant was added to 200 µL of 1.2% thiobarbituric acid. Next, the mixture was again incubated in a water bath at 100°C for 30 minutes. After cooling on ice, samples were read in a spectrophotometer at 535 nm.(15)

2.7.2 Determination of catalase activity.

The skin of mice was homogenized in a 0.1 M sodium phosphate buffer solution, pH 7.0, at an equivalent volume of 200 times its weight. Then, the homogenate was centrifuged at 5,800 rpm, 10 min at 4°C, the upper layer was discarded and the bottom layer was used for spectrophotometric measurements of H_2O_2 at 230 nm (28). In the cuvette, were added 980 µL of the reaction medium (15% H_2O_2, in 1 M Tris-HCL buffer), containing 5 mM EDTA, pH 8.0, and 20 µL of the sample diluted in the Tris-HCl buffer. Initial and final absorbance was recorded at 230 nm, after 1 and 6 min, respectively. A standard curve was established using purified catalase (Sigma, MO, USA). Results were expressed in mmol/min/mg protein. Protein was determined by the Lowry method.(16)

2.7.3 Nitrite concentration

Nitrite concentration was measured from homogenates of the mice skin. Homogenates of the treated and control groups were diluted to 10% (w/V) in 50 mM phosphate buffer, pH 7.4 and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The concentration of nitrite was determined by a diazotization chromophore reaction that forms a pinkish color with a peak absorbance of 560 nm. 250 µL of the Griess reagent

(1% sulfanilamide, N-(1-naphthyl)-ethylenediamine 0.1%, 1.0% phosphoric acid and distilled water in the ratio of 1:1:1:1) were added to 250 µL of homogenate (supernatant) and the mixture was maintained at room temperature for 10 minutes. Thereafter, the absorbance of the samples was determined by spectrophotometry at 560 nm and the blank prepared by adding 250 µL of the Griess reagent to 250 µL of 50 mM phosphate buffer, pH 7.4. The absorbance values were interpolated on a calibration curve containing $NaNO_2$ concentrations ranging from 0.75 to 100 µM.(17)

2.8 Statistical Analysis

Data were expressed as mean and standard deviation and statistically analyzed by analysis of variance (ANOVA) one-way with Bonferroni post hoc test. The significance level was set at $p< 0.05$. We used the statistical software package SPSS, version 18.0.

3.0 RESULTS

3.1 Histopathological analysis

Representative histological sections of the four groups are shown in Fig. 3.I and 3.II. Five days post wounding, the NIC and IC groups (Figure 3A and 3B respectively) showed a discrete inflammatory infiltrate, with partial healing and a moderate amount of granulation tissue that was rich in newly formed blood vessels and fusiform fibroblasts. Masson staining showed a moderate deposition of a disorganized mature collagen matrix in the NIC group and a more organized and more intense degree of collagen deposition in the IC group (Figure 3E and 3F respectively). A moderate inflammatory infiltrate, a modest amount of fibroblasts and a granulation tissue that was poor in newly formed blood vessels was observed in the NID group (Figure 3C). On the other hand a moderate amount of fusiform fibroblasts and an increased density of blood vessels was observed in the ID group (Figure 3C). Masson red staining showed an intense deposition of a more organized collagen matrix in the ID group (Figure 3H) when compared with the NID group (Figure 3G).

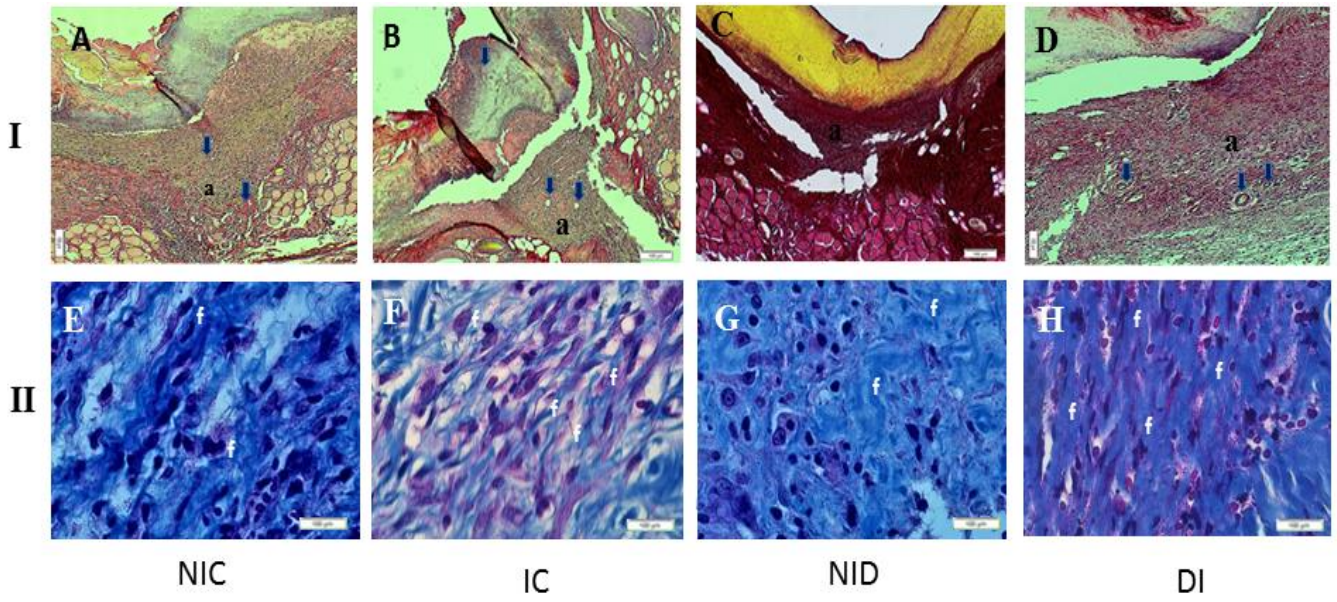

Figure 3.I. Photomicrograph showing granulation tissue (a); newly formed blood vessels (blue arrows) and fibroblasts (f) (HE 10X). Figure 3.II. Photomicrograph Masson staining showing organization and collagen deposition (c). (20X)

3.2 Collagen amount

The images were analyzed taking into account differences between thin and thick fibers and the relative percentage of collagen in the examined fields. The data showed significant differences between NIC versus ID groups ($p < 0,01$) and between the ID group versus NIC and NID groups ($p < 0,03$) (table 2).

GROUPS	*% Collagen (Mean+SE)*	*Angiogenesis (Mean+SE)*
NIC (a)	3.82 ± 0.18 (b,d) *	10.18 ± 1.09
IC (b)	4.99 ± 0.01	7.52 ± 1.32
NID (c)	3.90 ± 0.01(b,d)*	7.38 ± 0.99(d)*
ID (d)	4.09 ± 0.01(a,c)*	15.34 ± 1.14 (a,b,c)*

Table 2.Statistical analysis using morphometric computational systems to determine amount collagen. The significance level was set at p < 0.05.

3.3 Concentration of TBARS

As seen in Table 3, the average concentration of TBARS in the four groups was significantly different (F = 12.888, P ≤ 0.001). The Bonferroni test showed significant differences between the groups: ID had a decreased concentration of TBARS compared to NID (p< 0.05) and IC had an increased concentration of TBARS compared to NIC (p< 0.05). In addition, group ID showed significantly lower levels of TBARS than group IC (p< 0.05).

TBARS		*Mean + DP*	ANOVA
TBARS	NIC	2.38 ± 0.29	≤ 0,001
	IC	5.05 ± 1.52*	
	NID	3.57 ± 1.37	
	ID	1.65 ± 1.57*#	

Table 3.TBARS concentration levels in the skin of different groups: non-irradiated control (NIC), non-irradiated diabetic (NID), irradiated control (IC) and irradiated diabetic (ID). p < 0.001 compared to each respective control group; # relative to the group*

3.4 Catalase activity

As seen in Figure 4, the levels of catalase in the four groups were significantly different ($p \leq 0.001$). An increase in catalase activity was observed for IC mice, when their results were compared with those obtained from NIC and ID mice. The Bonferroni test (post hoc) showed that group IC had higher catalase activity compared to NIC group ($p \leq 0.05$). Other comparisons between groups were not statistically different ($p > 0.05$).

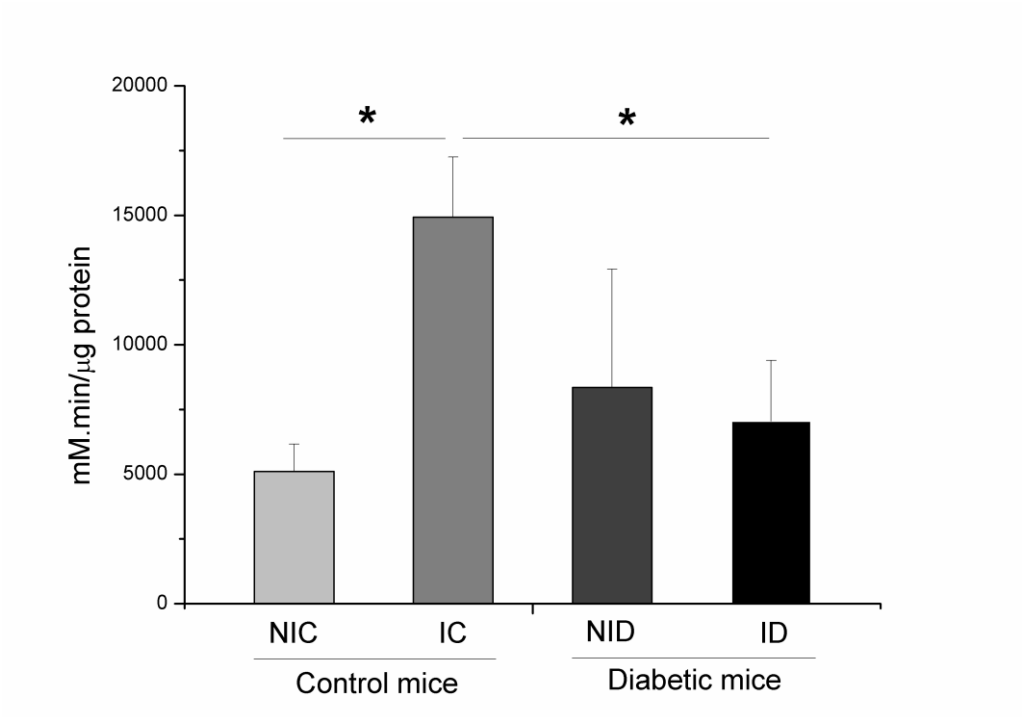

*Figure 4.Catalase concentration levels in the skin of different groups: non-irradiated control (NIC), non-irradiated diabetic (NID), irradiated control (IC) and irradiated diabetic (ID). *p ≤ 0.001Figure 4. Catalase concentration levels in the skin of different groups: non-irradiated control (NIC), non-irradiated diabetic (NID), irradiated control (IC) and irradiated diabetic (ID). *p ≤ 0.001).*

3.5 Nitrite concentration

As seen in Figure 5, the concentration of nitrite in the four groups was significantly different ($F = 54.128$, $p \leq 0.001$). Was observed a decreased concentration of nitrite in group ID compared to group NID ($p < 0.05$), and group IC had decreased nitrite concentration compared to group NIC ($p < 0.05$). There was no significant difference between groups ID and IC ($p > 0.05$).

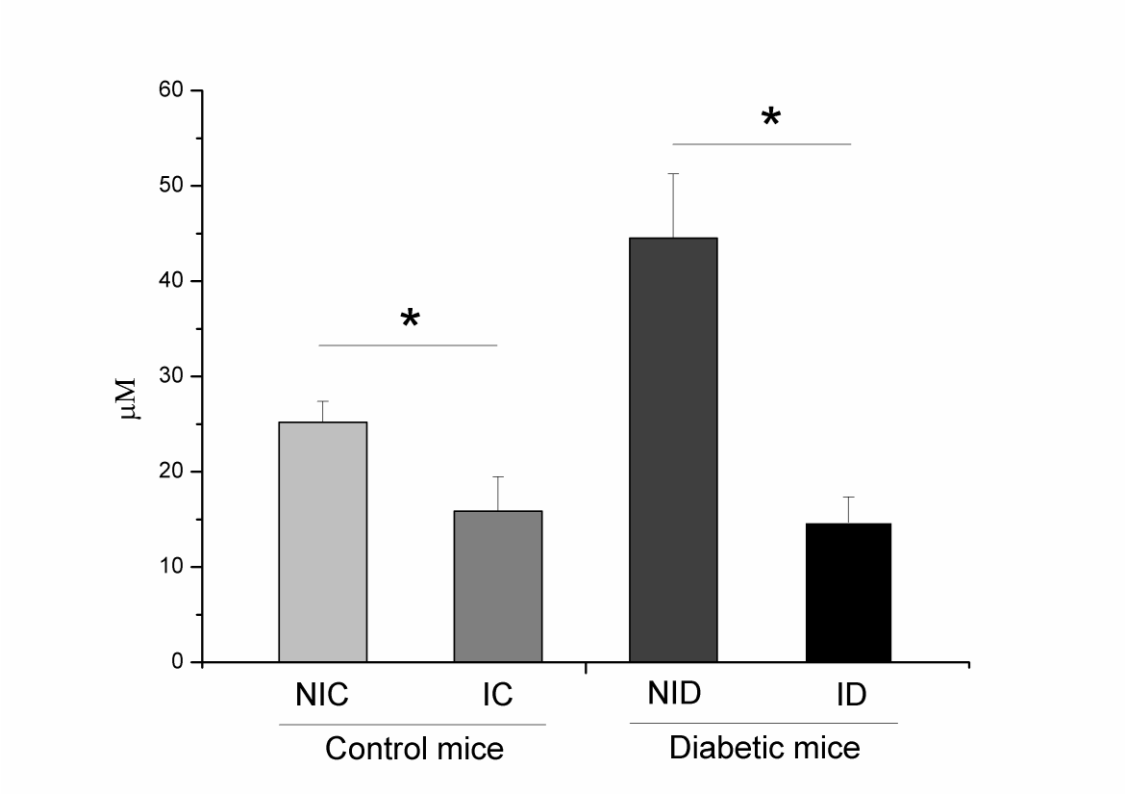

*Figure 5..Nitrite concentration levels in the skin of different groups: non-irradiated control (NIC), non-irradiated diabetic (NID), irradiated control (IC) and irradiated diabetic (ID) . *p ≤ 0.001.Figure 5. Nitrite concentration levels in the skin of different groups: non-irradiated control (NIC), non-irradiated diabetic (NID), irradiated control (IC) and irradiated diabetic (ID) . *p ≤ 0.001.*

4.0 DISCUSSION

Diabetes mellitus (DM) is an endocrine metabolic disease whose main clinical manifestation is hyperglycemia, which promotes oxidative stress due increased production of mitochondrial ROS and increased non-enzymatic glycosylation of proteins, as well as via the activation of several cellular transcription factors (18). It is well known that one of the main pathological abnormalities that are suffered by diabetic patients, is caused by difficulties in wound healing (19, 20), and new approaches are needed to accelerate tissue repair in diabetic patients (21-23). Photobiomodulation or LLLT has been studied extensively in several models of wound healing, and most previous reports have found beneficial effects for this modality of treatment. However, only a few studies have investigated the relationship between LLLT and its effects on oxidative and nitrosative stress in the wounded skin of chronic diabetic mice (24). Hyperglycemia-induced oxidative stress may cause tissue damage and induces hydroxyl radical generation that is correlated with the level of thiobarbituric acid (TBARS) reactive compounds used as an estimation of lipid peroxidation (25, 26). Oxidation is a

normal part of aerobic metabolism and ROS are produced naturally at a certain level, but at greatly elevated levels as a result of some pathological disease states. Antioxidants are substances that fight ROS and free radicals, and can act enzymatically, such as glutathione peroxidase, catalase and superoxide dismutase, or can act non-enzymatically for example: ascorbate, vitamin E, histidine peptides, and some iron-sequestering proteins (ferritin and transferrin). Inflammatory processes can generate ROS and reactive nitrogen species (RNS). The RNS include nitric oxide (NO•) in large amounts, and peroxynitrite (ONOO-). The presence of nitric oxide in biological systems promotes RNS production such as peroxynitrite (formed by spontaneous reaction between NO and superoxide) that reacts with tyrosine residues in proteins to form nitrotyrosine(27). Besides, nitrites and nitrates that circulate in the blood are produced from NO oxidation.

Photobiomodulation can produce NO through photodissociation of NO that is bound to cytochrome c oxidase (CCO). CCO is a photoacceptor located in the inner mitochondrial membrane whose function is to catalyze the oxidation of cytochrome c and the reduction O_2 to water, resulting in the pumping of protons out of the mitochondrial matrix. CCO has two heme centers (aa and a3) and two copper centers (CuA and CuB), of which the heme iron of cytochrome a3 and CuB together forms the O_2 binding site (28). Thus, NO may compete with oxygen to bind to the iron-sulfur complex and to the iron and copper centers in the respiratory chain and inhibit the mitochondrial ATP synthesis (29). It is proposed that the NO-CCO bond can be broken by visible and NIR photon absorption (30) to restore mitochondrial function and increase ATP synthesis (30, 31). Our results showed a significant reduction of nitrite levels by LLLT in the skin homogenate of wounded mice (Figure 5). This could be explained due to a possible protective effect that LLLT could have on the microvasculature because some studies (32) have suggested that hyperglycemia is a factor which naturally leads to injury of blood vessels and causes long-term microvascular and macrovascular complications. The excessive production of NO may also lead to endothelial injury. Volpe et al demonstrated that the hyperglycemia typical of diabetes exacerbated *in vitro* inflammatory responses (33). Their group observed that levels of NO, IL-6, TNF-α, and MDA were higher in supernatant of blood cells that had been stimulated with palmitate. Advanced studies have suggested that the effects of hyperglycemia and hyperlipidemia on peripheral blood mononuclear cells (PBMNC) include activation of the NADPH oxidase system leading to ROS production, enhanced

NF-kB activity, and increased levels of cytokines, chemokines, and circulating adhesion molecules (34-36). We believe that one important factor to explain our results may be mediated by NFkB, because a previous report (10) suggested that LLLT not only enhanced mitochondrial respiration, but also activated the redox-sensitive NF-kB transcription factor via brief generation of ROS. NF-kB regulates immune and inflammatory responses in endothelial cells, vascular smooth muscle cells, and macrophages (37) as well as production of inflammatory cytokines, TNF-alpha and NO (through iNOS expression) (38). Our findings agree with Kandolf-Sekulovic *et al* (24) who used LLLT (904 nm, irradiance 60mW/cm^2, fluence 3.6 J/ cm^2) in a model of contact hypersensitivity (CHS in albino Oxford rats) and observed a reduced release of nitric oxide by the inflammatory cells. These authors suggested that lower levels of NO were caused by fewer inflammatory cells in the dermis, and a possible lower capacity for NO production by skin resident cells (keratinocytes and Langerhans cells). These cells have been suggested to be the NO-producing cells in the CHS skin reaction (39). Eduardo et al (40) analyzed peroxynitrite formation in zymosan-induced arthritis in rats, and found inhibition of joint hyperalgesia that correlated with decreased nitric oxide levels and nitrotyrosine levels in the joint exudates, when compared to control rats,. Taken together, these results point towards a homeostatic role of low levels of NO derived from the constitutive NOS enzymes. Moreover other studies utilizing different experimental models have suggested that LLLT is able to induce SOD expression, decreasing the available concentration of superoxide anion and, as a result, reduce peroxynitrite production (24).

We found that catalase activity was increased significantly between groups IC versus NIC and ID. The suppression of catalase activity in diabetic animals is known to be involved in the susceptibility of diabetics to ROS (41). In addition, a certain level of oxidative stress is known to be required for the satisfactory induction of neovascularization in response to ischemia and tissue damage (42). A further possible mechanism to explain these results is that high glucose levels have been associated with low neutrophil chemotactic activity(43, 44) and reduction of the phagocytic activity of polymorphonuclear cells in diabetic patients(45, 46). Chamon et al(47) showed a 370% and 199% increase in ROS generation during the process of phagocytosis in healthy subjects and DM patients respectively. In the presence of pyruvate, these percentage increases were reduced to 81% and 80%, respectively. Therefore pyruvate exhibited a suppressive action on granulocytes both in healthy individuals and DM patients. One

effect of LLLT could be on pyruvate levels associated with a metabolic signaling pathway depending on the oxidizing profile of the target cell. Pyruvate is associated with protection of different cells against oxidative damage through non-enzymatic scavenging of ROS including H_2O_2 (48). Therefore, pyruvate could selectively cause reduction of H_2O_2 to prevent the generation of the hydroxyl radical (OH•). On the other hand, Karu(49) reported that laser photobiomodulation decreased production of superoxide anion and also increased catalase activity (antioxidant), leading to an increased protein synthesis in a culture of yeast cells.

An important factor to consider is that during oxidative stress, membrane lipids are continuously subjected to lipid peroxidation, shown by an increase in TBARS. In diabetes, lipid peroxidation is a possible factor that influences insulin resistance. Previous studies (41) found high TBARS levels in the blood and in the lung tissue in diabetes mellitus, suggesting that lipid peroxidation occurred in the first 60 days after onset of the disease. In this context, Silveira et al.(31) demonstrated a significant reduction of lipid peroxidation in rats treated with 2 J/cm^2 and 4 J/cm^2, suggesting that LLLT stimulated antioxidant mechanisms that protected against oxidative damage in lipid membranes. In our study, LLLT significantly reduced the amount of MDA generated in the skin of the mice (Table. 2). Many studies in experimental models have shown that LLLT can modulate ROS/RNS by lowering lipid peroxidation (TBARS and MDA) (50, 51) and reducing RNS by inhibiting synthesis of iNOS(51), and increasing the activity of respiratory (41) chain for increased ATP synthesis. However, it is important to emphasize that the effects of LLLT could depend on the total energy, power density, timing and frequency of irradiation, and the wavelength and other characteristics of the laser or light source used (7).

5.0 CONCLUSIONS

In this work our findings in the wounds of diabetic animals indicated a possible protective effect that 904 nm laser could have on the microvasculature, with lowered levels of nitrite, and increased protection against oxidative damage in lipid membranes. Besides, the better-organized and increased amount of collagen fibers demonstrated that LLLT could be effective in clinical practice with poorly healing diabetic wounds.

6.0 CONFLICTS OF INTEREST AND FUNDING SOURCES

The authors declare no conflicts of interest in this research. JCTR was supported by CAPES / PDSE (Coordenação de Aperfeiçoamento de Pessoal- Process no. 006648/2015-00). CF was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP-Process no. 2010/07194-7) in Brazil. MRH was supported by US NIH (grant R01AI050875). The authors express their thanks to professors Walberto dos Santos and Tatiana Sato and to the Laboratory of Pharmacology, Federal University of Ceara for all support with chemical reagents.

7.0 REFERENCES

1.Santos N, dos Santos J, dos Reis J, Oliveira P, de Sousa A, Carvalho C, et al. Influence of the use of laser phototherapy (λ660 or 790 nm) on the survival of cutaneous flaps on diabetic rats. Photomedicine and laser surgery [Internet]. 2010; 28(4):[483-8. pp.].

2.Ames BN, Shigenaga MK, Hagen TM. Oxidants, Antioxidants, And the Degenerative Diseases of Aging. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(17):7915-22.

3.Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: Role in inflammatory disease and progression to cancer. Biochemical Journal. 1996;313:17-29.

4.Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414(6865):813-20.

5.Dean RT, Fu SL, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochemical Journal. 1997;324:1-18.

6.Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004;84(4):1381-478.

7.Gupta A, Avci P, Sadasivam M, Chandran R, Parizotto N, Vecchio D, et al. Shining light on nanotechnology to help repair and regeneration. Biotechnology advances. 2013;31(5):607-631.

8.Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol B. 1999;49(1):1-17.

9.Chen AC-H, Huang Y-Y, Arany PR, Hamblin MR. Role of reactive oxygen species in low level light therapy. Proc SPIE. 2009;7165:doi: 10.1117/12.814890.

10.Chen AC, Arany PR, Huang YY, Tomkinson EM, Sharma SK, Kharkwal GB, et al. Low-Level Laser Therapy Activates NF-kB via Generation of Reactive Oxygen Species in Mouse Embryonic Fibroblasts. PLoS ONE. 2011;6(7):e22453.

11.Farias VX, Macedo FH, Oquendo MB, Tome AR, Bao SN, Cintra DO, et al. Chronic treatment with D-chiro-inositol prevents autonomic and somatic neuropathy in STZ-induced diabetic mice. Diabetes Obes Metab. 2011;13(3):243-50.

12.Collins TJ. ImageJ for microscopy. Biotechniques. 2007;43(1):25-40.

13.Smolle J. Computer recognition of skin structures using discriminant and cluster analysis. Skin Research and Technology. 2000;6(2):58-63.

14.Miot HA, Brianezi G. Morphometric analysis of dermal collagen by color clusters segmentation. Anais Brasileiros De Dermatologia. 2010;85(3):361-4.

15.Nobre-Junior HV, Oliveira RA, Maia FD, Nogueira MAS, de Moraes MO, Bandeira MAM, et al. Neuroprotective Effects of Chalcones from Myracrodruon urundeuva on 6-Hydroxydopamine-Induced Cytotoxicity in Rat Mesencephalic Cells. Neurochemical Research. 2009;34(6):1066-75.

16.Michiels C, Raes M, Toussaint O, Remacle J. Importance of SE-Glutathione peroxidase,catalase and Cu/Zn-SOD for cell-survival against oxidative stress. Free Radical Biology and Medicine. 1994;17(3):235-48.

17.Green LC, Tannenbaum SR, Goldman P. Nitrate synthesis in the germ-free and conventional rat. Science. 1981;212(4490):56-8.

18.Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414(6865):813-20.

19.Francis-Goforth KN, Harken AH, Saba JD. Normalization of diabetic wound healing. Surgery. 2010;147(3):446-9.

20.Adams SB, Jr., Sabesan VJ, Easley ME. Wound healing agents. Foot and ankle clinics. 2006;11(4):745-51.

21.Demidova-Rice TN, Hamblin MR, Herman IM. Acute and Impaired Wound Healing: Pathophysiology and Current Methods for Drug Delivery, Part 2: Role of Growth Factors in Normal and Pathological Wound Healing: Therapeutic Potential and Methods of Delivery. Adv Skin Wound Care. 2012;25(8):349-70.

22.Demidova-Rice TN, Hamblin MR, Herman IM. Acute and Impaired Wound Healing: Pathophysiology and Current Methods for Drug Delivery, Part 1: Normal and Chronic Wounds: Biology, Causes, and Approaches to Care. Adv Skin Wound Care. 2012;25(7):304-14.

23.Tatmatsu Rocha JC, Jansem de Almeida Catanho MT, da Mota DL. Application of the Laser Radiation in Patients of Pressure Ulcers: Clinical and Histomorphometric Analysis of the Derm. Brazilian Archives of Biology and Technology. 2008;51:231-4.

24.Kandolf-Sekulovic L, Kataranovski M, Pavlovic MD. Immunomodulatory effects of low-intensity near-infrared laser irradiation on contact hypersensitivity reaction. Photodermatology Photoimmunology & Photomedicine. 2003;19(4):203-12.

25.Ohkuwa T, Sato Y, Naoi M. Hydroxil Radical Formation in Diabetic Rats Induced by Streptozotocin. Life Sciences. 1995;57(13):1325-.

26.Winiarska K, Drozak J, Wegrzynowicz M, Fraczyk T, Bryla J. Diabetes-induced changes in glucose synthesis, intracellular glutathione status and hydroxyl free radical generation in rabbit kidney-cortex tubules. Molecular and Cellular Biochemistry. 2004;261(1-2):91-8.

27.Pacher P, Liaudet L, Soriano FG, Mabley JG, Szabo E, Szabo C. The role of poly(ADP-ribose) polymerase activation in the development of myocardial and endothelial dysfunction in diabetes. Diabetes. 2002;51(2):514-21.

28. Ferraresi, Cleber; Pires de Sousa, Marcelo Victor; Huang, Ying-Ying; et al. Time response of increases in ATP and muscle resistance to fatigue after low-level laser (light) therapy (LLLT) in mice. Lasers in Medical Science.2015;30(4): 1259-1267.

29.Huang Y-Y, Sharma SK, Carroll J, Hamblin MR. Biphasic dose response in low level light therapy- an update. Dose-Response. 2011;9(4):602-18.

30.Vladimirov YA, Osipov AN, Klebanov GI. Photobiological principles of therapeutic applications of laser radiation. Biochemistry-Moscow. 2004;69(1):81-90.

31.Silveira PC, Silva LA, Fraga DB, Freitas TP, Streck EL, Pinho R. Evaluation of mitochondrial respiratory chain activity in muscle healing by low-level laser therapy. J Photochem Photobiol B. 2009;95(2):89-92.

32.Bagasra O, Michaels FH, Zheng YM, Bobroski LE, Spitsin SV, Fu ZF, et al. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(26):12041-5.

33.Oliveira Volpe CM, Machado Abreu LF, Gomes PS, Gonzaga RM, Veloso CA, Nogueira-Machado JA. The Production of Nitric Oxide, IL-6, and TNF-Alpha in Palmitate-Stimulated PBMNCs Is Enhanced through Hyperglycemia in Diabetes. Oxidative Medicine and Cellular Longevity. 2014.2014:12-24.

34. Kolb H, Mandrup-Poulsen T. An immune origin of type 2 diabetes? (vol 48, pg 1038, 2005). Diabetologia. 2005;48(8):1677-1688.

35.Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, et al. Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. Diabetes. 2003;52(12):2882-7.

36.Guha M, Bai W, Nadler JL, Natarajan R. Molecular mechanisms of tumor necrosis factor alpha gene expression in monocytic cells via hyperglycemia-induced oxidant stress-dependent and -independent pathways. Journal of Biological Chemistry. 2000;275(23):17728-39.

37.Sena CM, Pereira AM, Fernandes R, Santos-Silva D, Faustino A, Ceica R. Novel therapeutic approach to target endothelial dysfunction in type 2 diabetes. Cardiovascular Research. 2014;103: S102–S141.

38.Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, et al. Role of the Toll-like receptor 4/NF-kappa B pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arteriosclerosis Thrombosis and Vascular Biology. 2007;27(1):84-91.

39.Ross R, Gillitzer C, Kleinz R, Schwing J, Kleinert H, Forstermann U, et al. Involvement of NO in contact hypersensitivity. International Immunology. 1998;10(1):61-9.

40.Eduardo FP, Mehnert DU, Monezi TA, Zezell DM, Schubert MM, Eduardo CP, et al. Cultured epithelial cells response to phototherapy with low intensity laser. Lasers Surg Med. 2007;39(4):365-72.

41.Dhaunsi GS, Bitar MS. Antioxidants attenuate diabetes-induced activation of peroxisomal functions in the rat kidney. J Biomed Sci. 2004;11(5):566-70.

42.Urbich C, Dernbach E, Aicher A, Zeiher AM, Dimmeler S. CD40 ligand inhibits endothelial cell migration by increasing production of endothelial reactive oxygen species. Circulation. 2002;106(8):981-6.

43.Mowat AG, Baum J. Chemotaxis of Polymorphonuclear Leukocytes from Patients with Diabetes Mellitus. New England Journal of Medicine. 1971;284(12):621-9.

44.Collison KS, Parhar RS, Saleh SS, Meyer BF, Kwaasi AA, Hammami MM, et al. RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs). Journal of Leukocyte Biology. 2002;71(3):433-44.

45.Chanchamroen S, Kewcharoenwong C, Susaengrat W, Ato M, Lertmemongkolchai G. Human Polymorphonuclear Neutrophil Responses to Burkholderia pseudomallei in Healthy and Diabetic Subjects. Infection and Immunity. 2009;77(1):456-63.

46.Lin J-C, Siu LK, Fung C-P, Tsou H-H, Wang J-J, Chen C-T, et al. Impaired phagocytosis of capsular serotypes K1 or K2 Klebsiella pneumoniae in type 2 diabetes mellitus patients with poor glycemic control. Journal of Clinical Endocrinology & Metabolism. 2006;91(8):3084-7.

47.Chamon JSF, Fagundes-Netto FS, Gonzaga RM, Gomes PS, Volpe CMdO, Nogueira-Machado JA. Absence of pyruvate anti-oxidant effect on granulocytes stimulated toll-like receptors. Free Radicals and Antioxidants. 2013;3, Supplement(0):S11-S5.

48.Moriguchi N, Hinoi E, Tsuchihashi Y, Fujimori S, Iemata M, Takarada T, et al. Cytoprotection by pyruvate through an anti-oxidative mechanism in cultured rat calvarial osteoblasts. Histology and Histopathology. 2006;21(9):969-77.
49.Karu T, Pyatibrat L, Kolyakov S, Afanasyeva N. Absorption Measurements of Cell Monolayers Relevant to Mechanisms of Laser Phototherapy: Reduction or Oxidation of Cytochrome c Oxidase Under Laser Radiation at 632.8 nm. Photomedicine and Laser Surgery. 2008;26(6):593-9.
50.Luo L, Sun Z, Zhang L, Li X, Dong Y, Liu TC-Y. Effects of low-level laser therapy on ROS homeostasis and expression of IGF-1 and TGF-beta 1 in skeletal muscle during the repair process. Lasers in Medical Science. 2013;28(3):725-34.
51.Rizzi CF, Mauriz JL, Freitas Correa DS, Moreira AJ, Zettler CG, Filippin LI, et al. Effects of low-level laser therapy (LLLT) on the nuclear factor (NF)-kappa B signaling pathway in traumatized muscle. Lasers in Surgery and Medicine. 2006;38(7):704-13.

ESTUDO II

Phototherapy Laser and LED acts over Mitochondrial Function and estimulates collagen organization in diabetic animals

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ABSTRACT

Diabetes Mellitus causing a delay on healing and its main complications are chronic inflammation, a decrease in collagen production and extracellular matrix. Objective: This study investigate Phototherapy (Laser and LED) effects on healing skin in diabetic animals and possible mitochondrial mechanisms involved. Methods: Twenty Wistar rats were randomized into groups: control group, incision without any treatment (SHAM), diabetic group, incision without any treatment (NID), diabetic laser group, incision irradiated group (DLASER) with Laser 904 nm, 40 mW, 1 min, 2,4 J, diabetic LED group, incision LED irradiated group (DLED), irradiated with LED 805 nm, 48 mW, 22 s, 1,056 J. Diabetic groups were submitted one injection with streptozotocin (70 mg/kg). 120 days after diabetes induction new glycemic analysis were performed to confirm chronic diabetes status. After that a cutaneous incision was made on the dorsum animal. Histological parameters, collagen with comparative study between picrosisrius and atomic force microscopy, VEGF, COX2, FIS1 e MFN2 imunoexpression were analyzed. Results: The comparative study between AFM and picrosirius red stain showed in NID a discrete deposition of immature collagen matrix. DLASER demonstrated an intense deposition of an organized and immature collagen. The DLED showed a moderate deposition of mature collagen matrix. The expression of VEGF demonstrated differences only 5^{th} day between NID versus DLED ($p < 0,005$). Immunoexpression of COX-2 showed on 5^{th} day there was differences between NID versus DLED ($p < 0,005$). Diabetic irradiated groups showed statistic increase in MFN2 during the experiment ($p=0,04$) and NID, DLASER and DLED increased FIS1 scores ($p=0,04$). Conclusion: We can see that the infrared wave beam, both laser and LED can have altered, expression of VEGF and COX2 and FIS1 and MFN2 and proliferation of mature collagen fibers in diabetic animals.

Keywords: Wound healing, diabetes mellitus type 1, LED, FIS1, MFN2

INTRODUCTION

Diabetes Mellitus (DM) defines a group of chronic metabolic diseases characterized by hyperglycemia due to insufficient production of insulin and / or insulin resistance (1). DM can cause impaired wound healing by affecting one or more biological mechanisms of the process (2, 3). However, the exact mechanism of poor healing of wounds in DM is still unclear but there is evidence that this process has been associated with poor angiogenesis and fibroplasias, small amounts of collagen fibrils, and low collagen production(2, 4, 5). In addition, wound healing with DM has been shown to be associated with persistent inflammatory state with elevated levels of pro-inflammatory cytokines and proteases together with impaired expression of growth factors such as platelet-derived growth factor (PNIDF), vascular endothelial growth factor (VEGF) which are essential for wound healing to progress as well as the induction of new blood vessels (3) 6). Still, diabetes is characterized by COX-2 expression up-regulated, which leads us to believe an important role in this prolonged inflammation (7). In this context, the clinical demand for therapeutic strategies to promote wound healing in diabetic patient has been growing in recent years

Innovative clinical approaches such as phototherapy by Low Level Laser Therapy (LLLT) or Light-Emitting Diode Therapy (LED) has been developed in order to stimulate the wound healing. Phototherapy increases mitochondrial proteins and improves function. Also it is an attractive strategy for promoting cell repair and regeneration, preserving organ function and treating a number of pathologies resulting from damage/inhibition of mitochondrial function(8). The use of light has been related the ability to improve collagen production and wound healing. There are a few decades LLLT is a light coherent and it has emerged as a safe and effective therapeutic in the inflammatory process (9) proliferative (10, 11) and remodeling (11, 12) that make up the wound healing process in the skin.

LED has been proposed as a new and inexpensive alternative to Phototherapy (13, 14). Despite the beneficial effects already observed by laser, these devices require high energy and are still relatively expensive. Light emitting diodes (LEDs) is a light a no coherent and it has been presented as therapeutic alternatives less expensive, LEDs are small and robust devices emit electromagnetic radiation which varies in wavelength ultraviolet to infrared normally generate light of low intensity in the mW range (15). Early studies, LED was implicated to promote pain relief (16), improve muscle performance (17) minimize muscle fatigue (18) and stimulate wound healing (19).

However, data on monotherapy with LEDs in wound healing is limited and new approaches are necessary.

Despite the positive effects of LLLT and LED on the stimulation of wound healing demonstrate by many authors (9, 11, 15, 19-21), there is a lack of information about the effects of these therapies on this process. Moreover, works describing the comparative effects of LLLT and LED on the tissue healing process in diabetic and non-diabetic ulcers is still scarce. Thus, with the techniques capable of detect structures and forces in the nanoscale range such as atomic force microscope (AFM) it has possible to examine collagen deposition during the wound healing. Atomic force microscope (AFM) helps to collect quantitative data from extracellular matrix molecules, which advantages include rapid specimen preparation and can provide valuable information on nano-mechanical properties of extracellular matrix components. In the present study were compared the effects of low power laser (904nm) and LED (805 nm) on the tissue healing process in diabetic and non-diabetic ulcers at two time points (3th and 5th days post lesion) having as parameters histologic analysis, extracellular matrix using AFM and collagen amount. COX-2, VEGF, FIS1 and MFN2 expressions to understand involved mechanisms in this repair mechanism.

METHODS

Experimental design

This study was conducted according to the Guiding Principles for the Use of Laboratory Animals and it was approved by the Animal Care Committee guidelines at Federal University of Ceará (protocol 01/2013). In this investigation, 20 male Wistar rats were used (45 days of age and weight 200-250 g), maintained under controlled conditions of temperature (24 ± 2°C) with light–dark periods of 12 h, with free access to water and commercial diet.

The experimental animals were randomly distributed into groups (n=5 each group): SHAM group, incision without any treatment (Sham), diabetic group, incision without any treatment (NID), diabetic laser group, incision irradiated group (DLASER), diabetic LED group, incision LED irradiated group (DLED).

Diabetes induction

The animals of Sham group were submitted an only one mesenteric injection with 0,022 mL NaCl 0,9% solution. Animals of diabetic groups (NID, DLASER and DLED) were submitted an only one injection with stretptozotocin (70 mg/kg citrate buffer, pH=4.5). Six hours after administration, the animals had their water supply

replaced by an aqueous solution of glucose (10%) for 24 hours, six days after the injection, blood sugar level was verified and only animals with blood sugar levels of 300 mg/dL or higher entered the study (22). In order to test blood glucose levels, the whole blood of each experimental rats were drawn from the tail vein and immediately determined using test strips read by a blood sugar machine (Accu-Chek Active, Roche, Basel, Switzerland).

Surgical procedures

Before surgical incision, new glycemic analysis was performed to confirm chronic diabetes status in diabetic groups. The animals were anesthetized by intraperitoneal injection of thiopental (40 ml/ kg i.p. Cristália®, Itapira, Brasil). Each animal was placed in prone position to perform trichotomy of dorsal region and disinfected with povidone iodine and were cut with a carbon steel surgical blade until the depth reached the hypodermis. In order to standardize the lesions, a surgical field with a gap of 2 mm width and 2 cm long was used, with reference to the animal's posterior iliac crest, which was labeled with pen soluble in alcohol (23). The health status of the animals was monitored on a daily basis. The animals were housed in pairs and the intake of water and food was monitored in the initial postoperative period. According to each experimental period, animals were euthanized according figure 1:

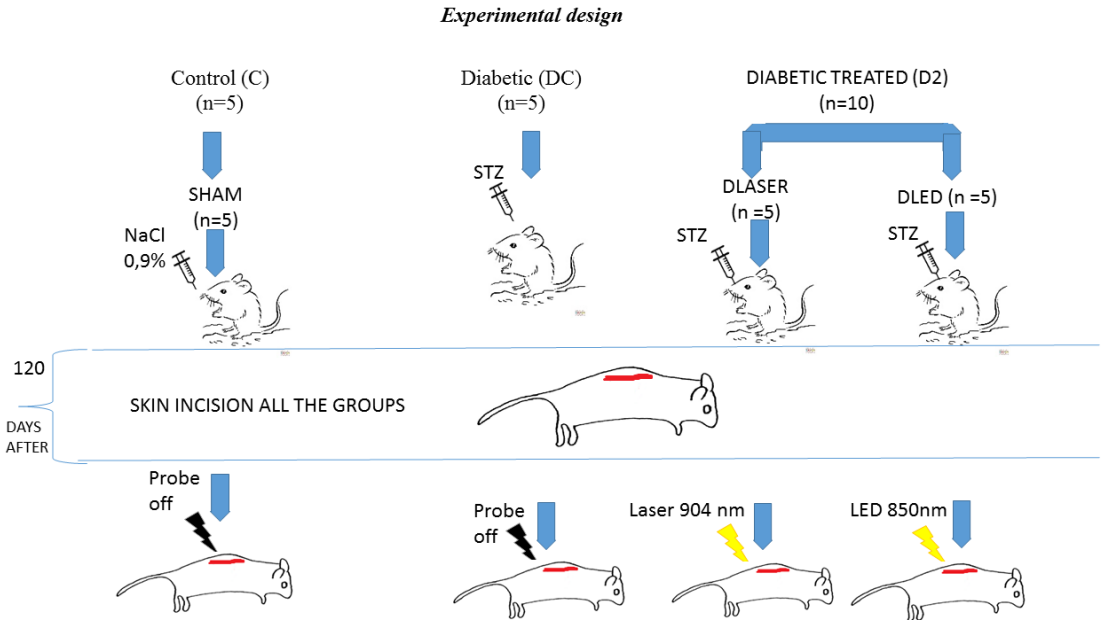

Figure . 1 Experimental design

Treatment

The animals denominated were irradiated with Laser (904 nm, 40 mW, 1 min, 2,4 J) and animals denominated DLED were irradiated with LED (805 nm, 48 mW, 22

s, 1,056J J). The application time varied according to the equipment used and has been created automatically (Table 1). The choice of the types of treatment was made due to lack of consensus in the literature on the effects of processing parameters on the outcome of LLLT.

Table. 1 Phototherapy treatment

	LLLT	LED
Λ	904 nm	805 nm
Average radiant power	40 mW	48 mW
Fluency	18,33 J/cm^2	14,69 J/cm^2
Time	1 min	22 s
Energy	2,4 J	1,056 J
Beam spot size at target	0,1309	0,196
Technique	contact	contact
Number of points irradiated	1	1
Number and frequency of treatment sessions	1 x / day/ 5 consecutive days	1 x / day / 5 consecutive days

Histopathological analysis

Rats were individually euthanized by carbon dioxide asphyxia in different set points (3 days and 5 days after incision). Skin samples were fixed in 10 % buffered formalin (Merck, Darmstadt, Germany) for 24 h. Afterwards, dehydrated and embedded in paraffin blocks. Three sections (5 μm) of each specimen were transversely sectioned (Microtome Leica Microsystems SP 1600, Nussloch, Germany) and stained with hematoxylin and eosin (H.E. stain, Merck, Darmstadt, Germany). The histopathological analysis was performed with an optical microscopy (Olympus Optical Co., Tokyo, Japan) and scoring according to the following parameters: granulation tissue, inflammatory process, area of fibrosis and angiogenesis. The criteria used on this analysis were used previously (22, 24). Each criterion was scored as absent, discrete, moderate, or intense according to the percentage of the phenomenon observed.

Collagen Analysis

For collagen study sections stained with picrosirius red were examined microscopically and the images used for analysis were captured by microscopy

(Olympus Optical Co., Tokyo, Japan) and light field 20X objective using a capture system AxioVision 4.7.2.0 software v (Carl Zeiss, Germany). The processing and image analysis were performed using the public domain software ImageJ 1:49, 64-bit version (National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/), using plugin color deconvolution. The images were analyzed and were collected data about relative percent on total tissue.

Atomic Force Microscopy

The images were investigated in a Flex Nano surf equipment. The samples were scanned vibrational mode using Nano sensors model NCSTR from Nano world cantilevers with typical resonance frequency of 160KHz. The histological samples were fixed in 10 % buffered formalin (Merck, Darmstadt, Germany) for 24 h. Afterwards, dehydrated and embedded in paraffin blocks. Three sections (5 μm) of each specimen were longitudinally sectioned (Microtome Leica Microsystems SP 1600, Nussloch, Germany) and deposited over glass slides. During the paraffin removal process the tissue was rehydrated.

Immunohistochemistry

Histological sections (5 μm) were dewaxing using xylene and rehydrated in graded ethanol. After, the material was pre-incubated with 0.3 % hydrogen peroxide (Labsynth®, Diadema, Brazil) in phosphate-buffered saline (PBS) solution for 10 min in order to inactivate endogenous peroxidase and then block with 5 % normal goat serum in PBS solution for 20 min. Three sections of each specimen were incubated for 2 h with polyclonal primary antibody anti-Cyclooxygenase-2 (1:500, Santa Cruz Biotechnology, Santa Cruz, USA), anti-Vascular endothelial growth factor (1:500, Santa Cruz Biotechnology, Santa Cruz, USA), Mitofusin 2 (MFN2) (1:900, Santa Cruz Biotechnology, Santa Cruz, USA) and Mitochondrial fission 1 protein (FIS1) (1:800, Santa Cruz Biotechnology, Santa Cruz, USA). Afterwards, the sections were incubated with biotin conjugated secondary antibody anti-rabbit IgG (Vector laboratories, Burlingame, CA, USA) at a concentration of 1:200 in PBS for 30 min, followed by the application of preformed avidin biotin complex conjugated to peroxidase (Vector Laboratories, Burlingame, CA, USA) for 30 min. A solution of 3-3'-diaminobenzidine solution (0.05 %) and Harris hematoxylin were applied. The expression of COX-2, VEGF was assessed qualitatively (presence and location of the immunotargets) and semi-quantitatively in five fields using an optical light microscope (Olympus Optical Co., Tokyo, Japan). According to previous studies, for quantify of positive cells per

field we used IHC Profiler plugin compatible with software ImageJ (25) and was described by a scoring scale from 1 to 4 (26) (1= negative, 2= low positive, 3= positive, and 4= high positive).

Statistical analysis

Histopathological, immunohistochemistry and collagen analysis were performed under double blind conditions for multiple comparisons, data were examined by the analysis of variance (ANOVA) repeat measures, followed by Tukey's test. The significance level was 5%. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Histopathological analysis

SHAM group

The groups were compared using hematoxylin eosin stain and was observed in ***SHAM group*** on 3th day a typical chronic ulcer, partial reepithelization, a moderate inflammatory infiltrate lymphocytic and a discrete granulation tissue. In 5th Day discrete inflammatory infiltrate, healing partially complete, discrete amount of fibroblasts. A moderate amount of granulation tissue rich in newly formed blood vessels was observed with fibroblasts fusiform.

Diabetic group (NID)

In 3th Day, the group presented a large chronic ulcer, an intense inflammatory infiltrate lymphocytic and a discrete granulation tissue. In 5th Day moderate inflammatory infiltrate, discrete amount of fibroblasts and a discrete granulation tissue poor in newly formed blood vessels. A few amount of fibroblasts fusiform and a discrete deposition of a disorganized and immature collagen matrix was observed. (Figure 1)

DLASER group

In 3th day, animals showed a chronic ulcer, with inflammatory infiltrate, granulation tissue and fibroblasts amount moderate. In 5th day discrete inflammatory infiltrate, moderate amount of fibroblasts and some moderate granulation tissue rich in newly formed blood vessels. An intense amount of fibroblasts fusiform and a moderate deposition of an organized and immature collagen matrix was observed. (Figure 1)

DLED group

In 3th day the DLED group showed discrete presence of granulation tissue and a typically discrete chronic inflammatory reaction, with few acute inflammatory cells and fibroblasts. The dermis showed an intense amount of newly formed blood vessels. In 5th day a discrete amount of granulation tissue and an intense amount of fusiform and triangular fibroblasts was also observed. The reepithelization process was advanced and a moderate to intense deposition of mature collagen matrix was showed. (Figure 1)

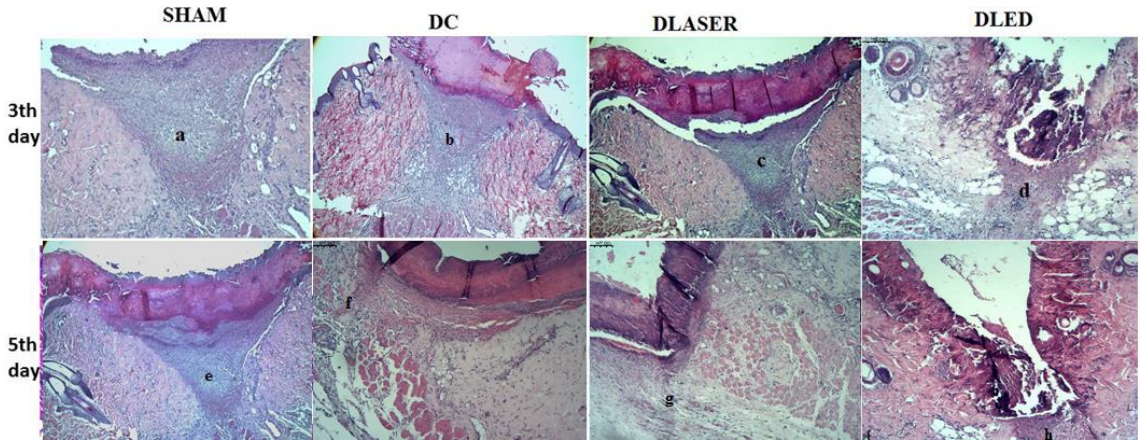

Figure . 2 comparative slides on 3 th and 5th day post treatment.On 3 th day showing a moderate inflammatory infiltrate lymphocytic and a discrete granulation tissue ***(a)****, an intense inflammatory infiltrate lymphocytic and a discrete granulation tissue* ***(b),*** *a chronic ulcer, with inflammatory infiltrate, granulation tissue and fibroblasts amount moderate* ***(c)*** *and discrete chronic inflammatory reaction, with few acute inflammatory cells and fibroblasts* ***(d)****. On 5th day Photomicrograph showing an discrete inflammatory infiltrate, healing partially complete, discrete amount of fibroblasts* ***(e)*** *discrete granulation tissue poor in newly vessels* ***(f),*** *moderate deposition of an organized and immature collagen matrix was observed (****g),*** *a moderate deposition of mature collagen matrix* ***(h).****SHAM=incision without any treatment; NID= diabetic group, incision without any treatment, DLASER=diabetic laser group, incision irradiated group, DLED=diabetic LED group, incision LED irradiated group.*

Collagen Analysis

Data about relative percent of content of collagen on total tissue revealed a statistical difference between the SHAM group x DLASER (p = 0.01) and between DLASER x DLED (p = 0,009). Another differences can be observed in table 1:

GROUPS	*% COLLAGEN FIBERS (Means)*
SHAM (a)	6.08 ± 0.80 [b]
NID (b)	3.01 ± 0.10 [a]
DLASER (c)	11.38 ± 0.1[b]
DLED(d)	3.56 ± 0.5[c]

Table. 2Statistical analysis using morphometric computational systems to determine amount collagen analyzed on 5th day by analysis of variance (ANOVA). Statistical analysis using morphometric computational systems to determine amount collagen analyzed on 5th day by analysis of variance (ANOVA) The 3th day period not showed because no significant differences were observed. Groups: non-diabetic control (Sham), diabetic non treated (NID), diabetic treated with LLLT (DLASER) and diabetic LEDT treatment (DLED). [a] versus Sham group; [b] versus NID group; [c] versus DLASER group. Date are showed mean ± SEM. The significance level was set at p < 0.05.

Atomic Force Microscopy

A comparative study between AFM and picrosirius red stain was made to analyses the interaction between collagen fibers and matrix cellular formation in hyperglycemic conditions. In the 3th day no differences were observed between groups. However, on 5th day SHAM group picrosirius red stain showed a disorganized collagen fibers and a discrete extracellular matrix with an incipient organization and the AFM discrete amount of fibers was observed but the network of interwoven fibers characteristic of the extracellular matrix has not yet formed. In diabetic control group (NID) picrosirius red stain evidenced a discrete deposition of a disorganized and immature collagen matrix and a few of fibers in extracellular matrix was observed in AFM. In other hand, DLASER group show a moderate deposition of an organized and immature collagen matrix was observed in AFM. In DLED group picrosirius red stain showed a deposition of mature collagen matrix and AFM showed an organized deposition (Figure 3).

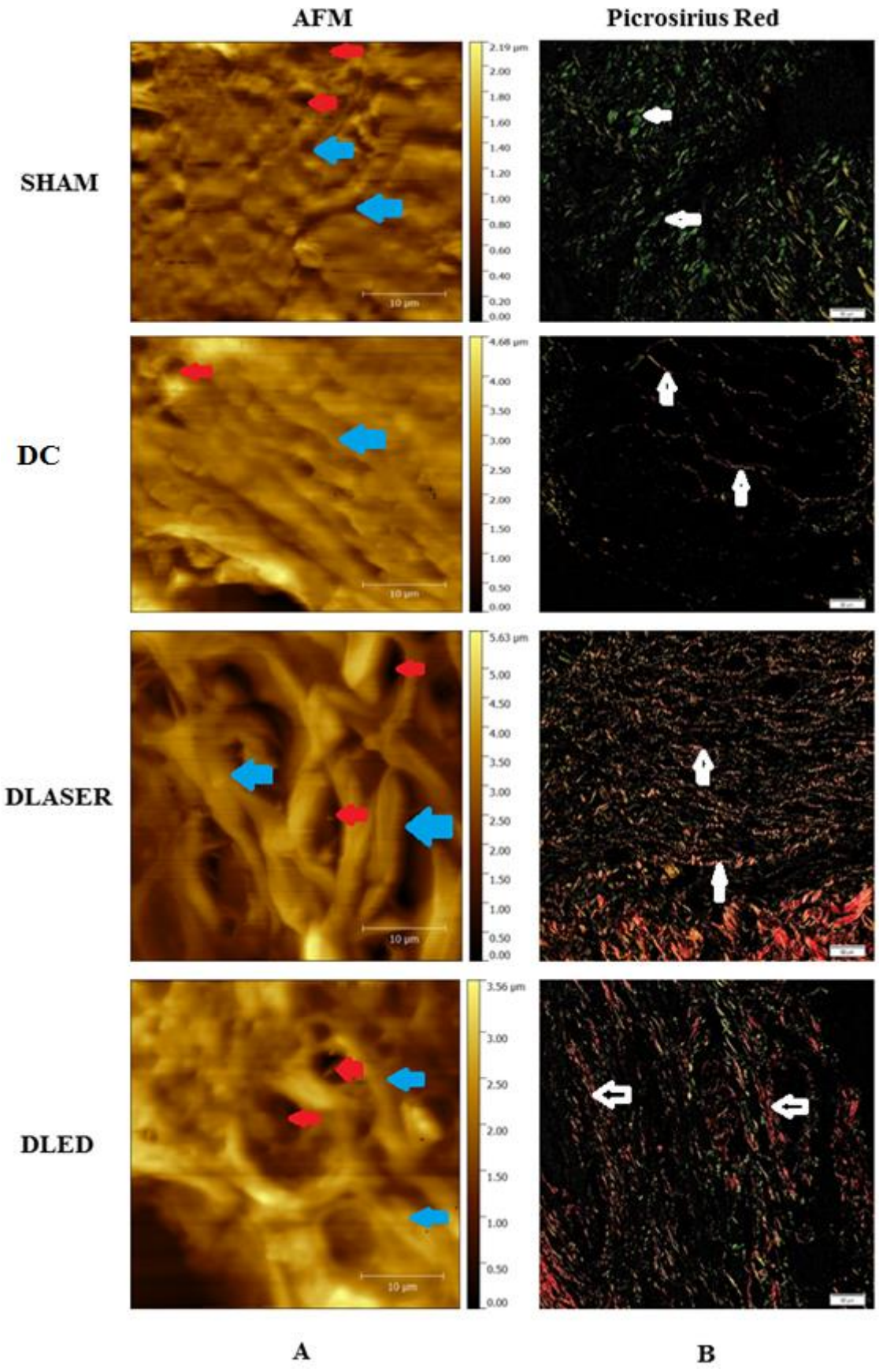

Figure . 3Histologic slides of skin diabetic mice and non-diabetics on the 5th day of post-surgical SHAM groups, NID. DLASER and DLED. A) Blades analyzed by atomic force microscopy (blue arrows indicate fibers that make up the ECM). Red arrows in show the formation of new blood vessels. B) Staining with picrosirius

red (white arrows links point to collagen fibers). SHAM=incision without any treatment, diabetic group, NID=incision without any treatment, DLASER=diabetic laser group, incision irradiated group, DLED=diabetic LED group, incision LED irradiated group.

Immunohistochemistry

VEGF. The findings indicated that the groups evolved differently between times 3th and 5th days. No differences were observed between groups at time 3th day. The DLED group demonstrated a statistically higher expression of VEGF than the NID (p = 0.000), DLASER (p = 0.02) and SHAM (p=0,002) after 5 days (Figure 4).

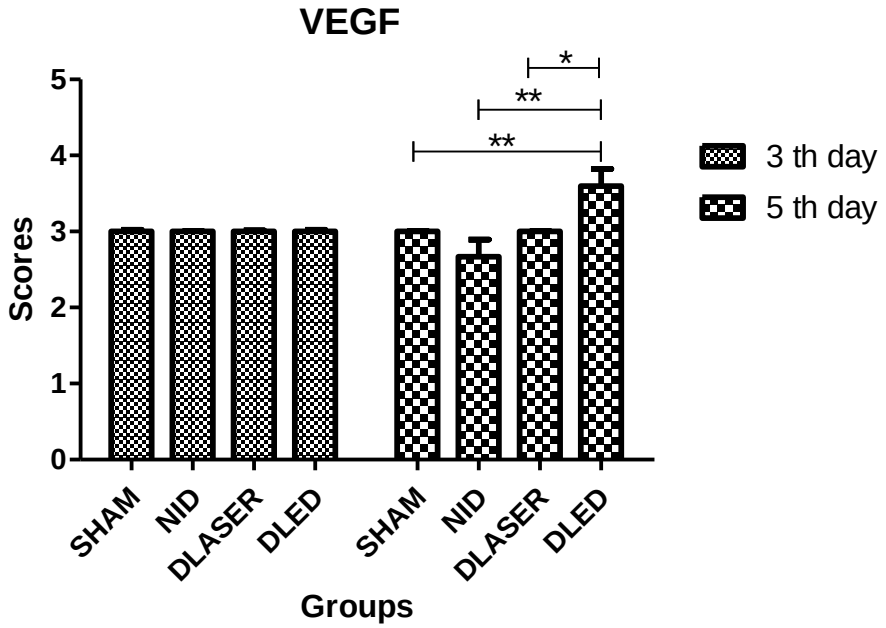

Figure . 4. Mean an SD scores for immunostaining of VEGFFigure 4. Mean an SD scores for immunostaining of VEGF. Significant differences of p = 0,00 are represented with a single asterisk; two asterisks p=0,002. SHAM=incision without any treatment; diabetic group, NID=incision without any treatment; DLASER=diabetic laser group, incision irradiated group; DLED=diabetic LED group, incision LED irradiated group.

COX-2. The labeling for COX-2 occurred in the DLED significantly lower marking than the DLASER (p= 0,001) after 3 days. Already in five days the DLED showed a significantly lower marking compared to the NID (p = 0.008) and DLASER (p = 0.005). In addition, SHAM group demonstrated significantly lower scores than the NID (p = 0.001) (Figure 5).

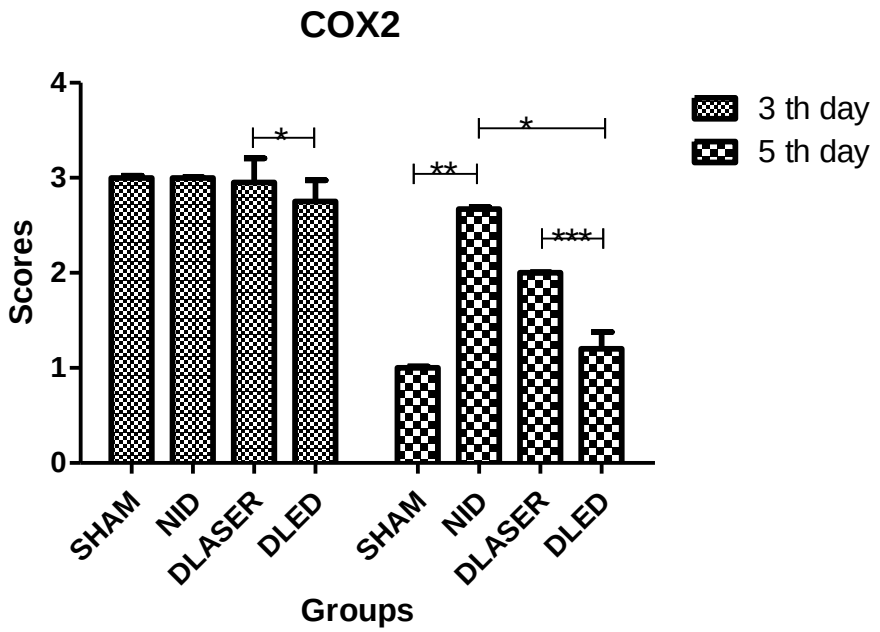

Figure . 5.Mean an SD scores for immunostaining of COX-2.Figure 5. Mean an SD scores for immunostaining of COX-2. Significant differences of p = 0,001 are represented with a single asterisk; two asterisks p=0,008; three asterisks p= 0,005. SHAM=incision without any treatment, diabetic group; NID=incision without any treatment; DLASER=diabetic laser group; incision irradiated group; DLED=diabetic LED group; incision LED irradiated group.

MFN2. On 3th day DLASER group showed lower scores compared to SHAM group (p = 0.02). On 5th day the NID group had the lowest scores with significant differences with DLED groups (p = 0.000), SHAM (p = 0.001). In addition, differences between the DLASER and DLED groups (p = 0.001) was observed. (Figure 6):

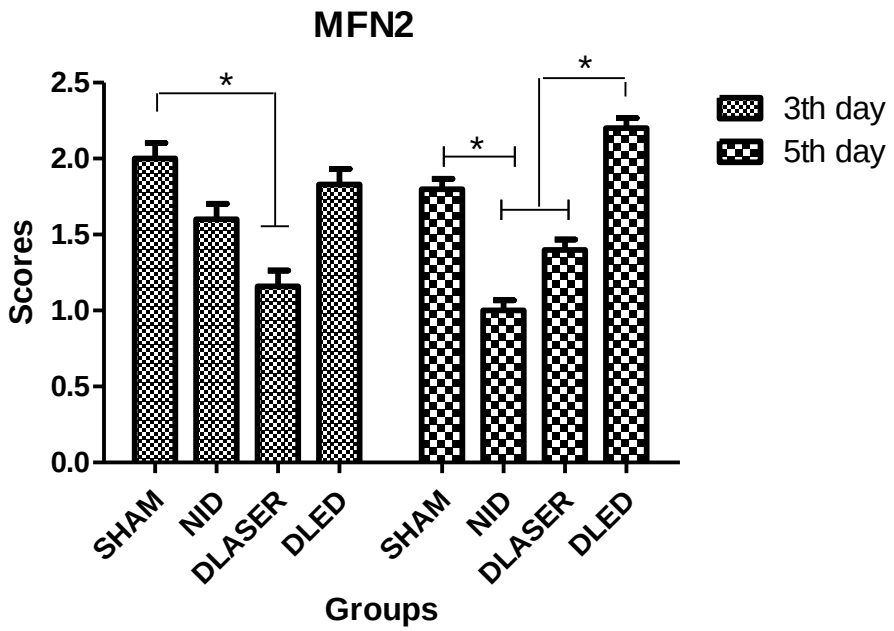

Figure . 6Mean an SD scores for immunostaining of MFN2Figure 6. Mean an SD scores for immunostaining of MFN2. Significant differences of p = 0,00 are represented with a single asterisk and p=0,04 by two asterisks. SHAM=incision without any treatment, diabetic group; NID=incision without any treatment; DLASER=diabetic laser group; incision irradiated group; DLED=diabetic LED group; incision LED irradiated group.

FIS1. On 3th day it's possible to observe that the LED group showed a statistically lower scores than the other groups (p = 0.000). In the five days DLED demonstrated statistically lower compared to DLASER (p = 0.000) and NID (p = 0.001). Also, DLASER showed statistically higher than the SHAM (p =, 000) (Figure 7).

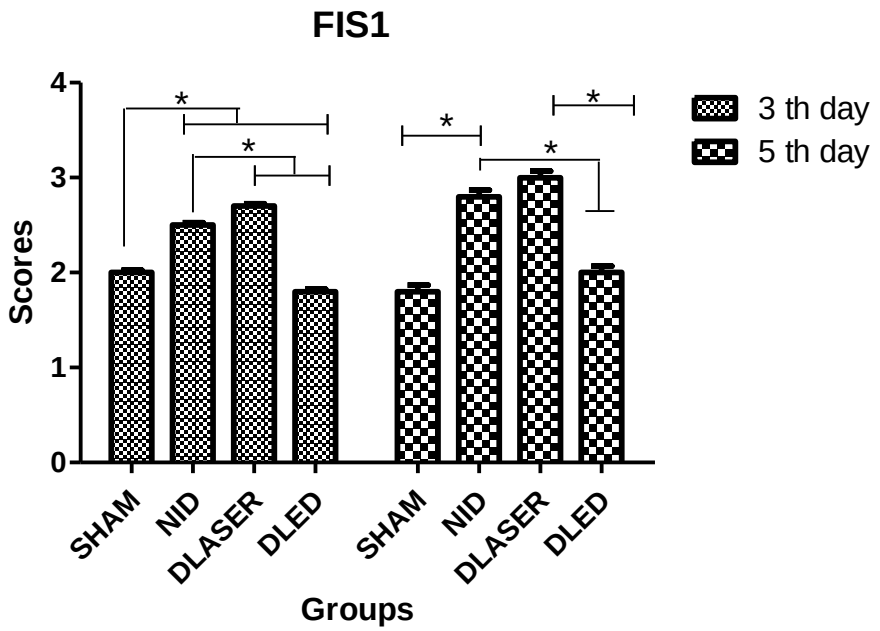

Figure . 7.Mean an SD scores for immunostaining of FIS1Figure 7. Mean an SD scores for immunostaining of FIS1. Significant differences of p = 0,04 are represented with a single asterisk. SHAM=incision without any treatment, diabetic group; NID=incision without any treatment; DLASER=diabetic laser group; incision irradiated group; DLED=diabetic LED group; incision LED irradiated group.

DISCUSSION

The objective of this study was to compare the use of LED and laser in the treatment of ulcers in diabetic rats at 3 and 5 days' post injury. Data showed a discrete presence of granulation tissue and a typically discrete chronic inflammatory reaction, with few acute inflammatory cells of inflammatory infiltrates in irradiated animals, especially DLED group on 3th and 5th days. The content of collagen was significantly higher in irradiated animals by Laser which was confirming with AFM that showed a larger organization of these fibers. Immunohistochemistry analysis demonstrated that the formation of new vessels, which was expressed by dialing VEGF increased in irradiated groups, especially the LED and a lower marker of COX-2 and showed indications that low-intensity phototherapy was able to produce changes in mitochondrial homeostasis (fission / fusion) through the changes of FIS1 and MFN2 levels when compared to control groups.

In diabetes, there a disturbance in clot formation and the inflammatory phase is deregulated (27) often with a prolonged and excessive inflammatory response. Our results using LLLT and LED show a decreased of inflammatory process in the injury

site and Phototherapy enhanced wound healing in diabetic mice, as evidenced by histopathological evaluation of wounds of treated and untreated animals. The results of this study indicate that an irradiation Laser 904 nm, 40 mW, 1 min, 2,4 J, and LED 805 nm, 48 mW, 22 s, 1,056 J accelerates wound healing in induced diabetic rats. The treated animals showed a better quality of scar tissue and inhibitory effects were not found in any of the therapies employed. Our findings agree with Peplow et al (20) that used Laser photobiomodulation of wound healing in diabetic and non-diabetic mice. The authors showed that non-irradiated animals on the 14th day had a partial inflammatory exudate, with the inflammatory response of the grouping of moderate to intense. By contrast, the irradiated animals with LLLT 1.6 J demonstrated no inflammatory exudate and only minimal inflammatory response, with collagen fibers that filled the whole edge of the wound in his work.

The healing of a wound requires a well-orchestrated integration of the complex biological and molecular events of extracellular matrix (ECM) deposition and angiogenesis. Collagen is a major component ECM and is synthesized due to the balance between synthesis and degradation of matrix(28). Healing in patients with diabetes is associated with the loss of collagen, which can be due to a change in synthesis of metalloprotease enzymes, increased metabolism, or a combination of both (45). Our comparative study between picrosirius red stain and AFM showed convergent results with collagen deposition increased and matrix extracellular formation it was more organized, respectively. These results corroborate with Singer et(29) al that links angiogenesis and wound contraction and matrix deposition, aided by such growth factors as fibroblast growth factors, and vascular endothelial growth factor.

Despite the enormous amount of research on the pathogenesis of delayed healing diabetes(6, 11, 20, 21, 30) still there no clear answer and seems to be a result of disease and micro / macrovascular (21) as well as inadequate angiogenesis. There evidence that diabetic animals have reduced vascular density, damage to VEGF signaling and reduced collateral perfusion, which demonstrates an impaired endogenous angiogenic response (31). Our findings indicated that on 3th day the effects of LLLT and LED on VEGF of expression were not noticed but over the five days of treatment there was increase expression of this factor in DLED compared to Sham, NID and DLASER. These results are important because some authors noted that diabetic db / db mice possessed altered sensitivity to exogenous signals such as VEGF and IGF 1 as well as the ability to change the release of cytokine and indicates a stimuli effect over

proliferation and migration of endothelial cells according another authors (32). Moreover, expression of COX-2 showed that DLED was higher compared to DLASER 3 th day but on 5th day the diabetic animals treated with LED showed the less levels compared to DLASER. These data may partly be explained by the fact that COX-2 have been confirmed to participate in this process and regulate the expression of VEGF (7). Besides, COX-2 has been intensely studied for many years as an inflammatory mediator, and the up-regulation of COX-2 expression has been associated with the up-regulation of VEGF in various tissues (33, 34). These results support the results on the reduction of the inflammatory infiltrate in DLED animals and the formation of new blood vessels observed in atomic force microscopy.

In addition, mitochondrial dysfunction is considered to be a critical component in the development of insulin resistance and type 1 diabetes (35, 36). It has been known that altered mitochondrial fusion promotes mitochondrial elongation and disorganized fission leads to exacerbated increased fragmentation of mitochondria, both hurting mitochondrial function (37). Diabetes and oxidative stress it generates excessive mitochondrial fission activate leading to mitochondrial damage and injury cellular (38). In mammals, mitochondrial fusion is controlled by MFN1 and MFN2 on the mitochondria outer membrane and the inner membrane. Bach et al (2005) and Hernandez-Alvarez (2010) reported that MFN2 expression is reduced in skeletal muscle of obese and diabetes type 2 and that deficiency of MFN2 produces mitochondrial dysfunction, increases the concentration of H2O2, leading to insulin resistance in skeletal muscle and liver (39, 40). The mitochondrial fission is regulated by cytoplasmic protein dynamin-related protein 1 (Drp1), which moves the outer mitochondrial membrane and participates in the fragmentation reaction. Furthermore, FIS1 protein that presumably functions as the adapter in external mitochondrial fission protein Drp1 (37). Our findings showed increased MFN2 scores on irradiated diabetic animals with Laser and LED and which leads us to think that a possible mechanism of action of phototherapy on the tissue healing process is the modular homeostasis between fusion and fission mitochondrial.

Although healing in mice skin not accurately portrays what happens on human skin, countless studies using mouse models to examine the laser interactions in the skin(20, 41). Our findings lead us to believe that LLLT infrared wave spectrum was effective in stimulating collagen production well as in the formation of extracellular matrix in animals with chronic diabetes, and the LED showed significant results as to

minimize inflammation and stimulate the formation of new vessels, even though the laser showed a very effective action on the formation and organization of the ECM and collagen fibers. What draws our attention were the differences between Laser and LED on the scheduling of the factors involved in mitochondrial function (FIS1 and MFN2), suggesting different mitochondrial mechanisms of action, with the LED modulating mitochondrial fusion and laser on mitochondrial fission. A possible explanation of differences between immunoassayed FIS1 and MFN2 related phototherapy given by the laser and the LED is that, although the wavelengths are the same, except LED consistency could be an interesting variable in order to promote an outcome adaptation of mitochondria new energy offers (quanta), also explaining the differences between the LED and Laser in the organization and distribution of the fibers of the matrix observed in AFM.

We believe that this study can provide information to help ensure that new research be conducted in order to understand whether there is indeed a difference between action mechanisms between Laser and LED related mitochondrial dysfunction function

CONCLUSIONS

It was demonstrated that the delay in healing of diabetic ulcers was dependent phototherapy, by angiogenesis incremented (VEGF) and inflammation decreased (COX-2), effects which resulted in a more organized collagen formation in irradiated groups. A possible explanation about this better healing over irradiated tissue can be attributed to a photobiomodulation mechanism on mitochondrial homeostasis, especially between mitochondrial fusion and fission (FIS1 and MFN2). When comparing the effects of Laser and LED could identify the LED was more beneficial in reducing the inflammatory process by biomodulation mitochondrial fusion. We cannot completely rule out other mechanisms, because there are other intricate signaling pathways involved. However, these observations provide a basis for future research on the potential therapeutic implications of LBI and LED in the diabetic cure.

Acknowledgements

We acknowledge Dr. Karina Nogueira ZP Rossi (Pathology Department, Federal University of Sao Carlos-UFSCAR) that kindly provided her laboratory and Dr. Tatiana

de Oliveira Sato (Federal University of Sao Carlos) is thanked for her support with statistical analysis.

Funding

This study was supported by Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico – FUNCAP (Grant no. 0778929/2015) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Capes and Ministry of Education of Brazil - MEC.

REFERENCES

1.Alberti K, Zimmet PZ, Consultation WHO. Definition, diagnosis and classification of diabetes mellitus and its complications part 1: Diagnosis and classification of diabetes mellitus - Provisional report of a WHO consultation. Diabetic Medicine. 1998;15(7):539-53.

2.Meireles GCS, Santos JN, Chagas PO, Moura AP, Pinheiro ALB. Effectiveness of laser photobiomodulation at 660 or 780 nanometers on the repair of third-degree burns in diabetic rats. Photomedicine and Laser Surgery. 2008;26(1):47-54.

3.Akyol U, Gungormus M. The Effect of Low-Level Laser Therapy on Healing of Skin Incisions Made Using a Diode Laser in Diabetic Rats. Photomedicine and Laser Surgery. 2010;28(1):51-5.

4.Cruz J, Oliveira MA, Hohman TC, Fortes ZB. Influence of tolrestat on the defective leukocyte-endothelial interaction in experimental diabetes. European Journal of Pharmacology. 2000;391(1-2):163-74.

5.Black E, Vibe-Petersen J, Jorgensen LN, Madsen SM, Agren MS, Holstein PE, et al. Decrease of collagen deposition in wound repair in type I diabetes independent of glycemic control. Archives of Surgery. 2003;138(1):34-40.

6.Falanga V. Wound healing and its impairment in the diabetic foot. Lancet. 2005;366(9498):1736-43.

7.Li T, Hu J, Du S, Chen Y, Wang S, Wu Q. ERK1/2/COX-2/PGE(2) signaling pathway mediates GPR91-dependent VEGF release in streptozotocin-induced diabetes. Molecular Vision. 2014;20:1109-21.

8.Karu T. Mitochondrial signaling in mammalian cells activated by red and near-IR radiation. Photochemistry and Photobiology. 2008;84(5):1091-9.

9.Whelan HT, Smits RL, Buchman EV, Whelan NT, Turner SG, Margolis DA, et al. Effect of NASA light-emitting diode irradiation on wound healing. Journal of Clinical Laser Medicine & Surgery. 2001;19(6):305-14.

10.Kipshidze N, Nikolaychik V, Keelan MH, Shankar LR, Khanna A, Kornowski R, et al. Low-power helium : neon laser irradiation enhances production of vascular endothelial growth factor and promotes growth of endothelial cells in vitro. Lasers in Surgery and Medicine. 2001;28(4):355-64.

11.Reddy GK. Comparison of the photostimulatory effects of visible He-Ne and infrared Ga-As lasers on healing impaired diabetic rat wounds. Lasers in Surgery and Medicine. 2003;33(5):344-51.

12.Yu W, Naim JO, Lanzafame RJ. Effects of photostimulation on wound healing in diabetic mice. Lasers in Surgery and Medicine. 1997;20(1):56-63.

13.Al-Watban FAH, Andres BL. Polychromatic LED therapy in burn heating of non-diabetic and diabetic rats. Journal of Clinical Laser Medicine & Surgery. 2003;21(5):249-58.

14.Vinck EM, Cagnie BJ, Cornelissen MJ, Declercq HA, Cambier NID. Increased fibroblast proliferation induced by light emitting diode and low power laser irradiation. Lasers in Medical Science. 2003;18(2):95-9.

15.Adamskaya N, Dungel P, Mittermayr R, Hartinger J, Feichtinger G, Wassermann K, et al. Light therapy by blue LED improves wound healing in an excision model in rats. Injury-International Journal of the Care of the Injured. 2011;42(9):917-21.

16.Lizarelli RFZ, Miguel FAC, Freitas-Pontes KM, Villa GEP, Nunez SC, Bagnato VS. Dentin hypersensitivity clinical study comparing LILT and LEDT keeping the same irradiation parameters. Laser Physics Letters. 2010;7(11):805-11.

17.Ferraresi C, dos Santos RV, Marques G, Zangrande M, Leonaldo R, Hamblin MR, et al. Light-emitting diode therapy (LEDT) before matches prevents increase in creatine kinase with a light dose response in volleyball players. Lasers in Medical Science. 2015;30(4):1281-7.

18.Miranda EF, Pinto Leal-Junior EC, Marchetti PH, Dal Corso S. Acute effects of light emitting diodes therapy (LEDT) in muscle function during isometric exercise in patients with chronic obstructive pulmonary disease: preliminary results of a randomized controlled trial. Lasers in Medical Science. 2014;29(1):359-65.

19.Hunter S, Langemo D, Hanson D, Anderson J, Thompson P. The Use of Monochromatic Infrared Energy in Wound Management. Advances in Skin & Wound Care. 2007;20:265-6.

20.Chung T-Y, Peplow PV, Baxter GD. Laser Photobiomodulation of Wound Healing in Diabetic and Non-Diabetic Mice: Effects in Splinted and Unsplinted Wounds. Photomedicine and Laser Surgery. 2010;28(2):251-61.

21.Peppa M, Stavroulakis P, Raptis SA. Advanced glycoxidation products and impaired diabetic wound healing. Wound Repair and Regeneration. 2009;17(4):461-72.

22.Schindl A, Schindl M, Pernerstorfer-Schon H, Kerschan K, Knobler R, Schindl L. Diabetic neuropathic foot ulcer: Successful treatment by low-intensity laser therapy. Dermatology. 1999;198(3):314-6.

23.Manzi FR, Bóscolo FN, Almeida SMd, Tuji FM. Morphological study of the radioprotective effect of vitamin E (dl-alpha-tocopheril) in tissue reparation in rats.Estudo morfológico do efeito radioprotetor da vitamina E (dl-alfa-tocoferil) na reparação tecidual em ratos. Radiologia Brasileira. 2003;36(6):367-71.

24.Santos N, dos Santos J, dos Reis J, Oliveira P, de Sousa A, Carvalho C, et al. Influence of the use of laser phototherapy (λ660 or 790 nm) on the survival of cutaneous flaps on diabetic rats. Photomedicine and laser surgery [Internet]. 2010; 28(4):[483-8. pp.].

25.Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: An Open Source Plugin for the Quantitative Evaluation and Automated Scoring of Immunohistochemistry Images of Human Tissue Samples. Plos One. 2014;9(5).

26.Matsumoto MA, Caviquioli G, Biguetti CC, Holgado LdA, Saraiva PP, Muniz Renno AC, et al. A novel bioactive vitroceramic presents similar biological responses as autogenous bone grafts. Journal of Materials Science-Materials in Medicine. 2012;23(6):1447-56.

27.Chen J, Kasper M, Heck T, Nakagawa K, Humpert PM, Bai L, et al. Tissue factor as a link between wounding and tissue repair. Diabetes. 2005;54(7):2143-54.

28.Diegelmann RF, Evans MC. Wound healing: An overview of acute, fibrotic and delayed healing. Frontiers in Bioscience. 2004;9:283-9.

29.Singer AJ, Clark RAF. Mechanisms of disease - Cutaneous wound healing. New England Journal of Medicine. 1999;341(10):738-46.

30.Keswani SG, Katz AB, Lim FY, Zoltick P, Radu A, Alaee D, et al. Adenoviral mediated gene transfer of PDGF-B enhances wound healing in type I and type II diabetic wounds. Wound Repair and Regeneration. 2004;12(5):497-504.

31.Boodhwani M, Sellke FW. Therapeutic Angiogenesis in Diabetes and Hypercholesterolemia: Influence of Oxidative Stress. Antioxidants & Redox Signaling. 2009;11(8):1945-59.

32.Hu J, Wu Q, Li T, Chen Y, Wang S. Inhibition of high glucose-induced VEGF release in retinal ganglion cells by RNA interference targeting G protein-coupled receptor 91. Experimental Eye Research. 2013;109:31-9.

33.Nagoya H, Futagami S, Shimpuku M, Tatsuguchi A, Wakabayashi T, Yamawaki H, et al. Apurinic/apyrimidinic endonuclease-1 is associated with angiogenesis and VEGF production via upregulation of COX-2 expression in esophageal cancer tissues. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2014;306(3):G183-G90.

34.Zhou L-H, Hu Q, Sui H, Ci S-J, Wang Y, Liu X, et al. Tanshinone II-A Inhibits Angiogenesis through Down Regulation of COX-2 in Human Colorectal Cancer. Asian Pacific Journal of Cancer Prevention. 2012;13(9):4453-8.

35.Ohkuwa T, Sato Y, Naoi M. Hydroxil Radical Formation in Diabetic Rats Induced by Streptozotocin. Life Sciences. 1995;57(13):1325-30.

36.Winiarska K, Drozak J, Wegrzynowicz M, Fraczyk T, Bryla J. Diabetes-induced changes in glucose synthesis, intracellular glutathione status and hydroxyl free radical generation in rabbit kidney-cortex tubules. Molecular and Cellular Biochemistry. 2004;261(1-2):91-8.

37.Wu S, Zhou F, Xing D, editors. Mechanism study on mitochondrial fragmentation under oxidative stress caused by high fluence low-power laser irradiation. Conference on Mechanisms for Low-Light Therapy VII; 2012 Jan 21; San Francisco, CA2012.

38.Vincent A, Edwards J, McLean L, Hong Y, Cerri F, Lopez I, et al. Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy. Acta Neuropathol.120(4):477-89.

39.Bach D, Naon D, Pich S, Soriano FX, Vega N, Rieusset J, et al. Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle - Effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha, and interleukin-6. Diabetes. 2005;54(9):2685-93.

40.Isabel Hernandez-Alvarez M, Thabit H, Burns N, Shah S, Brema I, Hatunic M, et al. Subjects With Early-Onset Type 2 Diabetes Show Defective Activation of the Skeletal Muscle PGC-1 alpha/Mitofusin-2 Regulatory Pathway in Response to Physical Activity. Diabetes Care. 2010;33(3):645-51.

41.Byrnes KR, Barna L, Chenault VM, Waynant RW, Ilev IK, Longo L, et al. Photobiomodulation Improves Cutaneous Wound Healing in an Animal Model of Type II Diabetes. Photomedicine and Laser Surgery. 2004;22(4):281-U17.

ESTUDO III

Phototherapy regenerates pancreatic islets and modulates carbohydrate metabolism in diabetic animals

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ABSTRACT

Pancreatic lesions can produce diabetes. Laser Low Level Therapy and Light-Emitting Diode Therapy are seen as safe and effective Phototherapies over cell proliferation and regeneration. Aims/hypothesis: Were analyzed effects of Laser Low Level Therapy and Light-Emitting Diode Therapy on the pancreas after injection of streptozotocin and its implications on the carbohydrate metabolism. Methods: Twenty Wistar rats were randomized into groups: non diabetic control, diabetic control, diabetic treated with Laser and diabetic treated with Light-Emitting Diode. Except non diabetic control group all of animals were induced to diabetes type I by streptozotocin. Treated groups were irradiated by Low Level Laser Therapy and Light-Emitting Diode Therapy for 5 consecutive days and monitored for 30 days. Histological analysis, hepatic and muscle glycogen and intraperitoneal insulin tolerance test (IPITT) and intraperitoneal glucose tolerance test (IPGTT) were performed. Results: Low Level Laser Therapy and Light-Emitting Diode Therapy altered Metabolic features**.** Diabetic treated with Laser group and diabetic treated with Light-Emitting Diode group showed hypertrophy in islets and ducts ($p = 0,001$). Intraperitoneal insulin tolerance test and Intraperitoneal insulin tolerance test showed differences between diabetic control and diabetic treated. In diabetic control group the hepatic glycogen content was 232 % and 296% smaller than diabetic treated with Laser and diabetic treated with Light-Emitting Diode group respectively. Furthermore, in diabetic control group the glycogen content of the gastrocnemius muscle was 680 % and 706 % smaller when compared with diabetic treated with Laser and Laser and diabetic treated with Light-Emitting Diode group respectively. Conclusions: this study shows that Phototherapy was able to modify histologic and metabolic features as well as altered carbohydrate metabolism on diabetic animals.

Keywords: glycogen. Pancreatic ducts. islets of Langerhans. Phototherapy. Diabetes

Abbreviations

LLLT - Low Level Laser therapy

T1DM -Type 1 diabetes mellitus

T2DM-Type 2 diabetes mellitus

STZ - streptozotocin

LEDT - Light-Emitting Diode Therapy

i.p. -intraperitoneal

IPITT- intraperitoneal insulin tolerance test

IPGTT- intraperitoneal glucose tolerance test

MSCs - mesenchymal stem cells

CSCs - cardiac stem cells

MFN2 - mitofusin

ROS- reactive oxygen species

INTRODUCTION

Pancreas injury treatment remains a challenge of the world. Advances are necessary to reduced morbidity and mortality rates following this severe injury and Phototherapy can be a possible treatment to optimize the tissue repair process [1, 2]. The application of light in diseases that increases stress conditions, preventing cell death and tissue damage has been known but, some mechanisms involved in this response remain unclear, notably in laser effects on mitochondrial mechanism[3]. The biological mechanism the effects of Phototherapy is absorption of red and infrared light by cytochrome c oxidase at complex IV of mitochondrial chain [4, 5]. Also enhances mitochondrial respiration, ATP production and increases proton gradient, leading to an increased activity of the Ca^{2+}/Na^{+} antiporters, Na^{+}/K^{+} ATPase and Ca^{2+} pumps[3] .

Type 1 diabetes mellitus [T1DM) occurs due loss of endocrine beta cells via autoimmune destruction and type 2 diabetes mellitus [T2DM] beta cell failure, due increased stress what makes us believe that diabetes in fact results from an inadequate beta cell mass[6]. Regardless of, hyperglycemia and type-2 diabetes can develop when beta cell compensations fail to adapt to the increasing insulin requirements imposed by insulin resistance [7]. In this way, some studies propose that insulin resistance and type 2 diabetes mellitus (T2DM) are caused by mitochondrial dysfunction. This is supported by an association between mitochondrial dysfunction and reduced insulin sensitivity, as well as an impaired beta cell function in the pancreas [8, 9].

An early study[8] evidenced that newly generated beta cells came from beta cells, and showed endocrine cell plasticity under physiological circumstances. In other hand, Bonner-Weir [10] et al assess the ability human ductal cells to differentiate into islet pancreatic using human pancreatic remnants of pancreas transplant donors. However, it is not known whether similar cell fate conversions could occur after streptozotocin-induced injury. Additionally, the present findings seek to elucidate the plasticity and pivotal adjustment of cell secretion process in compensatory beta answers the endocrine

pancreas when subjected to phototherapy. The aim of this study was analyzed effects of two types of phototherapy (LLLT and LEDT) on the pancreas after injection of streptozotocin (STZ) and its implications on the carbohydrate metabolism**.**

MATERIALS AND METHODS

Animals

This work was approved by the Animal Care Committee guidelines at Federal University of Sao Carlos (protocol 052/2013) and managed according to the *Guide for the care and use of laboratory animals*. In this study, 40 male Wistar rats were used (10 weeks old and weighing $\pm$ 200 g), and were maintained under controlled conditions of temperature (25 ± 2 C) with light–dark periods of 12 h, with free access to water and commercial diet, except in experiment days when were submitted fasting.

Diabetes induction

Wistar rats with 8 weeks, 250g, were fasted 12 hours before intraperitoneal injection of streptozotocin (solution 70mg/kg - STZ)[11] with citrate buffer (pH 4.5) and six hours after administration, animals had their water supply replaced by an aqueous solution of glucose (10%) for 24 h. The state diabetic was confirmed by determination of blood glucose level on the 7th day of STZ administration. Rats with fasting blood glucose level > 13,88 mmol/l were selected for experimentation.[7]

Light Treatments (LLLT / LED Treatments)

A low-power Ga-Al-As laser (Photon Laser III, DMC® São Carlos, São Paulo, Brazil) was used with parameters: 808 nm, continuous wave diode, with a 0.028 cm^2 spot area, a power output of 100 mW, fluence at 78 J/cm^2 (irradiation time of 22s, energy per point 2.2 J), The LEDT equipment was a prototype and following parameters were used: $\lambda = 850$ nm; 48 mW, 22 s; spot diameter 5mm, spot area 0,196cm^2,0,098 J) was applied on pancreas projection area LEDT using the same conditions of the LLLT technique. To assure that irradiation occurred on the same local in all of animals, a plastic artifact was made with 30 x 30 cm and was localized over the hip articulation (Figure 1). After the last day of irradiation, the animals were killed and pancreas removed for subsequent analysis. The treatment parameters can have observed in Table 1:

Table . 1. Device Information, Irradiation and Treatment Parameters

	LLLT	**LED**
Type	Ga-Al-As Semiconductor Diode Laser	Diode LED
Model	Photon Laser III, DMC® São Carlos, São Paulo, Brazil	prototype
Wavelength	808nm	850 nm
Operating mode	Continuous wave	Continuous wave
Spot diameter	0.1309	5mm
Spot area	0.028 cm2	0,196cm²
Output Power	100mW	45 mW
Fluence	78,5 J/cm^2	5,51 J/cm^2
Irradiation time per session	22 seconds	22 seconds
Energy per point per session	2.2 J	0,99 J
Application technique	Over hip joint. Was used a plastic artifact to assure that irradiation occurred in the same local in all of animals.	
Number and frequency of treatment sessions	Five consecutive days totalizing 5 sessions of treatment.	

Groups

After diabetes induction the animals were randomly divided into four groups:

Sham group (n=5)**:** Non-diabetic control rats that receive LLLT treatment on mode off during 22s for five consecutive days and follow up for thirty days (Placebo effect).

NID group (n=5)**:** Diabetic rats that receive LEDT treatment on mode off during 22s for five consecutive days and follow up for thirty days (Placebo effect in diabetic animals).

DLASER group (n=5): Animals were diabetic induced with a single injection of streptozotocin and after seven days' post streptozotocin injection they were irradiated with Photon Laser III (DMC) at one point over the pancreas region using the punctual contact technique for five consecutive days and following for thirty days.

DLED group: Animals were diabetic induced with a single injection of streptozotocin and after seven days' post streptozotocin injection they were irradiated with LEDT (prototype) at one point over the pancreas region using the punctual contact technique for five consecutive days and following for thirty days.

Figure. 1 Application technique shows plastic artifact used for standardization of the application of LLLT technique and LED. Note is that the animals were not anesthetized during treatment so as to not change the metabolic profile of the treated animals. The same technique was used for the SHAM group being the LED in the off mode.

Experimental Procedure

Measurements

Body weight and food intake were recorded weekly.

Tolerance tests

For the establishment of diabetic state, seven days after the administration of STZ by injection, blood glucose was measured with a glucometer (Accu-Check, Roche®) fasted condition. Tolerance tests were realized before streptozotocin induced (baseline) and after, weekly, totalizing three measurements. Were collected blood samples in the tail. In the intraperitoneal glucose tolerance test (IPGTT) the animals were fasted for 12 hours, after this period they received an intraperitoneal (i.p.) glucose injection (2 g/kg). Blood glucose was measured at 0, 15, 30, 60, 90, 120 and 180 minutes with a glucometer (Accu-Check, Roche®). Two days after the IPGTT, we realize the intraperitoneal insulin tolerance test (IPITT) then fed animals were submitted to an i.p. insulin injection (1 UI/kg) and then, blood glucose was measured at 0, 15, 30, 45 and 60 min with a glucometer (Accu-Check, Roche®) [7]

Analysis

In the 30 th day of experimental protocol fasted animals were killed by decapitation. Pancreas and Liver were removed and weight for subsequent analysis.

Hepatic and Muscular Glycogen Content

The muscle and hepatic glycogen content was measured using the colorimetric method through spectrophotometer at 480 nm absorbance. The concentration of the glicosil-glucose was determined using with reference a standard glucose sample (1mM concentration). [12]The glycogen content was expressed in μmol/g. Tissue samples from the liver and gastrocnemius muscle were dissolved in 1ml of 6N KOH in a boiling-water bath for 5 minutes. After the extract was transferred 1:10 v/v to ethanol; 1 volume of 10% K_2SO_4 was added to improve precipitation. The pellet was re-suspended in 2.5 ml of distilled water and glucose was determined in a proper aliquot using phenol-sulfuric acid (500 μL of phenol and 2 ml of sulfuric acid (H2SO4). After this, the concentration of sugar was estimated to be a standard glucose concentration 1 mM and the sample was read in a spectrophotometer at 480 nm absorbance [12]

Histopathological analysis

The samples of pancreas were fixed in 10% buffered formalin (Merck, Darmstadt, Germany) for 24 h. After this, the samples were dehydrated and embedded in paraffin blocks. Three sections (5μm) of each specimen were longitudinally sectioned (Microtome Leica Microsystems SP 1600, Nussloch, Germany) and stained with hematoxylin and eosin (H.E. stain, Merck, Darmstadt, Germany). The morphological description of the pancreas areas was performed with an optical microscopy (Olympus Optical Co., Tokyo, Japan). Histopathological alterations in the pancreas were evaluated by two blinded observers. For descriptive analysis, the samples were stained with HE to evaluate cellular organization, density of islets and density ducts per area (μm^2. The specimens were examined using a light microscopy (×4 and ×40; Leica Microsystems AG, Wetzlar, Germany)

Data analysis

Data shown as mean ± SE unless otherwise stated. Statistical comparisons between groups were performed using ANOVA test and Tukey post hoc. Differences were considered significant when $p < 0.05$.

RESULTS

3. Results

Metabolic features

Compared to the non-diabetic animals, diabetic rats had decreased body weight with typical diabetic symptoms i.e. polyuria, polydipsia and thickened urine smell (Figure 2). Treatment of Laser and LEDT improved these general features. Chronic treatment with Laser and LEDT prevented the body weight loss, polydipsia, and polyphagia and also showed a certain downward trend in water intake (Figures 2 and 3):

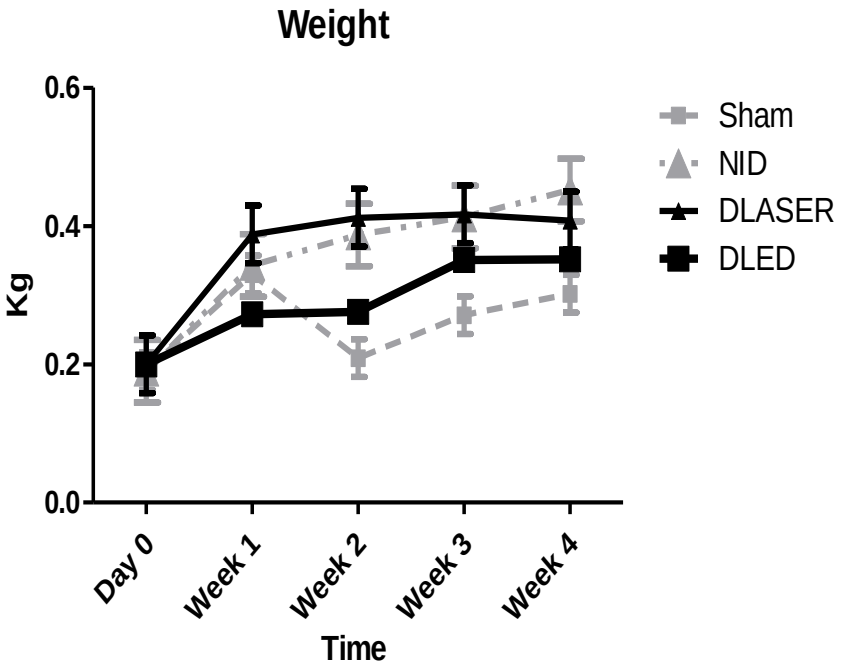

Figure. 2.Weight variation throughout the experiment time in non-diabetic control (Sham), diabetic non treated (NID), diabetic treated with LLLT (DLASER) and diabetic LEDT treatment (DLED) Results are expressed as mean and standard error of the mean.

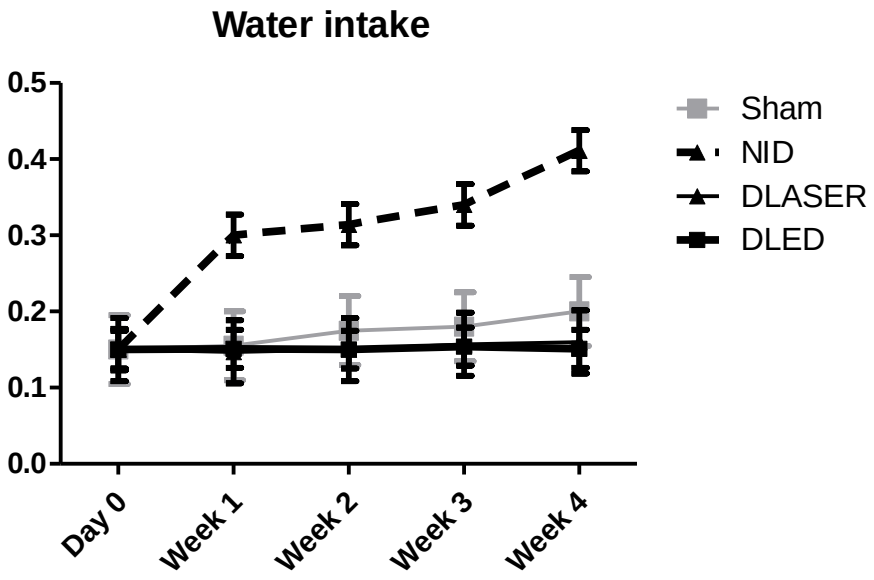

Figure. 3.Water intake variation throughout the experiment time in non-diabetic control (Sham), diabetic non treated (NID), diabetic treated with LLLT (DLASER) and diabetic LEDT treatment (DLED). Results are expressed as mean and standard error of the mean.

Histological descriptive analysis

Some studies have reported the regeneration of the pancreas after pancreatectomy [13] and streptozotocin treatment[14]. This regeneration has been implicated new training of preexisting endocrine cells from epithelial cells or ductal epithelium. The islanders survivors in pancreatic tissue undergo extensive hypertrophy to compensate for the pancreas removed function compared to normal pancreas[15]. Examples of the histologic changes in each of the groups in pancreatic islets are shown in Figure 4. The Sham group showed a normal lobular architecture of the pancreas. The pancreas had relative amount of islets of Langerhans (one or two per area) between the exocrine pancreatic acini (Figure 4A). Pancreatic sections diabetic group (NID) showed border region between the endocrine and exocrine became indistinguishable and some islets were completely destroyed and showed empty sites (Figure 4B). The group DLASER showed improvement on the previous morphological changes, the border between exocrine and endocrine portions have become distinct. Many acini showed an increase in density ducts (Figure 4 C black arrows) with significant difference between the NID group (Table 2). Some ducts were noticed near islets with some cellular connection and neovascularization. DLED group showed hypertrophy in islets and ducts. (Figure 4 D):

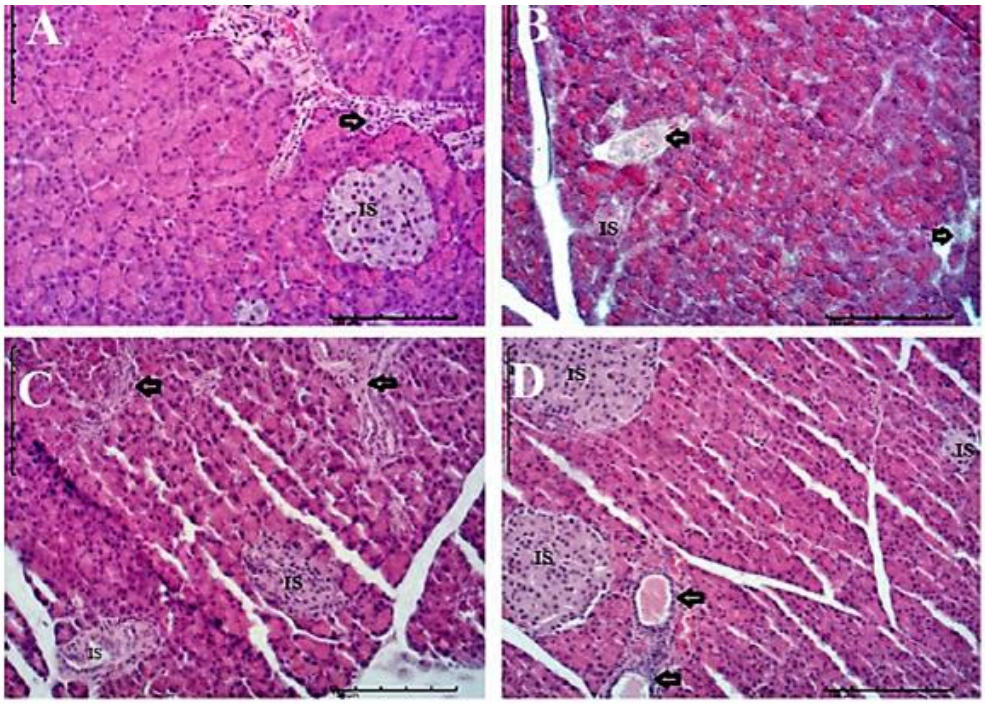

Figure. 4 Hematoxylin and eosin-stained sections of normal mouse pancreas and after streptozotocin induced (A) Normal pancreas section showing the presence of acinar cells, ducts, blood vessels as well as islets (IS). (B) Group NID: Endocrine pancreas showing islet cell atrophy with a drastic decrease in number of cells per islet and total disorganization of islet cells due streptozotocin injection. (C) Group DLASER: Endocrine pancreas showing histologic features of increased ducts (black arrows) number and islets (IS). (D) Pancreas on DLED group showing prominent 'hypertrophied' islets.(40 X)

DLED animals showed great amount of ducts and islets around the acini's tissue. Ulcers, necrosis or tumors not were detected any animals of all the groups. Histologic comparisons of duct density and islets density data between groups are shown in Table 2.

Table . 2.Table 2: Duct density and islet Langerhans density analysis of LLLT in streptozotocin (STZ)-induced diabetes

GROUPS	Duct Density (Mean ± SEM)	Islets Density (Mean ± SEM)
Sham[a]	0.165 ± 0,002	0.067 ± 0.010
NID[b]	0.005 ± 0,001 [a]	0.025 ± 0,008 [a]
DLASER[c]	0.014 ± 0.000 [b]	0.067 ± 0.055
DLED[d]	0.016 ± 0.007 [b]	0.028± 0.006

Density of islets and density ducts per area (μm^2). The specimens were examined using a light microscopy (×4 and ×40; Leica Microsystems AG, Wetzlar, Germany). Groups: non-diabetic control (Sham), diabetic non treated (NID), diabetic treated with LLLT (DLASER) and diabetic LEDT treatment (DLED). [a] versus Sham group; [b] versus NID group; Date are showed mean ± SEM. Values are μm^2. * p = 0,005.

Tolerance tests

IPGTT. Groups reacted differently between them. Blood glucose reached the highest level at 60 min in NID and DLASER groups while NID and DLED groups this happened at 15 min and then showed a certain downward trend. DLED inhibited the increase of blood glucose level at 30 and 120 min after glucose loading when compared to the diabetic control group (NID) (Figure 5).

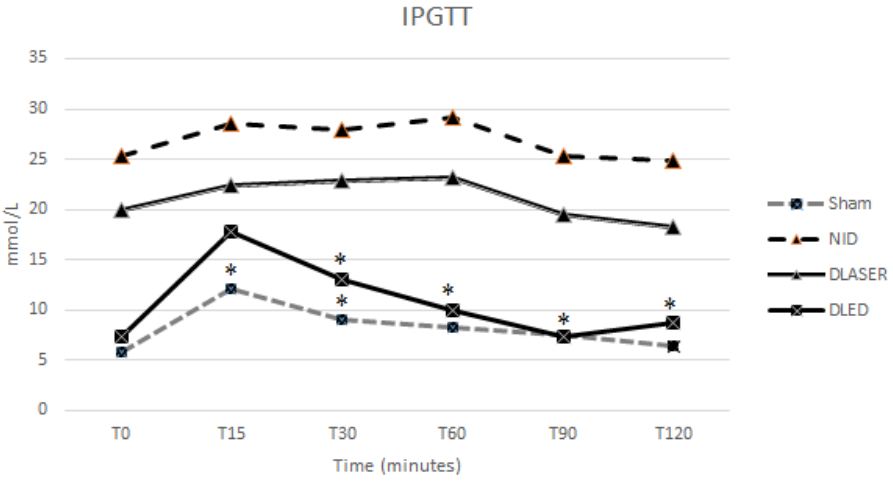

*Figure. 5.Effect of Laser or LED on intraperitoneal glucose tolerance test (IPGTT) in diabetic animals. Data express blood glucose levels (mmol/L, mean ± SD). Groups: Sham = no diabetic control; NID= diabetic control; DLASER= diabetic treated with Laser 808 nm; DLED = diabetic treated with LEDT 850 nm. *p< 0,05 compared with the diabetic control using ANOVA repeat measures.*

IPITT. Groups NID and DLED reacted similarly between them. Both NID and DLED decreased the blood glucose level after 15 and 20 min of insulin administration in comparison to diabetic control group (NID). (Figure 6)

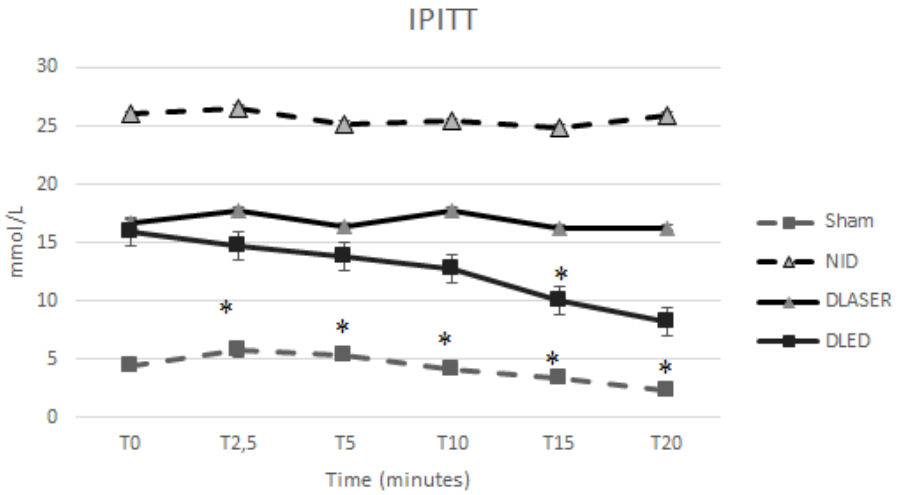

*Figure. 6.Effect of Laser or LED on IPITT diabetic animals. Data express blood glucose levels (mmol/L, mean ± SD). Groups: Sham = no diabetic control; NID= diabetic control; DLASER= diabetic treated with Laser 808 nm; DLED = diabetic treated with LEDT 850 nm. *p< 0,05 compared with the diabetic control using ANOVA repeat measures.*

The glycogen content in liver and gastrocnemius muscle was differ significantly across DLASER and DLED groups during the experimental period when compared with respective control groups. In the NID group, the hepatic glycogen content was 232 % and 296% smaller when compared with DLASER and DLED group respectively. No difference was observed in this parameter when compared DLASER versus Sham groups. Furthermore, in NID group the glycogen content of the gastrocnemius muscle was 680 % and 706 % smaller when compared with DLASER and DLED group respectively (Table 3).

*Table . 3. Glycogen hepatic and muscle (µml/g) in diabetic animals treated with Laser/LED**Table 3.** Glycogen hepatic and muscle (µml/g) in diabetic animals treated with Laser/LED*

Groups	Hepatic Glycogen	Muscle Glycogen
Sham[a]	5,44 ± 2,22	2,62 ± 0,28
NID[b]	2,81 ± 0,53 *a**	0,50± 0,10 *a**
DLASER[c]	6,52± 0,15 *b**	3,64 ± 0,22 *b**
DLED[d]	8,33± 1,65 *b**	3,53 ± ,018 *b**

Values are expressed as mean ± standard deviation (n=5/group). The differences are superscripts in italics. Groups: non-diabetic control (Sham), diabetic non treated (NID), diabetic treated with LLLT (DLASER) and diabetic LEDT treatment (DLED). [a] versus Sham group; [b] versus NID group; Date are showed mean ± SEM. Values are μm^2. * p = 0,005.

DISCUSS

In this study we can demonstrate that LLLT it could be a possibility of treatment for diabetes, which promoted histologic changes over injured pancreas and optimized carbohydrate metabolism performance in a model of diabetes mainly when used LEDT. The metabolic features like body weight with typical diabetic symptoms i.e. polyuria, polydipsia and thickened urine smell were minimized in treated groups (DLASER and DLED). Moreover, the rise of ducts and islets of Langerhans in DLED and DLASER groups respectively attest that light acted positively on the pancreatic tissue.

Cell proliferation promoted by LLLT is a physiological effect on already well established and it has been shown in vitro embryonary cells, including mesenchymal stem cells and cardiac stem cells [16] as well as many cell types another to fibroblasts, human osteoblasts[17] keratinocytes[18], home satellite cells [19]. It is believed that spectrums of light ranging from red to infrared is absorbed by the mitochondrial respiratory chain components resulting in increased adenosine triphosphate (ATP) / or cyclic AMP and reactive oxygen species (ROS) thereby initiating a signaling cascade that promotes cell proliferation [18, 20, 21]. Sequentially to this increase in ATP and protein synthesis after LLLT, the expressions of cytokines and growth factors to

stimulate and increase cell proliferation[22, 23]. In our results, the histological analysis showed that irradiation produced a proliferation cell represented in the number of islets and hypertrophy of preexisting islets that agree with some studies that indicates that most post-natal beta cells are derived from pre-existing beta cells. (6). Moreover, beta cells arise from precursor cells associated with ducts and is detected on or near the primitive epithelium conduit [24].The differentiation of the ductal precursors also plays a significant role in expanding beta cells during fetal life [24] but it remains unclear whether the ductal precursors participate in the expansion of beta cells after birth. However, numerous insulin positive cells, which are associated with conduits have been observed in infants not only in the pancreas of childhood persistent hyperinsulinemic hypoglycemia , but without metabolic problems of the pancreas[8]. This indicates the possibility that the duct are precursors of the beta cell sources in the postnatal period. Our findings can contribute to the hypothesis ductal precursors participating in the expansion of beta cells after birth, the fact that there was a significant difference in density between the ducts irradiated groups (DLASER and DLED) compared to the untreated diabetic group (NID). These data lead us to believe that LLLT stimulated these germinative cells.

During food intake, blood glucose levels increase and there is the stimulation of the beta cells produce insulin. In sequence, inhibits glycogenolysis and gluconeogenesis promoting glucose uptake by muscle tissue and / or fat. In turn, when the blood glucose is low, the α cells secrete glucagon and stimulating hepatic glycogenolysis and gluconeogenesis, which causes the glucose to return to its normal level (25). In our study, high levels of hepatic glycogen in irradiated animals (DLASER and DLED groups) when compared to the control groups show that LLLT were able to promote glycogenesis, probably by increasing mitochondrial activity in beta cells that produced insulin and promoted glucose intake into the muscle and hepatic cells. Poitout et al. [25] suggests that the mechanism by which hyperglycemia can cause glucotoxicity in the beta cells is due to the fact that hyperglycemia may cause a disturbance in relation NADH / NAD +, activation of protein kinase C and stress of the endoplasmic the cell reticulum. These factors together produce increased production of reactive oxygen species leading to β-cells failure and generates diabetic state. Furthermore Lenzen et al[26] demonstrate that the islets express fewer genes for CuZnSOD enzymes, MnSOD, and catalase compared to the expression in the liver, kidney, brain, lung, skeletal muscle and cardiac and some glands. This imbalance in the formation of ROS and reduced the

efficiency of the antioxidant enzymes, possibly leave these islets more susceptible to ROS[27].

Photoexcitation induces changes in cytochrome c oxidase, and other components of the chain like NADH dehydrogenase [28], resulting in redox changes and modulations of biochemical reactions producing effects such as increased proliferation. Karu proposed that the absorption of light by chromophores of respiratory chain promotes oxidation of NADH pool leads to alterations in the redox state of both the cytoplasm and mitochondria [29]. In addition to that, early review (31) suggests that photons emitting LLLT are absorbed by mitochondria which in turn stimulate increased production of ATP with minor levels of ROS by activating transcription factors, such as NF-kB, capable of inducing genes responsible for these proliferative effects.

A possible explanation for our findings may be a modulation of LLLT on mitochondrial dysfunction. The fusion and mitochondrial fission acts maintaining the morphology and functionality of the organelle, but the mechanisms involved is not clear. Altered mitochondrial fusion promotes mitochondrial elongation and unorganized fission leads to exacerbated increased fragmentation of mitochondria, both damaging mitochondrial[30] function. Diabetes and oxidative stress it generates trigger excessive mitochondrial fission leading to mitochondrial damage and injury cellular[31]. Specific ablation of liver mitochondrial regulator fusion mitofusin (MFN2), causes mitochondrial dysfunction, insulin resistance and impaired glucose tolerance[32]. Althought we not analyzed mitochondrial biomarkers, Karu[3] related modulation of mitochondrial retrograde elements signaling by irradiation (reactive oxygen species ROS, changes in mitochondrial fusion-fission homeostasis of mitochondria and mitochondrial membrane potential) and showed that laser irradiation of He-Ne increased the respiratory activity modifying the ultrastructure of mitochondria and endoplasmic reticulum mitochondria associations in progeny cells [33].

We suggest that pancreas induced regeneration by LLLT is also a secondary response, but the connection between the light absorption and this effect is not fully understood. As a suggestion for future studies, we believe that LLLT could modulate mitochondrial dysfunction possibly like a regulator of mitochondrial fusion-fission. These modulations could improve the biochemical environment and the response of the beta cells and prevents oxidative stress generates due dyshomeostasis over beta cells.

CONCLUSIONS

Irradiation laser and LEDT played a role positive on the pancreatic tissue of diabetic streptozotocin-induced animals, generating an increase in density ducts, stimulating the liver glycogenesis and modifying carbohydrate metabolism. These results support evidence that post-natal beta cells are derived from pre-existing beta cells and LLLT stimulated these germinative cells.

Acknowledgements

We acknowledge Dr. Karina Nogueira ZP Rossi (Pathology Department, Federal University of Sao Carlos-UFSCAR) and Dr. Claudia Garcia de Oliveira Duarte (Nutrition and metabolism on exercise Laboratory) that kindly provided their laboratories and Dr. Tatiana de Oliveira Sato (Federal University of Sao Carlos) is thanked for her support with statistical analysis.

Funding

This study was supported by Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico – FUNCAP, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Capes and Ministry of Education of Brazil - MEC.

REFERENCES

1.Kido HW, Bossini PS, Tim CR, et al. (2014) Evaluation of the bone healing process in an experimental tibial bone defect model in ovariectomized rats. Aging Clinical and Experimental Research 26: 473-481

2.da Silva JP, da Silva MA, Figueiredo Almeida AP, Lombardi Junior I, Matos AP (2010) Laser Therapy in the Tissue Repair Process: A Literature Review. Photomedicine and Laser Surgery 28: 17-21

3.Karu T (2008) Mitochondrial signaling in mammalian cells activated by red and near-IR radiation. Photochemistry and Photobiology 84: 1091-1099

4.Gupta A, Avci P, Sadasivam M, et al. (2013) Shining light on nanotechnology to help repair and regeneration. Biotechnology Advances 31: 607-631

5.Avci P, Gupta A, Sadasivam M, et al. (2013) Low-Level Laser (Light) Therapy (LLLT) in Skin: Stimulating, Healing, Restoring. Seminars in Cutaneous Medicine and Surgery 32: 41-52

6.Stanger BZ, Hebrok M (2013) Control of Cell Identity in Pancreas Development and Regeneration. Gastroenterology 144: 1170-1179

7.Irles E, Neco P, Lluesma M, et al. (2015) Enhanced glucose-induced intracellular signaling promotes insulin hypersecretion: Pancreatic beta-cell functional adaptations in a model of genetic obesity and prediabetes. Molecular and Cellular Endocrinology 404: 46-55

8.Supale S, Thorel F, Merkwirth C, et al. (2013) Loss of Prohibitin Induces Mitochondrial Damages Altering beta-Cell Function and Survival and Is Responsible for Gradual Diabetes Development. Diabetes 62: 3488-3499

9.Tubbs E, Theurey P, Vial G, et al. (2014) Mitochondria-Associated Endoplasmic Reticulum Membrane (MAM) Integrity Is Required for Insulin Signaling and Is Implicated in Hepatic Insulin Resistance. Diabetes 63: 3279-3294

10.Weir S, Taneja M, Weir GC, et al. (2000) In vitro cultivation of human islets from expanded ductal tissue. Proceedings of the National Academy of Sciences of the United States of America 97: 7999-8004

11.Thakkar NV, Patel JA (2010) Pharmacological evaluation of "Glyoherb": A polyherbal formulation on streptozotocin-induced diabetic rats. International Journal of Diabetes in Developing Countries 30: 1-7

12.Moraes G, Altran AE, Avilez IM, Barbosa CC, Bidinotto PM (2005) Metabolic adjustments during semi-aestivation of the marble swamp eel (Synbranchus marmoratus, Bloch 1795) - A facultative air breathing fish. Brazilian Journal of Biology 65: 305-312

13.Hardikar AA, Karandikar MS, Bhonde RR (1999) Effect of partial pancreatectomy on diabetic status in BALB/c mice. Journal of Endocrinology 162: 189-195

14.Finegood DT, Weir GC, Bonner-Weir S (1999) Prior streptozotocin treatment does not inhibit pancreas regeneration after 90% pancreatectomy in rats. American Journal of Physiology-Endocrinology and Metabolism 276: E822-E827

15.Bhartiya D, Mundekar A, Mahale V, Patel H (2014) Very small embryonic-like stem cells are involved in regeneration of mouse pancreas post-pancreatectomy. Stem Cell Research & Therapy 5

16.Tuby H, Maltz L, Oron U (2007) Low-level laser irradiation (LLLI) promotes proliferation of mesenchymal and cardiac stem cells in culture. Lasers in Surgery and Medicine 39: 373-378

17.Stein A, Benayahu D, Maltz L, Oron U (2005) Low-level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. Photomedicine and Laser Surgery 23: 161-166

18.Grossman N, Schneid N, Reuveni H, Halevy S, Lubart R (1998) 780 nm low power diode laser irradiation stimulates proliferation of keratinocyte cultures: Involvement of reactive oxygen species. Lasers in Surgery and Medicine 22: 212-218

19.Ben-Dov N, Shefer G, Irinitchev A, Wernig A, Oron U, Halevy O (1999) Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. Biochimica Et Biophysica Acta-Molecular Cell Research 1448: 372-380

20.Stadler I, Evans R, Kolb B, et al. (2000) In vitro effects of low-level laser irradiation at 660 nm on peripheral blood lymphocytes. Lasers in Surgery and Medicine 27: 255-261

21.Castano AP, Dai T, Demidova-Rice TN, et al. (2007) Low-level light therapy for zymosan induced arthritis in rats. - art. no. 64280F. In: Conference on Mechanisms for Low-High Therapy II, San Jose, CA, pp F4280-F4280

22.Hawkins D, Abrahamse H (2006) Effect of multiple exposures of low-level laser therapy on the cellular responses of wounded human skin fibroblasts. Photomedicine and Laser Surgery 24: 705-714

23.Alexandratou E, Yova D, Handris P, Kletsas D, Loukas S (2002) Human fibroblast alterations induced by low power laser irradiation at the single cell level using confocal microscopy. Photochemical & Photobiological Sciences 1: 547-552
24.Meier JJ, Koehler CU, Alkhatib B, et al. (2010) beta-cell development and turnover during prenatal life in humans. European Journal of Endocrinology 162: 559-568
25.Poitout V, Robertson RP (2008) Glucolipotoxicity: Fuel excess and beta-cell dysfunction. Endocrine Reviews 29: 351-366
26.Elsner M, Guldbakke B, Tiedge M, Munday R, Lenzen S (2000) Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. Diabetologia 43: 1528-1533
27.Friederich M, Fasching A, Hansell P, Nordquist L, Palm F (2008) Diabetes-induced up-regulation of uncoupling protein-2 results in increased mitochondrial uncoupling in kidney proximal tubular cells. Biochimica Et Biophysica Acta-Bioenergetics 1777: 935-940
28.Ferraresi C, Hamblin MR, Parizotto NA (2012) Low-level laser (light) therapy (LLLT) on muscle tissue: performance, fatigue and repair benefited by the power of light. Photonics Lasers Med 1: 267-286
29.Karu T (1999) Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol B 49: 1-17
30.Wu S, Zhou F, Xing D (2012) Mechanism study on mitochondrial fragmentation under oxidative stress caused by high fluence low-power laser irradiation. In: Conference on Mechanisms for Low-Light Therapy VII, San Francisco, CA
31.Vincent A, Edwards J, McLean L, et al. Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy. Acta Neuropathol 120: 477-489
32.Sebastian D, Isabel Hernandez-Alvarez M, Segales J, et al. (2012) Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. Proceedings of the National Academy of Sciences of the United States of America 109: 5523-5528
33.Manteifel V, Bakeeva L, Karu T (1997) Ultrastructural changes in chondriome of human lymphocytes after irradiation with He-Ne laser: Appearance of giant mitochondria. Journal of Photochemistry and Photobiology B-Biology 38: 25-30

CONCLUSÕES GERAIS

Em conjunto os estudos mostraram que a Fototerapia:

- Gerou um efeito protetor contra danos oxidativos inerentes ao diabetes durante o processo de reparo tecidual;

- Acelerou o reparo de feridas e a expressão de VEGF e COX2 através da biomodulação da homeostasia mitocondrial (fusão e fissão);

- Desempenhou papel positivo sobre o tecido pancreático diabético, gerando aumento na densidade dos ductos pancreáticos, provável fonte de células germinativas das ilhotas pancreáticas. Além disso, houve estimulo na glicogénese hepática e modificação do metabolismo dos carboidratos.

DIFICULDADES E PERSPECTIVAS FUTURAS

A utilização de modelo experimental de DM não é um trabalho a curto prazo nem tampouco de fácil acompanhamento. Ao longo do percurso desta Tese, muitos percalços ocorreram no meio do caminho. Inicialmente a manutenção da sobrevida dos animais em estado diabético foi um grande desafio, pois não queríamos utilizar insulina exógena para que isso não interferisse no experimento do pâncreas, pois poderia haver um falso positivo em relação aos testes funcionais glicêmicos e, portanto, perdemos vinte por cento dos animais inicialmente utilizados, devido à não somente ao estado hiperglicêmico, mas também por morte durante anestesia, mesmo utilizando-se doses mínimas de tiopental, considerado o mais seguro e o melhor para modelos diabéticos, devido a não causar alteração dos níveis glicêmicos pós anestesia.

Outro grande problema foi devido ao atraso de 6 meses de entrega dos anticorpos FIS1 e MFN2, essenciais para a pesquisa e que caíram na malha fina da fiscalização da ANVISA em Janeiro de 2015 e que certamente gerou atrasos haja vista que tínhamos ainda que estabelecer as titulações necessárias para o uso desses anticorpos.

Nossa ida para o Massachussetts General Hospital também foi um desafio, pois precisamos alterar por 5 vezes o projeto inicial para que se adequasse as novas normas vigentes no Wellmann Center. Entretanto, o doutorado sanduiche nos fez amadurecer novas perspectivas para o uso da Fototerapia, não mais como paliativo para as consequências do DM, mas como agente Terapêutico no controle da glicemia, pois conseguimos visualizar e comprovar que a Fototerapia pode ser benéfica no estado hiperglicêmico atuando tanto em órgãos como células in vitro.

Acreditamos que podemos avançar numa perspectiva de desenvolver protótipos portáteis de fácil acesso a pacientes diabéticos que permitiram manter a glicemia em níveis dentro dos padrões de funcionalidade metabólica.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Grp IDFDA. Update of mortality attributable to diabetes for the IDF Diabetes Atlas: Estimates for the year 2013. Diabetes Research and Clinical Practice. 2015;109(3):461-5.
2. Pendsey SP. Understanding diabetic foot. International Journal of Diabetes in Developing Countries. 2010;30(2):75-9.
3. Francis-Goforth KN, Harken AH, Saba JD. Normalization of diabetic wound healing. Surgery. 2010;147(3):446-9.
4. Adams SB, Jr., Sabesan VJ, Easley ME. Wound healing agents. Foot and ankle clinics. 2006;11(4):745-51.
5. Demidova-Rice TN, Hamblin MR, Herman IM. Acute and Impaired Wound Healing: Pathophysiology and Current Methods for Drug Delivery, Part 1: Normal and Chronic Wounds: Biology, Causes, and Approaches to Care. Advances in skin & wound care. 2012;25(7):304-14.
6. Li N, Brun T, Cnop M, Cunha DA, Eizirik DL, Maechler P. Transient oxidative stress damages mitochondrial machinery inducing persistent beta-cell dysfunction. J Biol Chem. 2009;284(35):23602-12.
7. Mishra P, Chan DC. Metabolic regulation of mitochondrial dynamics. Journal of Cell Biology. 2016;212(4):379-87.
8. Ishihara N, Otera H, Oka T, Mihara K. Regulation and Physiologic Functions of GTPases in Mitochondrial Fusion and Fission in Mammals. Antioxidants & Redox Signaling. 2013;19(4):389-99.
9. Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photochem Photobiol B. 1999;49(1):1-17.
10. Karu T. Mitochondrial signaling in mammalian cells activated by red and near-IR radiation. Photochemistry and Photobiology. 2008;84(5):1091-9.
11. Organization WH. Global health risks: mortality

and burden of disease attributable to selected major risks. Geneve: World Health Organization; 2009.
12. Ribeiro Dias JC, Duarte Bonini Campos JA. Diabetes mellitus: reasons for prevalence in different geographic regions of Brazil, 2002-2007. Ciencia & Saude Coletiva. 2012;17(1):239-44.
13. Prevention. CfDC. A Snapshot: Diabetes In The United States 2014 [
14. Endocrinologia SBd. Numeros do Diabetes no Brasil 2016 [
15. Wild SH, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030 - Response to Rathman and Giani. Diabetes Care. 2004;27(10):2569-.
16. Ohlendieck K, Staunton L. Mass spectrometry-based proteomics of skeletal muscle aging. International Journal of Molecular Medicine. 2012;30:S5-S.
17. Freitas Lucia R S GLP. Evolução da prevalência do diabetes e deste associado à hipertensão arterial no Brasil: análise da Pesquisa Nacional por Amostra de Domicílios, 1998, 2003 e 2008. Epidemiol Serv Saúde2012. p. 7-19.

18. Angel A, Hux J, Anderson G, Deboer D. A population based analysis of diabetes (DM) related lower extremity amputations (LEA) in Ontario, Canada. Diabetes. 1997;46:538-.
19. Angel A, Hux J, Anderson G, DeBoer D. A time trend analysis of diabetes (DM) related lower extremity amputations (LEA) in Ontario, Canada. Diabetologia. 1997;40:1842-.
20. Coblentz J, Elias Pires ML, Oliveira D, Lebeis K, Terrezo L. Prevalence of type 2 diabetes mellitus and other associated risk factors in glaucoma patients. Revista Brasileira De Oftalmologia. 2010;69(1):33-5.
21. Malandrino N, Smith RJ. Personalized Medicine in Diabetes. Clinical Chemistry. 2011;57(2):231-40.
22. Poitout V, Robertson RP. Glucolipotoxicity: Fuel excess and beta-cell dysfunction. Endocrine Reviews. 2008;29(3):351-66.
23. K. F. Pathophysiology of type 2 diabetes and the role of incretin hormones and beta-cell dysfunction. JAAPA. 2007;20(12):3-8.
24. Kalra S. Recent advances in pathophysiology of diabetes: Beyond the dirty dozen. Journal of the Pakistan Medical Association. 2013;63(2):277-80.
25. Assmann TS, Duarte GCK, Rheinheimer J, Cruz LA, Canani LH, Crispim D. The TCF7L2 rs7903146 (C/T) polymorphism is associated with risk to type 2 diabetes mellitus in Southern-Brazil. Arquivos Brasileiros De Endocrinologia E Metabologia. 2014;58(9):918-25.
26. Prestwich TC, MacDougald OA. Wnt/beta-catenin signaling in adipogenesis and metabolism. Current Opinion in Cell Biology. 2007;19(6):612-7.
27. Lin CY, Gurlo T, Haataja L, Hsueh WA, Butler PC. Activation of peroxisome proliferator-activated receptor-gamma by rosiglitazone protects human islet cells against human islet amyloid polypeptide toxicity by a phosphatidylinositol 3 '-kinase-dependent pathway. Journal of Clinical Endocrinology & Metabolism. 2005;90(12):6678-86.
28. Vincent A, Edwards J, McLean L, Hong Y, Cerri F, Lopez I, et al. Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy. Acta Neuropathol.120(4):477-89.
29. Zorzano A, Isabel Hernandez-Alvarez M, Sebastian D, Pablo Munoz J. Mitofusin 2 as a Driver That Controls Energy Metabolism and Insulin Signaling. Antioxidants & Redox Signaling. 2015;22(12):1020-31.
30. Loson OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Molecular Biology of the Cell. 2013;24(5):659-67.
31. Supale S, Thorel F, Merkwirth C, Gjinovci A, Herrera PL, Scorrano L, et al. Loss of Prohibitin Induces Mitochondrial Damages Altering beta-Cell Function and Survival and Is Responsible for Gradual Diabetes Development. Diabetes. 2013;62(10):3488-99.
32. Tubbs E, Theurey P, Vial G, Bendridi N, Bravard A, Chauvin M-A, et al. Mitochondria-Associated Endoplasmic Reticulum Membrane (MAM) Integrity Is Required for Insulin Signaling and Is Implicated in Hepatic Insulin Resistance. Diabetes. 2014;63(10):3279-94.
33. Sebastian D, Isabel Hernandez-Alvarez M, Segales J, Sorianello E, Pablo Munoz J, Sala D, et al. Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(14):5523-8.

34. Cheng Z, Tseng Y, White MF. Insulin signaling meets mitochondria in metabolism. Trends in Endocrinology and Metabolism. 2010;21(10):589-98.
35. Bonnard C, Durand A, Peyrol S, Chanseaume E, Chauvin M-A, Morio B, et al. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. Journal of Clinical Investigation. 2008;118(2):789-800.
36. Bach D, Naon D, Pich S, Soriano FX, Vega N, Rieusset J, et al. Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle - Effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha, and interleukin-6. Diabetes. 2005;54(9):2685-93.
37. Isabel Hernandez-Alvarez M, Thabit H, Burns N, Shah S, Brema I, Hatunic M, et al. Subjects With Early-Onset Type 2 Diabetes Show Defective Activation of the Skeletal Muscle PGC-1 alpha/Mitofusin-2 Regulatory Pathway in Response to Physical Activity. Diabetes Care. 2010;33(3):645-51.
38. Youle RJ, van der Bliek AM. Mitochondrial Fission, Fusion, and Stress. Science. 2012;337(6098):1062-5.
39. Reaven GM. PATHOPHYSIOLOGY OF INSULIN-RESISTANCE IN HUMAN-DISEASE. Physiological Reviews. 1995;75(3):473-86.
40. Godbole VY, Grundleger ML, Thenen SW. EARLY DEVELOPMENT OF LIPOGENESIS IN GENETICALLY-OBESE (OB-OB) MICE. American Journal of Physiology. 1980;239(4):E265-E8.
41. Han S, Liang C-P, Westerterp M, Senokuchi T, Welch CL, Wang Q, et al. Hepatic insulin signaling regulates VLDL secretion and atherogenesis in mice. Journal of Clinical Investigation. 2009;119(4):1029-41.
42. Viollet B, Foretz M, Guigas B, Horman S, Dentin R, Bertrand L, et al. Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. Journal of Physiology-London. 2006;574(1):41-53.
43. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes. 2000;49(12):2063-9.
44. Presswala L, Shubrook J. What to do after basal insulin: 3 Tx strategies for type 2 diabetes. Journal of Family Practice. 2015;64(4):214-20.
45. Park SW, Goodpaster BH, Strotmeyer ES, de Rekeneire N, Harris TB, Schwartz AV, et al. Decreased muscle strength and quality in older adults with type 2 diabetes - The health, aging, and body composition study. Diabetes. 2006;55(6):1813-8.
46. Cetinus E, Buyukbese MA, Uzel M, Ekerbicer H, Karaoguz A. Hand grip strength in patients with type 2 diabetes mellitus. Diabetes Research and Clinical Practice. 2005;70(3):278-86.
47. Miyazaki Y, Mahankali A, Wajcberg E, Bajaj M, Mandarino LJ, DeFronzo RA. Effect of pioglitazone on circulating adipocytokine levels and insulin sensitivity in type 2 diabetic patients. Journal of Clinical Endocrinology & Metabolism. 2004;89(9):4312-9.
48. Falanga V. Wound healing and its impairment in the diabetic foot. Lancet. 2005;366(9498):1736-43.
49. Jorge AES, Hamblin MR, Parizotto NA, Kurachi C, Bagnato VS. Photodynamic therapy improves the ultraviolet-irradiated hairless mice skin. Photonic Therapeutics and Diagnostics X. 2014;8926.
50. Lau P, Bidin N, Krishnan G, AnaybBaleg SM, Sum MBM, Bakhtiar H, et al. Photobiostimulation effect on diabetic wound at different power density of near infrared laser. Journal of Photochemistry and Photobiology B-Biology. 2015;151:201-7.

51. Havlucu U, Bolukbasi N, Yeniyol S, Cetin S, Ozdemir T. Effects of Light-Emitting Diode Photobiomodulation Therapy and BioOss as Single and Combined Treatment in an Experimental Model of Bone Defect Healing in Rats. Journal of Oral Implantology. 2015;41(4):E110-E7.
52. da Cruz ED, Campos L, Pereira FD, Magliano GC, Benites BM, Arana-Chavez VE, et al. Clinical, biochemical and histological study of the effect of antimicrobial photodynamic therapy on oral mucositis induced by 5-fluorouracil in hamsters. Photodiagnosis and Photodynamic Therapy. 2015;12(2):298-309.
53. Camargo Catarino HR, de Godoy NP, Scharlack NK, Grosso Neves LM, de Gaspari de Gaspi FO, Marreto Esquisatto MA, et al. InGaP 670-nm laser therapy combined with a hydroalcoholic extract of Solidago chilensis Meyen in burn injuries. Lasers in Medical Science. 2015;30(3):1069-79.
54. Tsirogianni AK, Moutsopoulos NM, Moutsopoulos HM. Wound healing: immunological aspects. Injury. 2006;37 Suppl 1:S5-12.
55. Oliveira Volpe CM, Machado Abreu LF, Gomes PS, Gonzaga RM, Veloso CA, Nogueira-Machado JA. The Production of Nitric Oxide, IL-6, and TNF-Alpha in Palmitate-Stimulated PBMNCs Is Enhanced through Hyperglycemia in Diabetes. Oxidative Medicine and Cellular Longevity. 2014;2014:12.
56. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004;84(4):1381-478.
57. Rebelato E, Abdulkader F, Curi R, Carpinelli AR. Control of the intracellular redox state by glucose participates in the insulin secretion mechanism. PLoS One. 2011;6(8):e24507.
58. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414(6865):813-20.
59. Peppa M, Stavroulakis P, Raptis SA. Advanced glycoxidation products and impaired diabetic wound healing. Wound Repair and Regeneration. 2009;17(4):461-72.
60. Antoniades C, Tousoulis D, Tentolouris C, Toutouzas P, Stefanadis C. Oxidative stress, antioxidant vitamins, and atherosclerosis. From basic research to clinical practice. Herz. 2003;28(7):628-38.
61. Cuzzocrea S, Thiemermann C, Salvemini D. Potential therapeutic effect of antioxidant therapy in shock and inflammation. Curr Med Chem. 2004;11(9):1147-62.
62. Calles-Escandon J, Cipolla M. Diabetes and endothelial dysfunction: A clinical perspective. Endocrine Reviews. 2001;22(1):36-52.
63. Dhaunsi GS, Bitar MS. Antioxidants attenuate diabetes-induced activation of peroxisomal functions in the rat kidney. J Biomed Sci. 2004;11(5):566-70.
64. Cui Y, Guo Z, Zhao Y, Zheng Y, Qiao Y, Cai J, et al. Reactive effect of low intensity He-Ne laser upon damaged ultrastructure of human erythrocyte membrane in Fenton system by atomic force microscopy. Acta Biochim Biophys Sin (Shanghai). 2007;39(7):484-9.
65. Conlan MJ, Rapley JW, Cobb CM. Biostimulation of wound healing by low-energy laser irradiation. A review. J Clin Periodontol. 1996;23(5):492-6.
66. Ferraresi C, MR H, Parizotto NA. Low-level laser (light) therapy (LLLT) on muscle tissue: performance, fatigue and repair benefited by the power of light. (2193-0635 (Print)).
67. Huang Y-Y, Sharma SK, Carroll J, Hamblin MR. Biphasic Dose Response in Low Level Light Therapy - An Update. Dose-Response. 2011;9(4):602-18.
68. Chung T-Y, Peplow PV, Baxter GD. Laser Photobiomodulation of Wound Healing in Diabetic and Non-Diabetic Mice: Effects in Splinted and Unsplinted Wounds. Photomedicine and Laser Surgery. 2010;28(2):251-61.

69. Al-Watban FAH, Andres BL. Polychromatic LED therapy in burn heating of non-diabetic and diabetic rats. Journal of Clinical Laser Medicine & Surgery. 2003;21(5):249-58.
70. Vinck EM, Cagnie BJ, Cornelissen MJ, Declercq HA, Cambier DC. Increased fibroblast proliferation induced by light emitting diode and low power laser irradiation. Lasers in Medical Science. 2003;18(2):95-9.
71. Adamskaya N, Dungel P, Mittermayr R, Hartinger J, Feichtinger G, Wassermann K, et al. Light therapy by blue LED improves wound healing in an excision model in rats. Injury-International Journal of the Care of the Injured. 2011;42(9):917-21.
72. Lizarelli RFZ, Miguel FAC, Freitas-Pontes KM, Villa GEP, Nunez SC, Bagnato VS. Dentin hypersensitivity clinical study comparing LILT and LEDT keeping the same irradiation parameters. Laser Physics Letters. 2010;7(11):805-11.
73. Ferraresi C, dos Santos RV, Marques G, Zangrande M, Leonaldo R, Hamblin MR, et al. Light-emitting diode therapy (LEDT) before matches prevents increase in creatine kinase with a light dose response in volleyball players. Lasers in Medical Science. 2015;30(4):1281-7.
74. Miranda EF, Pinto Leal-Junior EC, Marchetti PH, Dal Corso S. Acute effects of light emitting diodes therapy (LEDT) in muscle function during isometric exercise in patients with chronic obstructive pulmonary disease: preliminary results of a randomized controlled trial. Lasers in Medical Science. 2014;29(1):359-65.
75. Eells JT, Wong-Riley MTT, VerHoeve J, Henry M, Buchman EV, Kane MP, et al. Mitochondrial signal transduction in accelerated wound and retinal healing by near-infrared light therapy. Mitochondrion. 2004;4(5-6):559-67.
76. Kim K-A, Shin Y-J, Akram M, Kim E-S, Choi K-W, Suh H, et al. High glucose condition induces autophagy in endothelial progenitor cells contributing to angiogenic impairment. Biological & pharmaceutical bulletin. 2014;37(7):1248.
77. Gavish L, Rubinstein C, Bulut A, Berlatzky Y, Beeri R, Gilon D, et al. Low-level laser irradiation inhibits abdominal aortic aneurysm progression in apolipoprotein E-deficient mice. Cardiovasc Res. 2009;83(4):785-92.
78. Kim YG, Pak SC, Lee SR. Hairless mouse epidermal antioxidants and lipid peroxidation assessed by He-Ne laser. Lasers in Surgery and Medicine. 2000;27(5):420-6.
79. Reinoso Rubio C, Carlos Simes J, Moya M, Soriano F, Atilio Palma J, Campana V. Inflammatory and Oxidative Stress Markers in Experimental Crystalopathy: Their Modification by Photostimulation. Photomedicine and Laser Surgery. 2009;27(1):79-84.
80. Fillipin LI, Mauriz JL, Vedovelli K, Moreira AJ, Zettler CG, Lech O, et al. Low-level laser therapy (LLLT) prevents oxidative stress and reduces fibrosis in rat traumatized Achilles tendon. Lasers Surg Med. 2005;37(4):293-300.
81. Eduardo FP, Mehnert DU, Monezi TA, Zezell DM, Schubert MM, Eduardo CP, et al. Cultured epithelial cells response to phototherapy with low intensity laser. Lasers Surg Med. 2007;39(4):365-72.
82. Marques MM, Pereira AN, Fujihara NA, Nogueira FN, Eduardo CP. Effect of low-power laser irradiation on protein synthesis and ultrastructure of human gingival fibroblasts. Lasers Surg Med. 2004;34(3):260-5.
83. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radical Biology and Medicine. 2000;28(10):1456-62.

84. Manteifel V, Bakeeva L, Karu T. Ultrastructural changes in chondriome of human lymphocytes after irradiation with He-Ne laser: Appearance of giant mitochondria. Journal of Photochemistry and Photobiology B-Biology. 1997;38(1):25-30.
85. Manteifel VM, Karu TI. Structure of mitochondria and activity of their respiratory chain in successive generations of yeast cells exposed to He-Ne laser light. Biology Bulletin. 2005;32(6):556-66.
86. Passarella S, Ostuni A, Atlante A, Quagliariello E. INCREASE IN THE ADP ATP EXCHANGE IN RAT-LIVER MITOCHONDRIA IRRADIATED INVITRO BY HELIUM-NEON LASER. Biochemical and Biophysical Research Communications. 1988;156(2):978-86.

ANEXOS

Submissão Revista Diabetologia no dia 16 de Abril de 2016

Gmail - Diab-16-0653: manuscript submitted to Diabetologia (SY-05) https://mail.google.com/mail/u/0/?ui=2&ik=5a844e6312&view=pt&q...

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Diab-16-0653: manuscript submitted to Diabetologia (SY-05)
1 mensagem

diabetologia-j@bristol.ac.uk <diabetologia-j@bristol.ac.uk> 16 de abril de 2016 23:11
Para: tatmatsu@gmail.com

16-Apr-2016

Dear Dr Tatmatsu Rocha

Re: Diab-16-0653
Phototherapy and Diabetes: Is there a light of the end tunnel?

Thank you for submitting this manuscript to Diabetologia. We will now begin processing your paper, and will be in touch once a decision has been reached. Since all our correspondence with you will be via e-mail it is essential that you notify us of any changes to your e-mail address.

Submissão para Revista Photochemistry and Photobiology B

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Evise <EviseSupport@elsevier.com> 24 de novembro de 2015 19:54
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Dear Prof. Tatmatsu Rocha,

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DECLARAÇÃO

Declaro que o protocolo para uso de animais em experimentação referente ao projeto intitulado: " Estudo imunohistológico e do estresse oxidativo na regeneração tecidual induzida por irradiação a laser em ratos diabetico-induzidos pela estreptozotocina ", de responsabilidade de José Carlos Tatmatsu Rocha, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA).

Declaro ainda que o referido projeto foi aprovado *ad referendum* da Comissão de Ética em Pesquisa Animal- CEPA.

Fortaleza, 31 de Janeiro de 2013

Profa. Dra. Nylane Maria Nunes de Alencar
Coordenadora da Comissão de Ética em Pesquisa Animal – CEPA

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Parecer da Comissão de Ética no Uso de Animais

nº 052/13

Protocolo nº. 052/13

A Comissão de Ética no Uso de Animais da Universidade Federal de São Carlos - CEUA/UFSCar **APROVOU** o projeto de pesquisa intitulado *"Efeitos do laser sobre o pâncreas: mecanismos de modulação da morfologia mitocondrial e da produção insulínica.*, submetido pelo pesquisador José Carlos Tatmatsu.

São Carlos, 29 de novembro de 2013

Azair Liane Matos do Canto de Souza

Profa. Dra. Azair Liane Matos do Canto de Souza

Presidente da Comissão de Ética no Uso de Animais