Efeito Antiinflamatório da Terapia Laser de Baixa Potência (660 nm) na Pleurisia em Ratos

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular – PPGBCM como requisito para a obtenção do grau de Mestre.

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RESUMO

A proposta deste estudo foi investigar o efeito potencial da terapia laser de baixa intensidade na modulação de mediadores pró-inflamatórios e antiinflamatórios na fase aguda da inflamação no modelo de pleurisia em ratos. No modelo clássico de pleurisia temos poucas evidencias do efeito antiinflamatório do laser e das características de dosagem, como o comprimento de onda, energia total, números e formas de tratamento, necessitando ainda de mais estudos. A amostra de 48 ratas Wistar foram divididas em grupos controle e experimentais. A inflamação foi induzida por carragenina (0.2 mL) injetada na cavidade pleural. Na 1ª, 2ª e 3ª hora após a indução o laser diodo de InGaAlP (660 nm) no modo contínuo (20 mW) foi usado nos grupos laser diferentes doses e formas de tratamento, com uma energia total de 0.9, 2.1 e 4.2 joules. Após 4 horas o volume de exsudato, leucócitos totais e diferencial, concentração de proteínas, oxido nítrico (NO), interleucina (IL)-6, interleucina (IL)-10, fator de necrose tumoral (TNF)-α e proteína quimioatrativa de monócitos (MCP)-1 foram mensuradas no líquido pleural aspirado. Todas as formas de tratamento e quantidade de energia reduzem significativamente o volume do exsudado ($P<0.05$). A energia final abaixo de 2.1 joules somente reduz significativamente ($P<0.05$) o NO, IL-6, MCP-1 e IL-10, enquanto que 2.1 joules reduz significativamente todas as variáveis independentemente da forma de tratamento. A migração de neutrófilos tem uma correlação significativa com a concentração de TNF-α ($r=0.551$). A terapia laser de baixa intensidade (660 nm) induz um efeito antiinflamatório caracterizado pela inibição de ambos leucócitos totais e diferencial, exsudado, proteínas totais, NO, IL-6, MCP-1, IL-10 e TNF-α, mas mantendo um alto perfil dose-dependente. Nestas condições acima, o laser com 2.1 joules foi mais efetivo que 0.9 joule e 4.2 joules

Palavras Chaves: Carragenina, Inflamação, LLLT, Pleurisia
ABSTRACT

The purpose of this study was to investigate the potential effect of low level laser therapy (LLLT) on modulating the pro-inflammatory and anti-inflammatory mediators of acute inflammation in rat pleurisy model. In the classic model of pleurisy there is little evidence of anti-inflammatory effects from LLLT and dosage characteristic, such as wavelength, total energy, number and pattern of treatment, with the necessity for more studies. A sample of 48 female Wistar rats were divided into control and experiential groups. An inflammation was induced by carrageenan (0.2mL) injected into the pleural cavity. At 1, 2, and 3 h after induction the continuous wave (20 mW) diode laser of the InGaAlP (660nm) type was used in laser groups different doses and treatment patterns and with a total energy delivered of 0.9, 2.1 and 4.2 joules. At 4 h after the exudate volume, total and differential leukocytes, protein concentration, nitric oxide (NO), interleukin (IL)-6, interleukin (IL)-10, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 were measured from the aspirated liquid. Any treatment pattern and quantity of energy can reduce a significantly volume of the exudate ($P<0.05$). The final energy lower than 2.1 joules reduces significantly ($P<0.05$) only NO, IL-6, MCP-1 and IL-10, while 2.1 joules reduce significantly all variables independently of the treatment pattern. The neutrophil migration has significant correlation with the TNF-α concentration ($r=0.551$). The LLLT (660nm) induced an anti-inflammatory effect characterized by inhibition of both total and differential leukocyte influx, exudation, total protein, NO, IL-6, MCP-1, IL-10 and TNF-α, but keeping a highly dose-dependent outline. Under these conditions, laser with 2.1 joules was more effective than 0.9 joule and 4.2 joules.

Keywords: Carrageenan, Inflammation, LLLT, Pleurisy
1. APRESENTAÇÃO DO TEMA

1.1 Introdução

A reação inflamatória é um mecanismo fisiopatológico de resposta à invasão por um agente infeccioso ou apenas reação a uma lesão de natureza variada (térmica, química e mecânica), sendo representada por um conjunto de reações locais e gerais do organismo. Este mecanismo é composto por vários fenômenos complexos que se associam e se complementam uns aos outros formando uma reação em cascata, que envolve uma complexa interação de células inflamatórias (neutrófilos, linfócitos, monócitos/macrófagos) e das células vasculares (endotelias e células da musculatura lisa) (TEDGUI & MALLAT, 2001).

A resposta inflamatória visa destruir, diluir ou isolar o agente agressivo, sendo, portanto uma reação de defesa e reparação do dano tecidual (CHANDRASSOMA & TAYLOR, 1993).

A inflamação é caracterizada, em sua forma aguda, pelos sinais cardíacos de dor, calor, rubor, edema e perda de função, envolvendo uma série de eventos como aumento do fluxo sanguíneo, aumento de permeabilidade vascular, exsudação de fluidos, migração de leucócitos, liberação de agentes algícicos e dos efeitos induzidos pelos mediadores químicos no foco inflamatório (KUMAR et al., 2005; RANG, 2001; GUALILIO, 2000).
A inflamação aguda tem duração relativamente curta, durando alguns minutos, horas ou dias e é independente da natureza do agressor, sendo a resposta muito similar, aos diferentes estímulos (SIQUEIRA JÚNIOR; DANTAS, 2000). A resposta fisiológica que ocorre imediatamente após um estímulo agressivo é referida como uma fase precoce (0-1 hora) ao contrário do que ocorre de 5-6 horas após a lesão (fase tardia da inflamação aguda), onde as células inflamatórias se acumulam no local lesado (ALBERTINI et al., 2004).

Evidências demonstram que vários fatores desempenham importantes papéis na modulação da resposta inflamatória de cada uma das fases da inflamação aguda. Na fase precoce, mediadores como a histamina e bradicinina modulam a resposta inflamatória aumentando o calibre e o fluxo vascular, responsável pelo calor e rubor presente no foco de inflamação (KUMAR et al., 2005; ALBERTINI et al., 2004).

Durante a fase tardia da inflamação aguda, há predominância de eventos celulares que se caracterizam pela marginação, adesão endotelial, diapedese e migração dos leucócitos para o foco da lesão, decorrentes dos estímulos quimiotáticos. Todos os granulócitos, monócitos e, em menor grau, os linfócitos respondem aos estímulos quimiotáticos com taxas variáveis de velocidade (KUMAR et al., 2005).

A inflamação é controlada (desencadeada, conduzida e extinta) pela presença de mediadores químicos cada um com um papel específico.
atuando em estágios definidos da reação inflamatória (DE PAOLA, 1988). Os mediadores podem originar-se do plasma, das células ou do tecido agredido, sendo divididos nos seguintes grupos: aminas vasoativas (histamina e serotonina); proteases plasmáticas (sistema de cinina - bradicinina, sistema complemento, sistema de coagulação-fibrinolítico); metabólitos do ácido araquidônico (via ciclooxigenase e via lipoxigenase); proteases lisossômicas; radicais livres derivados do oxigênio; fatores ativadores das plaquetas (FAP); quimiocinas, citocinas e óxido nítrico (KUMAR et al., 2000; ALBERTINI et al., 2004).

Durante a evolução do processo de reparo, os eventos que se sucedem são a infiltração de neutrófilos, infiltração de macrófagos, fibroplasia e deposição de matriz extracelular, angiogênese, cicatrização e reepiteliação.

A transmigração dos neutrófilos para tecidos lesados é um fenômeno precoce do processo de reparo. Ocorre quase que de imediato após sinalização dos neutrófilos retidos no coágulo, macrófagos residentes e células estromais. Citocinas, principalmente a interleucina (IL)-1 e o fator de necrose tumoral (TNF)-α, atuando sobre os receptores das células endoteliais, induzem a produção de óxido nítrico (NO), bem como a expressão de moléculas de adesão para neutrófilos. A expressão das proteínas de adesão é, neste momento, o elemento mais importante para a migração de neutrófilos (GERSZTEN et al., 1999).
A família das quimiocinas (citocinas com atividade atraente sobre leucócitos) é composta de aproximadamente 50 membros que se dividem em 4 famílias. Apesar da ação das quimiocinas ser mais evidente na quimiotaxia de macrófagos e linfócitos, alguns membros desta família de moléculas como a proteína quimioatraente de macrófagos (MCP)-1 exerce esta função também sobre neurófilos (CHRISTOPHERSON & HROMAS, 2001).

A interleucina (IL)-6 é considerada como um mediador fundamental em diversas etapas da inflamação (GALLUCCI et al., 2000). Dentre os vários efeitos pró-inflamatórios que lhe são atribuídos, os intimamente relacionados ao processo de reparo são, na etapa mais tardia, a indução mitótica de queratinócitos e, na fase mais precoce, os seus efeitos quimioatrativos sobre neutrófilos (SATO et al., 1999).

A interleucina (IL)-10 é um dos mais conhecidos sinalizadores negativos da inflamação. Acredita-se que ela seja o mais importante mediador com papel limitador e finalizador da resposta inflamatória. Adicionalmente, ela não só regula o crescimento ou diferenciação de várias células do sistema imune mas também de queratinócitos e células endoteliais (MOORE et al., 2001).

Ohshima (1998), encontrou um pico de RNAms de IL-10 nos 60 minutos após uma lesão. Sato et al. (1999) encontraram dois picos de IL-10 ao longo da evolução do reparo: o primeiro ocorreu três horas após a ferida e o segundo três dias após. O papel exato da presença do seu RNAms nos momentos iniciais da lesão e do segundo pico após três dias permanece por
ser desvendados. No entanto os efeitos conhecidos da IL-10 sobre os leucócitos ocorrem pela inibição da infiltração de neutrófilos e macrófagos ao sítio da injúria tissular (SATO et al., 1999).


O edema da pata induzido por carragenina é um modelo útil para avaliar a inflamação aguda, pois o pico do edema ocorre dentro de 3 a 5 horas (SALVEMINI, 1996). Entretanto esta técnica apresenta limitações na mensuração de células inflamatórias, proteínas e mediadores químicos onde não conseguimos extrair o exsudato inflamatório. Com o objetivo de avaliar
quantitativamente e qualitativamente, Spector (1956), descreveu primeiramente o modelo de pleurisia em ratos que mais tarde foi adaptado para o porco e o camundongo (MARTINS et al., 2005; MIKAMI, 1983).

A pleurisia em ratos induzida por carragenina permite a quantificação do volume e da concentração protéica do exsudato formado, além da avaliação da migração de células inflamatórias para a cavidade pleural (SHIVKAR, 2004). Este tipo de pleurisia é utilizado na investigação da fisiopatologia da inflamação aguda e avaliação da eficácia de terapias antiinflamatórias (ARRUDA et al., 2003).

A inflamação não infecciosa é tradicionalmente tratada com drogas antiinflamatórias esteróides e não-esteróides. Os glicocorticóides são potentes agentes antiinflamatórios esteróides largamente utilizados que apresentam capacidade de inibir a enzima fosfolipase A-2 e a ciclooxigenase-2 (COX-2). Esta inibição reduz os níveis de ativação do ácido araquidônio e a produção de prostaglandinas, respectivamente, proporcionando um alto poder antiinflamatório (LUNARDELLI et al., 2006). Entretanto, muitas técnicas físicas alternativas como a estimulação elétrica, ondas curtas, infravermelho, ultra som e LASER têm sido usadas satisfatoriamente no tratamento de pacientes com doenças inflamatórias (ALBERTINI et al., 2004; MARTINS et al, 2005).

As raízes do mecanismo do LASER são modernas. Em 1900 o físico alemão Max Planck apresentou uma explanação do motivo pelo qual as
cores de um corpo quente reluzente mudam com a temperatura. Ele propôs que as radiações vêm em quantidades discretas ("quanta"). Assim, a radiação seria não apenas uma série de ondas mas, ao mesmo tempo, uma corrente de partículas ("fótons") (KITCHEN, 1998; TÜNER & HODE, 2002).

Por volta de 1917, Albert Einstein esboçou os princípios básicos para a produção da radiação LASER, a emissão estimulada da radiação, como parte da teoria quântica. Sobre este princípio está fundamentado o fenômeno LASER e desde então numerosos pesquisadores têm seguido as investigações até chegar os nossos dias. O processo tem sido complexo porque são vários os temas, os campos, as especialidades da ciência que se vão entrelaçando como a Física, Química, Biofísica, Medicina, Fisioterapia, Odontologia, Veterinária, entre outras (TÜNER & HODE, 2002).

A partir destas datas iniciou-se um período caracterizado pelo incremento das investigações no campo da Biofísica e Medicina. Como exemplo claro disso, foi criado em 1961 o centro de investigações e aplicação de LASER para uso dos médicos da Faculdade de Medicina da Universidade de Cincinnati sob o comando do dermatologista Dr. Leon Goldman (COLLS, 1987). Após 1961, muitos estudos propiciaram a construção de LASERS de Hélio-Neônio (He-Ne) e Neodímio-Yttrium Aluminium Garnet (Nd:YAG). Em 1962 o de Argônio (Ar), de 1964 o de Dióxido de Carbono e na última década o advento dos semicondutores como o Arseneto de Gálio (GaAs) (BRUNHEIRA & PINHEIRO, 1998).

Os primeiros LASERS médicos, desenvolvidos nas décadas de 1960 e 1970, eram usados para a destruição tecidual e coagulação. O primeiro emprego dos LASERS foi como fotocoaguladores, aproveitando que se obtinham descargas de alto poder energético que, em impulsos muito curtos, conseguiam efeitos térmicos locais capazes de vaporizar os tecidos. A cirurgia oftalmológica foi a primeira a adaptá-lo para a destruição de tumores muito localizados (KITCHEN, 1998).

Desta forma, no ano de 1961 em Nova York realizou-se, com sucesso, a primeira intervenção cirúrgica com LASER de alta potência para retirada de um pequeno tumor na retina. A partir deste momento foram incrementadas as experiências cirúrgicas e começou-se a observar de forma empírica, efeitos benéficos do laser de baixa energia como, por exemplo, aumento na
cicatrização e epitelização nos locais de aplicação, o que levou ao uso terapêutico de LASERS de baixa intensidade (COLLS, 1987).

Estes efeitos biológicos fizeram com que vários pesquisadores prestassem uma atenção especial para os efeitos bioestimulativos da radiação LASER de baixa energia. Entre eles destacamos os trabalhos de Endre Mester de Budapest e do professor Inyushin da Escola de Biofísica de Alma-Ata na Rússia. Eles produziram um grande volume de trabalhos científicos, experimentais e clínicos, tendo o LASER de He-Ne, como tema central. Contudo, devido à dificuldade do idioma, a pouca divulgação e a não reprodutividade de seus resultados, houve críticas e descrédito na utilização do LASER por mais de uma década (TÚNER & HODE, 2002).

Os primeiros LASERS empregados na medicina foram construídos tendo como base o rubi, emitindo-se na parte vermelha do espectro. Portanto, os efeitos biológicos que a radiação laser produzia foram estudados nesta parte do espectro lumínico. Atualmente, os lasers de rubi caíram em desuso devido ao seu complicado sistema de refrigeração. A medicina e a biologia empregam LASERS de alto rendimento como os lasers de CO₂, Argônio, Neodíneo, YAG e HeNe, buscando as características particulares do comprimento de onda de cada emissão para o tratamento de tecidos especificamente diferenciados (COLLS, 1987). A partir dos anos 90, diferentes substâncias foram introduzidas na tecnologia para obtenção de diodos LASER, gerando e ampliando a faixa de comprimento de onda. Com
estes dispositivos hoje podemos ter aparelhos pequenos, de fácil transporte e manuseio, com rara necessidade de manutenção e custo acessível.

Os comprimentos de onda mais utilizados estão entre 600 e 1200 nm (janela óptica terapêutica), pois os LASERS situados nesta região do espectro eletromagnético, são relativamente pouco absorvidos e consequentemente apresentam boa transmissão na pele e nas mucosas, já que a hemoglobina absorve comprimentos de onda menores que 600 nm enquanto a água absorve comprimentos de onda maiores que 1200 nm. (TÚNER & HODE, 2002).

A palavra LASER é um acrônimo de “Light Amplification by Stimulated Emission of Radiation” ou seja, amplificação da luz por emissão estimulada de radiação, a qual não é ionizante. As características que diferenciam a luz LASER das outras fontes luminosas, como a luz incandescente (branca) de uma lâmpada, são a monocromaticidade, colimação, coerência espacial e temporal. A monocromaticidade significa que a luz LASER emitida apresenta apenas um único comprimento de onda. A maioria dos LASERS apresentam feixes colimados, isto é, com um mínimo ângulo de divergência. A coerência é a sincronicidade das ondas de luz, demonstrando que ondas propagam-se com a mesma fase no espaço e no tempo (COLLS, 1987; KITCHEN, 1998; TÚNER & HODE, 2002).

Os LASERS são classificados em dois grandes grupos: os de alta potência e os de baixa potência. O LASER de alta potência, também

O termo “Low Level Laser Therapy” ou simplesmente LLLT, é o mais comumente utilizado na área terapêutica e caracteriza os LASERS de baixa potência (TÚNER & HODE, 2002). O LLLT emprega uma potência menor que 1 W (Watt) no tecido alvo (KITCHEN, 1998). Al-Watban e Zhang (1997) e Maegawa et al. (2000) corroboram que a amplitude de temperatura na LLLT é insignificante (máx. 1ºC), onde a energia dos fótons absorvidos por fotorreceptores de uma célula não se transforma em calor, o que provocaria efeito fototérmico ao invés de efeitos fotoquímicos e fotofísicos, orquestrando mudanças nestas moléculas, que por sua vez promovem respostas fisiológicas a nível molecular, celular e orgânico.

A interação da LLLT com o tecido é determinada por duas variáveis dependentes: comprimento de onda específico da emissão laser e características ópticas do tecido alvo (DEDERICH, 1991 apud
Algumas variáveis independentes também são importantes, tais como: o nível de potência (densidade de potência ou irradiância); energia total entregue sobre a área da superfície irradiada (densidade de energia ou fluência); duração da exposição e modo de entrega da energia ao tecido alvo, como: o tipo de regime operacional, frequência do pulso (taxa de repetição) e número de aplicações durante o tratamento (TÚNER & HODE, 2002; BAXTER, 1997).

A radiação luminosa ao atingir o tecido pode ser refletida, transmitida, absorvida ou espalhada. A absorção é o principal parâmetro da interação laser-tecido, pois dela depende a quantidade de energia entregue ao tecido e por sua vez o efeito provocado. Dependendo da energia do fóton, a radiação pode ser transferida a uma molécula por processos rotacionais, vibracionais ou eletrônicos, provocando no tecido os efeitos fotoquímicos, fototérmicos, fotomecânicos ou fotoelétricos (COLLS, 1987). O quantum de luz é apenas o desencadeador do início do processo regulatório do metabolismo celular, explicando a razão do uso de pequenas doses comumente empregadas na LLLT (KARU, 1989).

baseando-se em estudos prévios, constatou melhora no processo cicatricial quando utilizadas pequenas doses e observou que ao aumentar a densidade de energia estes efeitos eram inibidos.

São relatados por Karu (1989) como efeitos fotobiológicos associados a LLLT, o crescimento celular estimulado em tecidos conjuntivo, tendíneo e ósseo, a redução de formação do tecido fibroso após injúrias e também reparação de células nervosas. Outro efeito referido é o antiinflamatório que ocorre pela redução da capacidade dos linfócitos em reagir a estímulos antigênicos. Karu (1989) também mostrou que o LLLT reduz o edema, estimula a neoangiogênese, regenera os vasos linfáticos e veias, melhora a atividade tissular decorrentes de mudanças do conteúdo de prostaglandina e de enzimas específicas. Os efeitos podem ser explicados pelo aumento proliferativo das células em estágio G0 e G1 ou por mudança da atividade fisiológica de células excitáveis.

Nas células, ainda segundo Karu (1987), a presença de fotorreceptores primários caracterizados pelos co-fatores enzimáticos NADH-dehidrogenase e Citocromo C Oxidase encontrados no interior da crista interna das mitocôndrias, atuam na cascata de eventos da cadeia respiratória, causando desta maneira, regulação da oxidoredução no metabolismo celular. A fotoativação enzimática influencia as alterações metabólicas, aumentando a síntese de ATP e o consumo de oxigênio. Além disso, realizam a transdução do sinal a outras partes celulares, incorrendo
em maior duplicação do DNA e RNA e alterações no mecanismo da bomba
de Na+ e K+, inferindo uma fotorresposta

Além destes efeitos, outros pesquisadores têm demonstrado que a
radiação com LLLT (He-Ne; GaAlAs) também apresenta efeitos
antiinflamatórios (ENGLAND et al., 1989; CAMPANA et al., 1998; CAMPANA
et al., 2003) e efeitos analgésicos (HONMURA et al., 1993). Túner e Hode
(1998), mostram que o LLLT só promove efeitos bioestimulatórios
pronunciado em órgãos ou tecidos debilitados e a magnitude do efeito
depende do estado fisiológico em que as células são encontradas no
momento da irradiação.

Até o momento, os estudos em animais têm-se concentrado em duas
áreas principais de pesquisas: efeitos fotobioestimulantes na cicatrização de
feridas no reparo de tecidos em lesões experimentalmente induzidas e nos
efeitos sobre o processo inflamatório (KITCHEN, 1998).

Com relação ao processo inflamatório, Sattayut et al. (1999) e
Campana et al. (1998/1999) relataram que a ação da LLLT sobre os tecidos,
pode estar relacionada na possível inhibição do surgimento dos fatores
quimiotáticos nos estágios iniciais da inflamação, interferindo nos efeitos dos
mediadores químicos induzidos pela inflamação e provocando a inhibição da
síntese das prostaglandinas.
Testes laboratoriais controlados têm demonstrado que o LLLT pode reduzir a inflamação através da redução dos níveis PGE-2 e inibição da cicloxigenase-2 (COX-2) em culturas de células (CAMPANÃ et al., 1993; HONMURA et al., 1993), mas a efetividade clínica destes achados têm sido questionada em revisões sistemáticas de diversas condições patológicas (BASFORD, 1995).

Bjordal et al., (2003), sugere em sua revisão que o efeito da radiação com LLLT na redução da dor em doenças articulares crônicas é dependente da dose ajustada para inibir a inflamação ativa na cápsula articular. Eles assumiram que doses de 0,4 até 19 J/cm² e densidade de potência entre 5 e 21 mW/cm² são capazes de reduzir a inflamação sem comprometimento do metabolismo dos fibroblastos, pois doses acima de 20 mW/cm² podem temporariamente inibir os fibroblastos.

Outros trabalhos mostram que o LLLT com diodo de Ga-Al-As (650 nm) usado com uma potência contínua de 2,5 mW e densidade de energia de 2,5 J/cm² reduzem o edema na pata de ratos inflamadas com carraginina, apresentando um perfil na resposta anti-inflamatória semelhante ao diclofenaco de sódio administrado intraperitoneal na dose de 1 mg/Kg. Nesse estudo Albertini et al. (2004) mostraram que o LLLT não inibiu o edema induzido por carragenina em modelo de ratos adrenalectomizados sugerindo que o mecanismo de ação pode estar relacionado com a estimulação do eixo endócrino hipotálamo-hipófise-adrenal e, consequentemente, liberação de corticóides endógenos.
Recentemente, Martins et al. (2005) descreveram um efeito inibitório do LLLT (650 nm – 2,5 mW) sobre a migração de leucócitos (neutrófilos) para o espaço pleural no modelo de pleurisia em camundongos induzida por carragenina, com densidades de energia de 1 J/cm², 2,5 J/cm² e 5 J/cm² aplicadas na 1ª, 2ª e 3ª hora após a indução inflamatória.
1.2 Hipóteses

O LLLT pode apresentar uma ação antiinflamatória em modelo experimental de pleurisia em rato interferindo diretamente na liberação de mediadores inflamatórios e que as características de dosagem da energia luminosa utilizada podem modificar este efeito.
1.3 Objetivos

1.3.1 Objetivo Geral

- Avaliar o efeito do Laser de Baixa Potência (Ga-Al-In-P; 660 nm) no processo inflamatório agudo de pleurisia induzida por carragenina em ratos.

1.3.2 Objetivos Específicos

- Mensurar o volume do líquido e a quantidade de proteínas no exsudato inflamatório pleural dos grupos estudados.

- Verificar a migração de células inflamatórias para a cavidade pleural no processo inflamatório agudo nos grupos experimentais.

- Analisar a liberação de óxido nítrico (NO), interleucina (IL)-6, interleucina (IL)-10, fator de necrose tumor (TNF)-α e proteína quimioatrativa para monócitos (MCP)-1 no líquido pleural dos grupos estudados.

- Correlacionar as variáveis mensuradas com a migração de leucócitos polimorfonucleares para a cavidade pleural dos grupos estudados.
2. ARTIGO CIENTÍFICO

Original Article 03-10-2008

Anti-inflammatory effects of Low-Level Laser Therapy (660 nm) in the Early Phase in Carrageenan-Induced Pleurisy in Rat

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2.1 Abstract

Background and Objective: In the classic model of pleurisy there is little evidence of anti-inflammatory effects from low level laser therapy (LLLT) and dosage characteristic, such as wavelength, total energy, number and pattern of treatment, with the necessity for more studies. The purpose of this study was to investigate the potential effect of LLLT on modulating the pro-inflammatory and anti-inflammatory mediators of acute inflammation rat pleurisy model.

Study Design/Material and Methods: A sample of 48 female Wistar rats were divided into control and experiential groups. An inflammation was induced by carrageenan (0.2 mL) injected into the pleural cavity. At 1, 2, and 3 h after induction a continuous wave (20 mW) diode laser of the InGaAlP (660 nm) type was used in the four laser groups with different doses and treatment patterns. One group received a single dose of 2.1 joules and the other three groups received a total energy of 0.9, 2.1 and 4.2 joules. At 4 h later the exudate volume, total and differential leukocytes, protein concentration, NO, IL-6, IL-10, TNF-α and MCP-1 were measured from the aspirated liquid.

Results: Any treatment pattern and quantity of energy can reduce a significantly volume of the exudate ($P<0,05$). Energies below than 2.1 joules significantly reduces ($P<0,05$) only NO, IL-6, MCP-1 and IL-10, while 2.1 joules significantly reduce all variables independently of the treatment pattern. The neutrophil migration has a straight correlation with the TNF-α ($r=0,551$) and NO ($r=0,549$) concentration.
Conclusions: LLLT - 660 nm induced an anti-inflammatory effect characterized by inhibition of both total and differential leukocyte influx, exudation, total protein, NO, IL-6, MCP-1, IL-10 and TNF-α, but keeping a highly dose-dependent outline. Under these conditions, laser treatment with 2.1 joules was more effective than 0.9 joule and 4.2 joules.

Key Words: Inflammation, LLLT, Pleurisy
2.2 Introduction

Inflammation is a protective process essential for the preservation of the integrity of the organisms in the event of physical, chemical and infectious damage [1]. Acute inflammation is characterized by the classical sings of pain, heat, redness and swelling, involving a complex series of events including vasodilatation, increased permeability, fluid exudation and migration of leukocytes to the site of the inflammation [2].

Carrageenan-induced pleurisy is a well-characterized experimental model of inflammation that permits the quantification and correlation of both exudate and cellular migration with changes of other inflammatory parameters [3]. The major characteristic of this model in the rat is the biphasic profile of this inflammatory reaction, where early (4 h) and late (48 h) phases of both cell migration and exudation are clearly observed [4]. Thus, this model constitutes a biologic system suitable for the investigation of possible correlation occurring between cell migration, fluid leakage, nitric oxide (NO), chemokine, pro-inflammatory and anti-inflammatory cytokines.

One of the early cellular events in inflammation is the migration of leukocytes, primarily neutrophils. In addition, NO plays an important role in inflammation such as plasma exudation and leukocyte infiltration. The NO synthase (NOS) inhibitors can reverse several classic inflammatory symptoms [5].

The maintenance of leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes and the
endothelium. These events are mediated by the generation of early response cytokines, e.g., interleukin (IL-1) and tumor necrosis factor (TNF-α), the expression of cell-surface adhesion molecules and the production of chemotactic molecules, such as chemokines [6].

Chemokines are a family of structurally related glycoproteins with potent leukocyte activation and/or chemotactic activity. Most chemokines are produced in response to a variety of inflammatory stimuli, including the early-response cytokines, TNF-α, IL-1, C5a, leukotriene B4 and interferons [7]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that has been demonstrated to attract monocytes and neutrophils both in vitro and in vivo and appears to be a key part of leukocyte emigration, serving as a promoter of the transition from leukocyte rolling to adhesion on the endothelial surface [8].

Representative inflammatory cytokines include TNF-α, IL-1, IL-6, and IL-8. The TNF-α and IL-1 have been revealed to have many overlapping biological activities in the inflammatory reaction: both cause fever, accumulation of neutrophils in local tissues, induction of vascular adhesion molecules and stimulation of acute phase protein synthesis [9]. Some of these responses by TNF-α and IL-1 are now known to be mediated by other inflammatory mediators or secondarily induced cytokines such as IL-6 [10].

The IL-6 is widely produced by several cells such as fibroblasts, endothelial cells, keratinocytes, monocytes, T cells, mast and tumour cell lines, and cells
of neural origin. Thus, IL-6 is often used as a marker for systemic activation of pro-inflammatory cytokines [11]. However, evidence also indicates that, under certain conditions, IL-6 has both pro-inflammatory and anti-inflammatory properties [12]. Apart from this, there is also experimental evidence indicating that IL-6 down-regulates the synthesis of IL-1 and TNF-α [13].

The IL-10 is known as the most important anti-inflammatory cytokine found within the human immune response. Previous data has shown that IL-10 is capable of blocking the inflammatory response induced by several inflammatory stimuli in different models [11].

The conventionally used therapies for inflammation, non-steroidal anti-inflammatory drugs (NSAID’s), have a very important role in managing pain and acute inflammatory conditions [14], though with rather discouraging profile of side effects [13]. Even the anti-inflammatory drugs, cyclo-oxygenase 2 (COX-2) inhibitors, are not devoid of adverse effects [15].

Despite this, alternative physical techniques such as low-level laser therapy (LLLT) have been used clinically, among other indications for its proposed anti-inflammatory effects, pain relief and acceleration of the regeneration of damaged tissues [16]. Although in the past decade several studies have examined the effects of LLLT, the treatment protocols used included enormous variations in parameters (such as wavelengths, energy and power densities, wave modes, number of treatments), which makes it difficult to assess the optimum treatment parameters in each case [17].
However, more recent studies have been able to find some dose-dependent effects on TNF-α [18] and prostaglandin E₂ levels [19], besides reduction of the oedema [20,21], neutrophils migration [21], nitric oxide synthase (iNOS) expression [22] and COX-2 mRNA expression [23] in the different inflammatory experimental models after LLLT. The efficacy of LLLT radiation as an anti-inflammatory therapy is controversial.

Therefore, the present study was designed to explore the potential effects of LLLT applied at different points with dose variation on the modulation of the pro-inflammatory and anti-inflammatory mediators of acute inflammation in carrageenan-induced pleurisy in rat model.
2.3 Materials and Methods

2.3.1 Animals

Adult female Wistar rats (*Rattus norvegicus*) bred in our laboratory (around 3-4 months old, weighing 180 g - 220 g) were used, all of the same ancestry and socialization with free access to food and water. Animals were divided into six groups composed of eight rats each. The rats were maintained in accordance with the “Guiding Principles in the Care and Use of Animals” and the present study was approved by ethics committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

2.3.2 Carrageenan-induced pleurisy

Rats were anesthetized with a mix of Ketamine (80 mg/Kg) and Xilazine (12 mg/Kg). Saline 0.2 mL (saline group) or saline containing 2% λ-carrageenan (Cg) 0.2 mL (carrageenan group and Cg + laser groups) was injected into the pleural cavity at the level of the sixth left intercostal space. At 4 h after the intrathoracic injection, the animals were sacrificed with decapitation and the pleural cavity was opened. The liquid that had accumulated in the pleural cavity was washed with 2.0 mL of sterile saline solution (NaCl 0.9%) containing 1% EDTA in the aspirated liquid. All exudate contaminated with red blood cells was discarded [2].

2.3.3 Laser Irradiation

The continuous wave diode laser of InGaAlP type (model Endophoton-LLT-010 - KLD Biosistemas Equipamentos Eletrônicos Ltda) with an output power
of 20 mW and a wavelength of 660 nm (visible red) was used. The spot size was 0.035 cm$^2$ and the power density was 0.571 W/cm$^2$. Table 1 shows the laser parameters used in our experiment. Different doses were used for each of the four groups: the group with 1 point (Cg + 1 point laser with 21 J/cm$^2$), the group with 3 points (Cg + 3 points laser with 3 J/cm$^2$ each), the group with 7 points (Cg + 7 points laser with 3 J/cm$^2$ each) and the group with 14 points (Cg + 14 points laser with 3 J/cm$^2$ each). All the experimental groups were irradiated with LLLT from a spot in contact with the skin at 1, 2 and 3 h after pleurisy induction. Thus, the total energy delivered from the three treatment sessions was 2.1, 0.9, 2.1 and 4.2 joules during 36.75, 15.75, 36.75 and 73.50 sec, respectively. The animals received the irradiation at the left thoracic wall with different treatment methods according to the group: the group of 1 point was irradiated with LLLT where the pleurisy was induced; the group of 3 points received LLLT around the induction area; the group of 7 points was irradiated, in which 1 point was on the induction local and 6 points were symmetrically distributed around the area; in the group of 14 points they were distributed in a craniocaudal direction of crescent 2, 3, 4 and 5 points involving all thoracic wall.

2.3.4 Exudate Analysis

The volume of the exudates was measured and the result expressed by subtracting the volume injected into the pleural cavity (2.0 mL of solution) from the total volume aspirated. Total leukocytes were diluted in Thoma solution (1:20) and counted in a Neubauer chamber using light microscopy. Cytological slide smears stained with May-Grünwald/Giemsa were used for
differential leukocyte counts in a light microscope [24]. The pleural liquid removed from the rats was centrifuged at 1200 x g for 10 min and the protein concentration measured by the Biuret technique. NO is a very unstable radical, rapidly metabolized from nitrate to nitrite in the presence of oxygen [25]. Therefore, the amount of NO in the exudate was analyzed using the Griess reaction that measured nitrite, as previously described [26]. The frozen cell-free supernatant of the pleural fluid was thawed, and IL-6, IL-10, TNF-α and MCP-1 were determined using a rat enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Camarillo, CA).

2.3.5 Statistical Analysis

The results were evaluated statistically by analysis of variance (ANOVA) with LSD test post hoc using SPSS (Statistical Package for the Social Sciences) 12.0 software and were expressed as the means ± standard error of means (SEM). The correlation between the variables and the neutrophil infiltration into the pleural cavity was analyzed by using Pearson Correlation Linear. The level of statistical significance was defined as $P < 0.05$. 
2.4 Results

According to the analysis of this study, the rats injected with carrageenan in the pleural space developed an acute pleurisy after 4 h with a significant increase in all variables when compared to the saline group.

Figure 1 shows the protective effect of LLLT on the inflammatory parameters, such as: protein concentration, exudate volume, the amount of leukocytes and neutrophils in the pleural liquid of the saline group, carrageenan and in the four groups irradiated with different laser doses and treatment methods.

2.4.1 Protein

Protein concentration (fig 1A) showed a significant decrease ($P<0.05$) in laser groups with 1 point ($1.01 \pm 0.11$ g/dL), 7 points ($0.94 \pm 0.03$ g/dL) and 14 points ($1.13 \pm 0.10$) when compared to the carrageenan group ($1.42 \pm 0.04$ g/dL). It was observed that the laser group with 3 points ($1.47 \pm 0.07$ g/dL) did not modify the concentration of proteins that had migrated to the pleural cavity.

2.4.2 Exudate volume

In relation to the exudate volume (fig 1B) collected 4 h after the pleurisy induction, a significant reduction was observed in the four groups irradiated with LLLT when compared to the carrageenan group (1 point: $0.32 \pm 0.03$ mL; 3 points: $0.68 \pm 0.07$ mL; 7 points: $0.60 \pm 0.08$ mL and 14 points: $0.52 \pm 0.06$ mL vs Cg: $0.98 \pm 0.04$ mL, $P<0.001$). Besides that, the laser group with 1
point (2,1 joules) was more effective than the laser group with 7 points (2,1 joules) in reducing pleural exudate volume ($P=0.002$).

2.4.3 Total leukocytes

All groups irradiated with LLLT, except the group with 3 points, showed a significant decrease in the total number of leukocytes when compared to the carrageenan group (1 point: $35.03 \pm 4.07$; 3 points: $63.25 \pm 5.69$; 7 points: $37.21 \pm 2.35$ and 14 points: $46.22 \pm 3.81$ vs Cg: $70.67 \pm 3.90$, $P=0.001$), (fig 1C). Based on this, the group that received a final dose of 0.9 joule (group 3 points) did not demonstrate reduction in the leukocyte cells infiltration to the inflammatory site of the lesion ($P=0.176$).

2.4.4 Neutrophils

The reduction in the inflammatory acute phase response, represented by a lower migration of polymorphonuclear-neutrophil cells (PMNs), showed a mean of 50% reduction of these cells in the groups 1, 7 and 14 points that received higher or equal to 2.1 joules energy when compared to the carrageenan group or the laser group with 3 points (1 point: $31.09 \pm 3.60$; 7 points: $31.61 \pm 1.90$ and 14 points: $36.63 \pm 4.20$ vs Cg: $61.11 \pm 1.76$, $P<0.001$). The group 3 points did not demonstrate any significant reduction in the neutrophil infiltration ($58.30 \pm 5.60$, $P=0.568$), (fig 1D).

2.4.5 NO

The concentration of nitric oxide (NO) in the pleural cavity (fig 2A) presented a significant reduction in the groups irradiated with laser when compared to the
carrageenan group (1 point: 49.40 ± 2.53 nmol; 3 points: 55.25 ± 2.49 nmol; 7 points: 49.44 ± 2.22 nmol and 14 points: 55.70 ± 2.88 nmol vs Cg: 86.02 ± 3.86 nmol, \( P=0.001 \)). It is noticed that in laser groups with 1 and 7 points the reduction approximates the basal level values of the saline group (49.78 ± 5.63 nmol).

2.4.6 IL-6

In relation to the pro-inflammatory cytokine, a reduction was observed in the concentration of IL-6 in the irradiated groups after the application of energy when compared to the one that received only carrageenan (1 point: 7298.82 ± 178.99 pg, 3 points: 8596.92 ± 231.76 pg, 7 points: 7955.80 ± 478.74 pg and 14 points: 14587.74 ± 2613.69 pg vs Cg: 25357.77 ± 5451.93 pg, \( P<0.001 \)). This important mediator presented sensibility to LLLT irradiation independently of the chosen treatment method and energy dose (fig 2B).

2.4.7 MCP-1

The chemokine MCP-1 had a significant decrease in laser groups with 1 point (1131.25 ± 175.74 pg, \( P=0.019 \)), 3 points (1228 ± 209.39 pg, \( P=0.025 \)) and 7 points (1434.50 ± 171.89 pg, \( P=0.046 \)) when compared to the carrageenan group (3007.25 ± 1067.61 pg), but in the laser group with 14 points (2578.85 ± 741.09 pg, \( P=0.591 \)), where the total final energy was equal to 4.2 joules, a significant reduction was not verified in the measured values from the pleural liquid count (fig 2C).
2.4.8 *IL-10*

A significant reduction was verified of IL-10 concentration in the irradiated groups with 1 point (560.05 ± 70.18 pg, \(P=0.002\)), 3 points (690.76 ± 114.44 pg, \(P=0.007\)) and 7 points (855.87 ± 239.78 pg \(P=0.049\)) when compared to the carrageenan group (1436.15 ± 305.68 pg). In the group with 14 points (957.42 ± 216.25 pg), IL-10 did not demonstrate suggested any modification on the measured values showing that a more local treatment pattern tends to intervene in the final result (\(P=0.088\)), (fig 3A).

2.4.9 *TNFα*

The concentration of TNF-α (fig 3B) decreased significantly in the LLLT irradiated groups with 1 point (523.90 ± 122.21 pg), 7 points (298.05 ± 69.21 pg) and 14 points (509.05 ± 146.07 pg) when compared to the carrageenan group (1535.20 ± 402.81 pg, \(P<0.001\)). The laser group with 3 points did not present a decrease in TNF-α levels when compared to the carrageenan group (1206.27 ± 359.99 pg vs 1535.20 ± 402.81 pg, \(P=0.336\)).

2.4.10 Correlation between neutrophil cell migration with the study variables

At 4 h after carrageenan-induced pleurisy a significant increase was observed in neutrophil migration to the pleural cavity in the carrageenan group when compared to the saline group (fig 1D). With regard to all variables correlated to the neutrophil infiltration in the pleural cavity, only MCP-1 (\(P=0.060\)) and IL-10 (\(P=0.092\)) did not present a significant statistics-level. In our experimental model, the first inflammatory mediator to show a main correlation with
neutrophil infiltration was TNF-α (r=0.551) and the second was NO (r=0.549).

The results are summarized in Table 2.
2. 5 Discussion

Immediately after an acute injury the body initiates a series of biological responses. The inflammatory reaction consists of both vascular and cellular events. Injury responsive components such as mast cells, bradykinins and prostaglandins are activated along with the vascular responses and cellular membrane reactions. All of these combined processes and events are represented by the symptoms of edema, inflammation, pain and functional debility [4,11,27].

The carrageenan is a polysaccharide frequently used to induce acute inflammatory reaction in animal experimental models. Winter (1962) introduced the use of carrageenan as an irritant agent of rat paw edema, becoming the first and most popular method to evaluate new anti-inflammatory therapies with a hydroplethysmometer for inflammed paw volume measurement [28].

The paw edema induced by carrageenan is an useful method to evaluate acute inflammation, since edema peak occurs within 3 to 5 hours [29]. However, this technique has some limitations on measuring inflammatory cells, proteins and chemical mediators, once it is not possible to extract the inflammatory exudate.

Despite this, carrageenan-induced pleurisy in rats permits quantifying the volume and protein concentration of the exudate formed, besides the
evaluation of the inflammatory cell migration to the pleural cavity [30]. This kind of pleurisy is used to investigate acute inflammation pathophysiology and also to evaluate anti-inflammatory therapies efficacy.

The inflammatory response that occurs after carrageenan injection into the pleural cavity is characterized by cellular infiltration, mainly composed of neutrophils (approx. 90%) and to a lesser extent of monocyte/macrophages [27].

Phototherapy with LLLT has achieved high success in recent years. It has approved by the U.S. Food and Drug Administration (FDA) and is gradually earning its place in mainstream medical practice [33].

Many studies have suggested advantages of the biomodulatory effects of LLLT on the inflammatory process, wound healing and pain relief. LLLT has been shown to reduce the duration of acute inflammation and pain, just as to accelerate tissue repair in tendon and muscle injuries [32].

Laser therapy uses different wavelengths of the visible and near-infrared spectra including HeNe (632.8 nm), InGaAlP (630-685 nm), GaAlAs (780-870 nm) and GaAs (904 nm) [34,35]. Many researchers have attempted to understand the action of LLLT, as well as to determine the most appropriate wavelength, period of irradiation, number of treatments, energy density and energy total.
Albertini et al. (2004) have demonstrated that LLLT with InGaAlP (650 nm) employed with a continuous power of 2.5 mW and energy density of 2.5 J/cm² can reduce edema caused by carrageenan-inflammation in rat paw. In the anti-inflammatory response it has shown similar performance to the sodium diclophenac when intraperitoneally administrated with a dose of 1 mg/kg. In this study the authors verified that LLLT did not inhibit the carrageenan-induced edema in adrenalectomized rat models, suggesting that the mechanism of action can be related with the hypothalamo-hypophyseal-adrenal endocrine system and, as a consequence, with the release of endogenous corticoids [20].

Recently, Lopes-Martins et al. (2005) described an inhibitory effect of LLLT (650 nm – 2.5 mW) on leukocyte (neutrophil) migration to the pleural space in the carrageenan-induced pleurisy in mice with energy density of 1 J/cm², 2.5 J/cm² and 5 J/cm², applied on the 1st, 2nd and 3rd hour after the inflammatory induction. It was observed that the total dose of 7.5 J/cm² (0.6 joules) was more effective [21].

Based on these studies that have evaluated the anti-inflammatory action of LLLT, the dosage of 2.5 J/cm² with power of 2.5 mW has demonstrated to reduce edema and inflamed cells when local irradiation is applied on the inflamed site at 1, 2 and 3 h after the carrageenan induction, knowing to be dealing with experimental models in which only one point of laser would be sufficient to cover the rat paw or the mouse thoracic wall.
In our study a rat pleurisy model was used but we had no references about points quantity needed to irradiate the most thoracic wall of these animals. Thus, to accomplish our study, we used a continuous-wave power of 20 mW at energy dose of 3 J/cm² in 3 groups with 8 animals. These groups were irradiated at 3, 7 and 14 points distributed unequally (14 points symmetrically distributed within the rat’s thoracic wall; 7 points and 3 points located on the pleurisy induction site as described above in methods).

The analysis of cell counts (fig. 1D) showed a more significant decrease of neutrophil infiltration to the pleural space in the group irradiated at 7 points with 3 J/cm² in relation to the groups irradiated at 3 points and 14 points. Because our equipment presents a spot of 0.035cm² and the dose used was 3 J/cm², we concluded that each treatment point corresponds to 0.1 joule and, as the total number of applications on the thoracic wall was 7 points, the final energy was 0.7 joule. After that, we applied irradiation of 0.7 joule at 1 point on the inflammation spot, adding an experimental group to the sample which received a local treatment of 21 J/cm².

In the analysis result the energy of 0.7 joule applied at 1 point (21 J/cm²) showed similar or even better results than at 7 points (3 J/cm²) which surrounded the pleurisy-induced site. A significant disparity was noticed in the exudate volume in the group irradiated at 1 point with local treatment when compared to the laser group with 7 points in which the final energy dose was exactly the same. Thus, the local application of energy on the inflammatory
The transmigration of neutrophils to injured tissues is a precocious phenomenon of the repair process. It occurs right after the signalization activity mediated by congregated neutrophils. Cytokines (TNF-α, IL-1) act on the endothelial cell receptors inducing NO and cytokines production and cellular adhesion molecule expression in neutrophils.

The quantity of TNF-α (fig. 3B) in the pleural cavity of the 3 points group demonstrated a similarity between this study and the findings on protein concentration (fig. 1A), total leukocytes (fig. 1C) and neutrophils (fig. 1D), where we did not find a significant variation in the parameters in low doses of LLLT energy. This result gives a dose-dependent effect to this experimental model based on the quantity of energy deposited over the inflamed tissue. Probably the results observed in this group could be related to the fact that LLLT application was done around to the carrageenan injection point.

Shinomiya et al. (2001) reported earlier that TNF-α, IL-1, IL-6 were sequentially produced in the exudates of rats with carrageenan-induced pleurisy during the early stage of pleurisy. These chemokines in the inflammatory site cause further chemotaxis to attract granulocytes and monocytes. And the migration of leukocytes, in turn, produces further cytokines and other mediators [36].
The generation of TNFα, IL-6 and MCP-1 in the pleural exudates during the rapid increase in exudate volumes and leukocyte number suggest the involvement of these cytokines in triggering the inflammatory reaction and causing the subsequent responses such as neutrophil infiltration [6]. The cell types responsible for the production of these cytokines are probably both resident cells, mainly monocytes, and migrated neutrophils, because these cytokine levels were rapidly increased almost in parallel with the marked neutrophil influx [7], evincing in our study a moderate correlation between TNF-α and neutrophil infiltration (table 2).

Whether LLLT can modulate TNF-α in different animal models has been unclear, as previous studies did not find a reduction in TNF-α levels after high doses of LLLT (0.22 joule) [18]. Our study shows that LLLT reduces TNF-α levels with total energy dose higher than 2.1 joule, contradicting the findings of Aimbire et al. (2006) [18].

Non-steroidal anti-inflammatory drugs have been used as remedies for these inflammatory diseases, but several reports have warned that indomethacin increases the production of pro-inflammatory cytokines, such as TNF-α and IL-1, but suppresses IL-6 and IL-8 in the inflammatory exudates, and that the addition of PGE_{2} or PGI_{2} reverse these effects[36].

Recent studies, such as Bjordal et al. (2006) which investigated in situ if LLLT has an anti-inflammatory effect on activated tendinitis of the human Achilles tendon, concluded that PGE_{2} concentrations were significantly reduced after
active LLLT compared to concentrations before treatment [37]. Albertini et al. (2007) verified recently that LLLT with a wavelength of either 660 or 684 nm (30 mW - 7.5 J/cm²) diminished the formation of edema and the expression of COX-2 mRNA decreased in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation [23], suggesting a subsequent reduction in production of PGE₂.

Some studies suggest that PGE₂ regulates the cytokine production, such as IL-10, which then suppresses TNF-α production, while another study argues there is no involvement of IL-10 in the inhibition of TNF-α production [36]. In this research we did not evaluate the concentration of PGE₂, but we did not find any correlation between IL-10 and TNF-α and hence the action of laser does not seem to involve IL-10.

Karu et al. (2005) investigated the possible regulatory role of NO in the mechanism of cell attachment after LLLT and proposed the existence of signaling pathways between cell mitochondria, the plasma membrane and the nucleus that are activated by visible to near-infrared radiation [38]. Sakaguchi et al. (2006) demonstrated that the non selective NO inhibitor shows an anti-inflammatory effect and whether the combination of a NOS inhibitor and COX inhibitor exerts a synergistic anti-inflammatory effect on acute inflammation such as rat carrageenan-induced pleurisy. The combination of NOS and COX inhibitors showed greater decrease of the exudate volume (43%), leukocyte infiltration (31%) and exudate NOx level (37%). In our study we achieved a reduction of 67% in the exudate volume, 50% in the leukocyte migration and
42% in the NOx of the pleural exudate after the application of LLLT at one point with 21 J/cm². It is likely that LLLT causes the inhibition of NO production, resulting in the potentiation of anti-inflammatory effects with greater results than the combination of NOS and COX inhibitors.
2.6 Conclusion

We observed a distinct dose-response pattern for the anti-inflammatory effects of LLLT, which were number of points, dose, and total energy delivered in rat pleurisy model induced by carrageenan. The quantity of energy seems to be more determinant than the number of points irradiated with laser. Therefore, the healing of the wound in response to local application of LLLT could be an important mechanism which contributes to the results observed in the present report. Moreover, any treatment pattern and quantity of luminous energy can reduce an important volume of the exudate to the pleural cavity, but the local application of energy is more efficient than dividing it around the inflammation site.

The LLLT irradiation with total energy lower than 2.1 joules reduced only NO, IL-6, MCP-1 and IL-10, while an energy of 2.1 joules reduced all variables analyzed in this study, independently of the treatment pattern. The neutrophil migration had a straight correlation with the TNF-α and NO concentration released in the inflammation site, evidencing that the mechanism of the anti-inflammatory action of the laser irradiation can be through the TNF-α or NO. Our results confirm the anti-inflammatory effects of LLLT suggesting it as a clinical alternative to anti-inflammatory drugs.

However, many questions regarding molecular about and cellular mechanisms by which the cytokines exert these effects after LLLT irradiation remain to be answered.
2.7 References


2.8 Legends

**Table 1.** Protocol of laser radiation

**Table 2.** Correlation between neutrophil cell migration with the study variables

**Figure 1.** Effect of low-power laser on the total proteins concentration (A), exudate volume (B), total leukocytes (C) and polymorphonuclears (PMNs) (D) that accumulated in pleural cavity after 4 h of carrageenan injection on the saline, carrageenan (Cg) and laser groups. The Cg+laser groups were irradiated at 1, 2 e 3 h after induction of the pleurisy with 3J/cm$^2$ or 21J/cm$^2$. The groups with 3 J/cm$^2$ received the irradiation at 3 points (9J/cm$^2$), 7 points (21J/cm$^2$), 14 points (42J/cm$^2$) per session and the group with 21J/cm$^2$ received it at 1 point per session. Results are expressed as means ± SEM of 8 animals. *P<0.05 vs carrageenan group. **P<0.05 1 point vs 7 points.

**Figure 2.** Effect of low-power laser on the nitric oxide (NO) (A), interleukin (IL)-6 (B) and monocyte chemoattractant protein-1 (MCP-1) (C) that accumulated in pleural cavity after 4 h of carrageenan injection on the saline, carrageenan (Cg) and laser groups. The Cg+laser groups were irradiated at 1, 2 e 3 h after induction of the pleurisy with 3J/cm$^2$ or 21J/cm$^2$. The groups with 3 J/cm$^2$ received it at 3 points (9J/cm$^2$), 7 points (21J/cm$^2$), 14 points (42J/cm$^2$) per session and the group with 21J/cm$^2$ received it at 1 point per session. Results are expressed as means ± SEM of 8 animals. *P<0.05 vs carrageenan group.
Figure 3. Effect of low-power laser on the interleukin (IL)-10 (A) and tumor necrosis factor (TNFα) (B) that accumulated in pleural cavity after 4 h of carrageenan injection on the saline, carrageenan (Cg) and laser groups. The Cg+laser groups were irradiated at 1, 2 e 3 h after induction of the pleurisy with 3J/cm² or 21J/cm². The groups with 3 J/cm² received it at 3 points (9J/cm²), 7 points (21J/cm²),14 points (42J/cm²) per session and the group with 21J/cm² received it at 1 ponto per session. Results are expressed as means ± SEM of 8 animals. *P<0.05 vs carrageenan group.
Table 1. Protocol of laser radiation

<table>
<thead>
<tr>
<th>Groups</th>
<th>1 Point</th>
<th>3 Points</th>
<th>7 Points</th>
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<tr>
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<td>21</td>
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<td>3</td>
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<tr>
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<tr>
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<td>21</td>
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<td>21</td>
<td>42</td>
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<tr>
<td>Total energy per point (Joules)</td>
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<td>Total energy per treatment (Joules)</td>
<td>0.7</td>
<td>0.3</td>
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Table 2. Correlation between neutrophil cell migration with the study variables

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<td>Nitric oxide</td>
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<tr>
<td>IL-6</td>
<td>0,008*</td>
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<td>MCP-1</td>
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<td>IL-10</td>
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* Statistical significance
Figure 1
Figure 2

A. Graph showing NO (nmol/cavity) levels with different treatments:
- Saline
- Cg
- 1 point
- 3 points
- 7 points
- 14 points

Treatments:
- 21 J/cm² (0.7 J)
- 3 J/cm² (0.1 J)

B. Graph showing IL-6 (pg/cavity) levels:
- Saline
- Cg
- 1 point
- 3 points
- 7 points
- 14 points

C. Graph showing MCP-1 (pg/cavity) levels:
- Saline
- Cg
- 1 point
- 3 points
- 7 points
- 14 points

Legend:
- * indicates statistical significance.
Figure 3
3. CONSIDERAÇÕES FINAIS

A inflamação é uma resposta inespecífica do corpo as diferentes formas de agressão tecidual sendo um mecanismo protetor essencial para o início do processo de reparo.

Intervenções farmacêuticas incluindo drogas antiinflamatórias não-esteróides (NSAID) e injeções de esteróides são terapias comumente usadas para aliviar a dor e bloquear a inflamação em um curto espaço de tempo. O pequeno tempo de ação destas drogas, os mecanismos de ação biológica e as ótimas doses são frequentemente investigados em estudos de cultura de células e modelos animais. O modelo de pleurisia é um dos mais utilizado para estes propósitos pela facilidade de coletar o exsudato inflamatório e assim avaliar os mediadores e os mecanismos biológicos de ação de terapias antiinflamatórias.

Assim, intervenções terapêuticas não farmacológicas, como o LLLT apresentam a possibilidade de um promissor e efetivo recurso alternativo para as drogas antiinflamatórias e analgésicas.

Nosso estudo, confirmou o efeito antiinflamatório do LLLT no clássico modelo de pleurisia em ratos com a avaliação de diversos mediadores inflamatórios de fase aguda. Nossos resultados incrementam a base do conhecimento para o desenvolvimento e otimização do laser como uma alternativa clínica para drogas antiinflamatórias. Entretanto, muitas questões
recaem sobre os efeitos a longo prazo e a dependência da dose de energia para se observar os efeitos antiinflamatórios esperados.

Mais de 40 anos passaram desde a primeira apresentação dos efeitos biológicos do laser atémico. O tratamento com laser terapêutico passou assim rapidamente através de sua infância, adolescência e maturidade precoce e está agora na meia-idade. O debate inicial recaía sobre a eficiência do laser como agente terapêutico, porém evidências científicas e o seu gradual uso provaram a sua eficácia. Pesquisas atuais fortalecem o estado de maturidade do laser tanto de forma quantitativa como qualitativa. Hoje em dia pode-se facilmente dizer que o laser terapêutico tem efeitos biológicos importantes e satisfatórios. O que ainda não está claro são as ótimas doses, intervalo de tratamentos e muitos detalhes na complicada cadeia de mecanismos moleculares e celulares. Tem-se também um grau de incerteza quanto ao ótimo comprimento de onda a ser utilizado para os diferentes tecidos.

Extrapolar as pesquisas celulares e em animais para tratamentos clínicos em humanos não é ainda uma tarefa fácil, porém o laser terapêutico apresenta uma utilização constante na prática terapêutica nas crescentes áreas da medicina, fisioterapia, odontologia e veterinária.
4. REFERÊNCIAS BIBLIOGRÁFICAS


8. Campana VR, Moya M, Gavotto A, Soriano F, Juri HO, Palma JA. Effects of diclofenac sodium and HeNe laser irradiation on plasmatic fibrinogen


5. ANEXO 1: DOCUMENTO DE CONFIRMAÇÃO DE SUBMISSÃO
Effect Anti-inflammatory of Low-Level Laser Therapy (660 nm) in the Early Phase in Carrageenan-Induced Pleurisy in Rat

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Original Article 11-17-2007

Effect Anti-inflammatory of Low-Level Laser Therapy (660 nm) in the Early Phase in Carrageenan-Induced Pleurisy in Rat

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ABSTRACT

Background and Objective: In the classic model of pleurisy there is little evidence of anti-inflammatory effects from LLLT and dosage characteristic, such as wavelength, total energy, number and pattern of treatment, with the necessity for more studies. The purpose of this study was to investigate the potential effect of low level laser therapy (LLLT) on modulating the pro-inflammatory and anti-inflammatory mediators of acute inflammation rat pleurisy model.

Study Design/Material and Methods: A sample of 48 female Wistar rats were divided into control and experiential groups. An inflammation was induced by carrageenan (0.2mL) injected into the pleural cavity. At 1, 2, and 3 h after induction the continuous wave (20 mW) diode laser of the InGaAlP (660nm) type was used in laser groups with different doses and treatment patterns, and with a total energy delivered of 0.9, 2.1 and 4.2 joules. At 4 h after the exudate volume, total and differential leukocytes, protein concentration, NO, IL-6, IL-10, TNF-α and MCP-1 were measured from the aspirated liquid.

Results: Any treatment pattern and quantity of energy can reduce a significantly volume of the exudate ($P<0.05$). The final energy lower than 2.1 joules reduces significantly ($P<0.05$) only NO, IL-6, MCP-1 and IL-10, while 2.1 joules reduce significantly all variables independently of the treatment pattern. The neutrophil migration has straight correlation with the TNF-α ($r=0.551$) and NO ($r=0.549$) concentration.
**Conclusions:** LLLT-660nm induced an anti-inflammatory effect characterized by inhibition of both total and differential leukocyte influx, exudation, total protein, NO, IL-6, MCP-1, IL-10 and TNF-α, but keeping a highly dose-dependent outline. Under these conditions, laser with 2.1 joules was more effective than 0.9 joule and 4.2 joules.

**Key Words:** Inflammation, LLLT, Pleurisy
INTRODUCTION

Inflammation is a protective process essential for the preservation of the integrity of the organisms in the event of physical, chemical and infectious damage [1]. Acute inflammation is characterized by the classical signs of pain, heat, redness and swelling, involving a complex series of events including vasodilatation, increased permeability, fluid exudation and migration of leukocytes to the site of the inflammation [2].

Carrageenan-induced pleurisy is a well-characterized experimental model of inflammation that permits the quantification and correlation of both exudate and cellular migration with changes of other inflammatory parameters [3]. The major characteristic of this model in the rat is the biphasic profile of this inflammatory reaction, where early (4 h) and late (48 h) phases of both cell migration and exudation are clearly observed [4]. Thus, this model constitutes a biologic system suitable for the investigation of possible correlation occurring between cell migration, fluid leakage, nitric oxide (NO), chemokine, pro-inflammatory and anti-inflammatory cytokines.

One of the early cellular events in inflammation is the margination of leukocytes, primarily neutrophils. In addition, NO plays an important role in inflammation such as plasma exudation and leukocyte infiltration. The NO synthase (NOS) inhibitors can reverse several classic inflammatory symptoms [5].
The maintenance of leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes and the endothelium. These events are mediated by the generation of early response cytokines, e.g., interleukin (IL)-1 and tumor necrosis factor (TNF-α), the expression of cell-surface adhesion molecules and the production of chemotactic molecules, such as chemokines [6].

Chemokines are a family of structurally related glycoproteins with potent leukocyte activation and/or chemotactic activity. Most chemokines are produced in response to a variety of inflammatory stimuli, including the early-response cytokines, TNF-α, IL-1, C5a, leukotriene B4 and interferons [7]. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that has demonstrated to attract monocytes and neutrophils both in vitro and in vivo and appears to be a key part of leukocyte emigration, serving as a promoter of the transition from leukocyte rolling to adhesion on the endothelial surface [8].

Representative inflammatory cytokines include TNF-α, IL-1, IL-6, and IL-8. The TNF-α and IL-1 have been revealed to have many overlapping biological activities in the inflammatory reaction: both cause fever, accumulation of neutrophils in local tissues, induction of vascular adhesion molecule and stimulation of acute phase protein synthesis [9]. Some of these responses by TNF-α and IL-1 are now known to be mediated by other inflammatory mediators or secondarily induced cytokines such as IL-6 [10].
The IL-6 is widely produced by several cells such as fibroblasts, endothelial cells, keratinocytes, monocytes, T cells, mast and tumour cell lines, and cells of neural origin. Thus, IL-6 is often used as a marker for systemic activation of pro-inflammatory cytokines [11]. However, evidence also indicates that, under certain conditions, IL-6 has both pro-inflammatory and anti-inflammatory properties [12]. Apart from this, there is also experimental evidence indicating that IL-6 down-regulates the synthesis of IL-1 and TNF-α [13].

The IL-10 is known as the most important anti-inflammatory cytokine found within the human immune response. Previous data has shown that IL-10 is capable of blocking the inflammatory response induced by several inflammatory stimuli in different models [11].

The conventionally used therapies for inflammation; non-steroidal anti-inflammatory drugs (NSAID’s), have very important role in managing pain and acute inflammatory conditions [14], though with rather discouraging profile of side effects [13]. Even the anti-inflammatory drugs, cyclo-oxygenase 2 (COX-2) inhibitors, are not devoid of adverse effects [15].

Despite this, alternative physical techniques such as low-level laser therapy (LLLT) has been used clinically, among other indications, for its proposed anti-inflammatory effects, pain relief and acceleration of the regeneration of damaged tissues [16]. Although in the past decade several studies have examined the effects of LLLT, the treatment protocols used included enormous variations in parameters (such as wavelengths, energy and power...
densities, wave modes, number of treatments), which make it difficult to assess the optimum treatment parameters in each case [17].

However, more recent studies have been able to find some dose-dependent effects on TNF-α [18] and prostaglandin E₂ level [19], besides reduction of the oedema [20,21], neutrophils migration [21], nitric oxide synthase (iNOS) expression [22] and COX-2 mRNA expression [23] in the different inflammatory experimental models after LLLT. The efficacy of LLLT radiation as an anti-inflammatory therapy is controversial. The interaction of radiation with the biological system occurs at the cellular level, but the mechanisms involved are yet unknown.

Therefore, the present study was designed to explore the potential effects of LLLT applied at different points with dose variation on the modulation of the pro-inflammatory and anti-inflammatory mediators of acute inflammation in carrageenan-induced pleurisy in rat model.
MATERIALS AND METHODS

Animals

Adult female Wistar rats (*Rattus norvegicus*) bred in our laboratory (around 3-4 months old, weighing 180g-220g) were used, all of the same ancestry and socialization with free access to food and water. Animals were divided into six groups composed of eight rats each. The rats were maintained in accordance with the “Guiding Principles in the Care and Use of Animals” approved by the Council of the American Physiological Society.

Carrageenan-induced pleurisy

Rats were anesthetized with a mix of Ketamina (80mg/Kg) and Xilazina (12mg/Kg). Saline 0.2 mL (saline group) or saline containing 2% λ-carrageenan (Cg) 0.2mL (carrageenan group and Cg + laser groups) was injected into the pleural cavity at the level of the sixth left intercostal space. At 4 h after the intrathoracic injection, the animals were sacrificed with decaptation and the pleural cavity was opened. The liquid that had accumulated in the pleural cavity was washed with 2.0 mL of sterile saline solution (NaCl 0.9%) containing 1% EDTA in the aspirated liquid. All exudate contaminated with red blood cells was discarded [2].

Exudate Analysis

The volume of the exudates was measured and the result expressed by subtracting the volume injected into the pleural cavity (2.0 mL of solution) from the total volume aspirated. Total leukocytes were diluted in Thoma solution
(1:20) and counted in a Neubauer chamber using light microscopy. Cytological slide smears stained with May-Grünwald/Giemsa were used for differential leukocyte counts in a light microscope [24]. The pleural liquid removed from the rats was centrifuged at 1200 x g for 10 min and the protein concentration measured by the Biuret technique. NO is a very unstable radical, rapidly metabolized from nitrate to nitrite in the presence of oxygen [25]. Therefore, the amount of NO in the exudate was analyzed using the Griess reaction that measured nitrite, as previously described [26]. The frozen cell-free supernatant of the pleural fluid was thawed, and IL-6, IL-10, TNF-α and MCP-1 were determined using a rat enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Camarillo, CA).

**Laser Irradiation**

The continuous wave diode laser of InGaAlP type (model Endophoton-LLT-010 - KLD Biosistemas Equipamentos Eletrônicos Ltda) with an output power of 20mW and wavelength of 660 nm (visible red) was used. The spot size was 0.035 cm² and the power density was 0.571 W/cm². Table 1 shows the laser parameters used in our experiment. Different doses were used for each of the four groups: group with 1 point (Cg + 1 point laser with 21 J/cm²), group with 3 points (Cg + 3 points laser with 3 J/cm²), group with 7 points (Cg + 7 points laser with 3 J/cm²) and group with 14 points (Cg + 14 points laser with 3 J/cm²). All the experimental groups were irradiated with LLLT from a spot in contact with the skin at 1, 2 and 3 h after pleurisy induction. Thus, the total energy delivered from the three treatment sessions was 2.1, 0.9, 2.1 and 4.2 joules, respectively. The animals received the irradiation at the left thoracic
wall with different treatment methods according to the group: group of 1 point was irradiated with LLLT where the pleurisy was induced; group of 3 points received LLLT around the induction area; group of 7 points was irradiated, in which 1 point was on the induction local and 6 points were symmetrically distributed around the area; in group of 14 points they were distributed in a craniocaudal direction of crescent 2, 3, 4 and 5 points involving all thoracic wall.

Statistical Analysis

The results were evaluated statistically by analysis of variance (ANOVA) with LSD test post hoc using SPSS (Statistical Package for the Social Sciences) 12.0 software and were expressed as the means ± standard error of means (SEM). The correlation between the variables and the neutrophil infiltration into the pleural cavity was analyzed by using Pearson Correlation Linear. The level of statistical significance was defined as $P < 0.05$. 

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RESULTS

According to the analysis of this study, the rats injected with carrageenan in the pleural space developed an acute pleurisy after 4 h with a significant increase in all variables when compared to the saline group. This episode shows that carrageenan causes a potential inflammatory reaction.

Figure 1 shows the protective effect of LLLT on the inflammatory parameters, such as: protein concentration, exudate volume, the amount of leukocytes and neutrophils in the pleural liquid of the saline group, carrageenan and in the four groups irradiated with different laser doses and treatment methods.

Protein
Protein concentration (fig 1A) showed a significant decrease ($P<0.05$) in laser groups with 1 point ($1.01 \pm 0.11$), 7 points ($0.94 \pm 0.03$) and 14 points ($1.13 \pm 0.10$) when compared to the carrageenan group ($1.42 \pm 0.04$). It was observed that laser group with 3 points ($1.47 \pm 0.07$) did not modify the concentration of proteins that had migrated to the pleural cavity.

Exudate volume
In relation to the exudate volume (fig 1B) collected 4 h after the pleurisy induction, it was observed a significant reduction in the four groups irradiated with LLLT when compared to the carrageenan group (1 point: $0.32 \pm 0.03$; 3 points: $0.68 \pm 0.07$; 7 points: $0.60 \pm 0.08$ and 14 points: $0.52 \pm 0.06$ vs Cg: $0.98 \pm 0.04$, $P<0.001$). Besides that, laser group with 1 point (2.1 joules) was
more effective than laser group with 7 points (2,1 joules) in reducing pleural exudate volume (P=0.002).

**Total leukocytes**

All groups irradiated with LLLT, except the group with 3 points, showed a significant decrease in the total number of leukocytes when compared to the carrageenan group (1 point: 35.03 ± 4.07; 3 points: 63.25 ± 5.69; 7 points: 37.21 ± 2.35 and 14 points: 46.22 ± 3.81 vs Cg: 70.67 ± 3.90, P=0.001), (fig 1C). Based on this, the group that received a final dose of 0.9 joule (group 3 points) did not demonstrate reduction in the leukocyte cells infiltration to the inflammatory site of the lesion (P=0.176).

**Neutrophils**

The reduction in the inflammatory acute phase response, represented by a lower migration of polymorphonuclear-neutrophil cells (PMNs), showed a mean of 50% reduction of these cells in the groups 1, 7 and 14 points that received higher or equal to 2.1 joules energy when compared to the carrageenan group or the laser group with 3 points (1 point: 31.09 ± 3.60; 7 points: 31.61 ± 1.90 and 14 points: 36.63 ± 4.20 vs Cg: 61.11 ± 1.76, P<0.001). The group 3 points did not demonstrate significant reduction in the neutrophil infiltration (58.30 ± 5.60, P=0.568), (fig 1 D).

**NO**

The concentration of nitric oxide (NO) in the pleural cavity (fig 2A) presented a significant reduction in the groups irradiated with laser when compared to the
carrageenan group (1 point: 49.40 ± 2.53; 3 points: 55.25 ± 2.49; 7 points: 49.44 ± 2.22 and 14 points: 55.70 ± 2.88 vs Cg: 86.02 ± 3.86, P=0.001). It is noticed that in laser groups with 1 and 7 points the reduction approximates the basal level values of the saline group (49.78 ± 5.63).

**IL-6**

In relation to the pro-inflammatory cytokine, it was also observed a reduction in the concentration of IL-6 in the irradiated groups after the application of energy when compared to the one that received only carrageenan (1 point: 7298.82 ± 178.99, 3 points: 8596.92 ± 231.76, 7 points: 7955.80 ± 478.74 and 14 points: 14587.74 ± 2613.69 vs Cg: 25357.77 ± 5451.93, P<0.001). This important mediator presented sensibility to LLLT irradiation independently of the chosen treatment method and energy dose (fig 2B).

**MCP-1**

The chemokine MCP-1 had a significant decrease in laser groups with 1 point (1131.25 ± 175.74, P=0.019), 3 points (1228 ± 209.39, P=0.025) and 7 points (1434.50 ± 171.89, P=0.046) when compared to the carrageenan group (3007.25 ± 1067.61), but in laser group with 14 points (2578.85 ± 741.09, P=0.591), where the total final energy was equal to 4.2 joules, a significant reduction was not verified in the measured values from the pleural liquid count (fig 2C).
IL-10

It was verified a significant reduction of IL-10 concentration in the irradiated groups with 1 point (560.05 ± 70.18, \( P=0.002 \)), 3 points (690.76 ± 114.44, \( P=0.007 \)) and 7 points (855.87 ± 239.78, \( P=0.049 \)) when compared to the carrageenan group (1436.15 ± 305.68). In the group with 14 points (957.42 ± 216.25), IL-10 did not suffer any modification on the measured values showing that a more local treatment pattern tends to intervene in the final result (\( P=0.088 \)), (fig 3A).

TNFα

The concentration of TNF-α (fig 3B) decreased significantly in the LLLT irradiated groups with 1 point (523.90 ± 122.21), 7 points (298.05 ± 69.21) and 14 points (509.05 ± 146.07) when compared to the carrageenan group (1535.20 ± 402.81, \( P<0.001 \)). The laser group with 3 points did not present a decrease in TNF-α levels when compared to the carrageenan group (1206.27 ± 359.99 vs 1535.20 ± 402.81, \( P=0.336 \)).

Correlation between neutrophil cell migration with the study variables

At 4 h after carrageenan-induced pleurisy it was observed a significant increase in neutrophil migration to the pleural cavity in the carrageenan group when compared to the saline group (fig 1D). With regard to all variables correlated to the neutrophil infiltration in the pleural cavity, only MCP-1 (\( P=0.060 \)) and IL-10 (\( P=0.092 \)) did not present a significant statistics-level. In our experimental model, the first inflammatory mediator to show a main
correlation with neutrophil infiltration was TNF-α ($r=0.551$) and the second was NO ($r=0.549$). The results are summarized in Table 2.
DISCUSSION

Immediately after an acute injury event the body, in response to the disruption of vascular integrity, soft tissue, connective tissue and neurological processes, initiates a series of biological responses. The inflammatory reaction consists of both vascular and cellular events. Injury responsive components such as mast cells, bradykinins and prostaglandins are activated along with the vascular responses and cellular membrane reactions. All of these combined processes and events are represented by the symptoms of edema, inflammation, pain and functional debility [4,11,27].

The carrageenan is a polysaccharide frequently used to induce acute inflammatory reaction in animal experimental models. Winter (1962) introduced the use of carrageenan as an irritant agent of rat paw edema, becoming the first and most popular method to evaluate new anti-inflammatory therapies with a hydroplethysmometer for inflammed paw volume measurement [28].

The paw edema induced by carrageenan is an useful method to evaluate acute inflammation, since edema peak occurs within 3 to 5 hours [29]. However, this technique has some limitations on measuring inflammatory cells, proteins and chemical mediators, once it is not possible to extract the inflammatory exudate.
Despite this, carrageenan-induced pleurisy in rats permits quantifying the volume and protein concentration of the exudate formed, besides the evaluation of the inflammatory cell migration to the pleural cavity [30]. This kind of pleurisy is used to investigate acute inflammation pathophysiology and also to evaluate anti-inflammatory therapies efficacy.

The inflammatory response that occurs after carrageenan injection into the pleural cavity is characterized by cellular infiltration, mainly composed of neutrophils (approx. 90%) and to a lesser extent of monocyte/macrophages [27].

Phototherapy with LLLT has achieved high success in recent years. It has gained the approval of the U.S. Food and Drug Administration (FDA) and is gradually earning its place in mainstream medical practice [33].

Many studies have suggested advantages of the biomodulatory effects of LLLT on the inflammatory process, wound healing and pain relief, but the basic mechanism of these effects remains unclear. LLLT has been shown to reduce the duration of acute inflammation and pain, just as to accelerate tissue repair in tendon and muscle injuries [32].

Laser therapy uses different wavelengths of the visible and near-infrared spectra incluind HeNe (632.8nm), GaAlAs (780-870 nm), InGaAlP (630-685 nm) and GaAs (904 nm) [34,35]. Many researchers have attempted to understand the action of LLLT, as well as to determine the most appropriate
wavelength, period of irradiation, number of treatments, energy density and energy total.

Albertini et al. (2004) have demonstrated that LLLT with diode Ga-Al-As (650 nm) employed with a continuous power of 2.5 mW and energy density of 2.5 J/cm² can reduce edema caused by carrageenan-inflammation in rat paw. In the anti-inflammatory response it has shown similar performance to the sodium diclofenac when intraperitoneally administrated with a dose of 1 mg/kg. In this study the authors verified that LLLT did not inhibit the carrageenan-induced edema in adrenalectomized rat models, suggesting that the mechanism of action can be related with the hypothalamo-hypophyseal-adrenal endocrine system and, as a consequence, with the release of endogenous corticoids [20].

Recently, Martins et al. (2005) described an inhibitory effect of LLLT (650 nm – 2.5 mW) on leukocyte (neutrophil) migration to the pleural space in the carrageenan-induced pleurisy in mice with energy density of 1 J/cm², 2.5 J/cm² and 5 J/cm², applied on the 1st, 2nd and 3rd hour after the inflammatory induction. It was observed that the total dose of 7.5 J/cm² (0.6 joules) was more effective [21].

Based on these studies that have evaluated the anti-inflammatory action of LLLT, the dosage of 2.5 J/cm² with power of 2.5 mW has demonstrated to reduce edema and inflamed cells when applied local radiation on the inflamed site at 1, 2 and 3 h after the carrageenan induction, knowing to
be dealing with experimental models in which only one point of laser would be sufficient to cover the rat paw or the mouse thoracic wall.

In our study it was used a rat pleurisy model but we had no references about points quantity needed to irradiate the most toracic wall of these animals. Thus, to accomplish our study, we used a continuous-wave power of 20 mW at energy dose of 3 J/cm² in 3 groups with 8 animals. These groups were irradiated at 3, 7 and 14 points distributed unequally (14 points simetrically distributed within the rat’s thoracic wall; 7 points and 3 points located on the pleurisy induction site – check methods).

The analysis of cell counts (fig. 1D) showed a more significant decrease of neutrophil infiltration to the pleural space in the group irradiated at 7 points with 3 J/cm² in relation to the groups irradiated at 3 points and 14 points. Because our equipment presents a spot of 0.035cm² and the dose used was 3 J/cm², we concluded that each treatment point corresponds to 0.1 joule and, as the total number of applications on the thoracic wall was 7 points, the final energy was 0.7 joule. After that, we applied irradiation of 0.7 joule at 1 point on the inflammation spot, adding an experimental group to the sample which received a local treatment of 21 J/cm².

In the analysis result the energy of 0.7 joule applied at 1 point (21 J/cm²) showed similar or even better results than at 7 points (3 J/cm²) which surrounded the pleurisy-induced site. It was noticed a significant disparity in the exudate volume in the group irradiated at 1 point with local treatment.
when compared to the laser group with 7 points in which the final energy dose was exactly the same. Thus, the local application of energy on the inflammatory process seems to have a better effect on reducing the exudate volume instead of spreading it. The mechanism of this process is still unclear.

The transmigration of neutrophils to injured tissues is a precocious phenomenon of the repair process. It occurs right after the signalization activity mediated by congregate neutrophils. Cytokines (TNF-α, IL-1) act on the endothelial cell receptors inducing NO and cytokines production and cellular adhesion molecule expression in neutrophils.

The quantity of TNF-α (fig. 3B) in the pleural cavity of the 3 points group demonstrated a similarity between this study and the findings on protein concentration (fig. 1A), total leukocytes (fig. 1C) and neutrophils (fig. 1D), where we did not find a significant variation in the parameters in low doses of LLLT energy. This result gives a dose-dependent effect to this experimental model based on the quantity of energy deposited over the inflamed tissue.

Shinomiya et al. (2001) reported earlier that TNF-α, IL-1, IL-6 were sequentially produced in the exudates of rats with carrageenin-induced pleurisy during the early stage of pleurisy. These chemokines in the inflammatory site cause further chemotaxis to attract granulocytes and monocytes. And the migration of leukocytes, in turn, produces further cytokines and other mediators [36].
The generation of TNFα, IL-6 and MCP-1 in the pleural exudates during the rapid increase in exudate volumes and leukocyte number suggest the involvement of these cytokines in triggering the inflammatory reaction and causing the subsequent responses such as neutrophil infiltration [6]. The cell types responsible for the production of these cytokines are probably both resident cells, mainly monocytes, and migrated neutrophils, because these cytokine levels were rapidly increased almost in parallel with the marked neutrophil influx [7], evincing in our study a moderate correlation between TNF-α and neutrophil infiltration (table 2).

Whether LLLT can modulate TNF-α in different animal models has been unclear, as previous studies did not find a reduction in TNF-α levels after high doses of LLLT (0.22 joule) [18]. Our study shows that LLLT reduces TNF-α levels with total energy dose higher than 2.1 joule, contradicting the findings of Aimbire et al. (2006) [18].

Non-steroidal anti-inflammatory drugs have been used as remedies for these inflammatory diseases, but several reports have warned that indomethacin increases the production of pro-inflammatory cytokines, such as TNF-α and IL-1, but suppresses IL-6 and IL-8 in the inflammatory exudates, and that the addition of PGE₂ or PGI₂ reverse these effects[36].

Recent studies, such as Bjordal et al. (2006) which investigated in situ if LLLT has an anti-inflammatory effect on activated tendinitis of the human Achilles tendon, concluded that PGE₂ concentrations were significantly reduced after
active LLLT compared to concentrations before treatment [37]. Albertini et al. (2007) verified recently that LLLT with a wavelength of either 660 or 684 nm (30 mW - 7,5J/cm²) diminished the formation of edema and the expression of COX-2 mRNA decreased in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation [23], suggesting a subsequent reduction in production of PGE₂.

Some studies suggest that PGE₂ regulates the cytokine production, such as IL-10, which then suppresses TNF-α production, while another study argues there is no involvement of IL-10 in the inhibition of TNF-α production [36]. In this research we did not evaluate the concentration of PGE₂, but we did not find any correlation between IL-10 and TNF-α and hence the action of laser does not seem to involve IL-10.

Karu et al. (2005) investigated the possible regulatory role of NO in the mechanism of cell attachment after LLLT and proposed the existence of signaling pathways between cell mitochondria, the plasma membrane and the nucleus that are activated by visible to near-infrared radiation [38]. Sakaguchi et al. (2006) demonstrated that the non selective NO inhibitor shows an anti-inflammatory effect and whether the combination of a NOS inhibitor and COX inhibitor exerts a synergistic anti-inflammatory effect on acute inflammation such as rat carrageenan-induced pleurisy. The combination of NOS and COX inhibitors showed greater decrease of the exudate volume (43%), leukocyte infiltration (31%) and exudate NOₓ level (37%). In our study we achieved a reduction of 67% in the exudate volume, 50% in the leukocyte migration and
42% in the NOx of the pleural exudate after the application of LLLT at one point with 21 J/cm². It is likely that LLLT causes the inhibition of NO production, resulting in the potentiation of anti-inflammatory effects with greater results than the combination of NOS and COX inhibitors.
CONCLUSION

We observed a distinct dose-response pattern for the anti-inflammatory effects of LLLT, which were number of points, dose, and total energy delivered in rat pleurisy model induced by carrageenan. The quantity of energy seems to be more determinant than the number of points irradiated with laser. Any treatment pattern and quantity of luminous energy can reduce an important volume of the exudate to the pleural cavity, but the local application of energy is more efficient than dividing it around the inflammation site.

The LLLT irradiation with total energy lower than 2.1 joules reduces only NO, IL-6, MCP-1 and IL-10, while a final energy equals to 2.1 joules reduces all variables analyzed in this study, independently of the treatment pattern. The neutrophil migration has straight correlation with the TNF-α and NO concentration released in the inflammation site, evidencing that the mechanism of the anti-inflammatory action of the laser irradiation can be through the TNF-α or NO. Our results confirm the anti-inflammatory effects of LLLT suggesting it as a clinical alternative to anti-inflammatory drugs.

However, many questions regarding about molecular and cellular mechanisms by which the cytokines exert these effects after LLLT irradiation remain to be answered.
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Effect of low-power laser on the total proteins concentration (A), exudate volume (B), total leukocytes (C) and polymorphonuclears (PMNs) (D) that accumulated in pleural cavity after 4 h of carrageenan injection on the saline, carrageenan (Cg) and laser groups. The Cg+laser groups were irradiated at 1, 2 e 3 h after induction of the pleurisy with 3J/cm² or 21J/cm². The groups with 3 J/cm² received the irradiation at 3 points (9J/cm²), 7 points (21J/cm²), 14 points (42J/cm²) per session and the group with 21J/cm² received it at 1 point per session. Results are expressed as means ± SEM of 8 animals. *P<0.05 vs carrageenan group. **P<0.05 1 point vs 7 points.

283x203mm (150 x 150 DPI)
Effect of low-power laser on the nitric oxide (NO) (A), interleukin (IL)-6 (B) and monocyte chemoattractant protein-1 (MCP-1) (C) that accumulated in pleural cavity after 4 h of carrageenan injection on the saline, carrageenan (Cg) and laser groups. The Cg+laser groups were irradiated at 1, 2 e 3 h after induction of the pleurisy with 3J/cm2 or 21J/cm2. The groups with 3 J/cm2 received it at 3 points (9J/cm2), 7 points (21J/cm2), 14 points (42J/cm2) per session and the group with 21J/cm2 received it at 1 point per session. Results are expressed as means ± SEM of 8 animals. *P<0.05 vs carrageenan group.

284x203mm (150 x 150 DPI)
Effect of low-power laser on the interleukin (IL)-10 (A) and tumor necrosis factor (TNFα) (B) that accumulated in pleural cavity after 4 h of carrageenan injection on the saline, carrageenan (Cg) and laser groups. The Cg+laser groups were irradiated at 1, 2 e 3 h after induction of the pleurisy with 3J/cm² or 21J/cm². The groups with 3 J/cm² received it at 3 points (9J/cm²), 7 points (21J/cm²), 14 points (42J/cm²) per session and the group with 21J/cm² received it at 1 ponto per session. Results are expressed as means ± SEM of 8 animals. *P<0.05 vs carrageenan group.
Table 1. Protocol of laser radiation

<table>
<thead>
<tr>
<th>Model</th>
<th>Laser 660 nm + Carrageenan (4h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Output power (mW)</td>
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</tr>
<tr>
<td>Spot size (cm$^2$)</td>
<td>0.035</td>
</tr>
<tr>
<td>Power density (W/cm$^2$)</td>
<td>0.571</td>
</tr>
<tr>
<td>Groups</td>
<td>1 Point 3 Points 7 Points 14 Points</td>
</tr>
<tr>
<td>Energy density per point (J/cm$^2$)</td>
<td>21 3 3 3</td>
</tr>
<tr>
<td>Time per point (sec)</td>
<td>36.75 5.25 5.25 5.25</td>
</tr>
<tr>
<td>Dose per treatment (J/cm$^2$)</td>
<td>21 9 21 42</td>
</tr>
<tr>
<td>Total dose from all three treatments (J/cm$^2$)</td>
<td>63 27 63 126</td>
</tr>
<tr>
<td>Total energy per point (Joules)</td>
<td>0.7 0.1 0.1 0.1</td>
</tr>
<tr>
<td>Total energy per treatment (Joules)</td>
<td>0.7 0.3 0.7 1.4</td>
</tr>
<tr>
<td>Total final energy (Joules)</td>
<td>2.1 0.9 2.1 4.2</td>
</tr>
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</table>
**Table 2.** Correlation between neutrophil cell migration with the study variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level of significance (P)</th>
<th>Pearson correlation (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes</td>
<td>0,000*</td>
<td>0,940</td>
</tr>
<tr>
<td>Total proteins</td>
<td>0,000*</td>
<td>0,845</td>
</tr>
<tr>
<td>Exudate pleural</td>
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<td>0,818</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0,000*</td>
<td>0,551</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>0,000*</td>
<td>0,549</td>
</tr>
<tr>
<td>IL-6</td>
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<td>0,395</td>
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<tr>
<td>MCP-1</td>
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<td>0,286</td>
</tr>
<tr>
<td>IL-10</td>
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<td>0,257</td>
</tr>
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</table>

* Statistical significance