UNIVERSIDADE FEDERAL DE JUIZ DE FORA

RODRIGO GUERRA DE OLIVEIRA

Estudo da ação imunobiológica do laser de baixa intensidade sobre modelo de hipersensibilidade tardia à ovalbumina.

Juiz de Fora

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Tese apresentada ao Programa de Pós-Graduação em Saúde, área de concentração em Saúde Brasileira, da Universidade Federal de Juiz de Fora, como requisito parcial para obtenção do Título de Doutor em Saúde.

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RESUMO

O objetivo deste estudo foi avaliar o efeito do Laser de Baixa Intensidade (LLLT) em um modelo de Hipersensibilidade Tardia (RHT). LLLT vem sendo estudado há algum tempo e seus efeitos clínicos, aplicados no tratamento de várias doenças. O laser tem sido testado em diferentes modelos experimentais, mas seus efeitos permanecem obscuros. Tentou-se avaliar os efeitos do LLLT na RHT à ovalbumina (OVA), uma proteína que vem sendo utilizada como antígeno para sensibilizar cobaias. Esse é um modelo amplamente utilizado para avaliar os efeitos de substâncias com potencial para modular o sistema imunológico e as reações inflamatórias. Camundongos Balb/C foram divididos, randomicamente, em quatro grupos, (I) imunizado, não tratado e desafiado (n=6), (II) não imunizado, não tratado e desafiado (n=6); (III) imunizado, tratado com Azatioprina e desafiado (n=6); e o grupo (IV), imunizado, tratado com LLLT e desafiado (n=6). Passadas 48 horas do desafio, os animais foram submetidos a avaliação do edema da pata e foram eutanasiados para análise do coxim plantar. Testes de proliferação foram realizados (espontâneos, na presença de cocanavalina A e ovalbumina) para determinar a produção, em cultura de células, de TNF-α, INF-γ and IL-10. Análises imuno-histoquímicas para expressão de COX-2 também foram realizadas. No grupo de animais irradiados com laser e naqueles tratados com AZA, a medida da espessura da pata foi significativamente menor em comparação à do grupo controle. Tal fato foi acompanhado da redução significativa na densidade do infiltrado inflamatório, assim como a redução significativa nos níveis de TNF-α, INF-γ and IL-10 e na expressão de COX-2. Nossos resultados sugerem que o tratamento com laser possui efeito imunomodulador na RHT à OVA e podem contribuir para a imunoterapêutica do transplante renal.

Palavras-chaves: Reação de Hipersensibilidade Tardia. Imunossupressão. Laser de Baixa intensidade.

ABSTRACT

The aim of this study was to evaluate the effect of Low Level Laser Therapy (LLLT) in an experimental model of delayed hypersensitivity reaction (DTH). LLLT has been studied for some time and its clinical effects have been used to treat numerous diseases. LLLT has been tested in different experimental models and some of its effects have yet to be explained. We tried to assess the effects of LLLT on the DTH reaction to ovalbumin (OVA), a protein that has been used as an antigen to sensitize lab animals. This is a broadly used experimental model to assess the effects of substances that have the potential to modulate the immune system and inflammatory reactions. Balb/C mice were randomly divided into four groups, (I) immunized, untreated and challenged (n=6), (II) not immunized, untreated and challenged (n=6); (III) immunized, treated with Azathioprine (AZA) and challenged (n=6); and group (IV) immunized, treated with LLLT and challenged (n=6). Forty-eight hours after the challenge, the animals were submitted to a paw edema check and were euthanized for histopathology analysis of their plantar pads. Proliferations tests were made (spontaneous, in the presence of concanavalin A and Ovalbumin) to determine the production in cell cultures of TNF-α, INF-γ and IL-10. Immunohistochemical analyzes for expression of COX-2 were also performed. In the group of animals irradiated with lasers and those treated with AZA, footpad thickness measurements were significantly reduced in comparison to the control group. This was accompanied by a very significant drop in the density of the inflammatory infiltration as well as by a significant reduction in the levels of concentration of TNF-α, INF-γ and IL-10 and in the expression of COX-2. Our results suggest that treatment with LLLT has immunomodulatory effect and may have important contribution to the immunotherapeutic of kidney transplants.

Keywords: Delayed Hypersensitivity Reaction. Immunossupression. Low Level Laser Therapy.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina tri fosfato
APC	Células apresentadoras de antígeno (do inglês: *antigen presenting cells*)
CD95	Grupo de diferenciação (*cluster diferentiation*) 95
COX2	Ciclooxigenase 2
DAB	Dimetilbezantraceno
DNA	Ácido Desoxirribonucleico
DRC	Doença Renal Crônica
DTH	*delayed type hipersentitivity*
eNOS	Óxido Nítrico Sintase Endotelial
H_2O_2	Peróxido de Hidrogênio
HIV	Vírus da Imunodeficiência Humana
HOCl	Ácido Hipocloroso
IFN-γ	Interferon-*gamma*
IL-10	Interleucina 10
IL-2	Interleucina 2
IL-3	Interleucina 3
IL-1	Interleucina 1
IL-4	Interleucina 4
iNOS	Óxido Nítrico Sintase Induzível
Laser	Light amplification by stimulated emisión of radiation
LLLT	Terapia com laser de baixa intensidade (do inglês: *low level laser therapy*)
L TCD3	Linfócitos T
L TCD4 Th1	Linfócito T auxiliar (*helper*) 1
L TCD4 Th2	Linfócito T auxiliar (*helper*) 2
L TCD4	Linfócito T auxiliar (*helper*)
L TCD8	Linfócito T citotóxico / supressor
L Th0	Línfócito T auxiliar (*helper*) 0
LPS	Lipopolissacarídeo
MHC	Complexo principal de histocompatibilidade do inglês: *major histocompatibility complex*)

NCE	Nefropatia crônica do enxerto
NOS	Óxido Nítrico Sintase Neuronal
NO	Óxido Nítrico
NOS	Óxido Nítrico Sintase
NSAIDs	Drogas Anti-inflamatórias Não esteroidais Seletivas
O_2	Oxigênio
PBS	Solução Salina Tamponada
PG	Prostaglandina
PGE2	Prostaglandina E2
RHT	Reação de hipersensibilidade tardia
RNAm	Ácido Ribonucleico Mensageiro
ROS	Espécies Reativas de Oxigênio
SUS	Sistema Único de Saúde
SOD	Superoxidodismutase
STAT	*Signal Transducer and Ativator of Transcription*
TRS	Terapia Renal Substitutiva
TNF- α	Fator de necrose tumoral – alfa
TNF-β	Fator de necrose tumoral - beta
C_{TH}	*Célula T auxiliar*

SUMÁRIO

1 INTRODUÇÃO

1.1 Doença Renal Crônica

A doença renal crônica (DRC) é caracterizada pela manifestação clínica e laboratorial decorrente da perda lenta, progressiva e irreversível das funções renais. A DRC emerge hoje como um sério problema de saúde pública em todo o mundo, sendo considerada uma “epidemia” de crescimento alarmante. Estima-se que existam mais de 2 milhões de brasileiros portadores de algum grau de disfunção renal. A presença de disfunção renal eleva o risco de morrer prematuramente por doença cardiovascular em cerca de dez vezes em comparação à população normal (HOPKINS E BAKRIS, 2009; BROSNAHAN, 2010).

Independentemente da causa que levou à doença renal, a presença de obesidade, dislipidemia e tabagismo acelera a sua progressão, culminando com a necessidade de Terapia Renal Substitutiva (TRS). Atualmente, no Brasil, mais de 70.000 pacientes são dependentes dessa terapia, seja diálise ou transplante renal, com gasto anual de cerca de 2 bilhões de reais. Com base no grande número de grupos de risco, a previsão é que esse número possa duplicar nos próximos 5 anos, ultrapassando os 125 mil casos em 2010 (BOHLKE, 2008; FERNANDES *et al.*, 2008).

A DRC progride mesmo na ausência da causa inicial que determinou a lesão renal. Acredita-se que, com a redução inicial de certo número de néfrons, aqueles remanescentes tornam-se hiperfiltrantes, hipertrofiam-se, sofrem alterações da superfície glomerular com modificações na permeabilidade às proteínas (BROSNAHAN, 2010). Essas alterações levam à produção renal de fatores de crescimento, citocinas e hormônios. Esses agentes seriam responsáveis pelos processos de proliferação celular, coagulação intraglomerular, recrutamento e proliferação de células inflamatórias, aumento da matriz celular, proliferação colágena e fibrose.

Desse modo, a continuidade da presença de lesões fibróticas glomerulares e intersticiais acabaria por determinar perda progressiva dos néfrons e da filtração glomerular (COLLINS *et al.*, 2009). O organismo adapta-se continuamente a essa situação no sentido de manter a homeostase. Os mecanismos de adaptação glomerular e tubular permitem que o organismo sobreviva durante anos à redução

progressiva do parênquima renal. Porém, não há como sustentar indefinidamente essa situação. A partir de certo nível de destruição renal, é inexorável a progressão à fase terminal da doença, na qual o paciente passa a requerer terapias de substituição da função renal (FILIOPOULOS E VLASSOPOULOS, 2009).

Já estão estabelecidas com clareza as principais causas de DRC, sendo que diabete melito, hipertensão arterial, história familiar de DRC, e envelhecimento estão entre as principais. Além destas, outras moléstias podem estar relacionadas à perda de função renal como, glomerulopatias, rejeição crônica do enxerto renal, doença renal policística, doenças autoimunes, infecções sistêmicas, infecções urinárias de repetição, uropatias obstrutivas e neoplasias (ROSNER, 2009).

No Brasil, assim como em todo o mundo, a prevalência de pacientes mantidos em programa crônico de diálise vem crescendo substancialmente, afetando diretamente os gastos em saúde, o que tem causado preocupações em agências de saúde de todo o mundo quanto ao seu gerenciamento e financiamento de longo prazo (BOHLKE, 2008; FERNANDES *et al.*, 2008).

Segundo dados da Sociedade Brasileira de Nefrologia, em 1994, eram cerca de 24.000 pacientes mantidos em programa de diálise e, em 2006, mais de 70.000 pacientes; um crescimento médio no número absoluto de pacientes de cerca de 9% nos últimos anos, com uma taxa de incidência de 175 por milhão, e de prevalência de 383 por milhão (BOHLKE, 2008).

As taxas brasileiras de prevalência são cerca de quatro vezes menores que as dos Estados Unidos e Japão, e metade das taxas da Itália, França e Alemanha. Provavelmente, o baixo índice de diagnóstico da DRC, o acesso limitado à terapia renal substitutiva (TRS) e, principalmente, a alta taxa de mortalidade dos pacientes diabéticos e hipertensos ainda nas fases pré-dialíticas explicam esta baixa prevalência. É inquestionável, portanto, um aumento substancial no número de pacientes que irão necessitar de TRS nos próximos anos (GALLIENI *et al.*, 2009).

Uma TRS amplamente utilizada é a diálise, que tem por objetivos remover os resíduos sanguíneos, remover o excesso de líquidos e manter o equilíbrio dos sais no organismo (eletrólitos). Existem dois tipos principais de diálise: a hemodiálise e a diálise peritoneal. De acordo com as Diretrizes da SBN, para a maior parte dos indivíduos e na ausência de contraindicações, a escolha do método para a TRS pode se basear na preferência do paciente, uma vez que não há evidências que

suportem a superioridade de um dos métodos quanto à sobrevida do paciente(MORTON *et al.*, 2010). Segundo os dados do censo de 2006, 90,7% dos pacientes sob tratamento estavam utilizando a hemodiálise e o restante a diálise peritoneal. Esses dados contrastam com os de alguns países no mundo onde o percentual de diálise peritoneal chega a alcançar entre 18% a 83% (BOHLKE, 2008; FERNANDES *et al.*, 2008).

Em 2005, a taxa de mortalidade anual (número de óbitos/pacientes em diálise no meio do ano) foi de 13%. Não houve diferença de sobrevida em relação ao sexo e ao tipo de diálise. Entre os diversos fatores de risco, idade, diabetes e número de comorbidades associadas foram os mais importantes. Deve-se destacar que a DRC terminal é doença grave e, mesmo com a TRS, ela apresenta, em nosso meio, mortalidade superior em números absolutos a algumas neoplasias, como as de colo de útero, colo, reto e próstata, estando, mesmo, muito próxima à do câncer de estômago.

O custo do tratamento dos pacientes em estágio final de doença renal é substancial e representa um grande desafio para os serviços de saúde. Na Europa, menos de 0,1% da população necessita de TRS, entretanto, são gastos 2% do orçamento da saúde com este grupo. Nos Estados Unidos, estima-se gastar 29 bilhões de dólares com TRS em 2010. No Brasil, os gastos somente com a TRS já ultrapassam 2 bilhões de reais/ano. Avaliando-se esses números frente ao potencial crescimento do número de pacientes que necessitarão de TRS nos próximos anos, espera-se um crescimento explosivo nas despesas do SUS, comprometendo outros importantes programas de saúde governamentais ou aumentando o número de pacientes fora do sistema (FERNANDES *et al.*, 2008).

1.2 Transplante Renal

O transplante renal é uma importante opção terapêutica para o paciente com insuficiência renal crônica, tanto do ponto de vista médico, quanto social ou econômico. Ele está indicado quando houver DRC em fase terminal, estando o paciente em diálise ou mesmo em fase pré-dialítica (pré-emptivo). (RUSH *et al.*, 1998; SEIKKU *et al.*, 2005; SETOGUCHI et al., 2008; RUSH, 2010).

O transplante alogênico é, conceitualmente, o processo de transferência de células, tecidos ou órgãos de um indivíduo a outro da mesma espécie e é realizado nos casos em que há falência ou mau funcionamento irreversível de um órgão ou tecido. O principal desafio no sucesso de alotransplantes em humanos é a rejeição do enxerto (SEIKKU *et al.*, 2005). Para sua preservação e tratamento, há a necessidade do uso contínuo de imunossupressores. Apesar do extenso desenvolvimento de novas terapias imunossupressoras, o alto custo das drogas e, principalmente, seus efeitos colaterais ainda são um grande problema a ser superado.

A rejeição pode ser entendida como a deterioração funcional e estrutural do enxerto. De acordo com os padrões histológicos renais, a rejeição é classificada pelos critérios adotados após reunião em BANFF (Canadá) (SOLEZ *et al.*, 2007). Baseada nos critérios de BANFF, a biópsia do rim transplantado pode ser classificada em normal, rejeição mediada por anticorpos, rejeição *borderline* (limítrofe), rejeição aguda mediada por células T e rejeição crônica mediada por células T.

A rejeição mediada por anticorpos é caracterizada pela presença das alterações: características tissulares de rejeição aguda e piora na função renal. A *borderline* é caracterizada pela suspeita clínica de rejeição aguda mediada por células T, com focos de tubulite. A rejeição aguda mediada por células T caracteriza-se, histologicamente, pela infiltração intersticial de células mononucleares, rompimento da membrana basal tubular (tubulite) e necrose vascular, acompanhada por inflamação linfocitária. A rejeição crônica mediada por células T tem como característica a presença de fibrose intersticial, com infiltrado mononuclear e atrofia tubular (SOLEZ *et al.*, 2007).

Diversos autores têm documentado o aumento expressivo da sobrevida do enxerto renal ao longo da história do transplante. Atualmente, a sobrevida do enxerto renal no primeiro ano ultrapassa 80%, independentemente do tipo de doadores, e a vida média do enxerto renal também aumentou. Destaca-se que os benefícios observados na sobrevida do enxerto no primeiro ano após o transplante foram superiores àqueles observados após esse período (KLEIN *et al.*, 2002).

A despeito desse cenário bastante favorável no que tange à fase inicial do transplante, a perda tardia dos enxertos, quer por disfunção crônica, quer por morte

do receptor ou doença renal recorrente, permanece desproporcionalmente elevada em relação aos ganhos de curto prazo.

A nefropatia crônica do enxerto (NCE) é uma entidade clínicopatológica definida ainda precocemente, manifestada por uma perda gradual da função renal em meses ou anos após o transplante, acompanhada de hipertensão arterial e variados graus de proteinúria. Numerosos estudos clínicos e experimentais tentam elucidar os mecanismos envolvidos nesta complexa doença na tentativa de se obter terapêutica específica para prevenir ou interromper o processo patológico. Entretanto, os mecanismos exatos responsáveis pelos achados histopatológicos característicos ainda permanecem desconhecidos. Vários estudos experimentais demonstraram que fatores imunes (aloantígeno dependentes ou não) e fatores não imunológicos contribuiriam para a patogênese da NCE (WOMER *et al.*, 2000). Numerosos estudos clínicos confirmaram a rejeição aguda (RA) como um dos fatores de risco mais fortes para o desenvolvimento da NCE e posterior perda tardia do enxerto, daí a importância da biópsia do enxerto por ocasião do episódio de RA para que se possa acompanhar a evolução do transplante e o diagnóstico precoce da NCE. Inclusive determinando a natureza dos achados histopatológicos e a pesquisa de citocinas inflamatórias (SHISHIDO *et al.*, 2003).

Destacamos, aqui, o papel da TNF-alfa, citocina pró-inflamatória produzida principalmente por macrófagos, em menor proporção por linfócitos e outras células com papel imunológico. Foi, inicialmente, descrita como uma citocina presente em lesões neoplásicas malignas, diretamente associada à destruição tecidual local, debilitação sistêmica e caquexia (HOFFMAN, 2009).

Atualmente, é conhecida sua participação na resposta imunológica natural e adquirida, representando o principal mediador da resposta do hospedeiro contra bactérias Gram-negativas, além de estar associada, em níveis elevados, ao choque séptico e destruição de tecidos moles e duros (ISHIGAME *et al.*, 2006; KIM e MOUDGIL, 2008).

Outra citocina que merece destaque é a IL-10, que foi inicialmente descrita como “cytokine synthesis inhibitory factor” (CSFI), sendo reconhecida por sua capacidade de inibir a ativação e produção de citocinas por células Th1 (BETTINI e VIGNALI, 2009). Sua principal função parece, de fato, se limitar a respostas antiinflamatórias, papel que pode ser demonstrado pelo desenvolvimento espontâneo ou maior susceptibilidade a doenças inflamatórias em camundongos IL-

10 deficientes (KOBAYASHI *et al.*, 2001). A IL-10 também colabora no balanço responsável pelo crescimento e/ou diferenciação de um amplo espectro de células, como células B, células Natural Killer, células dendríticas, queratinócitos e células endoteliais (KOBAYASHI *et al.*, 2001; BROOKS *et al.*, 2006).

Como dito anteriormente, os macrófagos são importantes produtores de IL-10 e, devido ao seu papel na supressão de respostas celulares, muitas tentativas têm sido realizadas quanto ao emprego dessa citocina na terapia de transplantes, tentando-se induzir tolerância em enxertos alogênicos (SUTHANTHIRAN e STROM, 1995; OPELZ e DOHLER, 2009)

Os resultados obtidos nestes trabalhos, no entanto, são bastante contraditórios. Existem casos em que se observa aumento da sobrevida e casos em que não há alteração ou há diminuição da sobrevida de enxerto. Alguns trabalhos mostraram que enxertos de fígados alogênicos, tratados com IL-10 recombinante ou transfectados com o gene de IL-10, apresentaram aumento de sobrevida (SHINOZAKI *et al.*, 2000b; a; TASHIRO *et al.*, 2000).

Já no transplante cardíaco, são relatados casos em que o tratamento resulta em aumento de sobrevida (MIURA *et al.*, 2001; OSHIMA *et al.*, 2007; PAYNE *et al.*, 2007) ou em rejeição acelerada com agravantes de doença arterial (Furukawa, Becker *et al.*, 1999) e, no caso de ilhotas pancreáticas, o tratamento com IL-10 não aumenta a sobrevida dos enxertos, sendo que, em alguns casos, parece acelerar a rejeição (ZHENG *et al.*, 1995; EL-BAHNASAWY *et al.*, 2004; KUTTLER *et al.*, 2007) .

O motivo pelo qual esses efeitos diversos são observados ainda não é muito bem compreendido, mas parece ter relação com a quantidade de IL-10 produzida localmente (MOOR *et al.*, 2001) e o tipo de tecido transplantado (BERG *et al.*, 2001; TATEDA *et al.*, 2001); portanto não é possível admitir que a simples administração de IL-10 poderá suprimir a resposta imune contra halogênios.

Ao se discutir o sucesso de qualquer transplante, pensa-se diretamente no controle da resposta imune, permitindo a adaptação do transplante e evitando a sua rejeição. Os MHCs de classe I (HLA-A, HLA-B e HLA-C) encontram-se em praticamente todas as superfícies celulares. Esta classe de MHC reconhece antígenos protéicos externos, incluindo tecidos transplantados e é reconhecidos por linfócitos T com especificidade antigênica. Geralmente, as moléculas de classe I são reconhecidas por linfócitos T citotóxicos ou CD8+ (MIOSSEC *et al.*, 2009)

Ao se discutir a participação de células $TCD8^{+}$ na rejeição, é imprescindível

que se discuta também sobre o papel do IFN-γ nesse processo. Esta citocina, além de ser um potente ativador de células T $CD8^+$, aumenta a apresentação de antígenos por estar envolvida em cascatas proteolíticas que asseguram o processamento correto de peptídeos necessários para a apresentação via MHC classe I (STREHL *et al.*, 2005).

Além disso, a produção de IFN-γ por células T $CD8^+$ infiltrantes no enxerto pode estimular os neutrófilos a produzirem quimiocinas, que, por sua vez, recrutam linfócitos T ativados (MORITA *et al.*, 2001).

Outro mecanismo de rejeição mediado por IFN-γ ocorre através da produção dessa citocina por células T $CD8^+$ de memória. Há evidências de que a presença de células T $CD8^+$ $CD62L^{low}$ em pacientes pré-transplantados, leva a um maior risco de rejeição pós-transplante (BENICHOU *et al.*, 1999; HEEGER *et al.*, 1999; VALUJSKIKH *et al.*, 1999). Em camundongos, foi demonstrado que o recrutamento precoce de linfócitos T $CD8^+$ $CD62L^{low}$ preexistentes induz ao recrutamento de células polimorfonucleares e aumenta necrose do enxerto (EL-SAWY *et al.*, 2004). Há ainda, evidências que a presença na memória de células T pode ser responsável pela falha em induzir tolerância ao enxerto alogênico, tanto em animais quanto em humanos (BROOK *et al.*, 2006).

No transplante humano, a principal estratégia para reduzir a imunogenicidade dos enxertos tem sido minimizar as diferenças alogênicas entre doador e receptor mediante a seleção de doadores, além do uso de drogas imunossupressoras. Além disso, a análise de um painel de aloanticorpos presentes do receptor também é realizada para a melhor seleção do par receptor-doador e para diminuir a resposta imune ao enxerto.

O sucesso atual do transplante de órgão baseia-se, em grande parte, no uso de moléculas imunossupressoras que são capazes de bloquear a ação de células do sistema imune. Contudo, seus efeitos colaterais são um grande desafio a ser superado, tais como mielossupressão, cardiotoxicidade, nefrotoxicidade, desordens metabólicas, entre outros (DEMIRKIRAN *et al.*, 2008). Essas terapias são eficientes no tratamento da rejeição aguda, mas apresentam poucos efeitos em relação à rejeição crônica, Assim, o desenvolvimento de novas terapias para a nefropatia crônica do enxerto é, hoje, um importante foco das pesquisas cientificas mundiais.

1.3 Laser

Dessa forma, outras terapêuticas vêm sendo investigadas com o objetivo de modular a resposta inflamatória ou o próprio sistema imunológico em diversos processos patológicos. A palavra laser é um acrônimo com origem na língua inglesa: *Light Amplification by Stimulated Emission of Radiation* (Amplificação da luz por emissão estimulada de radiação). Esta radiação é eletromagnética não ionizante, sendo um tipo de fonte luminosa com características bastante distintas daquelas de uma luz fluorescente ou de uma lâmpada comum (KARU *et al.*, 1997; YIP *et al.*, 1999; GULSOY *et al.*, 2006).

A emissão laser é monocromática, ou seja, emite radiação em um único comprimento de onda. Por ser uma radiação com coerência espacial e temporal, suas ondas propagam-se com a mesma fase no espaço e no tempo e sua direcionalidade permite a obtenção de alta densidade de energia concentrada em pequenos pontos (AIMBIRE *et al.*, 2006; ROCHA JUNIOR *et al.*, 2009).

São justamente as características especiais desse tipo de luz que a fazem ter propriedades terapêuticas importantes (laser de baixa intensidade ou terapêutico), assim como ser utilizada em cirurgias (laser de alta intensidade). Através da indução de efeitos trófico-regenerativos, anti-inflamatórios e analgésicos, percebe-se que os efeitos terapêuticos dos lasers sobre os diferentes tecidos biológicos são muito amplos.

Apesar dos trabalhos que relatam aumento na microcirculação local (AGAIBY *et al.*, 2000; BJORDAL *et al.*, 2006), no sistema linfático (GAVISH *et al.*, 2008; UEBELHOER e ROSS, 2008), na proliferação de células epiteliais (AGAIBY, GHALI *et al.*, 2000) e fibroblastos (AIMBIRE *et al.*, 2006; BJORDAL *et al.*, 2006) assim como aumento na síntese de colágeno por parte dos fibroblastos (CAETANO *et al.*, 2009; FULOP *et al.*, 2009; MINATEL *et al.*, 2009) ainda permanecem sem esclarecimento alguns fenômenos importantes para o perfeito entendimento dessa terapêutica (ENWEMEKA *et al.*, 2004; WOODRUFF *et al.*, 2004; ENWEMEKA, 2005c; a; b; 2006; 2008; ROCHA JUNIOR *et al.*, 2009)

Os primeiros trabalhos que relacionam a utilização da radiação laser nas ciências médicas datam de 1960, exatos 43 anos após a publicação de Albert Einstein, que, em seu artigo “Zur Quantum Theories der Strahlung”, em 1917, expôs os princípios físicos da teoria da emissão estimulada de radiação. Theodore

Mainman desenvolveu a primeira fonte a emitir luz laser através de um rubi e, desde então, inúmeros trabalhos vêm conduzidos com o objetivo de comprovar os efeitos observados pela terapia com laser.

Os diversos tipos de Laser podem ser classificados em duas categorias: o LLLT (emite até 500 mW) e o Laser de alta intensidade de energia ou cirúrgico (emite acima de 100mW). Os primeiros interagem com a célula, respeitando seu limite de sobrevivência e acabam por induzir um aumento na produção de ATP e, consequentemente, produzir alterações metabólicas importantes no ambiente celular (STADLER *et al.*, 2000; ENWEMEKA, 2004; FULOP *et al.*, 2009). O laser de alta intensidade de potência promove destruição celular e, dessa forma, são utilizados para incisões, peelings, e remoção de tecido através do fenômeno de ablação (OLIVEIRA, GOUW-SOARES *et al.*, 2004).

Em relação ao processo de reparação, vários autores (ENWEMEKA *et al.*, 2004; WOODRUFF *et al.*, 2004; ENWEMEKA, 2006; 2008; CAETANO *et al.*, 2009; ENWEMEKA, 2009; FULOP *et al.*, 2009; MINATEL *et al.*, 2009; MINATEL *et al.*, 2009) observaram elevado efeito antiálgico, resposta óssea favorável, com diminuição do tempo de reparação óssea. Através de detalhada técnica de dosificação de síntese de ATP intracelular em culturas de linfócitos, observaram que o laser promove um aumento de aproximadamente 22% na síntese de ATP intramitocondrial. Tal fato, segundo os autores, provavelmente explica a efetividade do laser nos tecidos irradiados, promovendo um maior metabolismo celular e, consequentemente, melhor processo de reparação.

Com relação à interferência no processo de reparo tecidual, alguns autores observaram que a terapia com laser de baixa intensidade interfere nesse processo de reparo tecidual (ALBERTINI *et al.*, 2004; GONZAGA *et al.*, 2009; RIBEIRO *et al.*, 2009). Tais estudos sugerem que a irradiação laser muda o padrão normal de atividade fibroblástica durante o processo de reparação e estimula a síntese de colágeno logo no início da cicatrização, o que pode ser importante para seu resultado final, dependendo da orientação da polarização incidente.

O efeito anti-inflamatório do laser já foi, sem sucesso, estudado. Em 2009 FREITAS e colaboradores (GONZAGA *et al.*, 2009) buscaram demonstrar o efeito anti-inflamatório do laser através de proteína C reativa. Segundo os autores, não houve diferença estatisticamente significante entre os grupos experimentais e controle; conclusão que não foi corroborada por Fujimaki et al. (2003). Nesse

trabalho os autores, através de espécies reativas de oxigênio, sugerem que a LLLT deve ser utilizado para tratamento de tecidos com processo inflamatório instalado.

Um grupo de autores ressalta que o efeito do laser não seria modulando o processo inflamatório, mas teria, na verdade, um efeito bactericida, de proliferação celular e incremento de microcirculação vascular e sugere que o mecanismo de interação da radiação, principalmente com o óxido nítrico, deve ser mais bem investigado (VLADIMIROV *et al.*, 2004; VLADIMIROV *et al.*, 2004).

O entendimento dos efeitos do laser, principalmente nas inflamações agudas foi objeto de estudo de Ferreira et al. (2005). Esses autores observaram cerca de 68% a 95% de redução álgica em ratos Whistar e 54% de redução de edema. Concluíram que o laser de baixa intensidade inibiu a capacidade de sensibilização dos receptores durante o processo inflamatório, mas não esclareceram como tal efeito foi desencadeado.

Assim, Novoselova et.al. (2006) avaliaram o efeito da laserterapia na imunidade de ratos para tentar encontrar uma resposta para os efeitos no sistema imune. Sugeriram que o laser exerce influência na atividade celular de macrófagos e células T. Nesse trabalho, os autores monitoraram a produção de citocinas e óxido nítrico durante um mês. Concluíram que o laser induz favoravelmente uma resposta imunológica quando a exposição não ultrapassa dez dias e que o tratamento na região do timo gera maiores alterações na produção de citocinas.

Ainda tentando encontrar respostas dos efeitos do laser no sistema imune, Aimbire et.al. (2006) investigaram se a LLLT poderia modular o processo inflamatório agudo e o nível de fator de necrose tumoral (TNF-α). Utilizaram 35 ratos whistar, que receberam doses de tratamento diferentes para cada um dos cinco grupos. Concluíram que a laserterapia pode inibir a expressão de TNF-α pós-injúria.

Assim, pelo exposto faz-se necessário a pesquisa acerca do mecanismo de ação e dos efeitos do laser de baixa intensidade.

1.4 Estratégia do Estudo

Modelos animais têm sido amplamente utilizados em pesquisas biológicas como uma alternativa no estudo das doenças, pois eles reproduzem de forma similar tais condições (BREHM *et al.*, 2010; BREHM *et al.*, 2010). Nesse sentido, são usados na pesquisa da resposta imune e nos diferentes elementos envolvidos na defesa contra doenças, podendo servir também para a avaliação dos efeitos de substâncias químicas e físicas no desencadeamento da resposta imune normal (BREHM *et al.*, 2010).

A Reação de Hipersensibilidade Tardia (RHT) ou *"Delayed Type Hipersensitivity" (DTH)* é uma reação inflamatória imuno mediada pelo linfócito T; o termo tardia é aplicado porque o tempo de evolução da reação na pele é medido em dias ou mesmo semanas (BEETON e CHANDY, 2007; VAN BESOUW *et al.*, 2008).

A RHT é caracterizada, principalmente, por grande influxo de células inflamatórias inespecíficas, das quais o macrófago é o principal participante. Este tipo de reação foi, pela primeira vez, descrito em 1890, por Robert Koch, que observou que os indivíduos infectados com o *Mycobacterium tuberculosis* desenvolviam uma resposta inflamatória localizada quando um filtrado derivado de uma cultura de micobactéria era injetado intradermicamente. Ele denominou esta reação de pele localizada de uma "reação tuberculínica" (ANDRADE, 1997).

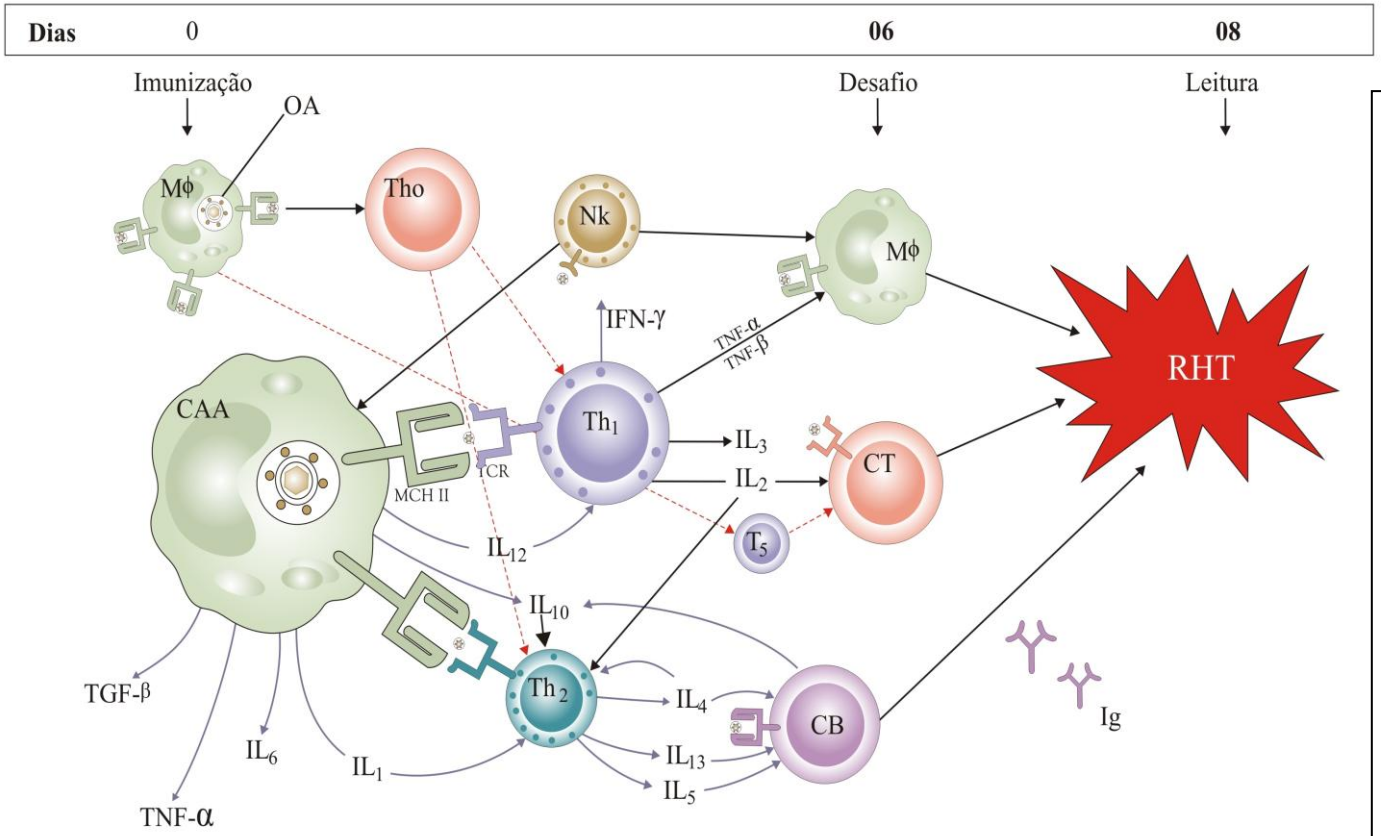
Mais tarde, tornou-se claro que uma variedade de outros antígenos também poderia induzir esta resposta, e o nome da reação foi alterado para hipersensibilidade do tipo tardio em referência ao início tardio da reação e ao extensivo dano tissular (hipersensibilidade) que está muitas vezes associado (KOBAYASHI *et al.*, 2001; BEETON e CHANDY, 2007).

O desenvolvimento de resposta de RHT requer uma fase inicial de sensibilização de uma a duas semanas após o contato primário com o antígeno. Durante este período, as células T_h são ativadas e clonalmente expandidas pelo antígeno apresentado por uma molécula do MHC de classe II em uma célula apresentadora de antígeno apropriada (Esquema 1).

Uma variedade de células apresentadoras de antígeno está envolvida na ativação de uma resposta da RHT, incluindo as células de Langerhans e os macrófagos. As células de Langerhans são células dendríticas encontradas na epiderme. Acredita-se que estas células interagem com os antígenos que penetram

através da pele e os transportam para os linfonodos regionais, onde as células T são ativadas pelo antígeno. O estudo da RHT em animais é uma maneira para analisar as etapas da resposta imune mediada pelas células T.

ESQUEMA 1 Fases da RHT



A resposta imune (RI) na RHT a ovalbumina (OVA). A Célula apresentadora de antígeno (CAA), incluindo o macrofaco (∅), Célula dendrítrica e célula B (CB) apresenta a OVA às células T e B, ativando-as. A célula T auxiliar (Th_0) será diferenciada em célula T auxiliar 1 (Th_1) e 2 (Th_2). Na RHT prevalece o clone Th_1, havendo maior secreção de interleucina (IL)12 em relação a IL-4 pela CAA. A RI perdura pela participação do ∅ e CB sensibilizados, respectivamente produzindo citocinas inflamatórias (IFN-γ, TNF-β e IL_s) e produção de anticorpos (Ig). A intensidade do processo inflamatório será exarcebada com o desafio e atenuada pela atividade das células T supressoras (T_s) e natural killer (NK).

FONTE: Adaptado de ANDRADE, UNIFESP, 1997.

Em algumas espécies, incluindo os humanos, as células endoteliais vasculares expressam as moléculas do MHC de classe II e, também, atuam como células apresentadoras de antígeno no desenvolvimento da resposta de RHT (IWASAKI e MEDZHITOV; TRIVEDI *et al.*, 2007). Geralmente, as células T ativadas durante a fase de sensibilização são as $CD4^+$, principalmente do subtipo T_h1, porém, em poucos casos, mostrou-se que as células $CD8^+$ induzem uma resposta de RHT. As células T ativadas são muitas vezes designadas como células T_{RHT} a fim de denotar a sua função na resposta da RHT, embora, na verdade, elas sejam, simplesmente, um subgrupo das células T_h. A geração da resposta imune está relacionada com a produção de várias citocinas inflamatórias como a interleucina-1, interleucina-2, IFN-γ e TNF-α (ZHANG *et al.*, 2009).

Em um segundo momento da resposta imunológica, o indivíduo já sensibilizado induz a fase efetora da resposta de RHT (Esquema 1). Na fase efetora, as células T_{RHT} secretam diversas citocinas que são responsáveis pelo recrutamento e pela ativação dos macrófagos e outras células inflamatórias inespecíficas. Normalmente, uma resposta de RHT não se torna aparente até, em média, 24 horas após o contato secundário com o antígeno; geralmente, a resposta atinge um pico entre 48 e 72 horas após o contato secundário.

O início tardio desta resposta reflete o tempo necessário para que as citocinas induzam o influxo localizado dos macrófagos e sua ativação. Uma vez iniciada a resposta de RHT, uma interação complexa de células inespecíficas e de mediadores é iniciada e pode resultar em uma amplificação. No momento em que a resposta de RHT está completamente desenvolvida, somente cerca de 5% das células participantes são células T_{RHT} específicas para antígeno; as células remanescentes são os macrófagos e outras células inespecíficas (CHEN *et al.*, 2005).

Os macrófagos funcionam como as principais células efetoras da resposta RHT. As citocinas produzidas pelas células T_{RHT} induzem a adesão dos monócitos sanguíneos às células endoteliais vasculares e a migração do sangue para os tecidos vizinhos. Durante este processo, os monócitos se diferenciam em macrófagos ativados. Os macrófagos ativados exibem elevados níveis de fagocitose e um aumento da habilidade de eliminar microrganismos através dos diversos mediadores citotóxicos. Além disso, os macrófagos ativados expressam níveis elevados das moléculas do MHC de classe II e moléculas de adesão celular e, desta forma, funcionam como células apresentadoras de antígenos mais eficazes

(HERNANDEZ-PANDO e ROOK, 1994; DAVIDSON e DIAMOND, 2001; EL-AGROUDY *et al.*, 2008)

Diversas citocinas desempenham um papel na geração de uma reação de RHT. As funções da IL-2 amplificam, de uma maneira autócrina, a população das células T produtoras de citocinas. Entre as citocinas produzidas pelas células T_{RHT} estão várias que atuam na ativação e atração dos macrófagos para o local de ativação. A IL-3 e o GM-CSF induzem a hematopoiese localizada da linhagem de granulócitos monócitos. O IFN-γ e o TNF-α (juntos com a IL-1 derivada dos macrófagos) atuam nas células endoteliais vizinhas, induzindo várias alterações que facilitam o extravasamento dos monócitos e de outras células inflamatórias inespecíficas.

Os neutrófilos e os monócitos circulantes se aderem às moléculas de adesão que estão expostas nas células endoteliais vasculares e extravasam para os espaços tissulares. Os neutrófilos aparecem em primeiro lugar na reação, atingindo um pico em cerca de 6 horas e, então, declinando em número. A infiltração do monócito ocorre dentro de 24 a 48 horas após a exposição do antígeno (IWASAKI e MEDZHITOV; KOBAYASHI *et al.*, 2001; ISHIGAME *et al.*, 2006; KIM e MOUDGIL, 2008).

Conforme os monócitos entram nos tecidos, para se transformarem em macrófagos, eles são quimiotaticamente atraídos para o local da resposta de RHT por algumas quimiocinas. Uma delas, denominada de fator de inibição da migração (MIF), inibe mais e mais a migração de macrófagos e, desta maneira, previne os macrófagos de migrarem além do sítio de uma reação de RHT. Conforme os macrófagos se acumulam no sítio de uma reação de RHT, eles são ativados pelas citocinas, principalmente pelo IFN gama e pelo TNF-α ligados à membrana, produzidas pelas células T_{RHT} (KOBAYASHI *et al.*, 2001; MOTTET e GOLSHAYAN, 2007; MIMORI, 2009; OUYANG *et al.*, 2009).

Inúmeros estudos têm sido desenvolvidos com o objetivo de compreender o papel do infiltrado inflamatório e qual a influência das células e das citocinas inflamatórias na destruição tecidual (GOODMAN e SERCARZ, 1983; JACYSYN *et al.*, 2001; BETTINI e VIGNALI, 2009; HOFFMAN, 2009). Iwasaki e Medzhitov (2010) sugerem que a destruição tecidual não é apenas o resultado da manutenção da resposta, mas também de alterações e de desequilíbrios no sistema imunológico.

Além dos linfócitos T e dos macrófagos, a presença de linfócitos B associada a estas células foi destacada por vários autores (WING e SAKAGUCHI; TRIVEDI *et al.*,

2007; ZHANG *et al.*, 2009). Os linfócitos B caracterizam resposta imunológica humoral local, e sua presença no sítio da lesão seria o resultado da expressão predominante de citocinas produzidas por células CD4+Th2. Além disso, alguns pesquisadores sugerem que o padrão da produção de citocinas é fator determinante da manutenção patológica da resposta inflamatória local (TANAKA e SAKAGUCHI, 2005; WONDERLICH *et al.*, 2006).

A população de linfócitos TCD4 é subdividida em grupos funcionais, conforme o padrão de produção de citocinas, em Th1 (linfócitos CD4 ou *helper* 1) e Th2 (linfócitos CD4 ou *helper* 2). Esta distinção é relevante, visto que sua função imunológica é distinta: células Th1 estão funcionalmente associados à ativação macrofágica e à síntese de opsoninas, enquanto que as células Th2 auxiliam a síntese de anticorpos e ativam eosinófilos (WING e SAKAGUCHI; TANAKA e SAKAGUCHI, 2005; STROM e KOULMANDA, 2009)

A partir de alterações quantitativas e funcionais em células Th1 e Th2, tem-se o predomínio de citocinas produzidas e liberadas por uma ou por outra subpopulação, a saber: IFN-γ e IL-2 secretadas por células Th1 e IL-4 e IL-10 secretadas por células Th2 (YIP *et al.*, 1999; TANAKA e SAKAGUCHI, 2005; ZIEGLER, 2006; SATYAM *et al.*, 2009; SUMMERS *et al.*, 2009; ZHANG *et al.*, 2009)

Mimori (2009) sugeriu que o padrão de citocinas, produzidas e liberadas no local da lesão inflamatória, dependerá diretamente da predominância populacional de células do infiltrado – se macrófagos ou linfócitos. Esta análise individualizada possibilitaria o desenvolvimento de terapia específica para cada paciente.

Uma enzima importante no desencadeamento do processo inflamatório é a COX-2. Ela faz parte de uma família de enzimas que convertem o ácido araquidônico em PG, sendo esta enzima a forma induzível cuja síntese é estimulada por citocinas inflamatórias, LPS e fatores de crescimento (Rofecoxib, diclofenac, and indomethacin increase risk of CVD, 2006; HARRIS, 2008; VOGT *et al.*, 2009).

Assim, os anti-inflamtórios não seletivos, isto é, que bloqueiam a COX-1 e a COX-2, possuem atividade anti-inflamatória, mas também causam uma série de efeitos adversos renais e, mais frequentemente, gastrintestinais(CASTELLSAGUE *et al.*, 2009) . Em 1997, nos Estados Unidos, foram registrados 16.500 óbitos de pacientes com artrite reumatoide e osteoartrite devido a complicações gastrintestinais relacionadas ao uso de anti-inflamatórios não esteroidais (AINE). A mortalidade foi de 5%-10% dos pacientes hospitalizados por causa desse problema

(CASTELLSAGUE *et al.*, 2009).

Na busca de alternativas menos tóxicas, sintetizou-se uma classe de anti-inflamatórios mais seletivos, isto é, que inibem preferencialmente a COX-2. Os inibidores seletivos da COX-2, denominados coxibs, possuem pouco ou nenhum efeito sobre a COX-1, quando empregados nas doses clínicas. O uso dos coxibs está baseado no princípio de aliviar a dor e a inflamação, pela ação das prostaglandinas geradas através da enzima COX-2, podendo-se assim reduzir os efeitos adversos gerados pela inibição da COX-1 (VOGT *et al.*, 2009).

As prostaglandinas (PG) são eicosanoides que, nos rins, exercem funções no tônus vascular, no balanço hidroeletrolítico e na liberação de renina (VOGT *et al.*, 2009). A ciclo-oxigenase, que é uma prostaglandina sintase G2/H2, tem como substrato o ácido araquidônico e produz a prostaglandina PGG2 e, subsequentemente, a PGH2, as quais são posteriormente metabolizadas pelas isomerases tecido-específicas, em prostaglandinas e tromboxanos.

Existem evidências *in vitro* e *in vivo* que sugerem a participação da COX na patogênese de doenças inflamatórias e autoimunes, principalmente correlacionando o aumento de seus níveis em presença de componentes bacterianos, IL-1 e TNF-α (PEREIRA, 2009; KEFALAKES *et al.*, 2009; SAHIN *et al.*, 2009).

Mais ainda, a idéia inicial de que os AINEs inibidores seletivos da COX-2 não apresentariam efeitos lesivos para o tecido renal vem sendo questionada. Estudos clínicos têm mostrado que o papel funcional intrarrenal da COX-2 está predominantemente associado à manutenção da homeostase hidroeletrolítica, enquanto a COX-1 parece estar mais correlacionada à manutenção da função de filtração glomerular. A expressão constitutiva da COX-2 em tecidos renais levanta a possibilidade de que seus inibidores específicos, como rofecoxib e celecoxib, possam causar os mesmos efeitos adversos renais que os AINEs não seletivos.

Apesar de uma variedade de medicações, a busca por novas modalidades terapêuticas permanece, principalmente para tentar minimizar os efeitos colaterais decorrentes da utilização das substâncias imunossupressoras. Com base nessa revisão da literatura, observa-se que a LLLT possui um efeito eminentemente analgésico, anti-inflamatório e biomodulador, e a perfeita compreensão dos fenômenos que são observados clinicamente precisa ser esclarecidos, principalmente no aspecto imunológico, em que se percebe na literatura especializada escassez de trabalhos científicos.

No presente estudo, serão investigados os efeitos da LLLT na resposta imune, utilizando-se um modelo experimental clássico de RHT em camundongos.

2 OBJETIVO

Analisar a ação imunobiológica da LLLT no modelo experimental de RHT sob os seguintes aspectos:

1. Determinar a ação da LLLT na reação de DTH à OVA;

2. Investigar a ação da LLLT sobre a produção de TNF-α, IFN-γ e IL-10 por células de linfonodos estimulados com Ovalbumina (OVA);

3. Determinar “in situ” a ação da LLLT sobre a expressão de COX-2 no modelo;

3 MATERIAL E MÉTODOS

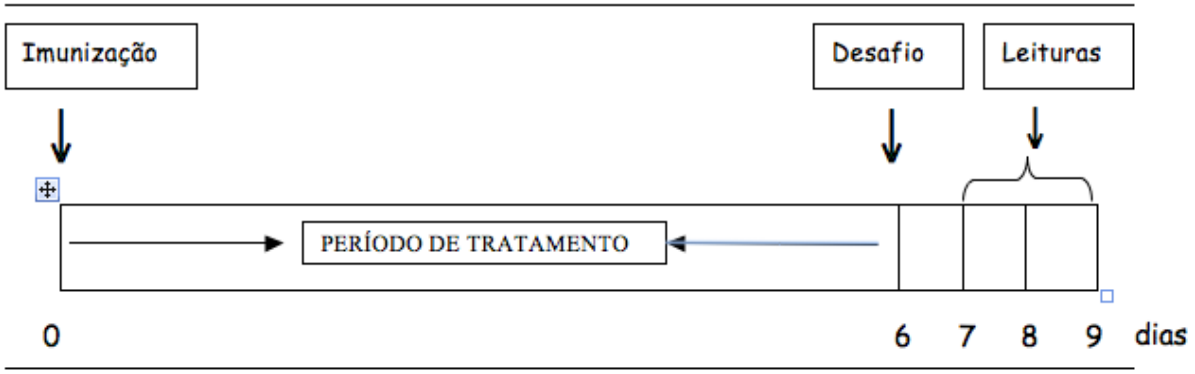
3.1 Animais

Foram utilizados 24 camundongos BALB/c, machos, com 6 a 8 semanas de idade e pertencentes à colônia do Centro de Biologia da Reprodução da Universidade Federal de Juiz de Fora. Os animais foram mantidos em local apropriado e climatizado, na temperatura de 19-25°C e com umidade relativa de 30-70% com ciclo de 12 horas de luz e 12 sem. Aos animais foi fornecido água e alimento *"ad libitum"*. Todo o experimento foi conduzido de acordo com o Guia de Princípios e Cuidados no uso de animais de laboratório e o protocolo de experimentação foi aprovado pelo comitê de ética da instituição (028/2009).

3.2 Delineamento Experimental

Os espécimes foram imunizados com Ovalbumina (OVA), uma proteína majoritária do ovo, com propriedades antigênicas e resistente à desnaturação térmica. Assim, uma aplicação de 100µg de OVA, produzida pela Sigma Chemical Co. St. Louis, MO, USA foi adminstrada, por animal, na base da cauda via subcutânea (sc). A ovalbumina foi emulsificada com adjuvante completo de Freund (CFA) (Sigma Co., St Louis). O esquema 2 representa o protocolo geral da RHT à OVA.

ESQUEMA 2 Protocolo Geral de RHT à OVA



A RHT foi induzida no coxim plantar posterior de camundongos imunizados e desafiados através da inoculação de 30µL de suspensão, contendo 600µg de OVA agregada, na pata posterior direita, após seis dias de imunização. O controle de fidelidade foi conseguido administrando de maneira similar 30µl de PBS na pata posterior esquerda (LOUIS *et al.*, 1984; TITUS *et al.*, 1984; TITUS *et al.*, 1984).

Segundo o protocolo de trabalho, o tratamento dos animais ocorreu no período compreendido entre o dia zero e o dia seis do experimento, independentemente de ser a Azatioprina ou o Laser. Nos dois grupos o tratamento foi realizado diariamente, respeitando o mesmo horário e o mesmo método, ou seja, azatioprina por gavagem e o laser na base da cauda. A intensidade de RHT, expressa pela diferença de espessura das patas posteriores, foi medida 24, 48 e 72 horas após o desafio, empregando-se um micrômetro (Starret, Athol, MA). No estudo piloto e nos experimentos, que foram repetidos por pelo menos três vezes, o ápice da reação ocorreu em 48 horas. Os resultados representam a média de 6 animais por grupo ± erro padrão, expressos em unidades RHT, que é definida como a diferença de 0,01mm na espessura das patas direita e esquerda de cada animal analisado.

QUADRO 1 Animais utilizados na RHT

GRUPOS	IDENTIFICAÇÃO
G I (n = 6)	Controle negativo
G II (n = 6)	Controle positivo
G III (n = 6)	tratado com AZA
G IV (n = 6)	tratado com Laser

3.3 Azatioprina

A azatioprina utilizada nos experimentos “in vivo” foi obtida da Microbiológica-Química e Farmacêutica (Rio de Janeiro, RJ). Inicialmente, foi preparada uma

solução estoque, dissolvendo 5mg em 0,03mL de hidróxido de sódio (0,1%) e 4,97mL de água bidestilada, corrigindo o pH para 7,6, sendo que, logo após, a solução foi esterilizada pela passagem em filtro milipore com 0,45 micra de diâmetro (Miliporo, Japão). A solução foi preparada no dia da administração e, nos experimentos “in vivo”, foram injetados 0,5mg/Kg/dia via oral.

3.4 O laser de baixa intensidade

O aparelho laser da empresa MM Optics (São Carlos – SP) com as seguintes características foi utilizado:

- Laser de emissão IR, pulsátil;
- Semicondutores: Arsênio e Gálio;
- Comprimento de onda: 870nm;
- Potência de Pico: 70mW;
- Potência média de saída: entre 0,5 a 3,5mW;
- Forma de aplicação: fibra óptica.

As aplicações foram feitas diariamente na base da cauda dos espécimes, respeitando inclusive o mesmo horário.

3.5 Análise Histopatológica da pata dos animais

O estudo teve como objetivo avaliar a possível influência da administração do laser na composição celular do coxim plantar dos animais submetidos a RHT à OVA. Seguiu-se o protocolo descrito no item 3.2 e, após a leitura da RHT, os camundongos foram sacrificados por dose letal de Quetamina (Francotar®) e Xilasina (Rompun®). Os coxins plantares posteriores foram dissecados, fixados em solução de formol a 10% por um período mínimo de 48 horas. O material fixado foi desidratado, diafanizado, incluído em parafina, submetido a microtomia (american optical mod. 820) e montados em lâminas. As lâminas foram corados pela hematoxilina – eosina para avaliação histopatológica, e outras foram separadas para realização de imuno-histoquímica.

A análise histopatológica foi realizada por meio do estudo cego, com o patologista, utilizando um microscópio bionocular (WILD, Alemanha). As características e a intensidade do infiltrado inflamatório foram descritas e a intensidade do infiltrado inflamatório será quantificada de acordo com o quadro abaixo:

QUADRO 2 Quantificação do infiltrado

SIMBOLOGIA	IDENTIFICAÇÃO
(-)	Ausência de infiltrado inflamatório
(+)	Infiltrado inflamatório leve
(++)	Infiltrado inflamatório moderado
(+++)	Infiltrado inflamatório intenso

Fotomicrografias foram realizadas utilizando-se o sistema NIKON MICROPHOT (Japão).

3.6 Obtenção dos órgãos e células para estudo

Para avaliar o perfil das citocinas produzidas após seis dias da imunização, procedeu-se à eutanásia dos animais para a remoção dos linfonodos inguinais e periaórticos. As células dos linfonodos foram obtidas através de trituração destes órgãos com homogeinizador em meio RPMI incompleto. As células foram ressuspensas em meio RPMI suplementado com 5% de soro fetal bovino, aminoácidos não essenciais, antibiótico e L-glutamina. Estas células foram estimuladas ou não *in vitro*, com a Ovalbumina (OVA – 200μg/mL) e ConA (10μg/mL), por 24, 48 e 72 horas. Após estes tempos, a viabilidade celular foi avaliada com azul de tripan, e os sobrenadantes coletados para dosagem de citocinas.

3.7 Dosagem de Citocinas por ELISA (TNF- α, IFN-γ e IL-10)

Placas de ELISA foram sensibilizadas com o anticorpo de captura, diluído em tampão carbonato-bicarbonato, incubadas 2 horas à temperatura ambiente e bloqueadas com PBS-Tween 20 (PBST) + 10% SFB, por 30 minutos. Após este período, as placas foram lavadas quatro vezes em PBST e, em seguida, adicionados os anticorpos de captura.

Após uma incubação de 2 horas à temperatura ambiente, foram colocados os padrões de citocinas recombinantes, em diluições seriadas. Nas fileiras seguintes, as amostras de sobrenadantes de cultura foram distribuídas. As placas foram então incubadas por 18 horas a 4^0 C. Terminada a incubação, as placas foram lavadas e o 2^0 anticorpo biotinilado foi incubado por mais 1 hora à temperatura ambiente. Mais quatro lavagens foram feitas e o conjugado enzimático, colocado para ser incubado por mais 1 hora. Após este período, a reação foi revelada pela adição do substrato contendo ácido cítrico 0,1 M, fosfato de sódio 0,2 M, água destilada, cromógeno ABTS e água oxigenada 30%. A reação foi bloqueada com ácido cítrico 0,2 M e a leitura feita em leitor de ELISA (SPECTRAMAX 190, Molecular Devices) a 410 nm. As quantidades de citocinas foram calculadas a partir das curvas-padrão, obtidas pelas diferentes concentrações das citocinas recombinantes.

3.8 Detecção da expressão de COX-2

O método do complexo avidina-biotina peroxidase antiperoxidase foi utilizado para verificar a expressão de COX-2. O método utilizado seguiu as etapas de fixação em acetona (10 min), reidratação dos cortes em PBS, pH 7,4 (10 min) e bloqueio da peroxidase endógena com solução de peróxido de hidrogênio a 0,4% por 30 minutos. Lavou-se em PBS (10 min) e a incubação foi feita com soro normal de cavalo (Vector Laboratory, Inc., Burlington, Califórnia, EUA) em câmara úmida (20 min). Por fim, procedeu-se incubação com os anticorpos primários policlonais (Quadro 3).

QUADRO 3 Anticorpos policlonais utilizados na reação

Anticorpo primário	Laboratório	Local	Diluição / incubação
Rabbit Anti-COX-2	Santa Cruz	Califórnia/EUA	1:100 / 1 hora

As lâminas foram então lavadas em PBS por 10 minutos e procedeu-se a incubação com anticorpo de cabra biotinilado de coelho (Dakopatts, Copenhagen, Dinamarca) diluído a 1:150 em PBS (30 min). Lava-se novamente em PBS (30 min) para promover a incubação com tampão acetato 0,2 M, pH 5,2 também por 10 minutos. A revelação do produto da reação imunológica foi obtida através do emprego da solução de 3-amino-9-eticarbazol (1 a 3 min). As lâminas foram lavadas em água corrente por 10 minutos e a contracoloração obtida com hematoxilina de Harris (1 min). Nova lavagem em água corrente por 10 minutos e a montagem da lâmina com Elvanol (polivinilálcool) e lamínula.

O controle negativo da reação imuno-histoquímica foi realizado omitindo-se em alguns cortes a etapa de incubação com o anticorpo primário.

3.9 Análises Estatísticas

Os resultados referentes ao estudo e comparação das variáveis interessadas nos ensaios realizados foram expressos em números inteiros ou média ± erro padrão (EP). Para comparar quatro ou mais animais nos ensaios “in vivo”, utilizou-se análise de variância (ANOVA); uma vez alcançada a significância estatística, utilizou-se o teste t de Bonferroni (PETRIE, 1982). Foi aceito como nível de significância de 0,05 ($p < 0,05$), ou seja, o limite de 5% de probabilidade de erro. Para realizar os cálculos, foi utilizado o programa de informática Primer of Biostatistics (GLANTZ, 1992).

4 RESULTADOS E DISCUSSÃO

Os resultados e a discussão serão apresentados na forma de três artigos científicos, intitulados "**Low level laser therapy reduces Ovalbumin-DTH in Balb/C mice**", aceito para publicação no periódico *Photomedicine and Laser Surgery*; "**Low Level Laser Therapy inhibits the expression of Cyclooxygenase 2 in a model of DTH to OVA"** submetido ao *Journal of Laser Applications* e "Low-level laser reduces production of TNF-α, IFN-γ and IL-10 induced by OVA" submetido ao *Lasers in Surgery & Medicine.*

Title: "Low level laser therapy reduces Ovalbumin-DTH in Balb/C mice"

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Running title: “Low level laser therapy reduces Ovalbumin-DTH in Balb/C mice”

ABSTRACT

The aim of this study was to evaluate the effect of Low Level Laser Therapy (LLLT) in an experimental model of delayed hypersensitivity reaction (DTH). LLLT has been studied for some time and its clinical effects have been used to treat numerous diseases. LLLT has been tested in different experimental models and some of its effects have yet to be explained. We tried to assess the effects of LLLT on the DTH reaction to ovalbumin (OVA), a protein that has been used as an antigen to sensitize lab animals. This is a broadly used experimental model to assess the effects of substances that have the potential to modulate the immune system and inflammatory reactions. Balb/C mice were randomly divided into four groups, (I) immunized, untreated and challenged (n=6), (II) not immunized, untreated and challenged (n=6); (III) immunized, treated with AZA and challenged (n=6); and group (IV) immunized, treated with LLLT and challenged (n=6). Forty-eight hours after the challenge, the animals were submitted to a paw edema check and were euthanized for histopathology analysis of their plantar pads. The results obtained in DTH units were as follows: Group I: 19.6±8.9; Group II: 5.8±2.6; Group III (AZA): 5.6±2.5; Group IV (laser): 5.2±2.6. DTH was less intense for the groups treated with AZA and Laser compared to Group I ($p < 0.05$). We observed no statistical difference between the AZA and LLLT-treated groups. The slides obtained from the footpads of the specimens showed that AZA and Laser acted in a similar way on the normal pattern of DTH triggering. Our results suggest that treatment with LLLT has an immuno-modulatory effect on the DTH reaction to OVA.

INTRODUCTION

Innate and acquired adaptive cellular or even antibody mediated immunity plays an active role in the pathogenesis of auto immune disorders (glomerulonephrities, hypersensitivity reaction, and organ transplants). The type IV hypersensitivity reaction, also known as delayed type hypersensibility, is triggered by sensitized T-Cells and is predominantly mediated by cell immunity. Usually cell immunity induces a protective response, with the destruction of foreign organisms. Late hypersensitivity is a type of cell-mediated response in which the ultimate action cell is the activated mononuclear phagocyte (macrophage) (Davidson e Diamond, 2001; Beeton e Chandy, 2007).

The experimental model of delayed hypersensitivity reaction (DTH) to ovalbumin (OVA) in mice has been used to demonstrate *in vivo* the immunosuppressive effect of drugs, such as steroids, azathioprine and cyclosporine A (Morris, 1995; Nambu, Nakae *et al.*, 2006; Beeton e Chandy, 2007; Van Besouw, Van Der Mast *et al.*, 2008; Yamashita, Sakai *et al.*, 2009).

Azathioprine (AZA) has been broadly used because it is an efficient immunosuppressant that precludes the specific proliferation of activated cells, and its efficacy has been proven for over 50 years (Atreya e Neurath, 2009; Prefontaine, Sutherland *et al.*, 2009). Azathioprine is a synthetic analogue of purine and was developed in an attempt to prevent the metabolic degradation of 6-mercaptopurine (6-MP) which removed its anti-leukemic effects. Currently, azathioprine is used in dermatology, gastroenterology, oncology, rheumatology, and in many other fields of medicine because of its anti-leukemic, anti-inflammatory and immunosuppressant properties (Gheith, Bakr *et al.*, 2008; Craig, Webster *et al.*, 2009). In several clinical situations the systemic

administration of azathioprine is used to treat localized lesions in skin and oral mucosa, such as atopic dermatitis, lichen planus and Pemphigus vulgaris (Thongprasom e Dhanuthai, 2008; Satyam, Khandpur *et al.*, 2009).

Low level laser therapy (600–980 nm) has been considered an adjuvant clinical treatment (Bensadoun, Franquin *et al.*, 1999; Carrasco, Mazzetto *et al.*, 2008; Gavish, Perez *et al.*, 2008). Clearly, its biomodulating, analgesic, and direct interference effects on the inflammatory process have drawn the attention of many researchers. Several studies in the last decade have examined the anti-inflammatory and immunomodulator effects of this therapeutic resource (Karu, Pyatibrat *et al.*, 1997; Albertini, Villaverde *et al.*, 2007; Rocha Junior, Vieira *et al.*, 2009). However, the mechanism of LLLT action is still not completely understood. This may be the reason why the treatment is not yet fully accepted, despite its satisfactory clinical performance which has been repeatedly proven since 1960.

In the present study, we used an ovalbumin hypersensitivity model of mouse footpad to compare the effects of LLLT and azathioprine on the inflammatory process. Our results suggest that LLLT significantly reduces the severity of the inflammatory process. In this experimental model, LLLT gave results similar to those obtained with azathioprine, the classical immunosuppressive drug.

Materials and Methods

I- Animals

Twenty four male Balb/C mice, aged between 4 and 6 weeks, weighing 30g, provided by the Animal House of the CBR [Reproduction Biology Center] of the

Federal University of Juiz de Fora (UFJF), were used. Mice at CBR-UFJF are housed in large dumping containers equipped with wire screens and two exhausters, as well as room heaters. The temperature was kept at approximately 22ºC by natural ventilation in summer and with the help of heaters in winter time. The lighting was mixed and consisted of natural light and fluorescent light bulbs, which were automatically controlled to turn on at 6:00 a.m. and off at 6:00 p.m. The animals were kept in individual polypropylene cages, equipped with beds of selected wood shavings, baby bottles with water, and troughs for palletized chow, under maintenance conditions which were in agreement with the criteria of the Brazilian College of Animal Experimentation (028/2009). The mice were subjected to daily macroscopic evaluations to detect signs of secondary infection.

II- Experimental model (OVA-DTH):

The DTH model used ovalbumin (OVA), a protein that has been used as an antigen to sensitize lab animals with or without adjuvant. DTH was induced by immunization with ovalbumin (OVA - Sigma, St. Louis, MO, USA). Briefly, mice were injected on the tail base with 100μL of 1.25mg/mL OVA (immunization) and six days after immunization, their footpads were injected with 30μL of 10mg/mL OVA emulsified with Complete Freund's Adjuvant (CFA) (Chondrex, Redmond, WA) (challenge). Animals were injected with an equal volume of PBS into another footpad as a control. The responses were evaluation as described elsewhere (Sarnstrand, Jansson *et al.*, 1999; Yoshimoto, Wang *et al.*, 2000). Forty-eight hours after challenge, footpad thickness was measured with a digital caliper (Starret, MA). The magnitude of the DTH response was determined as follows: [footpadswelling(mm) or DTH units] = [footpad thickness of OVA-injected footpad (mm)]−[footpad thickness of PBS-injected footpad (mm)].

The azathioprine used in the *in vivo* experiments was obtained from the Microbiológica- Química e Farmacêutica (Rio de Janeiro, RJ). Azathioprine was prepared with distillated water on the day of administration and administered at 0.5mg/Kg/per day in gavage. The tail base was irradiated with the laser at a power of 15mW and a dose of 3.8 J/cm^2 for 10s per day (irradiation area 0.04cm^2 and power density 0,72mW/cm^2). The laser (Twin Laser) device used was supplied by MM Optics (São Carlos – SP) and had the following characteristics: IR emission laser, pulsatile; semi-conductors, arsenic, alumminun and Gallium; wave length, 780nm; and means of administration, optic fiber.

The treatment with AZA or laser was done during the time between day zero and day six of the experiment. To compare the results with the DTH assays, we used ANOVA and the Bonferroni test. The significance level accepted was $p < 0.05$.

III – The groups

The animals were randomly divided into four groups, (I) immunized, untreated and challenged (n=6), (II) not immunized, untreated and challenged (n=6) (n=6) (these mice received only OVA emulsified with Complete Freund's Adjuvant); (III) immunized, treated with AZA and challenged (n=6); and group (IV) immunized, treated with LLLT and challenged (n=6) (see Table 1).

Table 1 – The groups

GROUP	IMMUNIZATION (200μL of 1.25mg/mL OVA - tail base)	TREATMENT	CHALLENGE (20μL of 10mg/mL OVA with CFA – footpad)
I - control +	X	-	X
II - control -	-	-	X
III - AZA	X	Azathioprine (0.5mg/Kg/day per os)	X
IV - Laser	X	Laser (3.8 J/cm^2 per day)	X

III- Histopatological and Histomorphometric Analysis

A histopatological analysis was made of the animals' footpad to assess the possible influence of LLLT and AZA administration on the animals showing a DTH reaction to OVA. After measuring DTH, the animals were euthanized with an overdose of intraperitoneal ketamine (100ml/kg) plus xylazine (10mg/kg). The footpads were dissected and fixed in a 10% formaldehyde solution for a minimum of 48 hours. The fixed material was submitted to routine histological analysis and the slides were stained with hematoxilin-eosin. Histopathology analysis was carried out by means of a double blind study performed by two different pathologists. Inflammatory infiltrate characteristics and intensity were assessed. Inflammatory infiltrate intensity was classified according with the follow criteria (Table 2):

(-) = No inflammatory infiltrate

(+) = Mild inflammatory infiltrate

(++) = Moderate inflammatory infiltrate

(+++) = Intense inflammatory infiltrate

Table 2 - Inflammatory infiltrate intensity analysis in DTH to OVA in BALB/c mice: Group I (positive control) and Groups treated with LLLT and AZA.

Animal groups	DERMA		
	Superficial	Medium	Deep
I (positive control)	++	+++	+++
III (AZA)	+	++	++
IV (LLLT)	+	++	++

Micro-photographs were taken using the NIKON MICROPHOT system (Japan).

IV- Statistical analysis

Statistical significance was calculated by ANOVA and the Bonferroni test. The significance level accepted was $p < 0.05$.

RESULTS

Footpad edema analysis

DTH was analyzed 48 hours after aggregated OVA inoculation using a digital caliper (Starret, MA). LLLT mice and AZA-treated mice showed a significant reduction in the DTH reaction compared to non-treated mice (Figure 1). The hypersensitivity reaction was expressed in DTH units.

The results for DTH were as follows: Group I: 19.6±8.9; Group II: 1.5±2.6; Group III (AZA): 5.6±2.5; Group IV (Laser): 5.2±2.6. DTH was lower for groups II and III than

for the control groups ($p < 0.05$). There was no statistical difference between group III and group IV (AZA and LLLT).

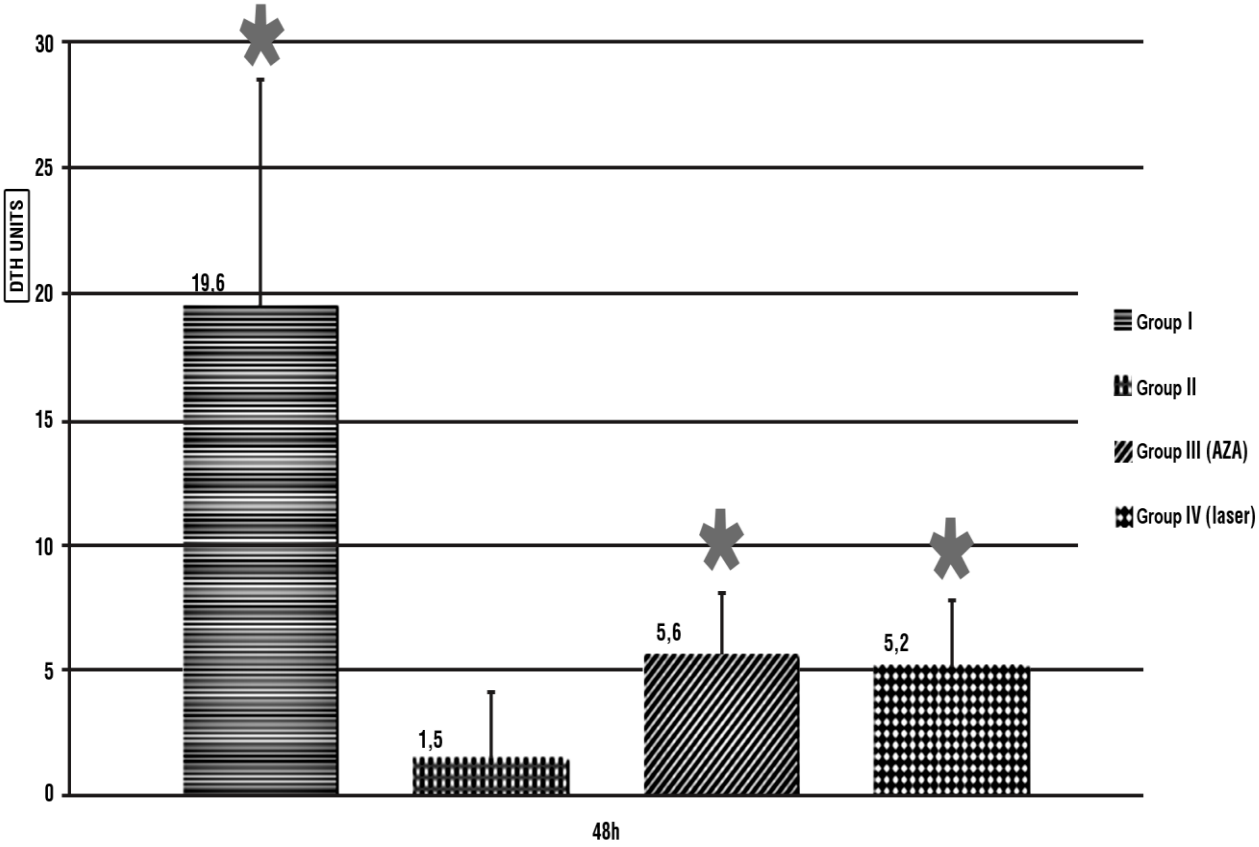

Figure 1 - Mean values of DTH to OVA. The magnitude of the DTH response was determined as follows: [footpadswelling(mm) or DTH units] = [footpad thickness of OVA-injected footpad (mm)]−[footpad thickness of PBS-injected footpad (mm)]. ANOVA and Bonferroni Test ($p < 0.05$) were used. 169x97mm (300 x 300 DPI)

Histopathological analysis

The histopathological analysis of the footpad samples from mice of Group I (positive control) revealed the presence of mononuclear inflammatory infiltrates, which were mainly represented by multifocal lymphocytes in the superficial, middle and deep dermis. In some microscopic fields mononuclear cells with a morphology suggestive of macrophages were observed. Polymorphonuclear cells with a

morphology suggestive of neutrophils were rarely observed (Figure 2A). Table 2 shows a graphic representation of the quantities of inflammatory infiltrate in the different experimental groups.

The animals treated with AZA or LLLT presented a significant reduction ($p<0.05$) in infiltrate intensity when compared to the positive control group. In Figure 2B (AZA Group) we observed a scarce mononuclear infiltrate diffusely distributed throughout the connective tissue. A similar finding can also be seen in animals treated with LLLT (Figure 2C).

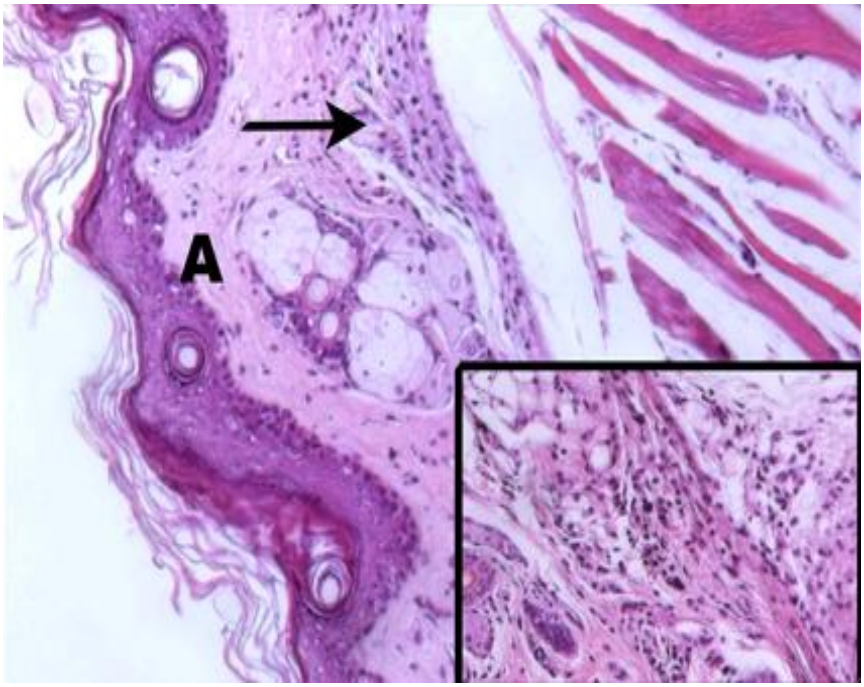

Fig 2A – Microphotography of the footpad from a group I animal (positive control): histopathology suggests the presence of an intense mononuclear inflammatory infiltrate scattered in the superficial, middle and deep derma. In the detail we see a build up of mononuclear cells with morphology suggesting lymphocytes (original magnification100x. Inset: in detail mononuclear cell, original magnification 400x). 149x99mm (72 x 72 DPI)

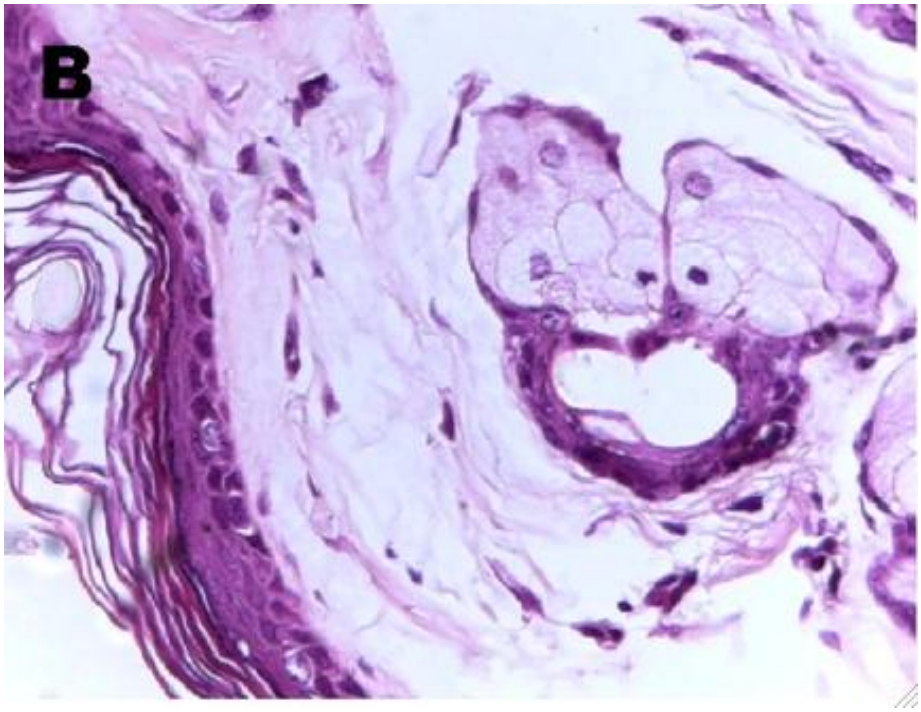

Fig 2B – Microphotography of the plantar pad of a group III animal (AZA): histopathology reveals no inflammatory infiltrate. Normal footpad epithelium architecture and connective tissue (original magnification 400x).152x113mm (72 x 72 DPI)

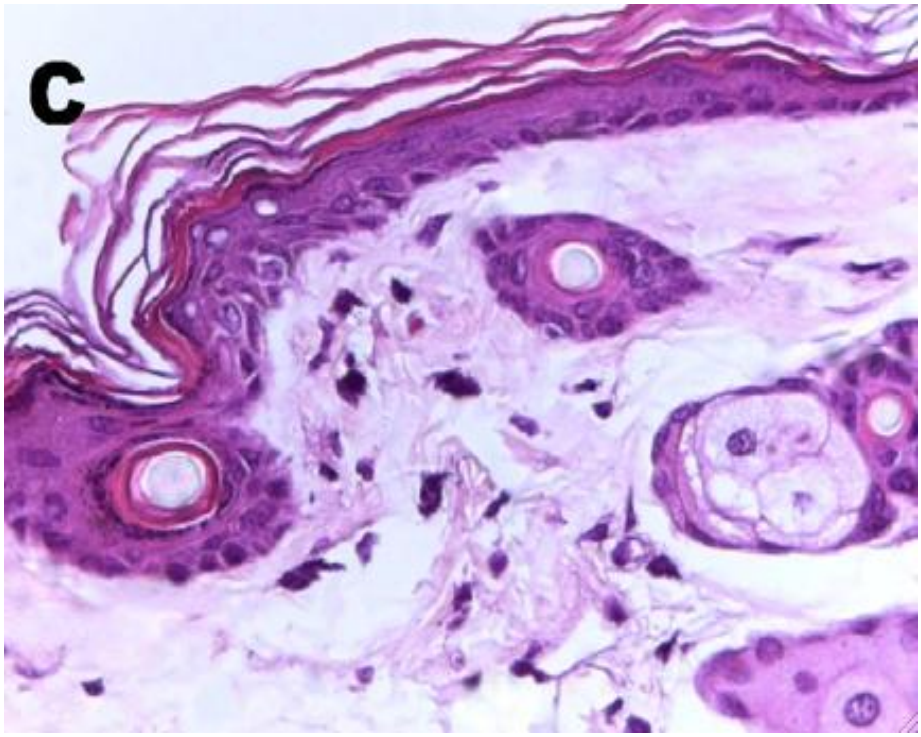

Fig 2C – Microphotograph of the plantar pad from a group IV animal (LLLT): we see some mononuclear inflammatory cells. Normal epithelium architecture and connective tissue of the footpad (original magnification 400x). 152x110mm (72 x 72 DPI)

DISCUSSION

The experimental model of the DTH reaction to OVA allows an assessment and quantification of the T-cell immune response to immunogens and haptens. It also allows a simulation of the acute rejection that happens sometimes after allografting and even during certain autoimmune diseases. Thus, it is ideal for the study of the immunosuppressive potential of new therapeutic agents.

Efficient immunofsupression is key to a better quality of life for transplanted patients. Today, the prevention of acute rejection episodes is achieved by inducing generalized immunosupression, which can be obtained through the administration of a combination of nonspecific drugs acting on different immune response sites (Gheith, Bakr *et al.*, 2008; Atreya e Neurath, 2009; Craig, Webster *et al.*, 2009; Gelber, 2009). However, these immunosuppressive agents have many and severe side effects, such as a greater susceptibility to infection, liver toxicity, myelotoxicity, oncogenicity and nephrotoxicity, especially after long term treatment (Maddox e Soltani, 2008; Mimori, 2009; Nevins e Thomas, 2009; Webb, Prokurat *et al.*, 2009).

Thus, since the FDA acknowledged the effectiveness of LLLT, especially with respect to its ability to enhance wound healing (Rocha Junior, Vieira *et al.*, 2009), increase collagen synthesis (Yu, Naim *et al.*, 1997) and keratocyte mobility (17), stimulate the release of growth factors (Bensadoun, Franquin *et al.*, 1999; Albertini, Villaverde *et al.*, 2007; Ahmed, Radwan *et al.*, 2008; Brill, Budnik *et al.*, 2008), and promote the transformation of fibroblasts into myofibroblasts (Karu, Pyatibrat *et al.*, 1997; Carvalho Pde, Silva *et al.*, 2006; Brill, Budnik *et al.*, 2008; Mafra De Lima, Costa *et al.*, 2009), researchers have become increasingly interested in the immunomodulator effects of this therapy.

As previously mentioned, the immunosuppressive effects of LLLT are still unknown; however, studies suggest that the mechanism responsible is similar to that seen when chemical substances are administered (Albertini, Villaverde *et al.*, 2007). It is known that steroids regulate pro-inflammatory proteins and affect gene expression, thereby interfering with the entire inflammatory process (Albertini, Villaverde *et al.*, 2007; Brill, Budnik *et al.*, 2008; Carrasco, Mazzetto *et al.*, 2008; Gavish, Perez *et al.*, 2008; Hagiwara, Iwasaka *et al.*, 2008). It is possible that LLLT irradiation changes RNA expression at the level of mRNA or protein synthesis, as seen in preceding studies of the expression of IL-1a, IL-2, TNF-a, IFN-g, ICAM-1 and IL-2R in mononuclear cell cultures from peripheral blood (Mafra De Lima, Costa *et al.*, 2009; Matsumoto, Ferino *et al.*, 2009).

The capacity of LLLT to drain lymphatic cells can be explained by the direct effects of laser light on the production of cytokines, because laser light can penetrate to 50mm below the tissue surface (Wasik, Gorska *et al.*, 2007; Uebelhoer e Ross, 2008). We speculate that when laser light hits cells it results in reduced antigen presentation in draining lymphatic cells and damages the activation and proliferation of lymphocytes. Lymphocyte migration to the area challenged by antigen could be equally altered. Thus, new studies are needed to try and explain how production of inflammatory and non-inflammatory cytokines is related to the immunosupression following LLLT.

It has been shown (Yu, Naim *et al.*, 1997; Rocha Junior, Vieira *et al.*, 2009) that laser irradiation can accelerate TGF-ß (ß growth factor) expression and facilitate changes in leukocyte activity. Moreover, ß1 tumor necrosis factor is a chemotactic agent for neutrophils, macrophages and fibroblasts (Brill, Budnik *et al.*, 2008; Gavish, Perez *et al.*, 2008). These findings suggest that LLLT can directly impact the triggering of DTH and consequently induce multiple changes in the dynamics of the immune response.

In the present investigation we have shown that the model of DTH to OVA was efficient in assessing LLLT as an immunosuppressive agent. DTH and the cell groups involved are in agreement with what has been published in the literature; lymphocytes and macrophages were found in the group without treatment and in those treated, there was a clear therapeutic effect that impacted on the normal process. LLLT altered the normal triggering of the DTH reaction to OVA, and seemed to work similarly to AZA.

It has been shown that a fluence of 3.8 J/cm^2 caused the same degree of DTH inhibition as an azathioprine dose of 0.5mg/Kg/day. In prior clinical and experimental studies, many laser doses were used, with fluences ranging from 0.01 J/ cm2 to more than 50 J/cm^2 with distinct effects [24–26]. In this study, a fluency of 3.8 J/cm^2 and a power of 15mW were used, which were the same as those used in prior anti-inflammatory studies with LLLT (Yu, Naim *et al.*, 1997; Carvalho Pde, Silva *et al.*, 2006; Gavish, Perez *et al.*, 2008; Uebelhoer e Ross, 2008; Mafra De Lima, Costa *et al.*, 2009; Matsumoto, Ferino *et al.*, 2009; Rocha Junior, Vieira *et al.*, 2009).

To the best of our knowledge, this is the first paper using the DTH reaction to OVA experimental model to test the immunosuppressive effects of laser therapy. The results suggest that LLLT can contribute to the treatment of mucosa and skin lesions associated with autoimmune disorders and help avoid or reduce the use of steroids and immunosuppressive agents. We hope this study will stimulate other experimental and clinical studies designed to understand the molecular basis of LLLT.

CONCLUSIONS

In this study of the effect of LLLT on DTH to OVA in mice we observed the following:

a) LLLT caused DTH inhibition, and resulted in an immunosuppressive reaction similar to that of azathioprine;

b) LLLT appreciably reduced inflammatory infiltrate composition and intensity.

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Low-Level Laser Therapy Inhibits the Expression of Cyclooxygenase 2 in a Model of DTH to OVA

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Low-Level Laser Therapy Inhibits the Expression of Cyclooxygenase 2 in a Model of DTH to OVA

Objective: Low-level laser therapy (LLLT) is being used as an adjuvant therapy in medicine, dentistry and physical therapy; however, its applicability is not yet completely accepted because some of its effects have not been properly clarified, primarily with regards to its anti-inflammatory action. In this study, the in situ effects of LLLT on Cyclooxygenase 2 (COX-2) expression after irradiation were examined in an animal model of delayed hypersensitivity reaction (DTH) to ovalbumin (OVA).

Methods: In this study, the experimental model of DTH to OVA was used to evaluate the anti-inflammatory effects of LLLT. The animals were randomly divided into 3 groups: control - (I), not immunized, not treated and challenged (n=6); control + (II) immunized, not treated and challenged (n=6); and (III) immunized, treated with laser and challenged (n=6). The treatment was given after the induction phase and before the sensitization phase in the animals. Footpad thickness and immunohistochemical analyzes for expression of COX-2 were performed.

Results: In the group of animals irradiated with the laser, footpad thickness was significantly reduced compared to animals from the control group. This reduction was accompanied by a significant drop in the expression of COX-2. The results obtained showed that the laser inhibited the DTH. Additionally, low-level laser therapy, in this model, inhibits the expression of COX-2.

Conclusion: Low-level laser therapy showed a reduction in the expression of COX-2 in the DTH model in the footpads of mice.

Key words: COX-2, delayed hypersensitivity reaction, low-level laser

Low Level Laser Therapy Inhibits the Expression of Cyclooxygenase 2 in a Model of DTH to OVA

INTRODUCTION

Non-steroidal selective cyclooxygenase-2 inhibitors were developed in order to avoid the side effects of the non-selective inhibitors, especially for the gastrointestinal tract and kidneys. However, recent studies have shown that cyclooxygenase-2 is constitutively expressed in the kidneys and is highly regulated in response to alterations in intravascular volume. When the volume of blood is compromised, the prostaglandins derived from cyclooxygenase-2 play an important role in renal circulation. It is supposed that the drugs that selectively inhibit COX-2 can interfere with renal function in a manner similar to those that are not selective. Studies developed with the state of the art “coxibs” have shown that they are not exempt from aggravating pre-existing problems and causing kidney damage to their users.

Low-level laser irradiation (800–1000 nm) has been considered an adjuvant therapy in clinical treatments. Its analgesic, modulating and direct interference effects in the inflammatory process have been widely disclosed; however, few studies in animal models have examined the possible anti-inflammatory effect of this therapeutic resource. In addition, the results of these studies are inconclusive, and the possible mechanism of action has not yet been totally elucidated. This inconclusiveness may be the reason why this therapy has not yet been totally accepted, even though its satisfactory clinical performance has been shown since 1960.

Low-level laser therapy was considered by the Food and Drug Administration (FDA) to be an important therapeutic resource for the treatment of inflammatory diseases. The results of some studies suggest that low-level laser therapy can be used as an efficient method of tissue repair, in inflammatory processes and in processes involving modulation of the immunological system.

Therefore, in this study, the effects of laser irradiation on the expression of COX-2 were evaluated in a classic animal model of delayed type hypersensitivity reaction (DTH) to ovalbumin (OVA).

Materials and Methods

I - Animals

Eighteen male BALB/c mice, aged between 4 and 6 weeks and weighing 30 g, from the Reproduction Biology Center (CBR) at Federal University de Juiz de Fora, were used. The animals were kept in climate-controlled cages, with the temperature maintained around 22ºC and room lighting controlled automatically to produce a 12: 12 hour light: dark cycle. The animals were kept in individual cages, with water dispensers and fed *ad libidum.* All the animals were kept in individual polypropylene cages equipped with beds of selected wood shavings, baby bottles with water and troughs for palletized chow under maintenance conditions, which are in agreement with the criteria of the Brazilian College of Animal Experimentation. They were submitted to daily macroscopic evaluations in order to observe signs indicating secondary infection.

II – Groups

The animals were randomly divided into three groups: control - (I), not immunized, not treated and challenged (n=6); control + (II) immunized, not treated and challenged (n=6); and (III) immunized, treated with laser and challenged (n=6), as follows:

GROUP	IMMUNIZATION (tail base)	TREATMENT	CHALLENGE (footpad)
I - control -	-	-	X
II - control +	X	-	X
III - laser	X	Laser (3.8 J/cm^2 per day)	X

Figure 1 – The groups

III - Experimental model

The delayed hypersensitivity reaction (DTH) model uses ovalbumin (OVA), a protein that has been used as an antigen for the sensitization of mice with or without an adjuvant solution. For the DTH to OVA model, the following methodology was used: immunization with ovalbumin (Sigma Chemical Co. St. Louis, MO, USA) on the tail base (day zero), challenge with aggregated OVA (Sigma Co., St Louis, MO, USA) in the footpad six days after immunization and reading of the thickness of the footpad. Accuracy was controlled by administrating, in a similar manner, 30 µl of saline solution in the left back paw (TITUS and col., 1984). The DTH reaction was evaluated 48 hours after the challenge.

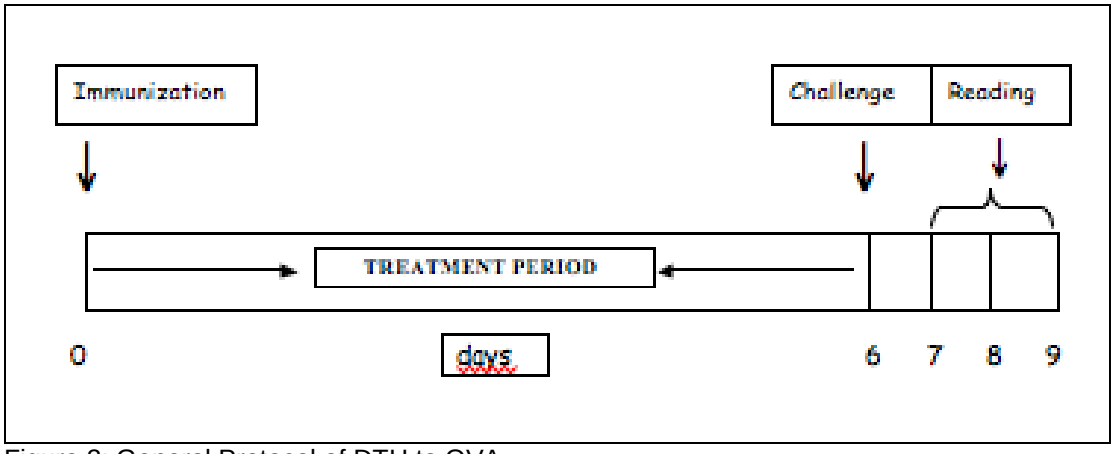


Figure 2: General Protocol of DTH to OVA

The laser device was provided by MM Optics (São Carlos – SP) and has the following characteristics: IR laser, pulsat; semi conductors: arsenic and gallium; wavelength: 780 nm; peak power: 70 mW; average exit power: between 0.5 and

3.5mW; and form of application: fiber optics.

The laser treatment was given between day zero and day six of the experiment, daily. The results represent the average of six animals per group ± standard error, expressed in DTH units and defined as the difference of 0.01 mm in thickness of the right and left paws of each animal as measured by a micrometer (Starret, MA, USA).

To compare the results in the DTH tests, we used ANOVA and the Bonferroni test. The level of significance accepted was $p < 0.05$.

IV - Immunohistochemical study

An analysis of the animals' foot pads was done to evaluate the possible influence of the use of the laser in the cell composition of the animals submitted to DTH to OA. After reading the DTH, the animals were euthanized with an overdose of anesthesia with intraperitoneal ketamine (100 ml/kg) + xylazine (10 mg/kg). The footpads were dissected and set in a 10% formaldehyde solution for at least 48 hours. The immunohistochemical study was done using the avidin-biotin peroxidase anti-peroxidase (ABC) complex method to verify the expression of COX-2 in order to evaluate the effect of LLLT on the delayed hypersensitivity reaction in the footpads of mice.

The method was carried out in the following stages: a) placement in acetone (10 min); b) rehydration of the cuts in PBS, pH 7.4 (10 min); c) blockage of endogenous peroxidase with a hydrogen peroxide solution at 0.4% (30 min); d) wash in PBS (10 min); e) incubation with normal horse solution (Vector Laboratory, Inc., Burlington, California, USA) in a humid chamber (20 min); f) incubation with a primary

polyclonal antibody for one hour – COX-2, rabbit polyclonal IgG (Santa Cruz Biotechnology, California, USA; dilution 1:100); g) wash in PBS (10 min); h) incubation with biotinylated goat anti-rabbit antibody (Dakopatts, Copenhagen, Denmark) diluted in PBS (30 min); i) wash in PBS (10 min); j) incubation with a 0.2 M, pH 5.2 acetate buffer (10 min); l) revelation of the product of the immunological reaction using a solution of 3-amino-9-eticarbazol (1 to 3 min); m) wash in running water (10 min); and p) assembly of the slide with Elvanol (polyvinyl alcohol) and a coverslip.

The immunohistochemical reaction was controlled by omitting, in some slides, the incubation stage with the primary antibody. Images of the fields analyzed were obtained using the Axio Vision computerized image analysis system (Axiostar Zeiss, Carl Zeiss, Hamburg, Germany). The intensity of the COX-2 expression was quantified as follows:

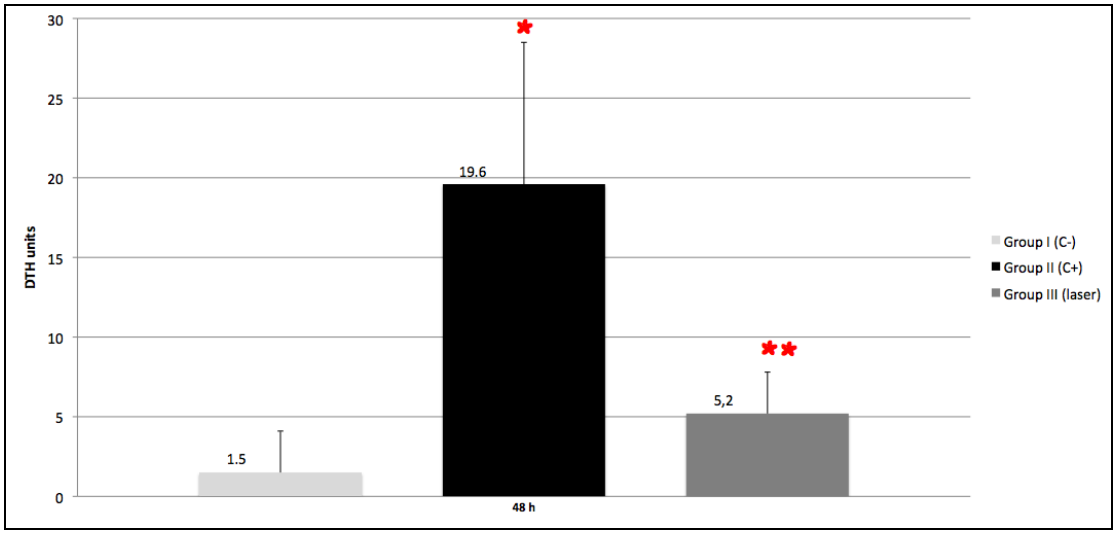
	Intensity of the COX-2 expression
(+)	Light, with less than 20% of the cells positive for immunomarkings for COX-2.
(++)	Moderate, with 20% to 40% of the cells positive for immunomarkings for COX-2.
(+++)	Intense, with more than 60% of the cells positive for immunomarkings for COX-2.

Table 1: The intensity of the COX-2 expression

RESULTS

Analysis of delayed hypersensitivity reaction in the animals

DTH was evaluated 48 hours after inoculation of aggregated OVA in the footpad of the specimens and was significantly reduced in the animals irradiated with lasers (Fig. 1). The lower intensity of DTH to OVA observed in the treated animals compared to the control + animals enabled us to conclude that in this model, the laser must work through an anti-inflammatory action.



Graphic 1: Average of units of DTH to OVA 48 hours after the challenge. * $p < 0.05$ when compared to group I (control -); ** $p < 0.05$ when compared to group II (control +);

Treatment with LLLT (group III) significantly reduced ($p<0.05$) the intensity of DTH compared to the control groups.

Immunohistochemical analysis

The animals submitted to LLLT presented with a significant reduction ($p<0.05$) in the intensity of the infiltration compared to the immunohistochemical findings of the positive control group. In Figure 2C (laser group), we can see the presence of a scarce mononuclear inflammatory infiltrate that is diffusely distributed by the conjoining tissue with few cells expressing COX-2.

Table 2 – Analysis of the intensity of the inflammatory infiltration in DTH to OVA in BALB/c mice treated with LLLT.

	Group of animals
I (positive control)	+++
III (LLLT)	+

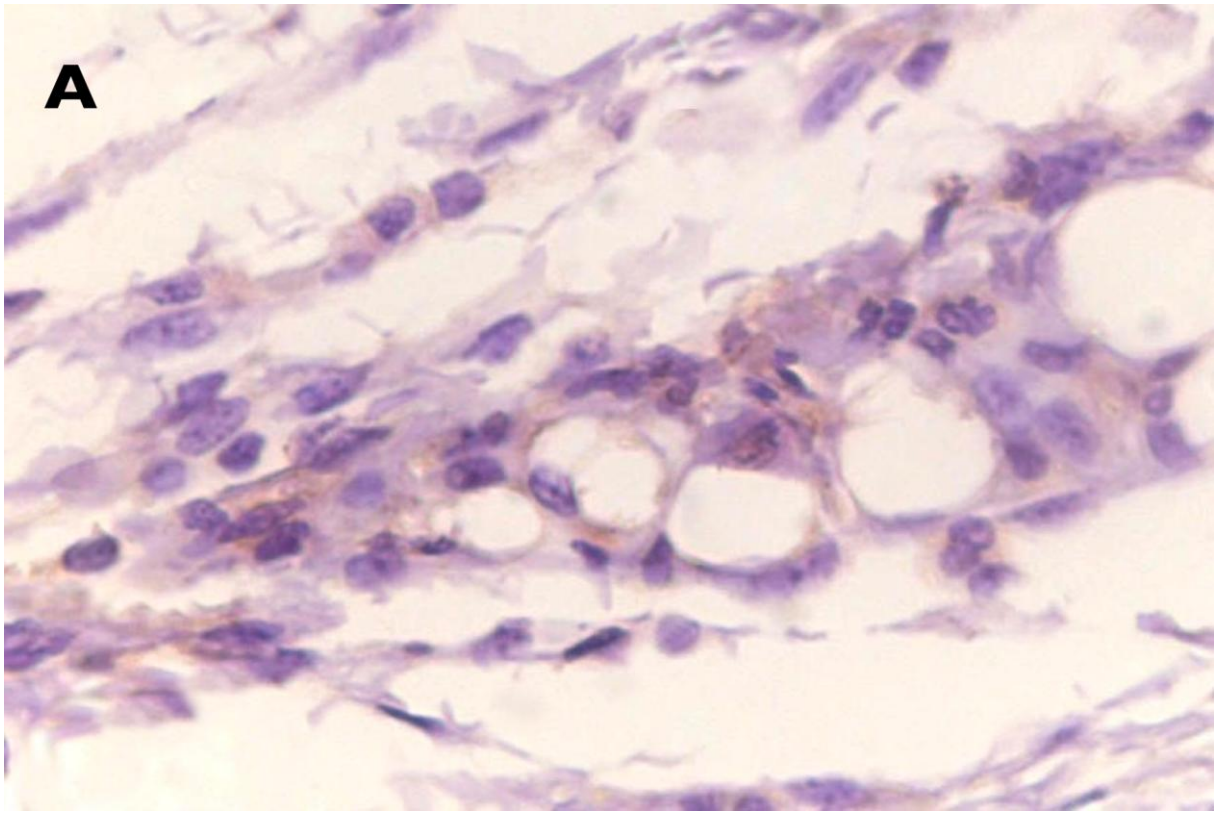


Fig 2A – Photomicrograph of the footpad of an animal in group I (positive control). Histopathological evaluation suggests the presence of an intense mononuclear inflammatory infiltrate.

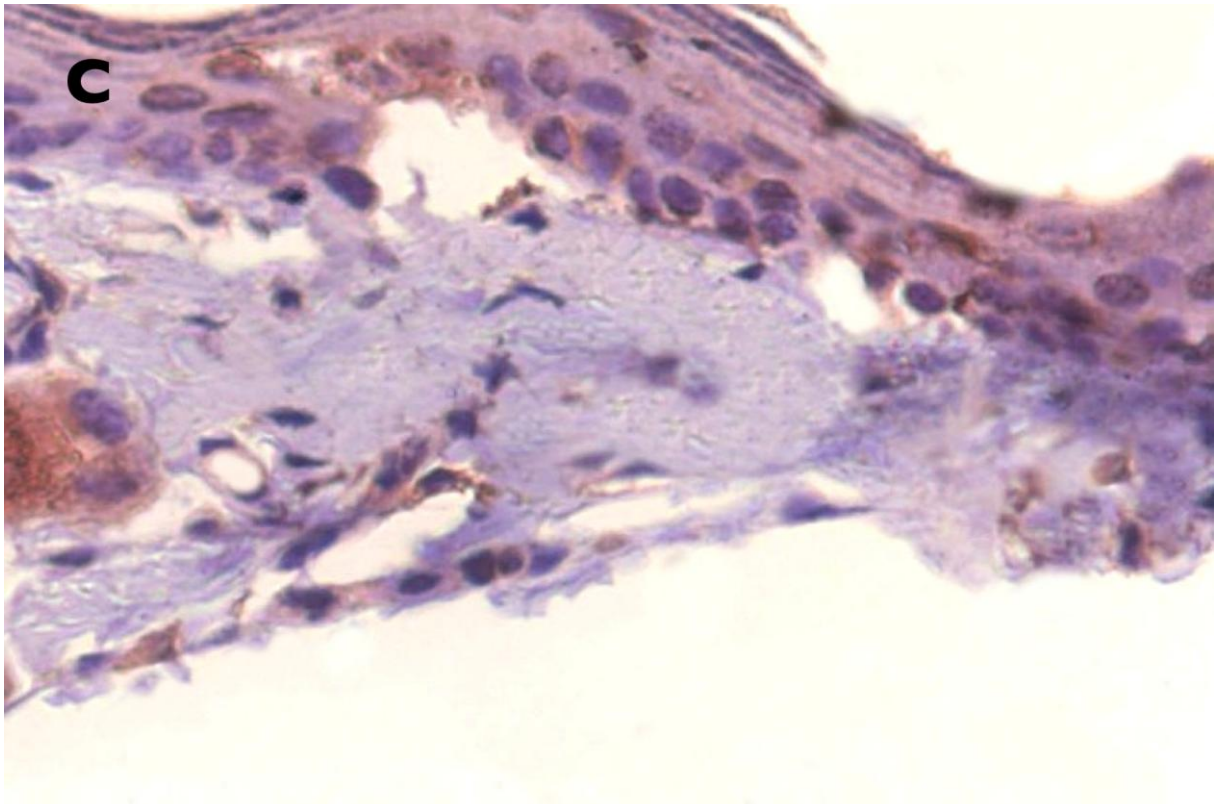


Fig 2C – Photomicrograph of the footpad of an animal in group III (LLLT), Some mononucleated cells and a few cells expressing COX-2 are seen.

DISCUSSION

The initial idea that selective nonsteroidal anti-inflammatory inhibitors of COX-2 do not have harmful effects on kidney tissue is being questioned. Clinical studies have shown that the intra-renal functional role of COX-2 is predominantly associated with the maintenance of hydrostatic homeostasis, whereas COX-1 appears to be more related to the maintenance of the glomerular filtration function.

Studies clearly demonstrate that COX-2 and COX-1 play an important role in renal function and that the selective inhibition proposed by the coxibs is not exempt from causing harm to this function, especially under the aforementioned conditions. Thus, interest in new therapies with minimum side effects continues to be the target of many studies.

The anti-inflammatory action of coxibs is due to the selective inhibition of COX-2, while preserving the function of COX-1. Thus, they block the isoform of COX that is responsible for inflammation (COX-2) and do not interfere with COX-1, which protects the stomach. In this manner, it would be possible to prescribe AINE much more safely, with better tolerance and with the same analgesic and anti-inflammatory response.

Ten years later, however, these expected results did not hold true. The inhibition of COX-2 and the maintenance of COX-1, for those who requited the use of AINES for longer periods of time (a year or more) showed, through several clinical studies, a significant increase in the incidence of acute thrombotic events, in addition to adverse hematological, hepatic, cardiovascular and renal effects.

Thus, since the FDA began to recognize the effectiveness of low-level laser therapy (LLLT), principally in improving the pattern of wound healing, increasing collagen synthesis, increasing the motility of keratinocytes (17), stimulating the release of growth factors and promoting the transformation of fibroblasts into myofibroblasts, researchers have begun to investigate the anti-inflammatory effects of this therapeutic resource.

As considered previously, the possible anti-inflammatory effects of the laser are still unknown; however, studies suggest that the mechanism of the anti-inflammatory action is similar to that observed in chemical substances. It is known that corticoids regulate pro-inflammatory proteins and affect the expression of genes, which interfering in the inflammation process as a whole. It is possible that LLLT irradiation changes the expression of RNA at the level of mRNA synthesis of the proteins, as

was observed in prior studies for the expression of IL-1a, IL-2, TNF-α, IFN-γ, ICAM-1 and IL-2R in the mononuclear cell cultures of peripheral blood.

It has been shown that laser radiation can accelerate the expression of transforming growth factor-beta (TGF-ß) and facilitate alterations in the activity of leukocytes. Thus, these findings suggest that the effects of laser therapy may directly influence the initiation of DTH and consequently cause multiple effects on the dynamics of the immune system response.

Based on the studies that evaluated the anti-inflammatory effects of LLLT, the dose of 2.5 to 3.8 J/cm2 has been shown to reduce DTH and the number of cells when applied at the inflammation site. The experimental model used in this study does not precisely translate to an acute inflammation but does simulate the migration of cells to the location of the reaction well. LLLT altered the pattern of normality of this phenomenon, indicating that low-level laser therapy can be used to reduce the expression of COX-2.

In this study, it was shown that the DTH to OVA was efficient for evaluating the influence of the low-level laser to inhibit the production of COX-2. Induction of the delayed hypersensitivity reaction and the cell profile found on the back of the animals' paws are in accordance with the literature; that is, lymphocytes and macrophages were found in the untreated and treated, and the action of the low-level laser was clearly seen in the normal course of the process. Low-level laser therapy altered the normal initiation of DTH to OVA.

The results suggest that LLLT can contribute to the treatment of lesions on skin and mucous membranes, whether or not they are associated with autoimmune diseases, and allow patients to avoid or reduce the use of corticoids, immunosuppressive drugs and principally selective inhibitors of COX-2. This finding

encourages us to carry out other experimental and clinical studies to evaluate this hypothesis.

CONCLUSION

In the delayed hypersensitivity study to ovalbumin in mice the following was found:

The laser significantly reduced the “in situ” expression of COX-2 in the experimental model of DTH to OVA.

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Low-level laser reduces the production of TNF-α, IFN-γ and IL-10 induced by OVA

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ABSTRACT

Background and Objective: Delayed, or type IV, hypersensitivity reactions are a useful model to study the effects of new substances on the immune system. In this study, the experimental model of the delayed type hypersensitivity (DTH) reaction to ovalbumin (OVA) was used to evaluate the immunomodulating effects of low-level laser therapy (LLLT), which is used as an adjuvant therapy in medicine, dentistry and physical therapy because of its potential anti-inflammatory and analgesic effects observed in several studies.

Materials and Methods: The effects of LLLT (λ 780 nm, 60 mW/cm2 of radiation and fluency of 3.8 J / cm2) in reaction to ovalbumin in Balb/C mice were examined after the induction phase of the hypersensitivity reaction. The animals treated with Azathioprine (AZA), the animals that received a vehicle instead of ovalbumin and those not immunized served as controls (n=6 for each group) . Footpad thickness measurements and hematoxylin-eosin histopathological exams were performed. Proliferation tests were also performed (spontaneous, in the presence of concanavalin A and ovalbumin) to determine the production in mononuclear cells cultures of TNF-α, INF-γ and IL-10. **Results:** In the group of animals irradiated with lasers and in the group treated with AZA, footpad thickness measurements were significantly reduced in comparison to the control group($p < 0,05$). This reduction was accompanied by a very significant reduction in the density of the inflammatory infiltrate and by a significant reduction in the levels of TNF-α, INF-γ and IL-10.

Conclusion: LLLT radiation was shown to have an immunomodulating effect on DTH to OVA in Balb/C mice.

Keywords: Cell proliferation; Delayed hypersensitivity reaction; infrared radiation, laser.

INTRODUCTION

Infrared radiation (600-980 nm) is used in low-level laser therapies (LLLT) to help wounds heal, relieve pain and reduce inflammation. However, there are few studies that have examined its anti-inflammatory and immunosuppressive effects (1-3). The results of these studies are often controversial, and the possible mechanism of the photobiological effects observed has not been completely clarified. Thus, this modality of treatment is not widely accepted, although it has been in use since 1960. Most of the studies on the biostimulation and anti-inflammatory effect obtained with low-level laser near infrared radiation (IR) were conducted on cell cultures (4-6). However, few studies have examined its possible immunosuppressive effects in animal models. In this study, the effects of laser radiation were evaluated in an animal model of delayed hypersensitivity reaction (DTH), a classic model to test the effect of new substances on the capacity of an animal or a human being to create an immune response (7). In the induction phase (sensitization or afferent phase), application of the haptene is made at the base of the tail, which induces the activation and proliferation of T lymphocytes in the regional lymph nodes. In the efferent phase, ovalbumin is applied on the back of the footpad, and the T lymphocytes are recruited to the location of the challenge. These lymphocytes produce a variety of inflammatory mediators, enhancing the context of the inflammatory response in a more vigorous process (7-10). Because this process begins and ends at the location of the episode, the experimental DTH model is appropriate to study immune and inflammatory reactions. The use of animal models in studies on the immunomodulating and anti-inflammatory effects of a variety of physical and chemical agents is widely accepted (8, 10-12). The immunosuppression induced by Azatiophrine (AZA) was widely evaluated in this same experimental model (13-15). Local immunomodulating effects on the subcutaneous hypersensitivity reaction were observed with LLLT radiation at a 635 nm wavelength, before and immediately after the induction of the reaction (16-18). The LLLT suppressed the delayed hypersensitivity reaction to the antigen applied intradermally in guinea pigs, not only in the irradiated location but also in distant locations. These results indicate possible systemic effects of this wavelength (19, 20).

However, there are no studies that have examined the possible effects of the irradiation on the delayed hypersensitivity reaction (DTH). In this study, we tested the hypothesis that LLLT has immunomodulating effects similar to those of AZA, an immunosuppressant agent used since 1960 (13, 21). Balb/C mice were irradiated after the DTH induction phase, and the intensity of the reaction was measured in the footpad of the animals 48 hours after the reaction began. To evaluate the observed effects, measurements of the swelling on the footpad and histopathological analysis were performed based on a culture of lymphocytes obtained from the groin and periaortic lymph nodes. The production of TNF-α, INF-γ and IL-10 were investigated in these lymphocyte cultures (22-24). In this study, a fluency of 3.8 J/cm2 was used, which is in agreement with the doses used in previous studies on the anti-inflammatory effects of LLLT (1, 20, 25-27).

MATERIALS and METHODS

*Animals*Twenty-four male Balb/C mice, aged between 4 and 6 weeks, weighing 30 g and provided by the Animal House of the CBR [Reproduction Biology Center] of the Federal University of Juiz de Fora (UFJF), were used. Mice at CBR-UFJF are housed in large dumping containers equipped with wire screens, two exhausters and room heaters. The temperature was kept at approximately 22ºC by natural ventilation in the summer and with the help of heaters in wintertime. The lighting was mixed and consisted of natural light and fluorescent light, which were automatically controlled to turn on at 6:00 a.m. and off at 6:00 p.m. The animals were kept in individual polypropylene cages equipped with beds of selected wood shavings, baby bottles with water, and troughs for palletized chow under maintenance conditions that were in agreement with the criteria of the Brazilian College of Animal Experimentation (028/2009). The mice were subjected to daily macroscopic evaluations to detect signs of secondary infection.

Experimental model (OVA-DTH):

The DTH model used ovalbumin (OVA), a protein that has been used as an antigen to sensitize lab animals, with or without adjuvant. DTH was induced by immunization with OVA (Sigma, St. Louis, MO, USA). Briefly, mice were injected in the tail base with 200 μL of 1.25 mg/mL OVA (immunization), and six days after immunization, their footpads were injected with 20 μL of 10 mg/mL OVA emulsified with complete Freund's adjuvant (CFA) (Chondrex, Redmond, WA) (challenge). Animals were injected with an equal volume of PBS into another footpad as a control. Forty-eight hours after challenge, footpad thickness was measured with a digital caliper (Starret, MA). The magnitude of the DTH response was determined as follows: [footpad

swelling (mm) or DTH units] = [footpad thickness of OVA-injected footpad (mm)]−[footpad thickness of PBS-injected footpad (mm)].

The AZA used in the *in vivo* experiments was obtained from the Microbiológica-Química e Farmacêutica (Rio de Janeiro, RJ). AZA was prepared with distillated water on the day of administration and administered at 0.5 mg/Kg/per day by gavage. The tail base was irradiated with the laser at a power of 15 mW and a dose of 3.8 J/cm^2 for 10 s per day (irradiation area 0.04 cm^2 and power density 380 mW/cm^2). The laser (Twin Laser) device used was supplied by MM Optics (São Carlos – SP) and had the following characteristics: IR emission laser: pulsatile; semi-conductors: arsenic and gallium; wave length: 780 nm; and means of administration: optic fiber.

The treatment with AZA or laser was done during the time between day zero and day six of the experiment. To compare the results with the DTH assays, we used ANOVA and the Bonferroni test. The significance level accepted was $p < 0.05$.

Groups

The animals were randomly divided into four groups: Group I (- control) not immunized (n=6); Group II (+ control) immunized, untreated and challenged (n=6); these mice received only OVA emulsified with Complete Freund's Adjuvant; Group III (AZA), immunized, treated with AZA and challenged (n=6); and Group IV (Laser) immunized, treated with LLLT and challenged (n=6) (see Table 1).

Table 1 Groups

GROUP	**IMMUNIZATION** 200 μL of 1.25 mg/mL OVA – tail base	**TREATMENT**	**CHALLENGE** 200 μL of 1.25 mg/mL OVA – tail base
I: - Control	-	-	X
II: + Control	X	-	X
III: AZA (0.5 mg/Kg/per day by gavage)	X	X	X
IV: Laser (3.8 J/cm2 for 10 s per day)	X	X	X

Obtaining the organs and cells studied

An analysis of the levels of TNF-α, INF-γ and IL-10 was performed to evaluate the possible influence of the laser and AZA on the cellular composition of the animals submitted to DTH to OVA. After six days of immunization, the animals were sacrificed with an overdose of anesthesia with intraperitoneal ketamine (100 ml/kg) + xylazine (10 mg/kg). The mesenteric and periaortic lymph node cells were obtained by grinding these organs with a homogenizer in an incomplete RPMI medium. The cells were resuspended in unsupplemented RPMI with 5% fetal bovine solution, non-essential amino acids, antibiotics and L-glutamine. These cells were placed in 24-well culture plates (3×10^6 cells) and either stimulated *in vitro* with OVA and ConA for 24, 48 or 72 hours or left unstimulated. After these time periods, the supernatants were gathered for cytokine measurement.

Measurement of cytokines by ELISA (TNF-α, IFN-γ and IL-10)

ELISA plates were sensitized with the capture antibody, diluted in a carbonate-bicarbonate buffer, incubated for two hours at room temperature and blocked with

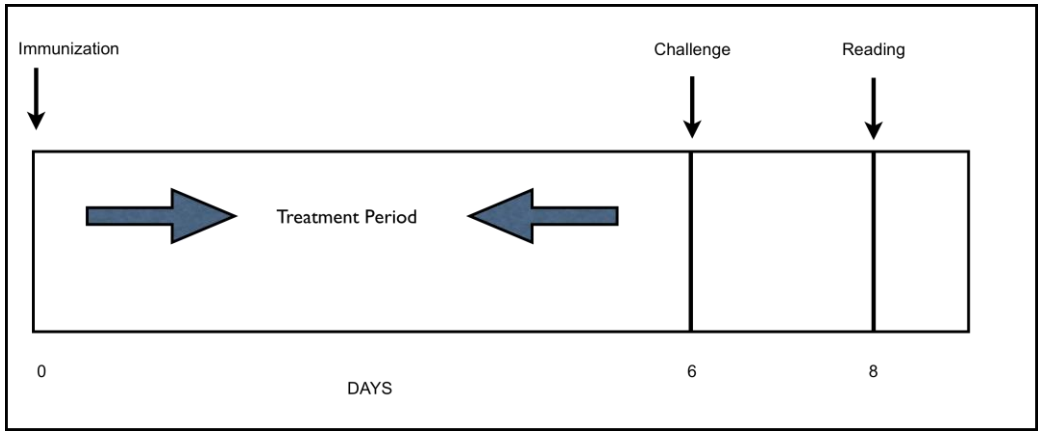
PBS-Tween 20 (PBST) + 10% FCS (fetal calf serum) for thirty minutes. After this period, the plates were washed four times in PBST and then added to the capture antibodies. After incubation for two hours at room temperature, the recombinant cytokine standards were serially diluted. The culture supernatant samples were distributed. The plates were then incubated for 18 hours at 4°C. The plates were washed four more times, and, after placement of the enzyme conjugate, they were incubated for another hour. After this period, the reaction was detected by the addition of a substrate containing 0.1 M citric acid, 0.2 M sodium phosphate, distilled water, ABTS chromogen and hydrogen peroxide 30%. The reaction was blocked with 0.2 M citric acid, and the reading was made in an ELISA reader (SPECTRAMAX 190, Molecular Devices) at 410 nm. The quantities of cytokines were calculated based on standard curves obtained by different concentrations of recombinant cytokines.

RESULTS

Delayed hypersensitivity reaction

DTH was analyzed 48 hours after aggregated OVA inoculation using a digital caliper (Starret, MA, USA). LLLT-treated mice and AZA-treated mice showed a significant reduction in the DTH reaction compared to non-treated mice (Figure 1). The hypersensitivity reaction was expressed in DTH units.

Figure 1 General protocol of DTH to OVA



The results for DTH were as follows: group I: 1.5±2.6; group II: 19.6±8.9; group III (AZA): 5.6±2.5; and group IV (laser): 5.2±2.6. DTH was lower for groups III and IV than for the control groups ($p < 0.05$). There was no statistical difference between group III and group IV (AZA and LLLT) (Figure 2).

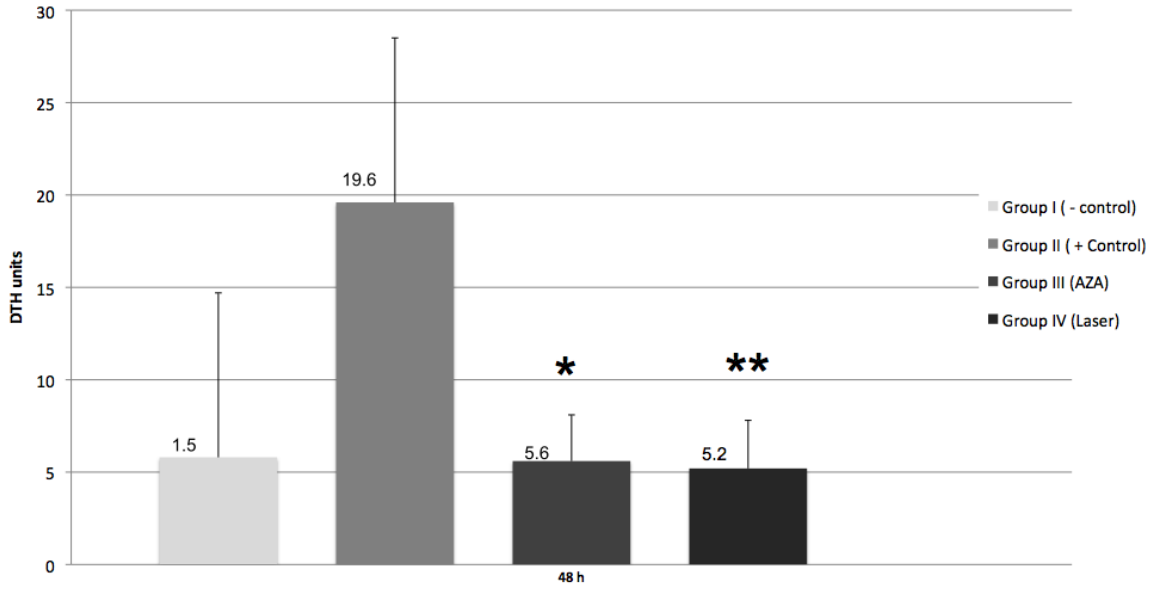


Figure 2 Average of DTH to OVA 48 hours after the challenge. * p < 0,05 when compared with Group II (+ control); ** p < 0,05 when compared with Group II (+ control)

Histopathological analysis

The histopathological analysis of the footpad samples from mice of group II (positive control) revealed the presence of mononuclear inflammatory infiltrates, which were mainly represented by multifocal lymphocytes in the superficial, middle and deep dermis. In some microscopic fields, mononuclear cells with morphology suggestive of macrophages were observed. Polymorphonuclear cells with morphology suggestive of neutrophils were rarely observed (Figure 3A).

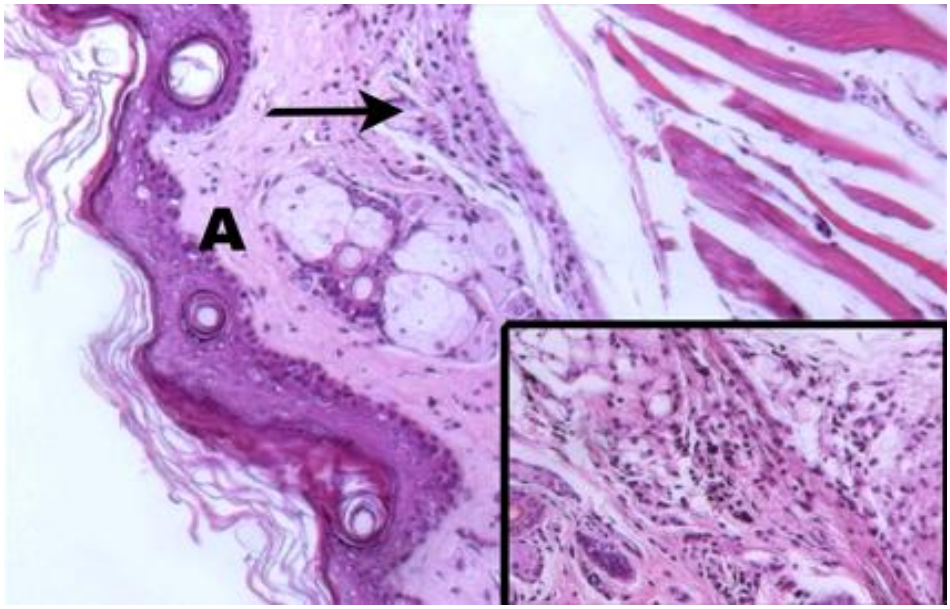


Figure 3A Microphotography of the footpad from a group I animal (positive control): histopathology suggests the presence of an intense mononuclear inflammatory infiltrate scattered in the superficial, middle and deep derma. In the detail we see a build up of mononuclear cells with morphology suggesting lymphocytes (original magnification100x. Inset: in detail mononuclear cell, original magnification 400x).

The animals treated with AZA or submitted to LLLT presented a significant reduction ($p<0.05$) in infiltrate intensity when compared to the histopathological findings of the positive control group. In Figure 3C (laser group), we see a scarce mononuclear infiltrate diffusely distributed throughout the connective tissue.

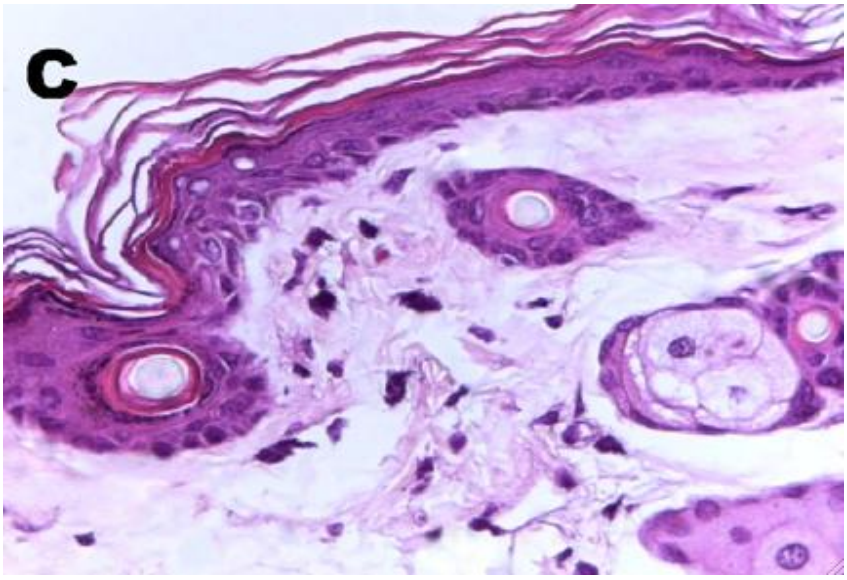


Figure 3C Microphotograph of the plantar pad from a group IV animal (LLLT): we see some mononuclear inflammatory cells. Normal epithelium architecture and connective tissue of the footpad (original magnification 400x).

Similar results were found in the slides produced with animal tissue from the group treated with AZA (Figure 3B).

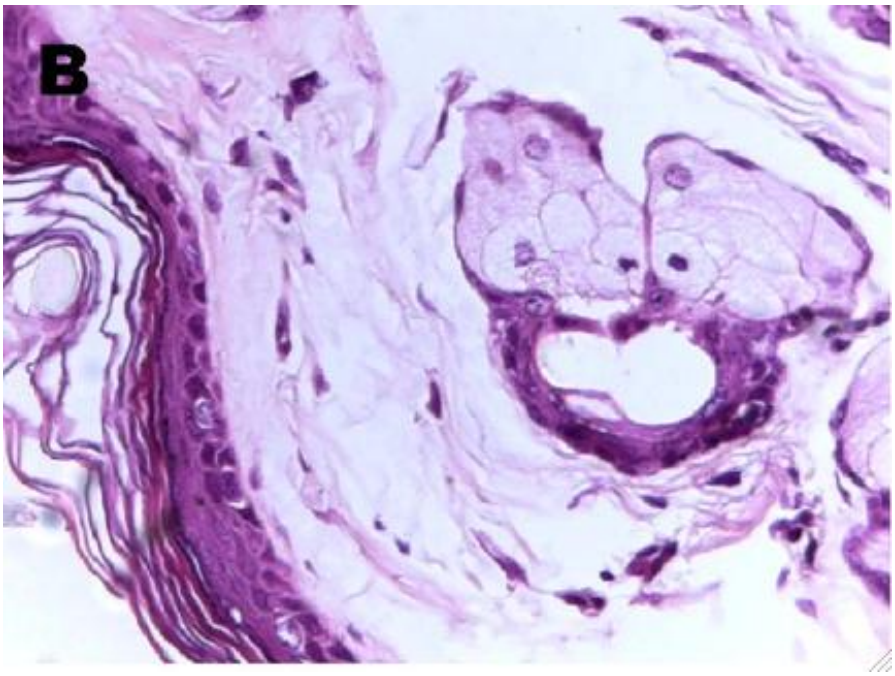


Figure 3B Microphotography of the plantar pad of a group III animal (AZA): histopathology reveals no inflammatory infiltrate. Normal footpad epithelium architecture and connective tissue (original magnification 400x).

*Production of cytokines (*TNF-α, INF-γ and IL-10)

In this study, the production of cytokines was evaluated through the ELISA test. The levels of these cytokines were used to evaluate the effects of the laser and of AZA. Levels of TNF-α, INF-γ and IL-10 were determined and are shown in Figure 4A-C.

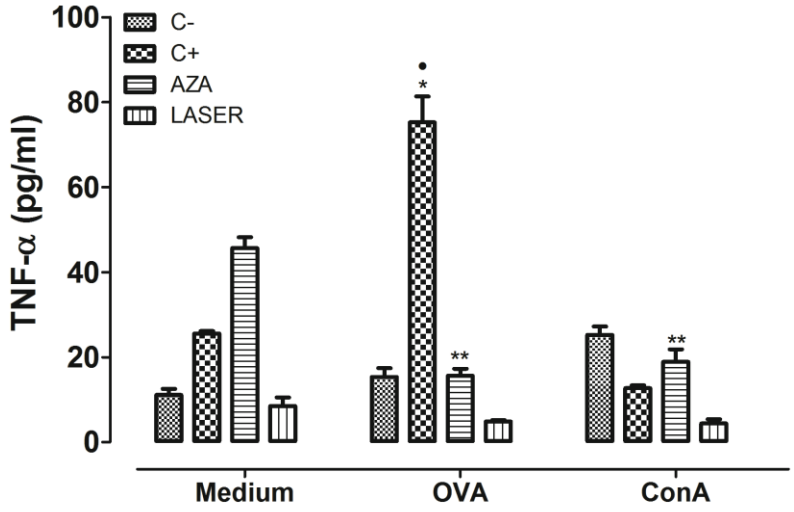


Figure 4A Production levels of TNF-α. • p < 0,05 when compared Group II (+ control) vs Group III (AZA); * p < 0,05 when compared Group II (+ control) vs Group IV (laser); ** p < 0,05 when compared Group III (AZA) vs Group IV (laser)

The animals treated with AZA or submitted to LLLT presented a significant reduction (p<0.05) in levels of TNF-α, INF-γ and IL-10. In Figure 4A (laser group) the animals presented a siginificant reduction (p<0.05) in production of TNF-α when compared to the AZA group (Figure 4A).

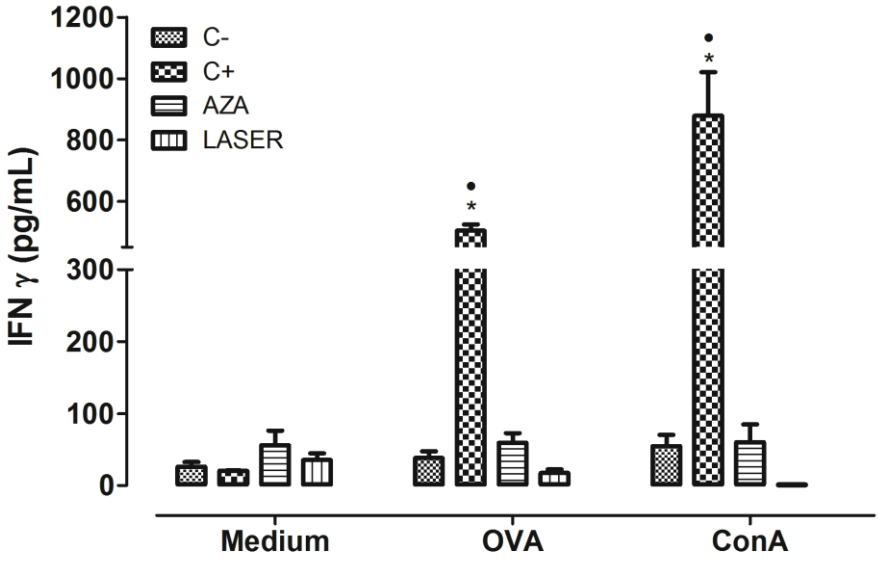


Figure 4B Production levels of IFN-γ. • p < 0,05 when compared Group II (+ control) vs Group III (AZA); * p < 0,05 when compared Group II (+ control) vs Group IV (laser)

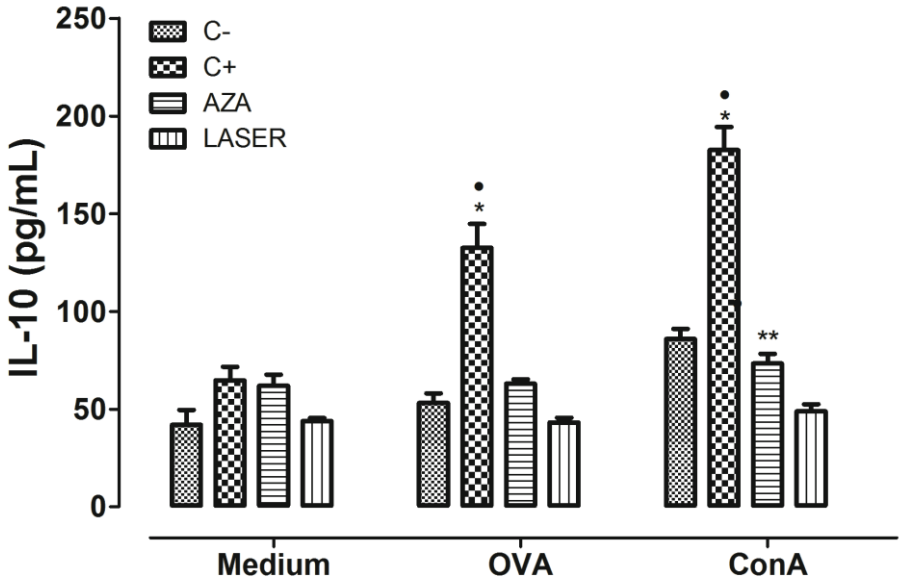


Figure 4C Production levels of IL-10. • p < 0,05 when compared Group II (+ control) vs Group III (AZA); * p < 0,05 when compared Group II (+ control) vs Group IV (laser); ** p < 0,05 when compared Group III (AZA) vs Group IV (laser)

DISCUSSION

The low-level laser is used as a therapeutic treatment due to its effectiveness in treating rheumatic diseases and in promoting the healing of wounds in dermatology (1, 22, 28, 29). In this study, there is no statistical difference between the effects of the laser and those obtained with AZA, a classic immunosuppressant in the treatment of variety of diseases as well as in cases of organ transplants (13, 30). Azathioprine and laser showed similar results in this study; as an immunosuppressant, azathioprine have important side effects and this time the laser appears as a good therapeutic option.

Anti-inflammatory and/or immunosuppressant effects of radiation were confirmed by the histopathological exam in the aforementioned animal model. Similar results were found in the rheumatoid synovia of patients submitted to treatment with laser irradiation at wavelengths of 904 nm (31, 32). However, there is little experimental data on the possible immunomodulating and anti-inflammatory effects of laser light in this wavelength. Therefore, in this study, a classic experimental model of delayed hypersensitivity reaction (DHT) was used to evaluate the possible immunomodulating effects of laser irradiation.

Measurement of footpad thickness is an efficient measure of the intensity of DTH, and a significant drop was found in the group of animals irradiated both with the laser and with AZA. Laser treatment was performed between immunization and the challenge of the animals, whereas the effects were observed in the phase after the challenge, showing possible systemic immunomodulating effects of the irradiation. Systemic effects of the LLLT were observed in previous studies. Improved healing of wounds was noted, not just for the irradiated wound but also in more distant locations (19). Inhibition of the tuberculinic reaction in animals was also observed in distant locations after a single LLLT irradiation (20). In this study, the *in vivo* effect observed was also documented by the histopathological analysis of the tissue of the back of the footpad and further examined in a lymph node culture system. The inflammatory infiltrate analyzed by the H&E and cytokine production levels were significantly lower in animals treated with AZA and in those that were irradiated with LLLT.

Tumor necrosis factor-alpha (TNF-α) is produced mainly by macrophages but

can also be released by lymphocytes and mastocytes. In addition to stimulatinf producing of collagenases and prostaglandins, it acts as a chemotactic for inflammatory cells (33).

TNF-α is an important mediator in the experimental model in question because it is one of the factors responsible for initiating the inflammatory reaction (34-36). The fact that the low-level laser in the delayed hypersensitivity reaction inhibited the levels of expression of TNF-α is fairly significant; however, because significant differences between the AZA-treated and laser-treated groups were not found, other cytokines, chemokines, and even cells are most likely also involved in the process of inhibition of the immune response in the model in question.

On the other hand, when the levels of IL-10 were compared to those of the Group II (+ control), these levels were significantly higher than those of the treated groups ($p < 0,05$). Increased levels of IL-10 suggest that immunomodulating properties could regulate either the inflammatory or immunosuppressant process, which is similar to the action of immunosuppressors such as AZA (37, 38).

Sensitization to the antigen is guided by the Th_1 lymphocytes, which secrete large quantities of interleukin-2 (IL-2) and IFN-γ that aid in the activation of the T CD8+ lymphocytes directed against cells that express HLA molecules. These sensitized T CD8+ cells, known as T cytotoxic lymphocytes, amplify the local response because they perpetuate the secretion of pro-inflammatory cytokines, such as interferon gamma (IFN-γ) and TNF-α (39-41). Thus, the results found in this study show that low-level laser therapy alters the normal immunological response pattern by inhibiting the production of cytokines responsible for initiating and maintaining the inflammatory response. This result is extremely relevant because it suggests that the laser may be used as an aid or a therapeutic resource for inflammatory and autoimmune diseases or even in cases of acute graft rejection.

The highest levels of IL-10 observed in the normal course of the immunological response could be inhibiting the effects of the IFN-γ and indirectly favoring a Th_2 deviation (42). In our study, we observed a decreased production of cytokines, which did not characterize the laser as an inhibitor of Th_2 immune response.

The delayed hypersensitivity reaction begins on the back of the tail, but the amplification of the response and the haptene-specific proliferation by the lymphocytes occurs in the lymph nodes (43). In order to examine other possible mechanisms of the laser light effect, the detection of serum levels of the inflammatory cytokines TNF-α and IFN-γ should also be conducted because the results presented by this work are in agreement with the results of in vitro studies that show a reduction in the proliferation rate of lymphocytes in cell cultures after LLLT irradiation (20, 44). We can also speculate that the lower DTH in the irradiated animals could be due to an alteration in the availability of certain cytokines responsible for the proliferation of lymphocytes, namely IL-6 and IL-2 (45). Funk and collaborators (46, 47) showed that LLLT irradiation reduced the production of IL-2 in peripheral monocytes in cell cultures. According to these authors, modulation of IL-1a, TNF-α and IFN-γ through laser irradiation was also found at the mRNA level. Therefore, alterations in the profile of cytokines in the DTH model could also be due to the reduction in cell proliferation.

The possible effects of LLLT on drainage lymph nodes could be explained by the direct action of the light on the groin lymph node itself because it penetrates up to 50 mm into tissue (26, 28, 29, 48, 49). On the other hand, we could speculate that laser light could affect Langerhans cells and keratinocytes, which could be presenting an antigen in an insufficient manner in draining lymph nodes and consequently causing inefficient activation and a lower proliferation of lymphocytes. The very migration of lymphocytes to the location of the challenge could also be altered. Studies to evaluate the existence of the suppressive effects of laser irradiation are necessary to better understand the phenomena obtained with the use of this therapy.

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5 CONCLUSÕES

A literatura apresenta de forma clara que a perda tardia do enxerto ainda é um capítulo a parte na história do transplante renal. Apesar dos inúmeros estudos em modelos aninais e clínicos, realizados na tentativa de elucidar os mecanismos envolvidos na complexa patogênese dessa condição, o índice de sucesso esperado ainda é baixo. As tentativas de encontrar terapêuticas para prevenir ou interromper a perda do enxerto são, sem dúvida, objetivos da ciência moderna e alvo de inúmeros trabalhos científicos. Os resultados aqui apresentados sinalizam que a LLLT, por demonstrar, no modelo experimental de RHT à OVA, efeito imunossupressor semelhante à Azatioprina, pode ser uma terapêutica a ser melhor estudada e quem sabe vir a ser utilizada para minimizar ou até impedir as conseqüências desastrosas da perda do enxerto.

As alterações na expressão de COX-2 e nos níveis de expressão de TNF-α, IFN-γ e IL-10 são observações objetivas de que o laser atua no sistema imunológico. Esse fato deve servir de estímulo para que estudos clínicos sejam consuzidos com o ojetivo de corroborar nossa impressão de que a terapia com LLLT pode atuar no sistema imunológico.

O sucesso atual do transplante de órgão baseia-se, em grande parte, no uso de moléculas imunossupressoras que são capazes de bloquear a ação de células do sistema imune. A terapêutica imunossupressora é eficiente no tratamento da rejeição aguda, embora apresente efeitos colaterais importantes. Na rejeição crônica os efeitos são muito mais modestos.

Os resultados apresentados neste trabalho demonstram que a LLLT pode vir a ser usado como opção terapêutica imunomoduladora no transplante renal, especialmente na nefropatica crônica do enxerto.

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