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EFEITOS DO LASER DE BAIXA INTENSIDADE DE 100 mW E 50 mW SOBRE OSTEOARTRITE EXPERIMENTAL

ANA CAROLINA ARARUNA ALVES

SÃO PAULO, SP
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Orientador: Prof. Dr. Paulo de Tarso Camillo de Carvalho

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Título da Dissertação: "Efeitos da radiação laser de baixa potência de 100 e 50 MW e comprimento de onda 808 nm sobre lesões cartilaginosas"

Presidente PROF. DR. PAULO DE TARSO CAMILO DE CARVALHO _____

Membro: PROF. DR. FLAVIO AIMBIRE SOARES DE CARVALHO _____

Membro: PROF. DR. JOSE ANTONIO SILVA JUNIOR _____

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RESUMO

A lesão da cartilagem e a sua destruição são comuns em osteoartrite (OA) e estão associadas com níveis elevados de metaloproteinases de matriz (MMPs), proteinases que podem degradar todos os componentes da matriz extracelular (ECM). O objetivo foi estudar o efeito do laser de baixa intensidade (LBI) com 50mW e 100mW em lesões articulares por meio da análise histopatológica, bem como pela expressão proteíca de metaloproteinases 2 e 9 no lavado articular. Utilizou-se 60 ratos Wistar machos, distribuídos aleatoriamente em 4 grupos de 15 animais, sendo: um grupo controle; um grupo lesão, e dois grupos tratados, um com LBI de 50mW, e outro com LBI de 100mW. Os animais foram submetidos a OA (solução de papaína a 4%) e, no dia da eutanásia, coletou-se o lavado articular, que foi imediatamente centrifugado e o sobrenadante armazenado para análise de expressão protéica por Western Blot. O material foi corado com HE para a descrição histopatológica e Picrosirius Red, para estimar o percentual de fibras colágenas. Como resultado, observou-se que os dois grupos laser foram eficientes na reparação tecidual, diminuindo a expressão de colágeno tipo III e aumentando a do tipo I em todos os tempos experimentais, no entanto, o grupo LBI 50mW foi melhor em relação à redução da metaloproteinase 9 em relação ao grupo LBPI 100mW em 21 dias. Podemos concluir que o LBI 50 mW foi mais eficiente na modulação de metaloproteinases de matriz e reparação do tecido cartilaginoso.

Palavras-Chave: Osteoartrite, Metaloproteinase de Matriz, Colágeno, Laser de Baixa Intensidade.

ABSTRACT

The cartilage damage and destruction are common in osteoarthritis (OA) and are associated with elevated levels of matrix metalloproteinases (MMPs), proteinases that can degrade all components of the extracellular matrix (ECM). The objective was to study the effect of low level laser therapy (LLLT) at 50mW and 100mW in joint damage evaluated by histopathological analysis, and protein expression of metalloproteinases (MMPs) 2 and 9 in the articular lavage. We used 60 male Wistar rats randomly divided into 4 groups of 15 animals each: a control group, an injury group, and two treated groups, one with LLLT 50mW and other with 100mW. The animals underwent OA induction (papain solution 4%) and, on the euthanasia day was collected the articular lavage, which was immediately centrifuged and the supernatant saved for analysis of protein expression by Western blot. The material was stained with hematoxylin and eosin for histopathologic description and Picrosirius Red, to estimate the percentage of collagen fibers. As a result, it was observed that both laser groups were efficient on tissue repair, decreasing the expression of collagen type III and increasing type I at all the experimental times, however, the group LLLT 50mW was better in reducing MMP - 9 in relation to the LLLT 100mW group in 21 days. In conclusion, LLLT 50 mW was more efficient on modulating matrix metalloproteinases and repair of the cartilaginous tissue.

Key Words: Osteoarthritis, Matrix metalloproteinase, Collagen, Low Level Laser Therapy.

ÍNDICE

LISTA DE ABREVIAÇÕES

MEC	**Matriz Extracelular**
TLBI	**Terapia a Laser de Baixa Intensidade**
MMP	**Metaloproteinase de Matriz**
OMS	**Organização Mundial de Saúde**
TNF- α	**Fator de Necrose Tumoral Alfa**
IL – 1	**Interleucina 1**
IL – 6	**Interleucina 3**
AINE	**Antinflamatório não-esteróide**
DNA	**Ácido Desoxirribonucléico**
COX – 2	**Ciclo-oxigenase-2**
GSH	**Glutathione**
NOS	**Sintase Óxido Nitrase**
ATP	**Adenosina Trifosfato**
NO	**Óxido Nítrico**
LBI	**Laser de Baixa Intensidade**
SDS	**Eletroforese em Gel de Poliacrilamida**
PVDF	**Polyvinylidene fluoride Membrane**
BSA	**Albumin bovine serum**
TBS-T	**Tris Buffer Saline**
AO	**Osteoartrite**
EA	**Espaço Articular**
CH	**Cartilagem Hialina**
MA	**Membrana Articular**
LLLT	**Low Level Laser Therapy**
HE	**Hematoxilina e Eosina**

1. CONTEXTUALIZAÇÃO

A cartilagem articular é um tecido conjuntivo especializado, avascular e aneural, composto exclusivamente por células chamadas condrócitos e uma grande quantidade de matriz extracelular (MEC). Os condrócitos são responsáveis pela homeostase deste tecido enquanto a MEC é o componente funcional da cartilagem articular, estruturalmente formada por fibras de colágeno. O colágeno tipo I corresponde por 90% do colágeno total em mamíferos, enquanto o colagéno do tipo III é predominante em tecidos com algum grau de elasticidade (AIGNER; SOEDER; HAAG, 2006; CALVI, 2012).

Degradação e remodelação da matriz extracelular em cartilagem são eventos chave no desenvolvimento da osteoartrite. Esse processo se deve ao aumento da atividade de algumas proteases, particularmente metaloproteinases de matriz (MMPs), que por sua vez, degradando constituintes da MEC podem sintetizar fibras colágenas dos tipos I e III (S. SANDYA, P. R. SUDHAKARAN, 2007; K NAITO, 1999).

As doenças que envolvem a lesão do tecido cartilaginoso estão freqüentemente associadas a um tópico controverso que é a capacidade de reparação da cartilagem articular. Acreditava-se que a cartilagem possuía pouca capacidade de regeneração (HUNZIKER, 2002), porém pesquisadores afirmam que esse tecido é metabolicamente ativo, tendo, portanto, capacidade para regenerar-se (PUGLIESE et al., 2003; BJORDAL et al., 2003; BOS et al., 2008).

O processo de reparo ocorre pela substituição do tecido cartilaginoso lesado por fibrocartilagem ou pela mitose dos condrócitos. Na literatura são citados diversos métodos para promover a reparação do tecido cartilaginoso, dentre esses, a terapia a laser de baixa intensidade (TLBI) (CAFALLI et al.,1993; TORRICELLI et al.,2001; SCHURMAN; SMITH, 2004; LIN et al., 2004; KUO et al., 2006; FROST-CHRISTENSEN, et al., 2008).

As lesões cartilaginosas (traumáticas ou degenerativas) são doenças ambulatoriais comuns na prática da Fisioterapia, e muitas vezes levam a perda da função articular, aumento da morbidade e diminuição da qualidade de vida dos pacientes acometidos.

A Osteoartrite (OA) é o problema de saúde mais frequente e sintomático na população idosa, de forma que mais de metade de todas as pessoas com mais de 65 anos de idade mostram alterações radiológicas dolorosas em joelhos e os sinais mais comuns além da dor são rigidez, hipertrofia e limitação na articulação amplitude de movimento. A OA afeta somente a cartilagem articular, mas toda a articulação incluindo ligamentos, músculos, osso subcondral, meniscos (no joelho), membrana sinovial, cápsula e fluido da articulação. Pode ocorrer como uma consequência de múltiplas causas, que vão desde trauma comum, sobrecarga biomecânica, erros inatos adquiridos de incongruência articular e defeitos

genéticos em componentes da matriz, a um desequilíbrio da homeostase sinovial (LONGO, 2012; TUHINA, 2012; BERG,2000).

Em 2004, a Organização Mundial de Saúde (OMS) estimou que mais de 150 milhões de pessoas apresentavam osteoartrite no mundo e que esta condição foi a quinta e nona causa de anos perdidos por incapacidade em países de baixa / média renda e alta renda, respectivamente. Além disso, dados recentes indicam um aumento de 30% na prevalência desta condição em uma década. Este grande aumento é devido a uma combinação de fatores, que incluem o envelhecimento da população, crescente prevalência de fatores de risco como a obesidade, por exemplo, (MIRANDA, 2012).

OA ocorre quando por um desequilíbrio entre as forças destrutivas e os mecanismos de reparação desestabilizando a homeostase. Este desequilíbrio pode produzir dor e incapacidade, e a sinovite conduz à secreção de citocinas pró-inflamatórias, tais como o fator de necrose tumoral alfa (TNF-α), interleucina1 ou 6 (IL-1 ou IL- 6). Esse desbalanço de citocinas no fluido sinovial leva à indução de proteinases, tais como metaloproteinases com subsequente degradação da cartilagem. O consenso atual é que a compressão prejudicial conduz à depleção de proteoglicanos, destruição da rede de colagéno e de degradação da cartilagem. Esses achados demonstram não só a indução da inflamação, mas também a falta de resolução da mesma, indícios importantes na OA. (EGLOFF, 2012; BERG, 2000; LEIJS, 2012).

É possível tratar os sintomas da AO conservadoramente com as mudanças de estilo de vida, analgésicos e anti-inflamatórios não esteróides (AINE), ou ainda com injeções intra-articulares de corticosteróides ou ácido hialurônico. Entretanto, o uso de contínuo de fármacos pode apresentar efeitos gastrointestinais adversos. Cirurgicamente, o tratamento final para OA severa é a substituição da articulação, e no momento, ainda não há um tratamento para curar OA. Considerando este panorama, atualmente a TLBI tem sido amplamente estudada, por apresentar características de atenuação da dor e inflamação, promovendo manejo da doença e até onde se sabe, sem efeitos deletérios a saúde do paciente (LEIJS, 2012).

A terapia a laser de baixa intensidade é uma forma de fototerapia que envolve a aplicação da luz laser de baixa potência nos comprimentos de onda vermelho ou infravermelho para tratar diversas doenças (CASTANO, 2007).

O mecanismo que utiliza o LBI não é baseado em aquecimento, ou seja, a energia dos fótons absorvidos não será transformada em calor, mas, sim, nos efeitos fotoquímicos, fotofísicos e/ou fotobiológicos nas células e nos tecidos irradiados.

Conforme Karu (1989) a interação da luz laser com os tecidos biológicos é determinada pelo seu comprimento de onda e pelas características ópticas de cada tecido. Cada tipo de laser resulta em luz de comprimento de onda específico e cada comprimento

de onda reage de uma maneira diferente com determinado tecido. Em baixas intensidades de luz, predomina a conversão da energia absorvida por fotoreceptores endógenos e também por moléculas fotoaceitadoras não especializadas. A este fenômeno denominamos de biomodulação. Dez anos mais tarde, Karu (1999) verifica que os mecanismos da TLBI são complexos, mas que essencialmente, ocorrem por meio da absorção da luz no espectro visível ou infravermelho por fotoreceptores dentro de componentes subcelulares, resultando na ativação de enzimas da cadeia respiratória, principalmente o citocromo c no interior da mitocôndria ou da bomba de sódio-potássio (Na- K).

Ogawa et al. (1991) afirmaram que a TLBI tem potencial para estimular a atividade enzimática, enquanto este aumento de energia, segundo Karu (1989), induz a aceleração da reprodução do DNA mitocondrial e na proliferação celular.

De acordo com Karu, Pyatibrat e Kaelendo (2003) as reações fotobiológicas do laser terapêutico dependem da absorção de um comprimento de onda específico para ativação das moléculas fotoreceptoras, e que este efeito fotobiológico natural significa que alguma molécula fotoreceptora deve absorver primeiramente a luz utilizada para a irradiação do tecido. Esta absorção de luz promove uma excitação eletrônica em nível celular e provoca mudanças na propriedade redox destas moléculas e, conseqüentemente, a aceleração na transferência dos elétrons (reações primárias). Após o início destas reações, iniciam-se as reações secundárias em cascata em nível celular, como por exemplo, o acréscimo na síntese de DNA.

De acordo com Laakso (1994), Hagiwara (2007), Chou e Huffman (2007) a resposta analgésica da fototerapia pode ser medida por mecanismos hormonais/opióides e suas respostas dependem diretamente da dose e do comprimento de onda utilizado para irradiar o tecido. Portanto, os parâmetros utilizados afetam diretamente os resultados.

A normalização da microcirculação e a capacidade de transmissão neural obtida através da TLBI têm sido relatadas como responsáveis pela interrupção do círculo vicioso que origina e perpetua a dor (POPE et al., 1994).

Kao e Sheen (2003) formularam uma hipótese de que o efeito não térmico do laser terapêutico acarreta em um aumento da concentração da enzima Glutathione (GSH) e estas, conseqüentemente, promovem a proteção das células contra os efeitos danosos oxidativos.

Em 2003, Martin relatou que em nível celular os citocromos podem ser definidos como proteínas ou transferentes de elétrons que transportam a energia produzida para as funções biológicas dos tecidos humanos. As enzimas citocromo c oxidase e sintase óxido nítrase (NOS) têm sido particularmente responsabilizadas pela reação à estimulação pela luz laser. A particular afinidade destas e de outras enzimas fotoreativas, aceleram suas

funções na presença do LBI e provocam aumento de ATP e óxido nítrico (NO) no interior da molécula, mudanças estas que acentuam o metabolismo celular e circulatório.

De acordo com estudos Gaida et al. (2004) a radiação com LBI emitida no intervalo do espectro visível ao infravermelho próximo ativa os efeitos celulares por três vias principais, embora seus efeitos ainda não estejam completamente estabelecidos e compreendidos:

1. O mecanismo fotobiológico de ação via ativação da cadeia respiratória, onde os fotoreceptores realizam o controle sobre o nível de ATP intracelular. Este evento pode significar uma alteração no metabolismo das células.

2. Ativação e mudanças na propriedade redox dos componentes da cadeia respiratória nas células: a fotoexcitação de certos cromófaros na molécula citocromo c oxidase influencia a condição redox destes centros e, conseqüentemente, a taxa de escoamento dos elétrons no interior da molécula.

3. Ativação indireta das células via liberação de mensageiros secundários das células ativadas diretamente: moléculas de oxigênio que reagem produzidas pelos fagócitos, linfocinas e citocinas produzidas através de várias subpopulações de linfócitos, ou ainda, a não produção de macrófagos ou como um resultado da não fotólise da hemoglobulina das células sanguíneas.

Diversos estudos experimentais *in vivo, in vitro* e clínicos têm demonstrado os efeitos positivos da fotobioestimulação por meio do laser de baixa intensidade (LBI) em: proliferação celular, incremento da microcirculação, neoformação vascular, estimulação da produção de colágeno pelos fibroblastos e reparação óssea (BAXTER, 1994; KLEBANOV et al., 2001; CARVALHO, et al. 2006). Entretanto, quando se trata de lesões cartilaginosas os resultados ainda são incipientes e controversos. Os resultados demonstram que tanto a bioestimulação como a bioinibição parecem estar relacionadas com o comprimento de onda e com a energia gerada. (ANDREU; ZALDIVAR, 1996; VLADIMIROV; OSIPOV; KLEBANOV, 2003; CAMPANA et al., 2004; FANDER, et al. 2006; BAYAT, et al., 2007; KAMALI, et al., 2007).

Bayat et al. (2007), estudaram o comportamento biomecânico da reparação de um defeito osteocondral no sulco patelo-femoral de 41 coelhos que receberam tratamento de LBI arsenieto de gálio (AsGa), com comprimento de onda de 890nm e densidade de energia de 4,8 J/cm^2. Os animais foram divididos em 3 grupos experimentais sendo um tratado, um placebo e outro controle. Os autores concluíram que nos animais tratados com LBI a cartilagem articular apresentava maior resistência mecânica quando comparados com os grupos placebo e controle.

Fander et al., (2006), realizaram um estudo em cartilagem articular de joelhos com 45 coelhos. Para tanto, utilizaram um modelo de lesão experimental e submeteram as

articulações à irradiação com LBI com comprimento de onda de 692,6 nm e densidade de energia de 1 e 4 J/cm^2. Por meio de análise imunohistoquímica observaram que os animais irradiados com densidade de energia menor (1J/cm^2) apresentaram resultados superiores na densidade de condrócitos e no índice de glicosaminoglicano. Os autores observaram também que nos animais não irradiados, não houve regeneração da cartilagem articular.

Lin, Huang e Chai (2006), utilizaram um modelo experimental de lesão articular em joelhos de 72 ratos Wistar, utilizando papaína, e pesquisaram a ação dos glicosaminoglicanos após tratamento com LBI HeNe, e relataram que o grupo tratado com laser HeNe apresentou um incremento da biossíntese da cartilagem artrítica constatado pela microscopia de luz.

Wong, et al., (2005), irradiaram cultura de condrócitos provenientes de cartilagem nasal humana com LBI neodímio dopado por ítrio e alumínio (Nd:YAG) com 6 J/cm^2, verificaram o aumento significativo na síntese de proteoglicanose na resposta proliferativa dos condrócitos.

Jia e Guo (2004), realizaram um estudo *in vitro* em cultura celular de condrócitos isolados da cartilagem articular do côndilo femoral de coelhos, com o objetivo de verificar a capacidade de respostas dessas células à irradiação de LBI HeNe 632,8nm com potência de 12mw e densidade de energia de 1 a 6 J/cm^2. Os autores observaram por meio de colorimetria que as doses entre 4 a 6 J/cm^2 foram responsáveis pelo aumento da atividade de proliferação celular dos condrócitos e que as doses de 4 e 5 J aumentavam a síntese e a secreção da MEC .

Tascioglu et al. (2004), realizaram um estudo clínico duplo cego, randomizado sobre a ação do LBI AsGa com comprimento de onda de 830 nm e potência de 50 mW no quadro álgico de pacientes portadores de osteoartrose. Os autores utilizaram duas densidades de energia sendo um grupo de 1,5 J/cm^2 e outro com 3 J/cm^2 e avaliaram os resultados por meio de um questionário específico para análise da dor. Os mesmos não constataram melhora significativa do quadro álgico nos pacientes tratados com LBI em comparação aos grupos controle e placebo.

Gur et al. (2003), realizaram um estudo clínico duplo cego, em pacientes com osteoartrose avaliando a dor, mobilidade articular e edema, com aplicação de um questionário. Após o período de tratamento com LBI AsGa 904nm com doses que variaram entre 2 e 3 J/cm^2, concluíram que em todos os grupos tratados com LBI independente da densidade de energia, os pacientes apresentavam melhora estatisticamente significativa em todos os aspectos clínicos avaliados, quando comparados com o grupo controle e placebo.

Torricelli et al. (2001), observaram em estudo *in vitro*, condrócitos derivados de cartilagem de seres humanos e de coelhos, irradiados com LBI arsenieto de gálio e alumínio

(AsGaAl) com comprimento de onda de 660nm e potência pulsada de 1W. Os autores utilizaram diversas formas de pulso e concluíram que histologicamente os grupos tratados com LBI independente do pulso, não desencadearam nenhuma lesão nas células cultivadas e estas apresentavam maior viabilidade e aumento na proliferação celular.

Nesse contexto, considerando os aspectos histopatológicos da doença, os efeitos positivos da fotobioestimulação obtidos por meio da TLBI relatados pela literatura em diversos tipos de tecido, e a não uniformização quanto aos parâmetros a serem utilizados para esta terapia, justifica-se o presente estudo de cunho comparativo entre duas potências diferentes de TLBI, com distintos e comprimento de onda de 808nm, em lesões cartilaginosas induzidas experimentalmente.

2. OBJETIVOS:

Analisar e comparar os efeitos do Laser de baixa intensidade com saída de 100 mW e 50 mW, em um modelo de osteoartrite induzido por papaína em ratos.

2.1 Objetivos Específicos

- Analisar os efeitos do LBI no reparo tecidual.
- Analisar os efeitos do LBI na expressão dos colágenos do tipo I e III.
- Analisar os efeitos do LBI sobre os níveis das MMPs 2 e 9 no lavado articular.

3. MATERIAS E MÉTODOS

3.1 Animais de experimentação

Foram utilizados 60 ratos (*norvergicos albinus*), de linhagem Wistar, machos com idade aproximada de 90 dias com peso corporal variando de 250 a 300 gramas, provenientes do Biotério da Universidade Nove de Julho - UNINOVE, mantidos em condições controladas de luminosidade e temperatura, com água e alimentação *ad libitum*.

Todos os procedimentos experimentais foram submetidos à avaliação do Comitê de Ética da Universidade Nove de Julho (AN 0016/2011) e estão de acordo com as normas do Colégio Brasileiro de Experimentação Animal – COBEA e aos padrões de experimentação animal do International Council for Laboratory Animal Science.

3.2 Grupos Experimentais

Para compor os grupos experimentais do projeto foi realizado um cálculo amostral com base nos estudos de Murat, et al., (2007) e Lin et al., (2004), considerando a aplicação do teste estatístico ANOVA para 3 tratamentos, com poder de teste de 80 e nível alfa de 0,05 resultando em uma amostra mínima de 60 animais, sendo 15 por grupo.

Os animais foram distribuídos de forma aleatória em quatro grupos distintos, contendo 15 animais em cada grupo, sendo: Grupo controle, Grupo Lesão, e dois grupos TLBP, onde um grupo foi tratado com laser de potência de 50 mW e outro tratado com laser de potência de 100 mW. Os grupos foram avaliados em quatro tempos experimentais distintos: 7, 14 e 21 dias.

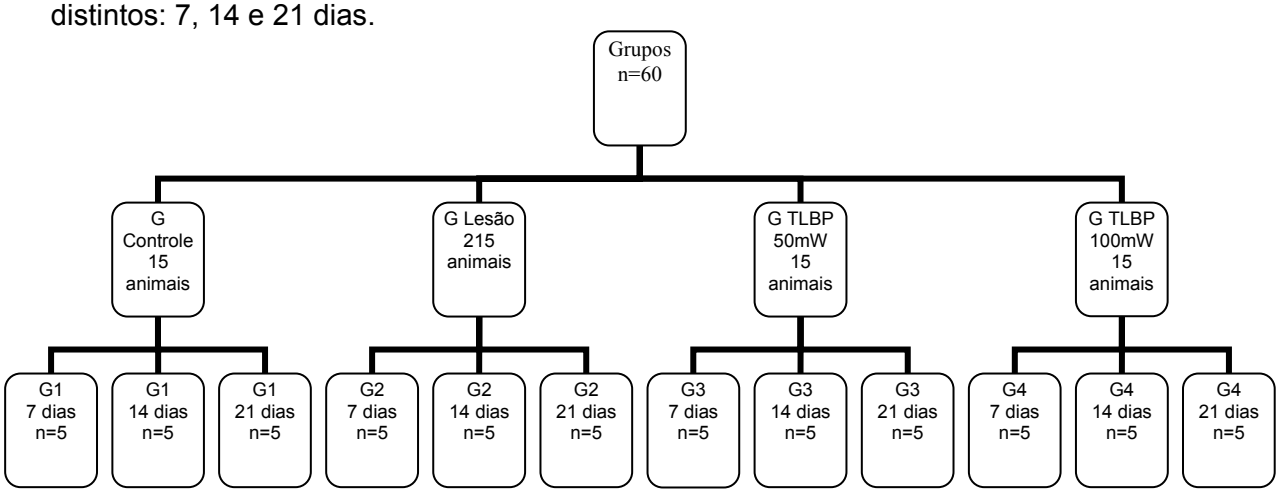

Figura. 1 Composição dos grupos experimentais.

3.3 Procedimentos

3.3.1 Indução das Lesões Cartilaginosas

Os animais foram anestesiados antes de cada infiltração da substância indutora da lesão cartilaginosa, com uma mistura de Quetamina (7%) e Xilasina (0,3%), numa mistura de 2:1 utilizando-se 0,2 ml/100g, por via intramuscular.

Após o procedimento anestésico foi realizada infiltração na articulação da pata traseira de cada animal com solução de papaína 4%, dissolvida em 10 ml de solução salina e adicionado 10 ml solução de cisteína (0,03 M). Essa solução foi usada como ativadora para produzir a lesão (LIN et al., 2004; MURAT, et al., 2007).

Após as infiltrações os animais foram imediatamente submetidos ao tratamento.

3.3.2 Aplicação do Laser

Foi utilizado o Laser da marca DMC® modelo Photon Laser III, com potência de 100 (densidade de potência de 3,5 W/cm2) e 50 mW (densidade de potência de 1,78 W/cm^2), área do feixe de 0,028cm^2, e comprimento de onda de λ 808 nm, meio ativo de Arsenieto de Gálio e Alumínio (AsGaAl). A aplicação deu-se sob forma de dois pontos pelo método transcutâneo nos compartimentos medial e lateral da articulação; com densidade de energia de 142 J/cm^2, tempo de 40 e 80 segundos, respectivamente e energia de 4J.

O tratamento foi realizado em dias alternados sobre o joelho direito nos grupos experimentais TLBI 50mW e TLBI 100mW, totalizando 4,7 e10 sessões até o dia da eutanásia de cada grupo, realizada em 7,14 e 21 dias, respectivamente .

3.3.3 Eutanásia

No dia de eutanásia, os animais foram identificados, pesados e, posteriormente, sofreram eutanásia por inalação de Dióxido de Carbono – CO_2. Ao final do protocolo de experimentação a câmara de CO_2 foi calibrada para um nível entre 70% e 80% de ocupação total da câmara. Os animais foram conduzidos individualmente até a câmara e colocados imediatamente em contato com o gás para perda rápida de consciência e na sequência, submetidos ao quadro de hipóxia atribuída à depressão dos centros vitais. Este método é relativamente rápido e necessita de uma câmara de CO_2 para ser realizado (LAPCHIK, 2009).

Após a eutanásia realizou-se a tricotomia do joelho direito e o animal foi, então,

posicionado em decúbito ventral, prendendo-se as patas dianteiras e traseiras em abdução. Foi desarticulada a articulação coxo-femoral da pata direita traseira de cada animal, para posterior análise do tecido cartilaginoso da articulação do joelho (imediatamente fixados por meio de solução de formol tamponado a 10% e encaminhados para procedimentos histológicos), e também foi realizada a coleta do lavado articular sendo que a cavidade articular foi lavada 2 vezes com 5µl de PBS contendo 1 mM EDTA e diluído posteriormente em 90µl de PBS + EDTA. O material foi imediatamente centrifugado (300Xg/10min) e o sobrenadante armazenado a -80°C para análise de expressão protéica. (DA ROSA et.al, 2012)

3.3.4 Procedimentos Histológicos e Análise Histomorfométrica

As articulação dos animais foram descalcificadas utilizando EDTA e submetidas à técnica histológica clássica para inclusão em parafina (MICHALANY, 1980), composta pela seguinte seqüência: desidratação em concentrações crescentes de álcool; diafanização com xilol que permitirá a penetração de parafina na peça; impregnação em banhos de parafina e inclusão em moldes; cortes transversais com uma espessura de quatro micrometros e montagem em bálsamo sintético. Os cortes histológicos foram corados com Hematoxilina e Eosina (HE) para análise do reparo tecidual, e Picrossirius Red para a quantificação das fibras colágenas.

A análise das lâminas foi realizada através da digitalização de imagens, por meio de um microcomputador com programa específico de Processamento e Análise de Imagem “Image Pro Plus ® 4.5”.

Para a quantificação das áreas representativas das fibras colágenas, foram digitalizados cinco campos, usando-se um microscópio (objetiva 40x) acoplado a uma câmara para captura de imagem, conectada ao microcomputador equipado com placa de vídeo.

Antes do processo de quantificação, todas as imagens foram digitalizadas padronizando-se a intensidade de luz do microscópio e a altura do condensador. As áreas representativas do colágeno foram separadas na imagem usando-se a distribuição de cor como parâmetro discriminante.

Para cada imagem quantificada, utilizamos o mesmo intervalo de cor, para separar a área a ser quantificada. O intervalo de cor padronizado foi definido de

forma empírica, no momento inicial do experimento. Através de tentativa e erro, uma faixa de cor foi ajustada, até separar as áreas representativas na imagem.

Posteriormente, o mesmo intervalo foi utilizado para identificar o colágeno a ser quantificado em todos os campos digitalizados. Na etapa seguinte, calculamos a área ocupada em cada um dos campos.

3.3.5 Análise da Expressão Protéica de Metaloproteinase de Matriz (MMP - 2 e MMP - 9) no Lavado Articular do joelho de ratos Wistar por Western Blot

Os lavados articulares foram diluídos em tampão Lammli (Tris 240 mM; Glicerol 40%, beta mercaptoetanol 200mM, SDS 1%, Azul de bromofenol 0,02%) e fervidos a 100ºC por 5 min. Quantidades iguais de proteínas (15 µg) foram separadas em gel de poliacrilamida a 10% (SDS-PAGE). As proteínas foram transferidas para membrana de PVDF (Polyvinylidene fluoride membrane) por *eletroblotting* e ainda a eficiência de transferência foi monitorada com o uso da coloração de Ponceau S a 0,5% da membrana do blot.

As membranas foram bloqueadas em solução de BSA 10% (albumin bovine serum, Sigma) por 2h em temperatura ambiente. Posteriormente, as membranas foram incubadas com anticorpo policlonal anti- MMP-9 e MMP-2 (ab58803 e ab2462) diluídos em BSA 3% com TBS-T por 48 h em câmara fria a 4^{o} C. Seguiram lavagens com TBS-T (3x 10 min) e incubação com anticorpo secundário anti-IgG de coelho (Invitrogem G21040, 1:2000), diluído em TBS-T com BSA 3% por 2 h. As membranas foram novamente lavadas com TBS-T (3x 10 min) e incubadas com solução ECL (2.5mM Luminol, 400uM ácido p-coumárico, 1M tris pH 8,5, 5,4mM H_2O_2, H_2O) por 10 min. e revelado com Image Quant LAS 400 (GE).

Como controle interno foi utilizada a beta actina (SC 47778), 1:200 e as imagens foram analisadas utilizando o software imageJ.

3.3.8 Análise Estatística

Os dados obtidos foram tabulados em Software Microsoft Excel 2007 e inicialmente avaliados quanto a sua normalidade pelo teste de Shapiro-Wilk, concluindo como resultado a distribuição normal. Foi então aplicado o teste de análise de variância ANOVA "*post hoc test*" de Tukey para comparações entre os períodos de 7,14 e 21 dias dentro de cada grupo, bem como entre os grupos. Todos os dados foram expressos como média e desvio padrão. Foi utilizado o software GraphPad Prisma 5, tomando-se como hipótese de nulidade $p<0,05$.

4. ARTIGO SUBMETIDO

O artigo **'Action of Low Level Laser Therapy – LLLT in expression of the metalloproteinases 2 and 9 (MMP2 and MMP9) and percentage of collagen type I and III in papain cartilage injury model'** foi submetido à revista *Lasers in Medical Science*.

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Action of Low Level Laser Therapy -LLLT in expression of the metalloproteinase MMP2 and MMP9 and percentage of collagen type I and III in papain cartilage injury model.

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Corresponding Author:	Paulo de Tarso Camillo Carvalho, PhD University Nove de Julho-UNINOVE Campinas , São Paulo BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University Nove de Julho-UNINOVE
Corresponding Author's Secondary Institution:	
First Author:	Ana Carolina Araruna Alves, Master
First Author Secondary Information:	
Order of Authors:	Ana Carolina Araruna Alves, Master
	Regiane Albertini
	Solange Almeida dos Santos
	Ernesto Cesar Pinto Leal-Junior, PhD
	Eduardo Tadeu Santana, Master
	Andrey Jorge Serra, PhD
	Jose Antonio Silva Junior, PhD
	Paulo de Tarso Camillo Carvalho, PhD
Order of Authors Secondary Information:	
Abstract:	Background: Cartilage injury and its destruction are common in osteoarthritis (OA) and are associated with increased levels of several matrix metalloproteinases (MMPs), proteinases wich can degrade all components of complex extracellular matrix (ECM).Objective: Investigate the effect of low level laser therapy (LLLT) operating at 50mW and 100mW power on joint damage in rats induced by papain, through histopathological analysis as well as protein expression of metalloproteinases 2 and 9. Materials and Methods: 60 male Wistar rats were randomly distributed into 4 groups of 15 animals which were: Control, was control negative group; Injury, control positive group; LLLT 50mW, was submitted to lesion and treated with LLLT at 50 mW; and LLLT 100mW were treated with LLLT at 100 mW. The animals were submitted to OA (4% papain solution) and then submitted to LLLT. At the euthanasia day, it was collected the articular lavage, which was immediately centrifuged and the supernatant stored for protein expression analysis by Western Blot. The material was stained with HE for the histopathological description and Picrosirius Red, was used to estimate the percentual of collagen fibers. As normal distribution was determined, ANOVA with Tukey's post hoc test was used for comparisons between periods 7, 14 and 21 days were compared within each group as well as between. All data are expressed as mean and standard deviation values, with the null hypothesis considered $p < 0.05$. Results: The both laser groups were efficient on tissue repair, decreasing collagen type III expression and increasing type I in all experimental times, however the LLLT 50mW group was better regarding reduction of metalloproteinase 9 comparing to LLLT

	100mW group in 21 days. Conclusion: LLLT at 50 mW was more efficient in the modulation of matrix metalloproteinases and tissue repair.

Action of Low Level Laser Therapy –LLLT in expression of the metalloproteinase MMP2 and MMP9 and percentage of collagen type I and III in papain cartilage injury model.

Ana Carolina Araruna Alves[1]; Regiane Albertine[1, 2]; Solange Almeida dos Santos;[3]; Ernesto Cesar Pinto Leal Junior[1, 2]; Eduardo Santana[1]; Andrey Jorge Serra[1]; Jose Antonio Silva Junior[1]; Paulo de Tarso Camillo de Carvalho[1,2]

[1] Universidade Nove de Julho (UNINOVE), São Paulo, SP, Brazil - Postgraduate Program in Rehabilitation Sciences,

[2] Universidade Nove de Julho (UNINOVE), São Paulo, SP, Brazil - Postgraduate Program in Biophotonics,

[3] Universidade Nove de Julho (UNINOVE), São Paulo, SP, Brazil - Departments of Physical Therapy,

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Address reprint requests to:
Paulo de Tarso Camillo de Carvalho
Rua Carlos Penteado Stevenson N. 700
Valinhos SP, Brazil
e-mail: ptpaulo@terra.com.br

Abstract

Background: Cartilage injury and its destruction are common in osteoarthritis (OA) and are associated with increased levels of several matrix metalloproteinases (MMPs), proteinases wich can degrade all components of complex extracellular matrix (ECM).**Objective:** Investigate the effect of low level laser therapy (LLLT) operating at 50mW and 100mW power on joint damage in rats induced by papain, through histopathological analysis as well as protein expression of metalloproteinases 2 and 9. **Materials and Methods:** 60 male Wistar rats were randomly distributed into 4 groups of 15 animals which were: Control, was control negative group; Injury, control positive group; LLLT 50mW, was submitted to lesion and treated with LLLT at 50 mW; and LLLT 100mW were treated with LLLT at 100 mW. The animals were submitted to OA (4% papain solution) and then submitted to LLLT. At the euthanasia day, it was collected the articular lavage, which was immediately centrifuged and the supernatant stored for protein expression analysis by Western Blot. The material was stained with HE for the histopathological description and Picrosirius Red, was used to estimate the percentual of collagen fibers. As normal distribution was determined, ANOVA with Tukey's post hoc test was used for comparisons between periods 7, 14 and 21 days were compared within each group as well as between. All data are expressed as mean and standard deviation values, with the null hypothesis considered $p < 0.05$. **Results:** The both laser groups were efficient on tissue repair, decreasing collagen type III expression and increasing type I in all experimental times, however the LLLT 50mW group was better regarding reduction of metalloproteinase 9 comparing to LLLT 100mW group in 21 days. **Conclusion:** LLLT at 50 mW was more efficient in the modulation of matrix metalloproteinases and tissue repair.

Introduction

Cartilage injury is a fairly common orthopedic problem. However, no satisfactory methods to repair cartilage defects have been developed to date. Trauma, osteochondritis, and osteoarthritis can cause damage to cartilage and subchondral bone. [1]

Articular cartilage degeneration in OA has been studied extensively. It has been demonstrated that collagens in OA articular cartilage display much less organized networks and that collagen content falls in advanced OA compared to early [2]

Cartilage destruction in osteoarthritis (OA) is associated with increased levels of several matrix metalloproteinases (MMPs), including the gelatinases MMP-2 and MMP-9. While increases in some MMPs may be destructive, up-regulation of others may result from increases in normal tissue turnover. Metalloproteinases, especially matrix metalloproteinases (MMPs), are considered to be the most important class of proteinase in terms of cartilage degradation, because collectively they can degrade all components of this complex extracellular matrix (ECM). Indeed, type I, II and III collagen is a major structural component of this ECM, and collagenolysis is an essentially irreversible step, making such proteolysis a major therapeutic target. [3,4,5]

According Eyre [6] the collagenous matrix of articular cartilage is a highly complex assemblage of multiple gene products. Neither the functions of the individual components nor the molecular mechanisms controlling the assembly, turnover or degradation in disease of the collagen heteropolymer are yet well understood. Collagen breakdown is considered to be a critical and perhaps irreversible step in the progression of osteoarthritis.

The literature reports a number of methods for promoting the repair of cartilaginous tissue, including low-level laser therapy. [7, 8]. Several experimental studies in vivo, in vitro clinical trials have demonstrated the positive effects of photobiostimulation through low-level laser (LLL) in: cell proliferation [9,10,11,12], increasing the microcirculation [13], stimulation of collagen production by fibroblasts [14], bone repair [15] and modulation of inflammatory markers such as interleukin [16] and metalloproteinases [17]. However, when it comes to cartilage lesions results are still preliminary and controversial [18, 19].

For the foregoing objective of the present study was to investigate the evolution of repair process, analysis of type collagen as well as protein expression of metalloproteinases 2 and 9 the effect of therapy with low-power laser operating at 50mW and 100mW power of joint damage in rats induced by papain.

Materials and Methods

Animals. The sample was composed by 60 male Wistar rats (Norvergicus albinus), aged from 90 days, wheighing 250-300g, The animals were obtained from the animal lodging facility of the Universidade Nove de Julho (Brazil) and kept under controlled conditions of light and temperature, with free access to water and chow. All experimental procedures were approved by Institutional Research Ethics Committee (AN 0016/2011) and followed the guidelines of the Brazilian College for Animal Experimentation as well as the standards of the International Council for Laboratory Animal Science.

Experimental Groups. Sixty animals were randomly distributed into 4 groups of 15 animals each. First group (Control) does not received any kind of intervention; second group (Injury), received induction but does not received any treatment; third group was treated with LLLT at 50 mW (LLLT 50mW), and rats of the fourth group were treated with LLLT at 100 mW (LLLT 100mW). All the groups were evaluated in 3 different experimental times: 7, 14 and 21 days (5 animals from the group, for each experimental time).

Papain induced osteoarthritis. The animals were anesthetized with an intramuscular injection of a 7% ketamine solution (Cetamin, Syntec, Cotia, SP) and 0.3% xyline solution (Xilazin, Syntec, Cotia, SP) at a proportion of 2:1 (0.2 mL per 100 g). The induction of OA was then performed following the methods described by others publications in literature [5,1]. For such, the 200 µL injections were performed in the right knee of the hind leg of each animal with a 4% papain solution dissolved in 10 mL of saline solution, to which 10 mL of a cysteine solution (0.03 M) was added. This solution was used as the activator to produce cartilage injury. The animals were then immediately submitted to the administration of low-level laser therapy.

Low Level Laser Therapy. An AsGaAl-type diode laser with a wavelength (k) of 808 nm, from Photon Laser III DMC (Sao Carlos, SP, Brazil) was used. The optical power was calibrated using a Newport multifunction optical meter, Model 1835C. The dose and parameters and are summarized in Table1.

Irradiation. Laser irradiation was given in the form of two points by transcutaneous compartments: medial and lateral, was performed immediately after the papain-cysteine injection, with a frequency of 3 times per week, on the right knee in groups LLLT 50mW and LLLT 100mW, until the day of sacrifice. The groups Control and Injury received no treatment and served as the control negative and positive groups respectively, for the comparative histomorphometric analysis. Animals were immobilized by means of grip and were irradiated at an angle of 90° to the surface of tissue area. LLLT was performed 3 times per week. Therefore, animals received 4, 7 and 10 treatment sessions regarding to 7, 14 and 21 days of experimental times.

Sample Colletctions. After receiving the treatment, at the euthanasia day, it was performed a procedure for obtaining the articular lavage. The articular cavity was washed with 1 mL of physiologic serum into the intracapsular knee space, the material was immediately centrifuged at 1500rpm/5min, as previously described [20] and the supernatant stored at -80 ° C for analysis of inflammatory mediators.

*Western Blotting.*The joint washes were diluted in Lammli buffer (240 mM Tris, 40% glycerol, 200mM beta-mercaptoethanol, 1% SDS, 0.02% Bromophenol Blue) and boiled at 100 ° C for 5 min. Equal amounts of protein (15 µg) were separated on polyacrylamide gels at 10% (SDS-PAGE). Proteins were transferred to PVDF membrane (Polyvinylidene fluoride membrane) by electroblotting and also the transfer efficiency was monitored using the Ponceau S staining of in 0.5% membrane blot. Membranes were blocked in a solution of 10% BSA (bovine serum albumin, Sigma) for 2 h at room temperature. Subsequently, the membranes were incubated with polyclonal anti-MMP-9 and MMP-2 (ab58803 and ab2462) diluted in 3% BSA in TBS-T for 48 h in cold room at 4 ° C. Followed washing with TBS-T (3x 10 min) and incubated with secondary anti-rabbit IgG (Invitrogem G21040, 1:2000) diluted in TBS-T with 3% BSA for 2 h. The membranes were again washed with TBS-T (3x 10 min) and incubated with ECL solution (2.5mM Luminol, 400um p-coumaric acid, 1M tris pH 8.5, 5.4 mM H2O2, H2O) for 10 min and revealed with Image Quant LAS 400 (GE). Beta actin (SC 47778), 1:200 was used as internal control and the images were analyzed using ImageJ software.

Euthanasia. At the end of each period (days 7, 14 and 21), the animals of each groups were identified, weighed, and subsequently undergo euthanized by inhalation of carbon dioxide - CO2. This method confers rapid loss of consciousness

in response to hypoxia attributed to depression of vital centers and requires a CO2 chamber to be performed [21]. The tibio-femoral articulation of the right hind leg of each animal was separated for analysis of the cartilaginous tissue of the knee. The material was immediately fixed using a 10% buffered formaldehyde solution and submitted to histological procedures.

Histological procedures, histopathological and morphometric analysis. The material was decalcified with EDTA and submitted to the classic histological method for embedment in paraffin: dehydration in increasing concentrations of alcohol; clearing with xylol in order to allow the penetration of paraffin; impregnation in paraffin baths and insertion in molds; cross-sectional cuts to a thickness of five micrometers; and mounting in a synthetic balsam. Then, it was stained with Hematoxylin and Eosin for the histopathological description and Picrosirius Red, an anionic compo site that distinguishes the thickness and density of collagen fibers through coloration emitted under polarized light, was used to estimate the percentual of collagen fibers. While the thin dissociated fibers typical of type III collagen are greenish, the thickest and strong associated fibers of type I collagen emit colors with bigger length wave as red and yellow Picrossirius stain for the quantification of collagen cells. Morphometric analysis was performed on the slides, by means of image digitization and computational analysis using a specific image processing and analysis program (Image Pro plus 4.5). To quantify the areas representing collagen, five fields observed using an microscope Nikon Eclipse E200 (40x lens) were digitized. The microscope was coupled to an image-capturing Sanyo digital active BLC camera, and this was connected to an microcomputer equipped with a video board. All the images were digitized before the quantification process, thereby standardizing the microscope light intensity and condenser height. The collagen areas were separated in the image, using the color distribution as the discriminating parameter [22,23,24].

Statistical analysis. The data were tabulated using the Microsoft Excel 2007 software and initially assessed for normality using the Shapiro-Wilk test. As normal distribution was determined, ANOVA with Tukey's post hoc test was used for comparisons between periods 7, 14 and 21 days were compared within each group as well as between control, injury, LLLT 50mW and LLLT 100mW. All data are expressed as mean and standard deviation values. The GraphPad Prism 5 software program was used, with the null hypothesis considered $p < 0.05$.

Results

Histopathologic analysis: 7 days

On histopathologic analysis performed seven days after the injury can be seen that the material obtained from the control group had joints with general characteristics of normality, with joint spaces without the presence of inflammatory exudate and synovial membrane with inner layer and exhibiting characteristics subintima behavior. Already in the joint injury group exhibited acute inflammation interspersed with areas with signs of chronicity in the region underlying the anterior cruciate ligament, consisting primarily of mononuclear cells, with joint spaces filled by hyaline material and fibrin adhered to the entire surface of the synovial membrane. The synovial membrane layer with subintima showing acute inflammatory infiltrate and dilated blood vessels. The bone marrow of the epiphysis was occupied by areas of cellular degeneration underlying hyaline cartilage. There was presence of cells involved in bone repair and neoformed bone trabeculae in the remaining portion of the epiphysis. The articular meniscus exhibited in its constitution thick collagen fibers and chondrocytes, with areas of ossification.

In the group treated with LLLT power of 50mW observed that the general characteristics showed synovial joint tissue repair, consisting primarily of mononuclear cells. Joint spaces with discrete presence of hyaline material and synovial membrane with intimal thickness usual. Layer subintima exhibited intense tissue repair process, consisting primarily of fibroblasts and macrophages. The articular surfaces exhibited homogeneous coating of hyaline cartilage. NA zone epiphyseal calcification observed signs of active resorption. Epiphyseal bone marrow showing normal pattern and filled with red bone marrow and intense presence of cells involved in bone repair, especially the underlying hyaline cartilage lining. Articular meniscus consists of thick collagen fibers and chondrocytes, with areas of ossification.

In the group treated with LLLT with the joint power of 100mW also presented general characteristics of tissue repair, consisting primarily of mononuclear cells. The joint spaces showed a slight presence of hyaline material and few leukocytes. The synovial membrane showed intimal thickness and habitual Layer subintima showing with intense tissue repair process, consisting primarily of fibroblasts and

macrophages. The articular surfaces showed homogeneous coating of hyaline cartilage with calcification zone of the epiphysis with signs of active resorption. Epiphyseal bone marrow exhibited normal standard. (Figure 1.)

Histopathologic analysis: 14 days

At 14 days the joints of the control group exhibited the general characteristics of normality, with joint spaces without the presence of inflammatory exudate. Synovial membrane with thickened intima and subintima showing typical characteristics.

At 14 days the lesion group had the joint presence of a chronic inflammatory process in the region underlying the anterior ligament, consisting primarily of mononuclear cells, joint spaces showed a slight presence of hyaline material. The synovial membrane layer subintima presented with intense tissue repair process, consisting primarily of fibroblasts and macrophages. The bone marrow of the epiphysis was occupied by areas of cellular degeneration underlying hyaline cartilage and the presence of cells involved in bone repair and neoformed bone trabeculae in the remaining portion of the epiphysis.

The group treated with LLLT 50mW presented general characteristics of tissue repair, with intense presence of fibroblasts, joint spaces without the presence of inflammatory exudate. Synovial membrane with intimal layer showing normal features, however, the subintima has high amount of fibroblast cell line. The articular surfaces exhibited homogeneous coating of hyaline cartilage. It also looked active endochondral ossification of the epiphysis, the epiphysis with bone marrow showing normal pattern, filled with red bone marrow.

In the group treated with LLLT 100mW articulation exhibited general features of tissue repair, with intense presence of fibroblasts and macrophages. , Joint spaces without the presence of inflammatory exudate. The synovial membrane with intima-subintima showing normal features, however, some areas present with villous synovial intimal layer thick. Surfaces coated articular hyaline cartilage homogeneous. Active endochondral ossification of the epiphysis. Epiphyseal bone marrow showing normal pattern and filled with red bone marrow. Articular meniscus consists of thick collagen fibers and chondrocytes, and the presence of ossification. (Figure 2.)

Histopathologic analysis: 21 days

At 21 days, the control group showed linkage with general characteristics of normality. Joint spaces without the presence of inflammatory exudate. Synovial membrane with thickened intima and subintima showing typical characteristics. Injury exhibited untreated synovial articulation with the general characteristics of the degenerative process, with joint spaces with inflammatory exudate .. Articular surfaces coated with hyaline cartilage evidence of fibrillation. Active endochondral ossification of the epiphysis. Epiphyseal bone marrow showing intense signs of tissue repair.

In the group treated with LLLT 50 mW articulation showed general signs of normalcy, with some areas showing signs of tissue repair comprising high amount of fibroblast cell line. Joint spaces without the presence of inflammatory exudate. Synovial membrane with inner layer and subintima showing normal features. Surfaces coated articular hyaline cartilage homogeneous. Epiphyseal bone marrow showing intense process of tissue repair. Articular meniscus consists of thick collagen fibers and chondrocytes, and the presence of ossification.

In the group treated with 100mW of power was also observed general characteristics of normal, with a few areas showing signs of tissue repair comprising high amount of fibroblast cell line. Joint spaces without the presence of inflammatory exudate. Synovial membrane with thickened intima and subintima showing typical characteristics. Surfaces coated articular hyaline cartilage homogeneous. Active endochondral ossification of the epiphysis. Epiphyseal bone marrow showing normal pattern and filled with red bone marrow. Articular meniscus consists of thick collagen.

Quantification of collagen types I and III by polarized light

The histological sections stained with picro-sirius red were photographed in polarized light microscope and percentage of collagen types I and III was obtained by subtracting image birefringent with Image Pro Plus 4.5. For seven days the group type I collagen quantification of the resulting averages show that the percentage of type I collagen was higher in the group had Injury when compared with the other groups. Injury vs Control ($p < 0.001$); Injury vs. LLLT 50mW ($p < 0.05$) and Injury vs LLLT 100mW ($p < 0.05$), whereas the comparison between the control group and those treated with laser was no statistical difference ($p > 0.05$) and the percentage of fibers as shown in Figure 1. (A, C, D, F and G). In the analysis for collagen type III

showed a tendency to increase this to control groups treated with LLLT and the averages of the groups tradados (LLLT 100mW vs LLLT 50mW) were higher and statistically different from the injury group (p <0.001); now to the analysis between the treated groups (LLLT 100mW vs LLLT 50mW) was obtained (P> 0.05). Figure 3. (B, C, D, F and G).

For analysis of the blades obtained after 14 days showed that a decrease in the percentage of type I collagen in the control group and the groups treated (LLLT LLLT vs. 50mW 100mW), however only group and the control group differ 50mw Statistical Injury compared to group (p <0.05). Figure 2. (A, C, D, F and G). In the analysis of collagen type III at 14 days showed an increase in the percentage for both groups treated LLLT (LLLT 50mW vs LLLT 100mW.) state values very close to the control group and not statistically different between the groups (p <0.05). In group Injury fibers photographed in green proved scarce and statistically different (p> 0.05) from other groups as can be seen in Figure 4. (B, C, D, F and G).

In group after 21 days showed that the trend of previous periods remained ie decrease in the percentage of type I collagen in the control and treated groups (LLLT 50mW vs. LLLT 100mW), remained with an index greater injury to the group. In the statistical analysis the control group and the treated groups showed statistical differences in relation to Injury group (p <0.05). Figure 3. (A, C, D, E and G). For analysis of collagen type III was observed an increased number of sites as green fibers with many fibers in transition as well as a good alignment of these fibers as can be seen in Figure 3. (C, D, E and G)., Statistical analysis in control and treated groups showed statistically significant differences in relation to Injury group (p <0.05). Figure 5. (B).

Analysis of protein expression of matrix metalloproteinases (MMP-2 and 9)

The expression levels of MMPs were evaluated by Western blot technique, followed by analysis of the relative density of bands corresponding to samples of articular lavage knee osteoarthritis induced by papain to 4%. As expected, we found only one band with a molecular weight of 72 kDa (active form of the enzyme) in the Western blot assays using primary anti-MMP-2 in the control groups, injury and treated with LLLT 50mw and 100mw. Likewise, it was detected predominately the active form of MMP-9 with molecular weight of 78 kDa. The analysis of the optical densities of the bands corresponding to MMP-2 indicated that the expression levels

of MMP-2 were significantly higher in samples injury group compared to the control group ($p < 0.05$) in all periods analyzed (7 days, 14 days and 21 days), also found that the injury group showed higher expression levels and statistically significant when compared with the treated groups (LLLT 50mW and 100mW) in all experimental periods ($p < 0.05$). Figure 6. Likewise, the expression of MMP-9 was detected at higher levels in group injury compared to the other groups (control, LLLT 50mW and 100mW LLLT) in all experimental periods. Figure 8. (A, B and C). However, in samples obtained at 21 days after injury the expression level of 100mW LLLT group was higher than 50mW and LLLT group showed a statistically significant difference. ($P < 0.05$). Figure 7.

Discussion

OA is the most common joint disease for middle-aged and older people and it's characterized by a complex and multifactorial process involving cartilage catabolism and anabolism. The cartilage metabolism is characterized by an adjustable balance between the synthesis and degradation of various components of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are considered to be key enzymes in the degradation of ECM and its progressive destruction causes damage to the articular cartilage, thereby, MMPs are able to attack interstitial collagen types (as I, II and III) [25,26,27,28]

In the present study we realized a histopathological analysis in which were demonstrated important results in testifying the papain induced OA model for cartilage injury, when comparing control group with injured in all experimental times [27, 24,29]. Primarialy, in injury group with 7 days it's possible to identify the intrinsec inflammatory process [26,27,30], which is gradually replaced for chronicity signs, proving the degenerative process achived by papain as inducing substance and the fibrinous aspect of joint space evidences the normal healing. When compared with the both treatment groups, it becomes evidenced that this healing process it's accelerated and intensified with presence of mononuclear and bone repair cells. In 14 days, the most relevant difference between the treatment groups and injury group is a delay in tissue repair process evidenced by the degeneration characteristics in bone marrow while there's a normal aspect for the same area in group 3 (LLLT 50 mW). Analysing group 3 and 4 (50 x 100mW) there's a presence of villous synovial in

group 4 only, and finally in 21 days, the injury group still presents intense signal of tissue repair while intervention groups are already presenting hyaline cartilage homogeneous with usual features.

Comparing our histopathological results to other studies with LLLT in chemically induced OA, we found similar outcomes. There are results in literature talking about the less ordered structured [25], what was seen in our results, mainly in injury group, and further, an ordered pattern reestablishment after the treatment with LLLT, best viewed in our case, in collagen analysis. Other study reports initial articular cartilage changes with flanking and fibrillation in injury group, and in the 2 months follow-up, progressive changes in articular cartilage for the same group including chondrocyte enlargement and hyalinization after 4 weeks, and deep fibrillation change and pitting of cartilage 2 months later. But when comparing to treated group, a nearly normal morphology of cartilage was found in the 2 months follow-up, what could be seen in our results too [31]. And finally, the existing results about the management of inflammatory process showed a significant reduction in the inflammatory infiltrate and in joint diameter, where the LLLT seems to increase microcirculation, which should help to reduce edema and to stimulate cell repair processes. In this study, a greater number of LLLT applications might lead to complete control of the arthritic process [30]. In this case, we were also able to see that LLLT reduced the inflammatory process in treated groups, especially in inflammatory infiltrate and cells in the injury site.

In this pathological context, it is still not well elucidated how LLLT improves cartilage wound healing. The results are controversial and not conclusive [22, 18, 31], but we might pay attention to some differences, for example in OA Induced Model and Laser parameters choice. But inversely, there are good results, showing that LLLT is effective in treating OA. One could conclude this, based on simple radiographs and three-dimensional computed tomographs (3D CT) images, gross observations and histopathology, mainly after 4 weeks of treatment. In radiography, it was observed that the bone density was close to normal, as were the joint surface and contour. In 3D CT the articular surface was close to normal and the overall damage was significantly improved, while no damage was observed in the femoral epicondyle. Significant cartilage proliferation was observed in gross appearance and in histopathological analysis the articular surface was replaced with chondrocytes but showed no organized alignment, as in normal articular cartilage, in the 4-week

treatment group [31].And other, saw the initial pathologic change of fibrillation and chondrocyte enlargement improved with LLLT and disappeared in the follow-up, 2 months later [32]. This results are according to ours, demonstrating that LLLT is an effective treatment for OA, decreasing pathological characteristics to normal patterns. However, LLLT mechanisms and even its application are been investigated, but it is already known the positive influence on wound healing (osteoblasts proliferation, increased microvascularization), tissue repair process (collagen synthesis), pain attenuation, and control inflammation [33,34, 9,35].

Of the existing MMPs, MMP-9 is most closely related to MMP-2 at the structural level. The C-terminal, hemopexin-like domain of MMP-2 is essential for its collagenolytic activity. MMP-2 cleaves all three of the interstitial collagen types I, II and III and MMP-9 rather cleaves collagen types I, III and V [36, 37].

The literature shows that OA is linked with degradation of MEC components, such as collagen fibers, and this degradation process occurs by the Matrix Metalloproteinases activity [38, 39, 25]. Our results demonstrated, high levels of both MMP 2 and MMP 9 for injury group compared to control group in each experimental time, and presented high degradation of collagen type III fibers while increased type I fibers, inverting the standard and corroborating with other results [36, 37] and certifying that our animal model can activate MMPs 2 and 9, degrading MEC and changing the normal aspect of collagen type I and III fibers distribution. In both treatment groups, 50 and 100 mW did not expressed differences comparing to control group, suggesting that LLLT reduced MMP levels until ordinary levels. When operating with 50 mW, LLLT was significantly different from injury group, reducing the expression of MMP – 2 and MMP – 9 in 7, 14 and 21 days, acting also in the collagen expression, reducing type I and increasing type III collagen fibers. On the other hand, LLLT at 100 mW also presented differences when compared with injury group, reducing MMP – 2 and MMP – 9 expression, but only in 7 and 14 days, not in 21 days and acting in the collagen distribution, declining collagen type I and enhancing type III fibers. All this results are statistically significant, except diminishing collagen type I fibers in 14days for LLLT – 100mW. The literature has other studies reporting the MMP – 9 cleaving characters for collagen type I and III, as well as MMP – 2: The role of MMP – 9 in wound healing, where increased levels of MMP-9 are linked with poorly healing [36,40]. We found reports with the same behavior for this metalloproteinases in human OA, showing increased levels for MMP – 9 but not for

MMP – 2 suggesting that the production of some of the crucial extracellular matrix-degrading enzymes might participate in the process of cartilage destruction in severe hip OA also in the late stage of the disease [41].

This results for collagen type are according to our findings for the matrix metalloproteinases, and with the related articles in literature where is described the activity of MMP -2 and MMP -9 acting in collagen cleaving and appearing at later stages in the progression of OA [36, 42,43,44].

The LLLT it's one of the alternative procedures that have been utilized and tested in the management of OA [30,9]. The World Association for Laser Therapy (WALT) has described the ideal parameters like time of irradiance, number of points, output, and energy for some pathological conditions in orthopedics area. So, for knee arthritis it's established 3-6 points and 12 Joules of Energy (minimum of 4 Joules/point) and also a mean output of 5- 500 mW and irradiation times ranging between 20 a 300 seconds. This guideline suggests that the irradiating time, the output and consequently the output density are directly related to the achived results and in this way, with our outcomes for the treatment groups. Despite of no statistically difference between group 3 and 4, there're better means results for 50 mW (with statistically significant difference in 21 days experimental time for MMP – 2 when compared to injury group and MMP – 9 when compared to 100 mW group), attesting the positive effect of LLLT: the longer the duration of exposure to light, the better the effect of therapy.

There are few studies (n=10) addressing LLLT and MMP, none about OA. But the results for other tissues (epithelial, muscular, tendineous, nervous central and peripheric) are similar to ours in some aspects: LLLT can modulate the inflammatory process, improving wound healing; stimulates collagen synthesis, modulates the equilibrium between regulatory matrix remodeling enzymes. The studies about LLLT effects on MMP – 2 and MMP – 9 even are lesser, showing that LLLT can stimulate the oxidative metabolism and the expression of matrix metalloproteinase (MMPs), which may indicate a matrix remodeling process, and that the influence above MMP expression it's dependent on the choice of the parameters. Thereby, there are various studies that used like us, an output power lesser than 50 mW [47, 29, 30,48,49], what justifies our option for chosing these parameters.

Conclusion

We found that LLLT operating at 50 mW in OA demonstrated better results as 100 mW in cartilage repair and healing, demonstrating that good results are directly related to chosen parameters, more specifically to output power, time of exposure to the light and finally, treatment duration.

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Figures

Figure 1. Montage photomicrographs of histological knees 7 days after induction of osteoarthritis with papain at 4% - In control group (A), note the presence of slightly thickened synovium (arrow), while in control group (B) is shown the articular surfaces under normal conditions meniscus and articular signs of ossification. Injury In group C, also note connective tissue underlying the articular capsule and synovial membrane showing intense cell proliferation consisting of fibroblasts and macrophages (stars), while in Injury group (D), also notice the tissue repair process medullary spaces of an epiphyseal joint (arrow). In LLLT 50mw In (E), is shown connective tissue underlying the synovial membrane and articular capsule consisting showing intense cell proliferation of fibroblasts and macrophages (stars), while in LLLT 50mw (F) is shown repair process of a spinal dosespaços epiphyseal articular with numerous osteoblast lineage cells forming trabecular immature bone (arrow). In LLLT 100mw (G) is shown connective tissue underlying the synovial membrane and articular capsule consisting showing intense cell proliferation of fibroblasts and macrophages. (stars). In LLLT 100mw (H) note that the meniscus and articular joint surfaces show signs of tissue integrity.

Figure 2. Montage photomicrographs of histological knees 14 days after induction of osteoarthritis with papain at 4% - In control group (A) is shown subsynovial integrates and synovial membrane under normal conditions (star), with the exception of some areas with thick intimal layer (arrow). In control group (B) is shown one of the few

areas with delayed repair process (arrow). In injury group (C) is shown some points of the intima thick and isolated areas with chronic inflammation (star) In injury while in group (D) is observed in bone marrow degenerative process celular.In LLLT 50mW (E), shown is the connective tissue underlying the articular capsule and synovial membrane. Note the presence of high amount of fibroblasts (star) and areas of the intima thickened synovial membrane (arrow). (arrow). In LLLT 50mW (F) is observed joint surfaces with hyaline layer thick red bone marrow (arrow). In LLLT 100mW (G) is shown the thickened synovial membrane and connective tissue repair process, which consists of intense proliferation of fibroblasts. (star). In LLLT 100mW (H) note that the meniscus and articular joint surfaces show signs of tissue integrity, with a thick layer of hyaline cartilage.

Figure 3. Montage photomicrographs of histological knees 21 days after induction of with osteoarthritis papain at 4% -. In control group (A and B) is shown the synovial membrane under normal conditions (arrow) as well as the articular surfaces and red bone marrow of the epiphysis. In injury group (CD) is shown subsynovial and synovial membrane under normal conditions, with the exception of some areas with thick intimal layer and fibrilição signals (arrow). In LLLT 50 mW group (E) is shown the underlying connective tissue in the synovium tissue repair process, however, with fewer fibroblasts (star). In LLLT 50 mW (F) is observed with articular surfaces hyaline layer thick and bone marrow repair process with newly formed trabecular bone (arrow). In LLLT group 100mW (G) is shown the thick synovial membrane (arrow) and in tissue repair process. In LLLT 100mW (H) note that the meniscus and articular joint surfaces show signs of tissue integrity, with a thick layer of hyaline cartilage.

Figure 4. (A) Graph relating to media and standard deviation of the percentage of type I collagen fiber to 7 days after the injury, note the existence of a statistical difference (ϕ $p < 0.05$) between groups injury group with LLLT (groups LLLT 50mw and LLLT 100mw)as well as control group and injury group** ($p < 0.001$). Note also that there is no difference between the treatment groups LLLT 50mw and LLLT 100mw. ($p > 0.05$) (B) Graph relating to media and standard deviation of the percentage of type III collagen fyber to 7 days after the injury; Note that the injury group is statistically different *($p < 0.05$), only control group. Tukey's Multiple Comparison Test. (C)Montage photomicrographs of histological knees 7 days after

induction of an OA process with papain at 4% - using polarized light illustrating the collagen quantification. (C) Control group observe the large amount of fibers that are shown in red color typified as type I collagen and the lack of staining greenish (collagen type III, marked by arrows). (D) The image obtained from the lamina Injury group, observe the rare areas of collagen marked by green color. (E) Treated with LLLT(50mw), this group noted that there is an increase of type III collagen fibers(arrows) in the in smaller quantities red fibers. (D) Group treated with LLLT(100mw), observe presence of the two types of collagen fibers as well as their interlacing(arrows). Stained with picro-sirius red x 40 - Scale bar 20μm.

Figure 5. (A) Graph relating to media and standard deviation of the percentage of type I collagen fiber to 14 days after the injury, note the existence of a statistical difference ($p < 0.05$) between groups injury group with control as well as injury group and LLLT 50mw group ϕ ($p < 0.05$). Note also that there is no difference between the treatment groups LLLT 50mw and LLLT 100mw. ($p > 0.05$) (B) Graph relating to media and standard deviation of the percentage of type III collagen fyber to 14 days after the injury; Note that the injury group is statistically different * ($p < 0.05$), only control group. Tukey's Multiple Comparison Test. (C)Montage photomicrographs of histological knees 14 days after induction of an OA process with papain at 4% - using polarized light illustrating the collagen quantification. (C) Control group observe the large amount of fibers that are shown in red color typified as type I collagen and the lack of staining greenish (collagen type III, marked by arrows). (D) The image obtained from the lamina Injury group, observe the rare areas of collagen marked by green color. (E) Treated with LLLT(50mw), this group noted that there is an increase of type III collagen fibers(arrows) in the in smaller quantities red fibers. (D) Group treated with LLLT(100mw), observe presence of the two types of collagen fibers as well as their interlacing(arrows). Stained with picro-sirius red x 40 - Scale bar 20μm.

Figure 6. (A) Graph relating to media and standard deviation of the percentage of type I collagen fiber to 21 days after the injury, note the existence of a statistical difference **($p < 0.001$) between groups control and group injury. Note also that there is difference between the injury group and treatment groups LLLT 50mw and LLLT 100mw. ϕ ($p > 0.05$) (B) Graph relating to media and standard deviation of the percentage of type III collagen fyber to 21 days after the injury; Note that the injury group is statistically different the treatment groups LLLT 50mw and LLLT 100mw. ϕ ($p < 0.05$). Tukey's Multiple Comparison Test. Montage photomicrographs of

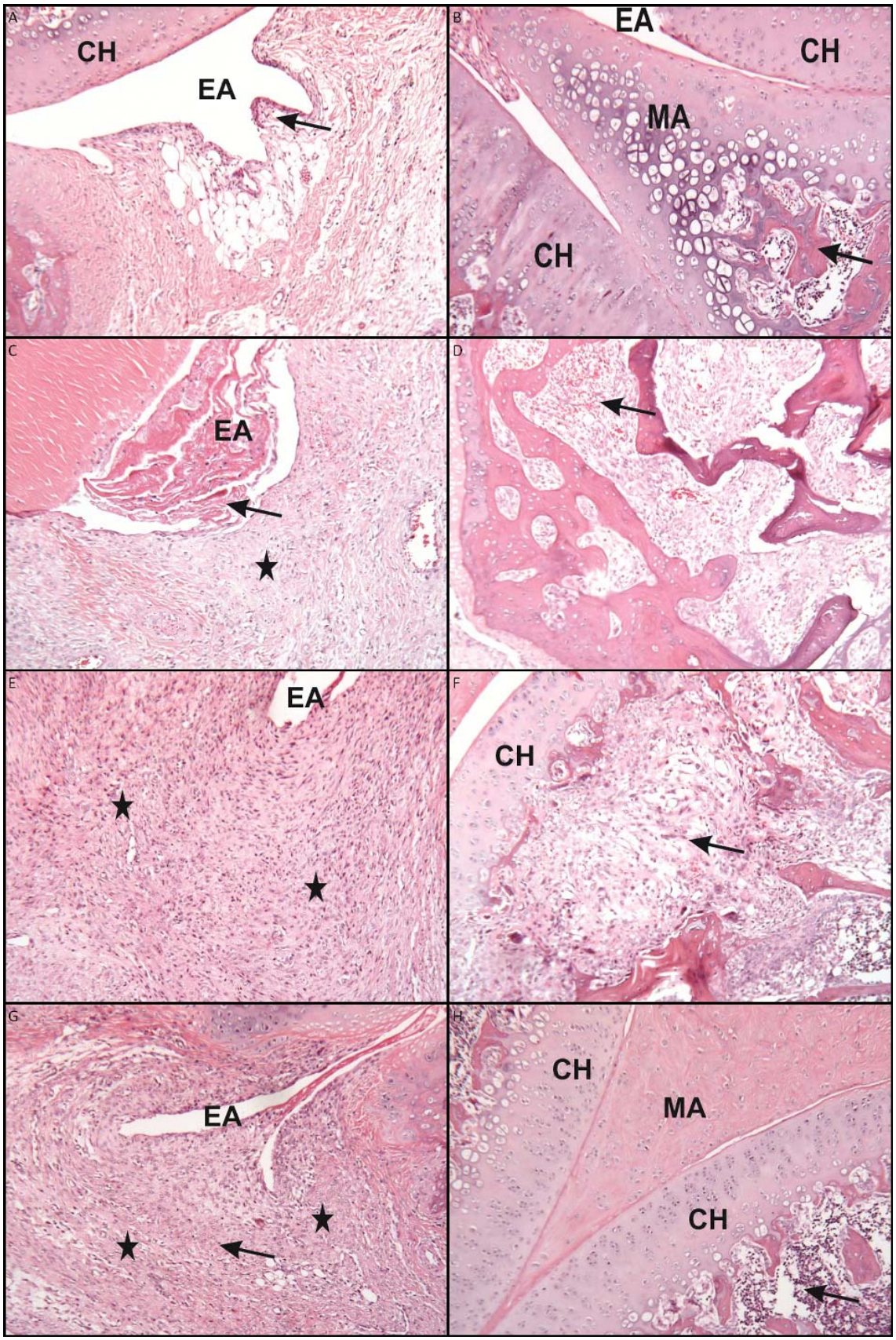
histological knees 14 days after induction of an OA process with papain at 4% - using polarized light illustrating the collagen quantification. (C) Control group observe the large amount of fibers that are shown in red color typified as type I collagen and the lack of staining greenish (collagen type III, marked by arrows). (D) The image obtained from the lamina Injury group, observe the rare areas of collagen marked by green color. (E) Treated with LLLT(50mw), this group noted that there is an increase of type III collagen fibers(arrows) in the in smaller quantities red fibers. (D) Group treated with LLLT(100mw), observe presence of the two types of collagen fibers as well as their interlacing(arrows). Stained with picro-sirius red x 40 - Scale bar 20μm.

Figure 7. Analysis of MMP-9 in the supernatant were Evaluated by Western blot. ß-actin was used to an internal control. (A) After 7 days of induction with 4% papain, the levels of MMP-2 proteins in articular synovial washed, showed statistical difference between the control group and ** injury group (P <0.001), and between the group and injury groups treated with LLLT - 50mw and 100mw ϕ (P <0.05), (B) After 14 days of induction with 4% papain, the levels of MMP-2 proteins in articular synovial washed, showed statistical difference between the control group and injury group ** (P <0.05) and between group injury and treated groups LLLT - 50mW and 100mW ϕ (P <0.05) (C) After 21 days of induction with 4% papain, the levels of MMP-2 proteins washed in articular synovial presented statistical difference between the control group and injury group ** (P <0.05), and between the group and injury group treated with LLLT - 50mw ϕ (P <0.05). However, in samples obtained at 21 days after injury the expression level of 100mW LLLT group was higher than 50mW and LLLT group showed a statistically significant difference. #(P <0.05).

Tables:

Table 1. Low Level Laser Therapy- LLLT parameters

	LLLT 50 mW (G3)	**LLLT 100 mW (G4)**
Laser	AsGaAl	AsGaAl
Equipment	DMC® - Photon Laser III	DMC® - Photon Laser II
Frequency	Continuous	Continuous
Power	100 mW	50 mW
Power Density	3,5 W/cm^2	1,78 W/cm^2
Spot Size (cm^2)	0,028 cm^2	0,028 cm^2
Energy Density	142 J/cm^2	142 J/cm^2
Energy	4 J	4 J
Time per Point	40 s	80 s
Number of Point	02	02
Method	Transcutaneous	Transcutaneous
Place	Knee Medial and Lateral Compartments	Knee Medial and Latera Compartments
Total Energy	8J	8J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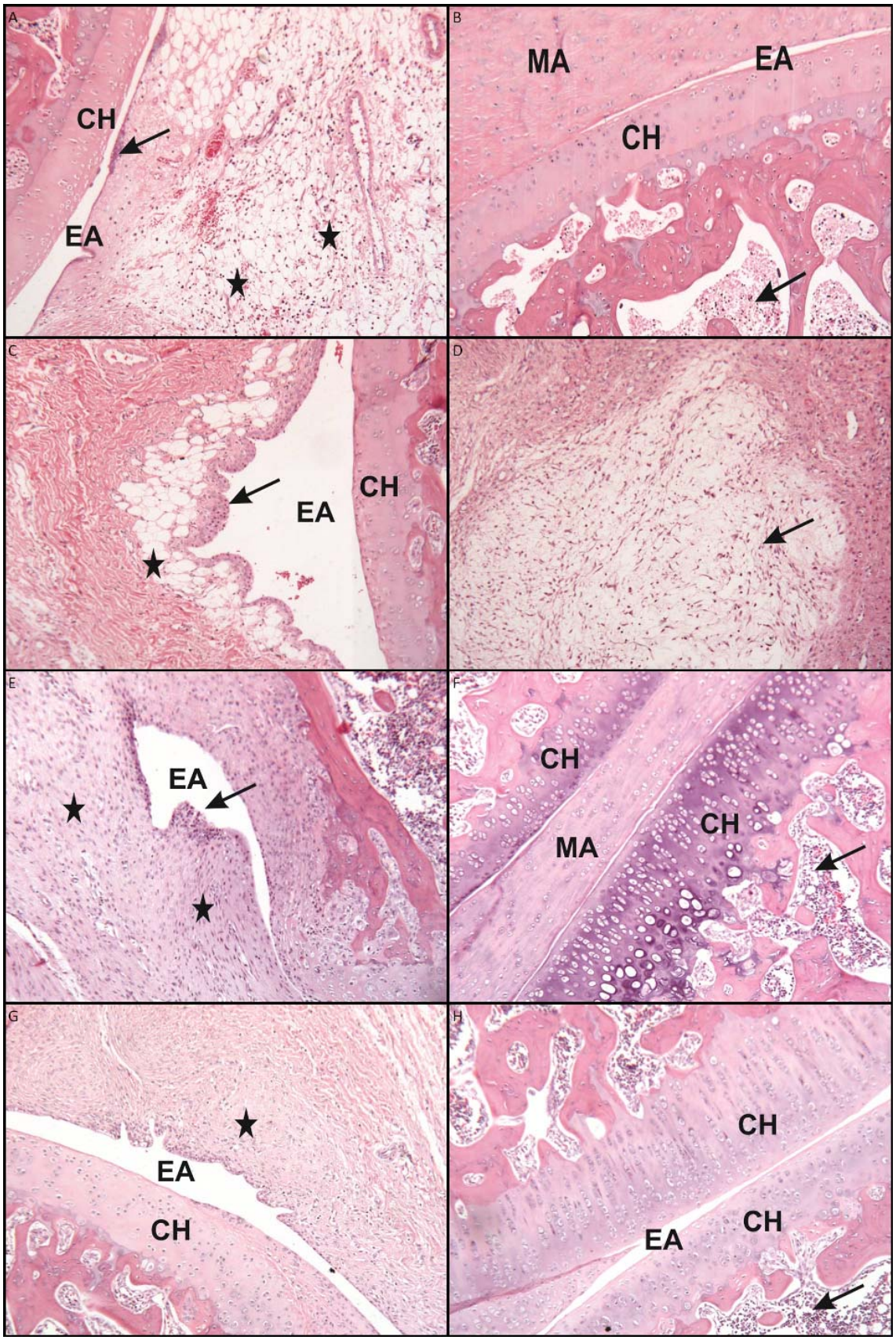
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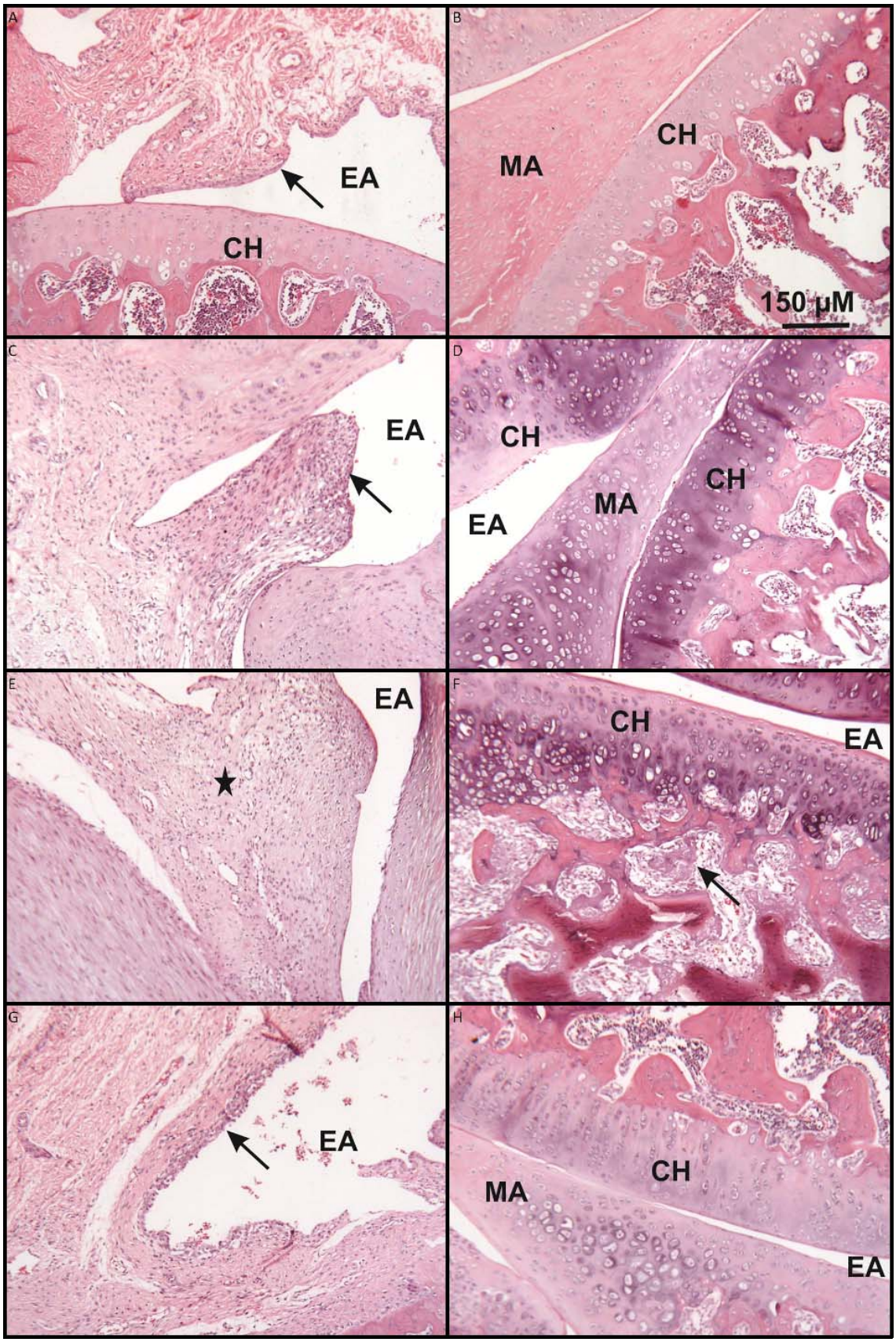
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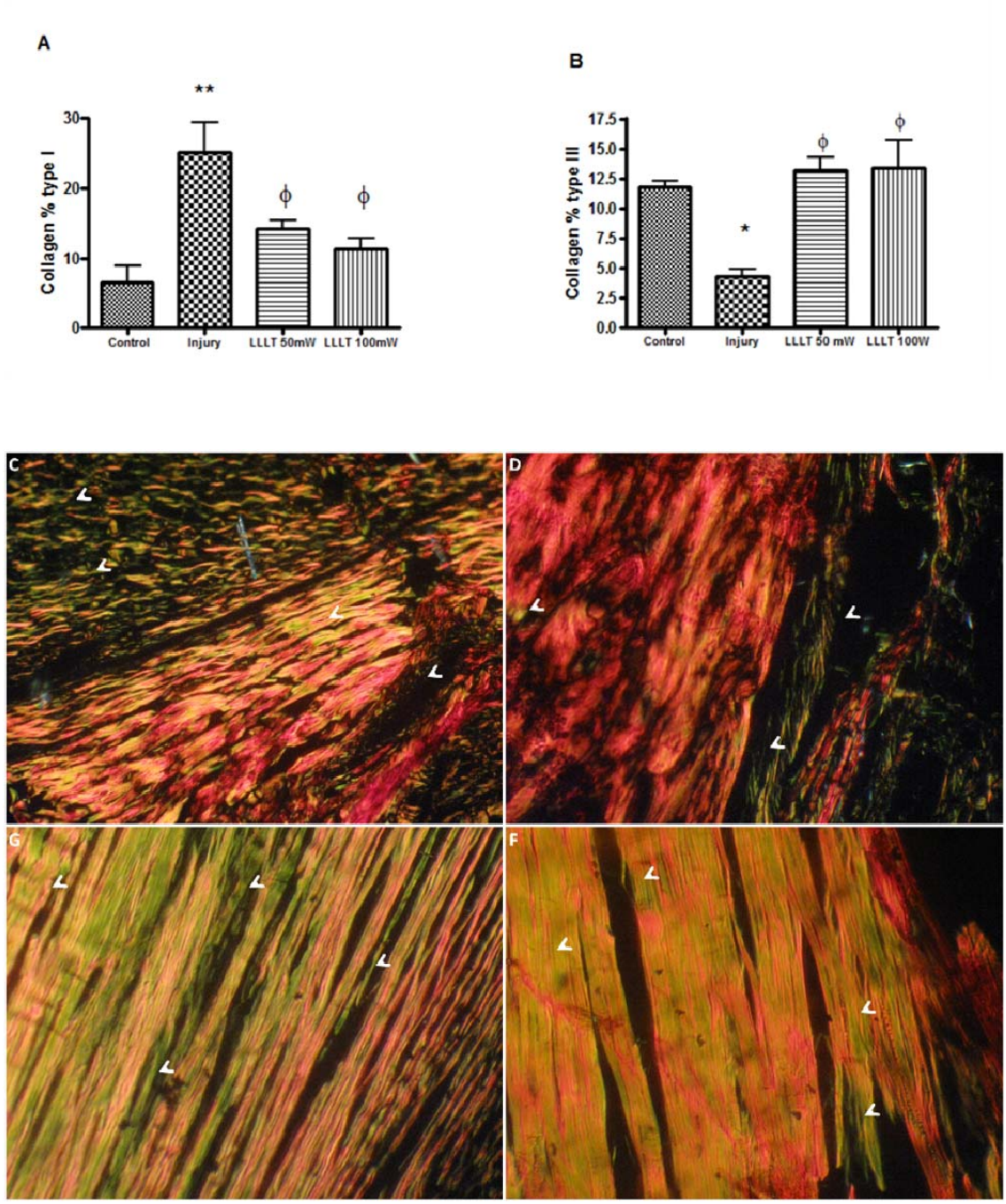
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LLLT 100mW
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12.5
10.0
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*
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ϕ
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Injury
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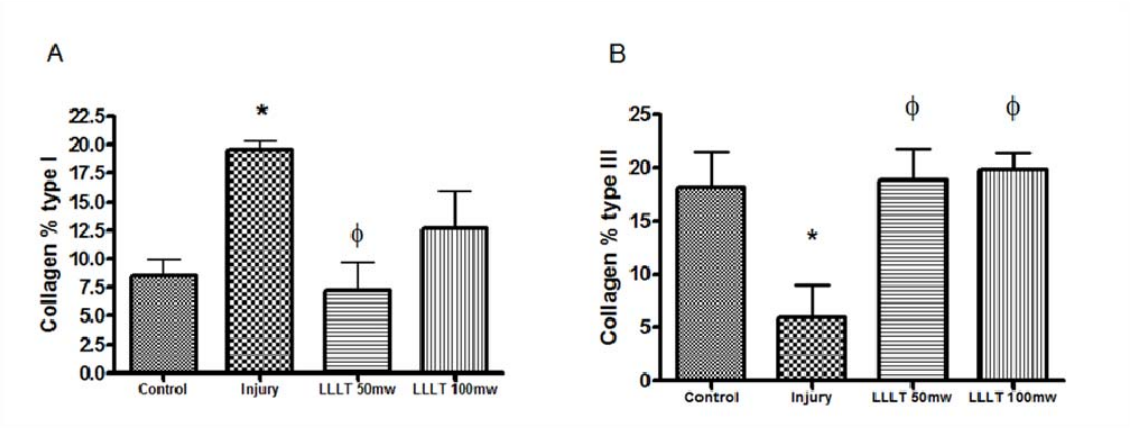
D
G
F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ϕ
Control
Injury
LLLT 50mw
LLLT 100mw
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ϕ
ϕ
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Injury
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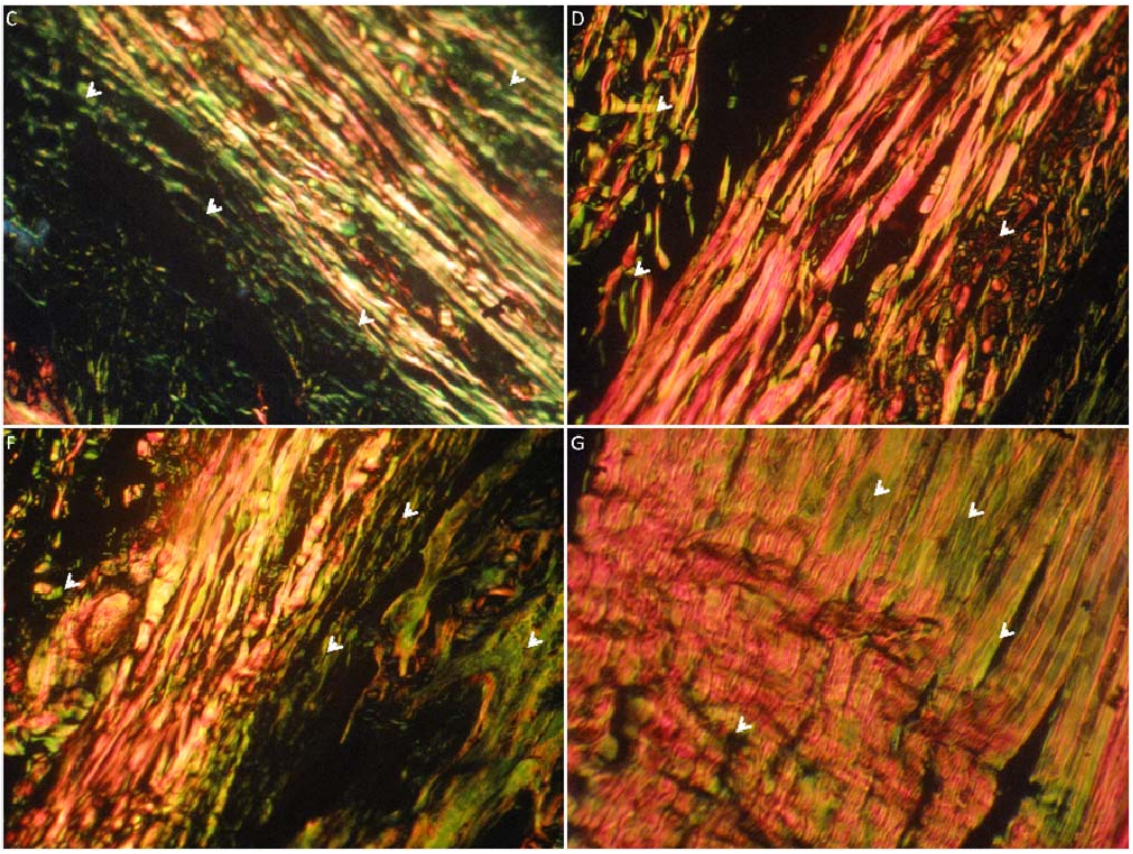
LLLT 100mw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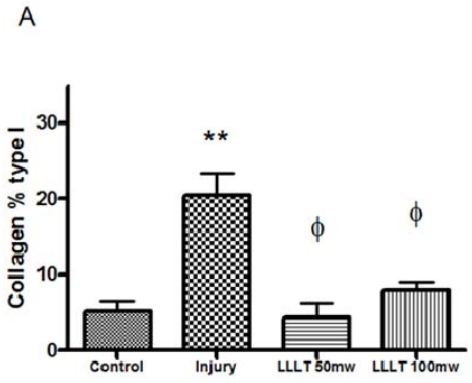
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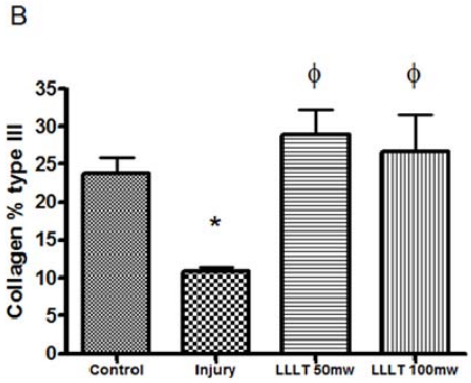
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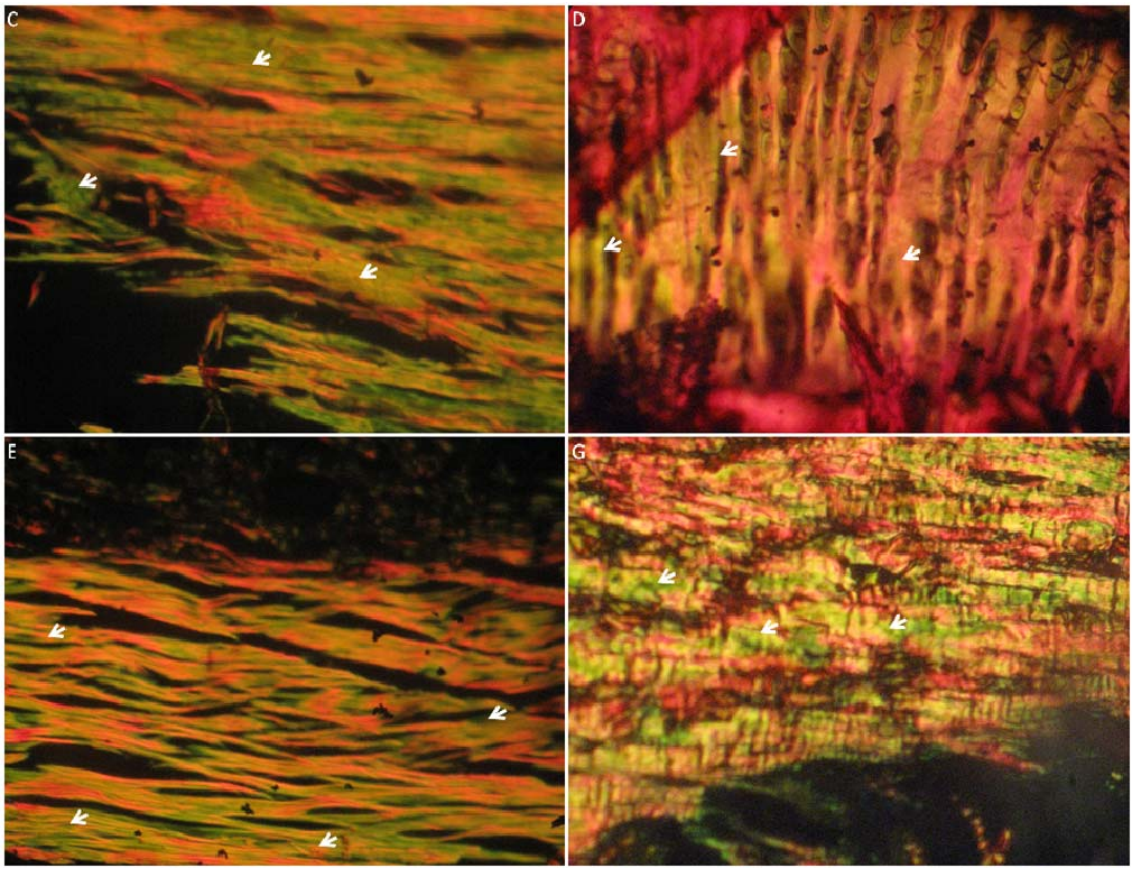
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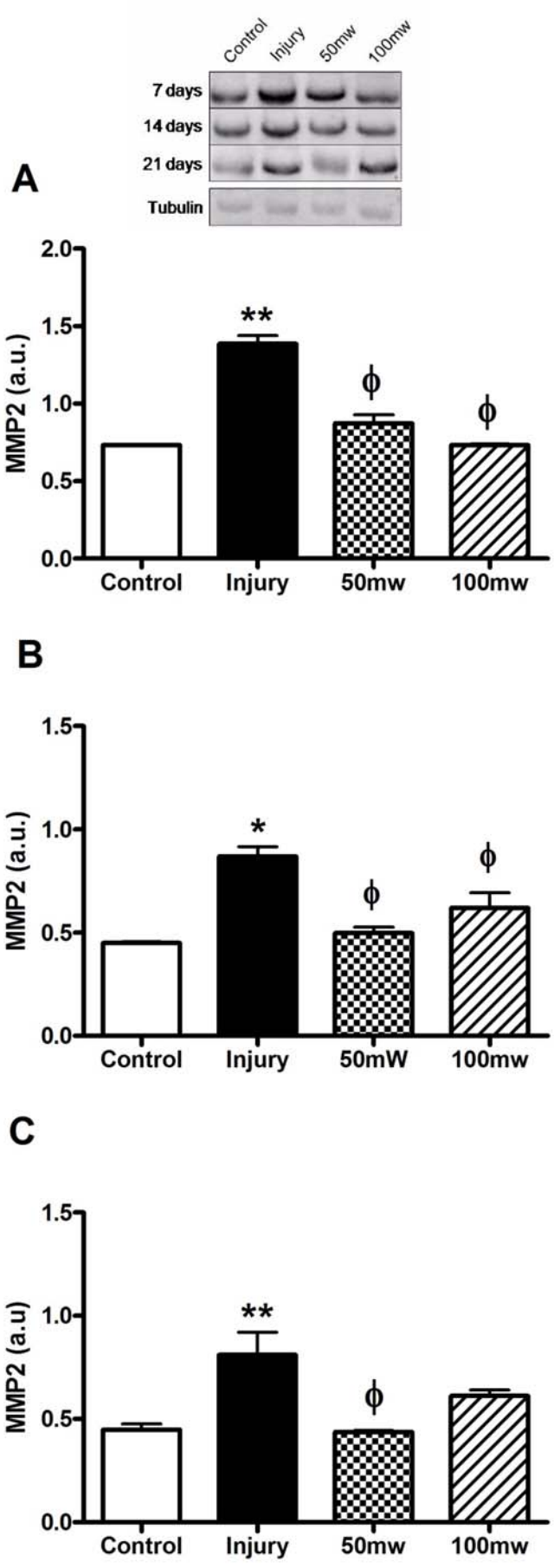
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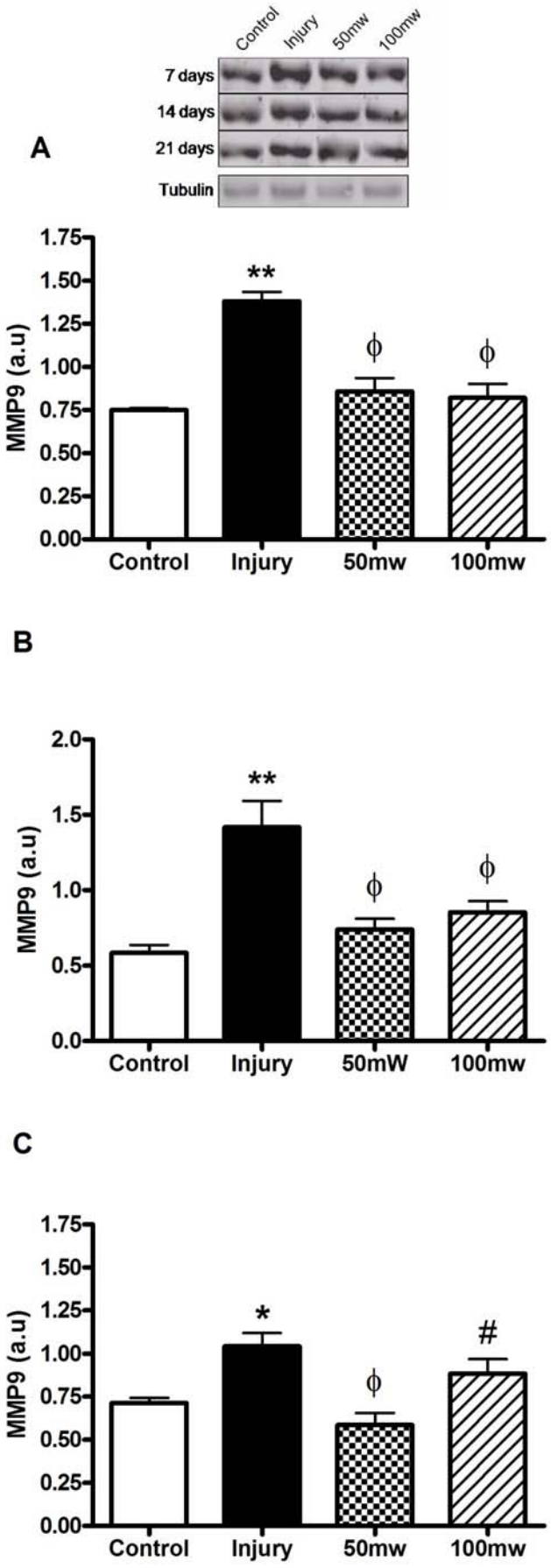
Control
Injury
50mw
100mw
7 days
14 days
21 days
Tubulin
MMP2 (a.u.)
2.0
1.5
1.0
0.5
0.0
**
ϕ
ϕ
Control
Injury
50mw
100mw
B
MMP2 (a.u.)
1.5
1.0
0.5
0.0
*
ϕ
ϕ
Control
Injury
50mW
100mw
C
MMP2 (a.u)
1.5
1.0
0.5
0.0
**
ϕ
Control
Injury
50mw
100mw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7 days
14 days
21 days
Tubulin
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1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
ϕ
ϕ
Control
Injury
50mw
100mw
B
MMP9 (a.u)
2.0
1.5
1.0
0.5
0.0
**
ϕ
ϕ
Control
Injury
50mW
100mw
C
MMP9 (a.u)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
ϕ
#
Control
Injury
50mw
100mw

5. CONSIDERAÇÕES FINAIS

A OA caracteriza-se por um processo complexo e multifatorial, envolvendo o catabolismo e anabolismo da cartilagem. Este processo realiza-se por um balanço entre a síntese e degradação de vários componentes da matriz extracelular (MEC). As metaloproteinases de matriz (MMPs) são considerados como sendo enzimas-chave na degradação de MEC, onde a destruição progressiva desta, provoca danos na cartilagem articular, pois as MMPs são capazes de atacar colagénio intersticial – como os tipos I, II e III (AKTAS, 2011; CHEN,2011;LIN, 2006; MICHALANY, 1980).

No presente estudo, realizamos uma indução química através de papaína que pôde degradar o tecido cartilaginoso, o que foi visto na análise histopatológica. Os dois grupos tratados aceleraram e intensificaram o processo de reparo, mas 50 mW alcançou um aspecto semelhante ao normal previamente(14 dias). E aos 21 dias, os grupos de intervenção já estão apresentavam cartilagem hialina homogênea com características habituais, enquanto o grupo lesão ainda apresenta intenso sinal de reparação tecidual. Este restabelecimento do aspecto padrão através dos grupos laser também está relacionado em outros artigos, bem como redução dos sinais inflamatórios, principalmente do infiltrado inflamatório, com o aumento da microcirculação (LIN, 2006; PFANDER, 2006; POMONIS, 2005).

Das MMPs existentes, MMP-9 é a mais estreitamente relacionada com a MMP-2 em níveis estruturais. Funcionalmente, a MMP-2 cliva todos os três tipos de colágeno I, II e III e a MMP-9 ,por sua vez cliva I, III e V (GUO, 2011; NAITO, 1999). A literatura mostra que a OA está relacionada com a degradação dos componentes da MEC, bem como das fibras de colagénio, e este processo de degradação ocorre pela atividade metaloproteinases da matriz (LIU, 2009; OTERO, 2012; PFANDER, 2006). Os grupos de tratamento, 50 e 100 mW não expressaram diferenças quando comparados ao grupo controle, indicando que a TLBI reduziu os níveis das MMPs estudadas até níveis normais. Quando operando com 50 mW, a TLBP reduziu a expressão de MMP - 2 e MMP - 9 em 7, 14 e 21 dias, atuando também na expressão de colágeno, reduzindo tipo I e aumentando as fibras de colágeno do tipo III. Os resultados de expressão de colágeno corroboram com outros artigos, onde é descrita a atividade de MMP-2 e MMP -9 degradando colágeno e aparecendo em fases tardias da OA (HULEJOVÁ, 2007; LIU, 2009; OTERO, 2012; POMONIS, 2005). Já a laserterapia de 100 mW teve resultados semelhantes aos da TLBP com

50mW, incluindo em relação a expressão do colágeno, entretanto não houve diferença somente na expressão da MMP - 2 em 21 dias. Estudos relacionam o aumento dos níveis de MMP-9 a cicatrização ruim (BIGG, 2007; POMONIS, 2005). Encontramos relatórios com o mesmo comportamento para este metaloproteinases em OA humana, mostrando aumento dos níveis de MMP -9 (POMONIS, 2005).

A TLBI é um dos procedimentos alternativos que têm sido utilizados e testados no tratamento de OA (GUO, 201; ROCHA JÚNIOR, 2009). A Associação Mundial de Laser Therapy (WALT) descreveu os parâmetros ideais para o tratamento. Esta orientação sugere que o tempo de irradiação, a potência de saída e, consequentemente, a densidade de potência estão diretamente relacionadas com os resultados alcançados e, também com os nossos resultados para os grupos de tratados. Assim, existem vários estudos que utilizaram como nós, uma potência de saída menor do que 100 mW (CASTANO, 2007; DE MORAIS, 2010;KAMALI, 2007; PFANDER, 2006), o que justifica nossa opção por comparar estes parâmetros.

Durante todo o experimento não houve diferença estatisticamente significativa entre os grupos tratados com TLBI com 100 e 50 mW. Exceto para 2 resultados: o primeiro referente a MMP – 2 em 21 dias, onde a TLBI com 50 mW diminui a sua expressão quando comparado ao grupo lesão e o segundo, refere-se a MMP – 9 no mesmo tempo experimental, no qual os grupos tratados apresentam diferenças, com melhor resultado para o grupo TLBP 50 mW. Este grupo, portanto, apresentou melhores médias, o que sugere um indício positivo para a TLBP: quanto maior o tempo de exposição à luz, melhor o efeito do tratamento. Existem poucos estudos LBI e MMP, nenhum abordando OA. Os estudos sobre os efeitos da TLBP sobre MMP - 2 e MMP - 9 são ainda mais excassos, ilustrando o quanto essa área ainda necessita de pesquisas.

6. CONCLUSÃO

Nossos resultados demonstram que a TLBP estimula o metabolismo tecidual durante o processo de reparo, e atua diretamente na expressão de metaloproteinases de matriz (MMPs), atenuando o processo de remodelamento da MEC em casos de lesão da cartilagem, e que a sua influência na expressão das MMPs 2 e 9 é dependente da escolha dos parâmetros. Nesse estudo obtivemos bons resultados com ambas as potências de saída, porém a TLBP com 50mW demonstrou melhores resultados.

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8. APÊNDICE

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ORIGINAL ARTICLE

Low-level laser therapy in different stages of rheumatoid arthritis: a histological study

Ana Carolina Araruna Alves · Paulo de Tarso Camillo de Carvalho · Marcio Parente · Murilo Xavier · Lucio Frigo · Flávio Aimbire · Ernesto Cesar Pinto Leal Junior · Regiane Albertini

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Abstract Rheumatoid arthritis (RA) is an autoimmune inflammatory disease of unknown etiology. Treatment of RA is very complex, and in the past years, some studies have investigated the use of low-level laser therapy (LLLT) in treatment of RA. However, it remains unknown if LLLT can modulate early and late stages of RA. With this perspective in mind, we evaluated histological aspects of LLLT effects in different RA progression stages in the knee. It was performed a collagen-induced RA model, and 20 male Wistar rats were divided into 4 experimental groups: a non-injured and non-treated control group, a RA non-treated group, a group treated with LLLT (780 nm, 22 mW, 0.10 W/cm^2, spot area of 0.214 cm^2, 7.7 J/cm^2, 75 s, 1.65 J per point, continuous mode) from 12th hour after collagen-induced RA, and a group treated with LLLT from 7th day after RA induction with same LLLT parameters. LLLT treatments were performed once per day. All animals were sacrificed at the 14th day from RA induction and articular tissue was collected in order to perform histological analyses related to inflammatory process. We observed that LLLT both at early and late RA progression stages significantly improved mononuclear inflammatory cells, exudate protein, medullary hemorrhage, hyperemia, necrosis, distribution of fibrocartilage, and chondroblasts and osteoblasts compared to RA group ($p<0.05$). We can conclude that LLLT is able to modulate inflammatory response both in early as well as in late progression stages of RA.

Keywords Phototherapy · Rheumatoid arthritis · Inflammation

A. C. A. Alves · P. T. C. de Carvalho · M. Parente · E. C. P. Leal Junior (✉) · R. Albertini
Post Graduate Program in Rehabilitation Sciences, Nove de Julho University (UNINOVE), Rua Vergueiro, 235, 01504-001 São Paulo, São Paulo, Brazil
e-mail: ernesto.leal.junior@gmail.com

P. T. C. de Carvalho · E. C. P. Leal Junior · R. Albertini
Post Graduate Program in Biophotonics Applied to Health Sciences, Nove de Julho University (UNINOVE), São Paulo, São Paulo, Brazil

M. Xavier
Biomedical Engineering Center, Camilo Castelo Branco University, São José dos Campos, São Paulo, Brazil

M. Xavier
Department of Physical Therapy, Vale do Jequitinhonha and Mucuri Federal University, Diamantina, Minas Gerais, Brazil

L. Frigo
Biological Sciences and Health Center, Cruzeiro do Sul University, São Paulo, São Paulo, Brazil

F. Aimbire
Federal University of São Paulo (UNIFESP), São José dos Campos, São Paulo, Brazil

Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease of unknown nature and etiology. It is characterized by peripheral symmetric polyarthritis, which leads to deformity and destruction of joints. With disease progression patients develop inability to perform daily life activities, resulting in significant personal, professional, and social impact [1].

The RA is staged in acute and chronic phases. In acute phase after inflammatory stimulus, the vascular endothelium begins to express adhesion molecules, which facilitates the migration of inflammatory cells. Inflammatory mediators derived from these cells enhance the number of inflammatory

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cells, principally neutrophils, at the site of injury, modulate vascular tone via vasodilatation and increased vascular permeability and consequently the edema. The chronic phase represents the perpetuation of inflammatory response with the presence of cells and mediators responsible by late phase of inflammation, which culminates in necrosis and posteriorly destruction of articular cartilage [2].

The signs, symptoms, and clinical course of RA can be extremely variable, ranging from mild, self-limiting arthritis to rapidly progressive disease that is associated with significant physical and psychosocial morbidity and premature mortality [2].

Current literature refers to some animal experiments [3–6] and clinical trials [7–10] showing positive effects in favor of low-level laser therapy (LLLT) for treatment of several kinds of joint inflammation. On the other hand, few authors have studied the use of LLLT in treatment of RA [11–14].

In spite that Cochrane Database [15] concludes that LLLT could be considered for short-term treatment for relief of pain and morning stiffness for RA patients, recent studies have reported mixed results about the therapy. For instance, Meireles et al. [16] applied LLLT (at a wavelength of 785 nm, dose of 3 J/cm^2, and mean power of 70 mW) in the hands of patients with RA and do not observed positive effects. On the other hand, Bálint et al. [17] irradiated synovial membrane samples obtained from five people who had RA and were undergoing knee surgery with a near-infrared diode laser at a dose of 25 J/cm^2, and observed that LLLT resulted in decreases in both α-enolase and vimentin expression in the synovial membrane.

With these factors in mind, in the current study we sought to investigate the LLLT anti-inflammatory effect on histological features of knee from animals subjected to model of rheumatoid arthritis.

Materials and methods

Animals

Twenty male Wistar rats (*Norvegicus albinus*), approximately 90 days of age, weighing 250–300 g were used in this experiment. Animals were obtained from Central Animal House of Research and Development Institute of Vale do Paraíba University (UNIVAP)—Brazil, and they were kept under controlled conditions of light and temperature, with water and food ad libtum. All experimental procedures were approved by Institutional Research Ethics Committee (protocol number A014/2007/CEP) and followed the guidelines of the Brazilian College for Animal Experimentation as well as the standards of the International Council for Laboratory Animal Science

Experimental groups

Animals were randomly distributed into four groups of five animals each. First group does not received any kind of intervention (control group), second group received RA induction but does not received any treatment (RA), third group was treated with laser in early RA stage (early RA+LLLT group), and rats of the fourth group were treated with LLLT in late RA stage (late RA+LLLT group).

Collagen-induced arthritis

For immunization, type II collagen (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.01 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. An emulsion was made with incomplete Freund's adjuvant (Sigma Aldrich, St. Louis, MO, USA) and collagen solution at a ratio of 6:4 using a homogenizer at high speed for 2 min. Wistar rats were given 0.1 ml of cold emulsion by knee joint injection and received a booster injection in the tail 1 week later using another identically prepared emulsion [18].

Low-level laser therapy

It was used a laser device manufactured by (MMoptics, São Carlos, Brazil). For laser irradiation, animals were immobilized by means of grip and were irradiated at an angle of 90° to the surface of tissue area. LLLT was performed once per day. Therefore, animals of early RA+LLLT group were treated 14 times, and animals of late RA+LLLT group were treated 7 times. Before the experiments started, the laser equipment was checked with a power checker (13PEM001/J, Melles Griot, The Netherlands). All rats were placed in common cages and randomly divided into groups of five. Parameters employed are shown in Table 1.

Table 1 LLLT parameters

Wavelength, 780 nm
Laser frequency: Continuous output
Power output, 22 mW
Spot size, 0.214 cm^2
Power density, 0.10 W/cm^2
Energy delivered per treatment, 1.65 J
Total energy delivered, 23.1 J (early RA+LLLT group) and 11.55 J (late RA+LLLT group)
Energy density, 7.7 J/cm^2
Irradiation time per treatment, 75 s in each point
Number of points treated, 1 (at middle of front side of knee joint)
Application mode: probe held stationary in skin contact with a 90 ° angle and slight pressure

Euthanasia and histological procedures

The animals submitted to euthanasia for the histological procedure to be carried out. For such they were identified, weighed, and afterwards submitted to euthanasia with lethal dose of 200 mg/kg of sodium pentobarbital via intraperitoneal injection. Animals from all experimental groups were sacrificed at 14th day after RA induction.

The segments to be used in the histology were fixed in 10 % formaldehyde for 24 h. After this period, they were decalcified using EDTA, embedded in paraffin blocks, and submitted to 5 μm transversal sections. Slides containing two sections each were prepared, and they were also stained using hematoxylin–eosin. The whole defects extension was sequentially sectioned in order to the surgical defect central region could be evaluated.

The histological evaluation of the inflammatory events was done in a descriptive way using a semi-quantitative method based on the knowledge about the aspects related to the normality and following the requisites used previously by Leonel et al. [19], Ré Poppi et al. [20], and AboElsaad et al. [21]. Samples were stained with hematoxylin–eosin and were subjected to morphological analysis with light microscopy (Zeiss, USA) by two pathologists who evaluated samples blindly and independently. In order to quantify the outcomes analyzed in histological evaluation (exudate protein, distribution fibrocartilage, hyperemia, mononuclear inflammatory cells, medullary hemorrhage, necrosis, and chondroblasts and osteoblasts), blinded assessors used a standard scoring intensity system (Table 2). Laser treatment in early RA stage group started at 12th hour after RA induction and at 7th day for late RA stage group.

Statistical analysis

The data were initially evaluated considering the distribution normality hypothesis. The normality was defined through the application of the Shapiro–Wilk test. The decision level was established at $p<0.05$. The test used for comparison between groups was ANOVA with Tukey post hoc test and significant level was established at $p<0.05$.

Table 2 Parameters employed to make histological score

Event intensity	Score equivalence
Absent	1
Rare	2
Discrete and focal	3
Discrete and diffuse	4
Moderate and focal	5
Moderate and diffuse	6
Intense and focal	7
Intense and diffuse	8
Severe and focal	9
Severe and diffuse	10

Results

Figure 1 shows a general picture of histological pattern presented in all 4 experimental groups.

The presented data allow us to observe that groups treated with LLLT showed reduction of inflammatory cells when compared with RA group in both periods (early RA stage and late RA stage—$p<0.05$). There was no difference between the groups treated with LLLT ($p>0.05$; Fig. 2).

Data presented in Fig. 3 shows it was a significant difference regarding intensity of exudate between groups ($p<0.05$). The comparative analysis of the mean and standard deviation of the scores obtained by semi-quantitative analysis of the exudate showed that groups treated with LLLT had a lower exudate than in the RA group ($p<0.05$). There was no significant difference between treated groups ($p>0.05$).

The same pattern was observed in intensity score for the variable medullary hemorrhage, it was noted difference between groups. Tukey post test shows that both LLLT treated groups had statistically significant difference from the RA group ($p<0.05$), and there was no significant difference between LLLT treated groups ($p>0.05$; Fig. 4).

In Fig. 5 is possible to observe the comparison between the mean scores obtained from intensity of hyperemia. Statistical analysis between groups shows significant difference between both LLLT groups when compared to the RA group ($p<0.05$). Group treated early with LLLT showed significant difference compared to control group ($p<0.05$). Additionally, it was also found that significant differences between the LLLT treated groups ($p<0.05$).

In Fig. 6, we can see the comparison between the mean scores obtained from the semi-quantitative analysis for necrosis. We found significant statistical difference between both groups treated with laser compared to RA group ($p<0.05$). Groups treated both early than late with LLLT showed significant difference compared to control group ($p<0.05$). There was no difference between treated groups ($p>0.05$).

Figure 7 shows the comparison between the mean scores obtained from the semi-quantitative analysis for the variable distribution fibrocartilage. It was observed statistically significant differences between groups in ANOVA test ($p<0.05$). In Tukey post test analysis groups treated with LLLT group showed statistically significant difference ($p<0.05$) compared to the RA group. There was no difference between LLLT treated groups ($p>0.05$).

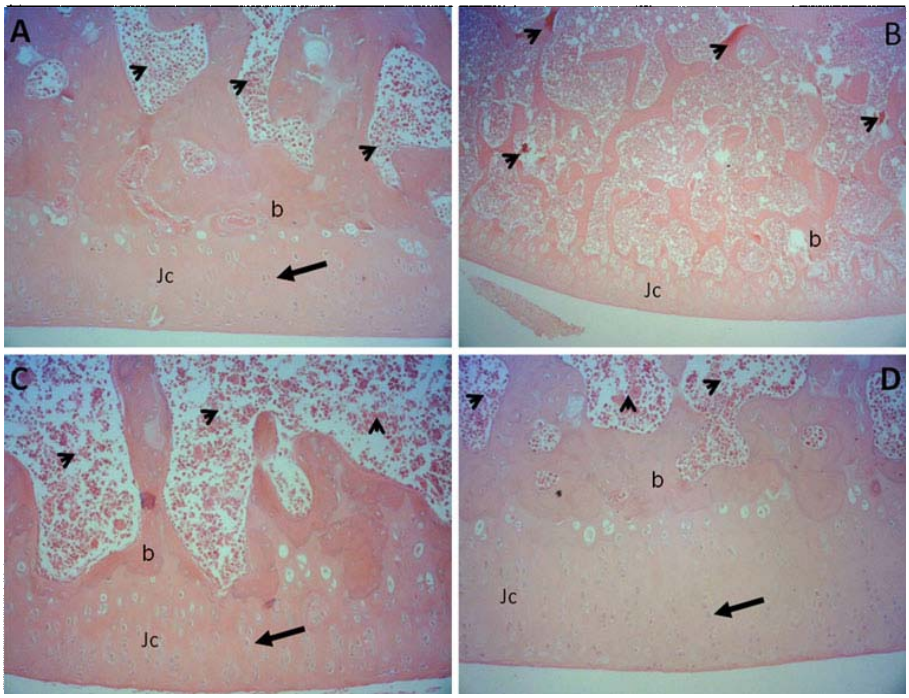

Fig. 1 Representative photomicrographs the experimental groups. **a** Represents group where saline were injected in animals' knees, normal morphology of joint cartilage (*Jc*) can be observed. Undisrupted organization of chondrocytes (*arrow*) and extra cellular matrix, absence of inflammatory cells are marked. **b** Represents untreated RA group, thinner cartilage joint and subchondral bone (*b*) disruption can be observed, there are also hemorrhage areas (*arrowhead*). **c** Group treated with laser at the early RA phase presents presence of numerous blood vessels at subchondral bone (*arrowhead*), which is also observed at joint cartilage. The structure of articular cartilage has a similar pattern of cell alignment observed in healthy animals (*arrow*). **d** Group treated with laser at the late RA phase, increased vascular beds are conspicuous (*arrowhead*), and the structure of articular cartilage has a similar pattern of cell alignment and extra cellular matrix as observed in healthy animals

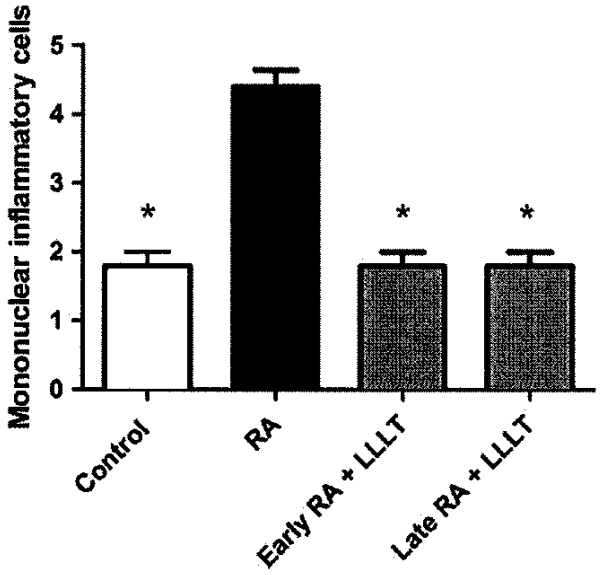

Fig. 2 Score of intensity of mononuclear inflammatory cells in experimental groups; Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group—ANOVA and Tukey post test)

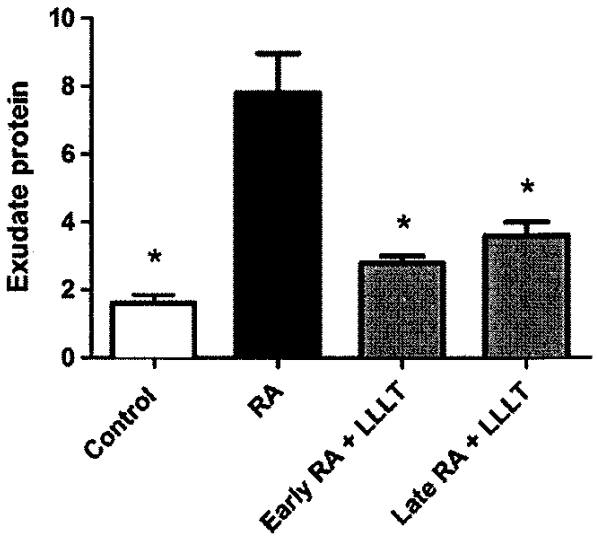

Fig. 3 Score of intensity of the exudate protein in experimental groups. Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group—ANOVA and Tukey post test)

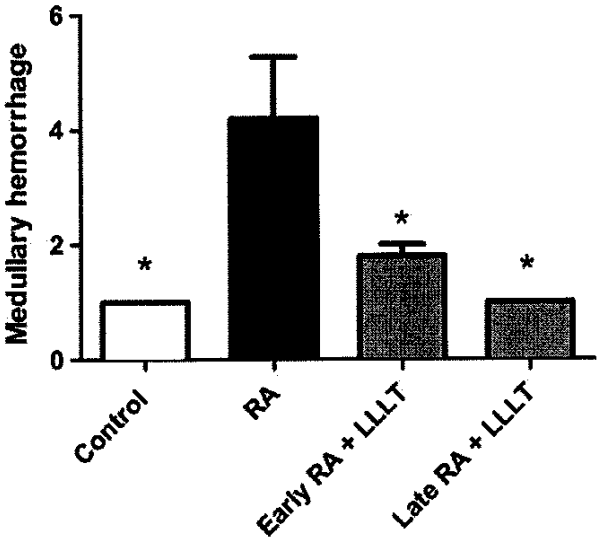

Fig. 4 Score of intensity of medullary hemorrhage in experimental groups. Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group—ANOVA and Tukey post test)

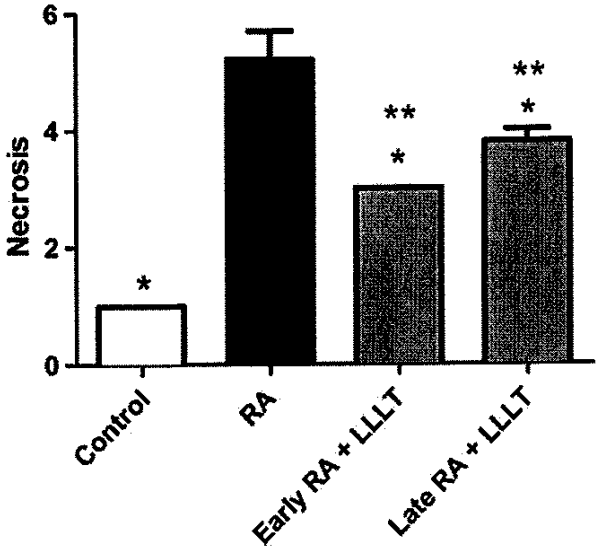

Fig. 6 Score of necrosis in experimental groups. Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group and **$p<0.05$ compared to control group—ANOVA and Tukey post test)

In Fig. 8, we can see the comparison between the mean scores obtained from the semi-quantitative analysis of chondroblasts and osteoblasts. We found statistically significant differences between groups ($p<0.05$). In Tukey post test analysis groups treated with LLLT group showed statistically significant difference ($p<0.05$) compared to the RA group. One more time there was no difference between the treated groups ($p>0.05$).

Discussion

Rheumatoid arthritis (RA) is the most common kind of chronic joint inflammation and has a large incidence in general population in several countries. In the last years, several animal and human trials have shown that LLLT has modulatory effects on inflammatory markers (prostaglandin E_2, tumor necrosis factor-α, interleukin-1β, plasminogen activator, cyclooxygenase 1, and cyclooxygenase 2), reduces the inflammatory

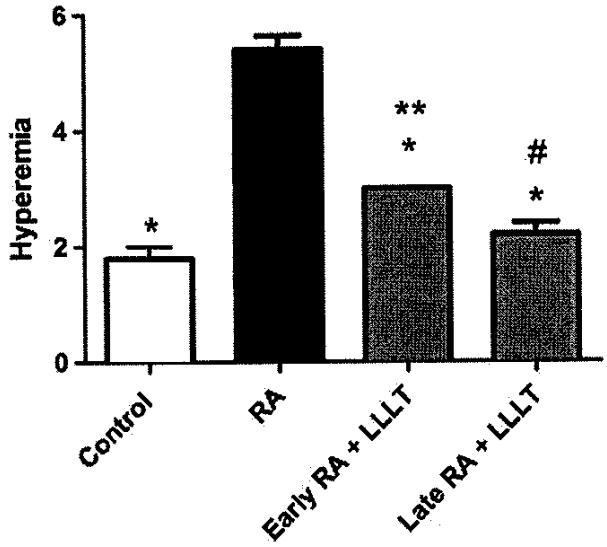

Fig. 5 Score of intensity of hyperemia in experimental groups. Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group, **$p<0.05$ compared to control group, and #$p<0.05$ comparing LLLT groups—ANOVA and Tukey post test)

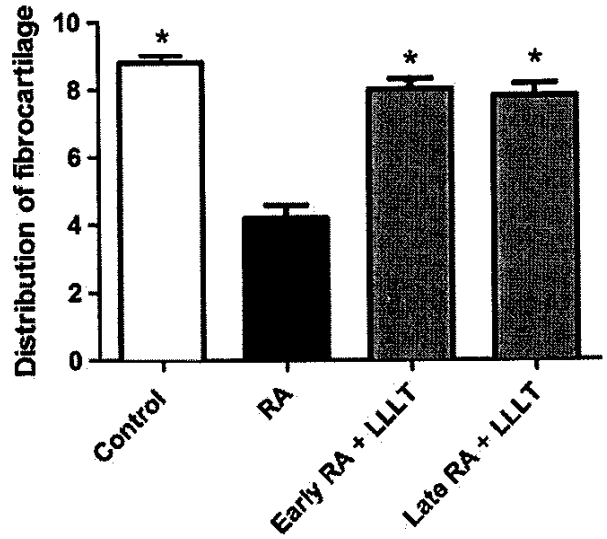

Fig. 7 Score of intensity of distribution fibrocartilage in experimental groups. Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group—ANOVA and Tukey post test)

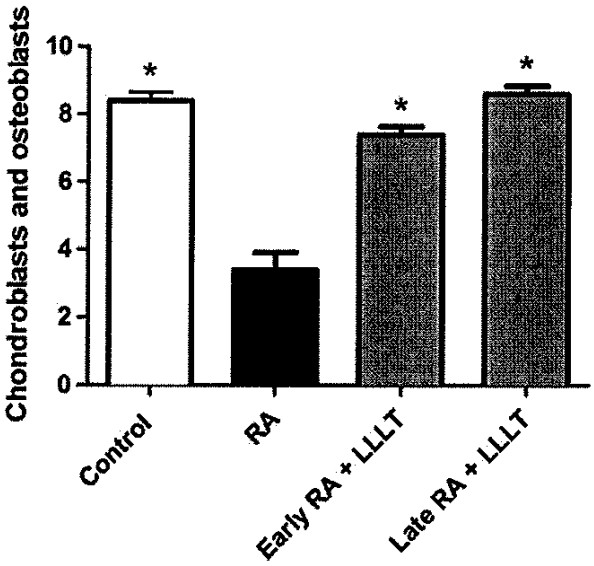

Fig. 8 Score of intensity of chondroblasts and osteoblasts in experimental groups. Values are expressed as mean and standard deviation (*$p<0.05$ compared to RA group—ANOVA and Tukey post test)

process itself (edema, hemorrhagic formation, necrosis, neutrophil cell influx), and modulates leucocyte activity (macrophages, lymphocytes, neutrophils) [22–29].

In this study, the periods were chosen in according to histological aspects observed in the experimental model commonly used to mimics the clinical signs of rheumatoid arthritis. Although the experimental model used in the present manuscript reproduces the clinical signals of rheumatoid arthritis is important note that the time course of development and perpetuation of this syndrome is different that observed in humans. Additionally, considering that rheumatoid arthritis is an autoimmune disease, the experimental model used herein was induced through of immunization which requires 14 days for that the chronic inflammation can be recognized histologically.

Regarding parameters used in this research, the choice of energy density as well as wavelength was based on a previous study from our group [30] that demonstrated a significant anti-inflammatory effect of laser on collagenase-induced tendinitis.

In our outcomes was observed a decrease of inflammatory cells comparing LLLT treated groups with RA group, and there was no difference between the groups treated with LLLT. This shows that laser irradiation in both RA progression stages showed anti-inflammatory effects, corroborating with previous studies that have reported significant reduced inflammatory influx in acute phase as well as in chronic phase of inflammatory process due phototherapy [30, 31].

It has been reported that one of the main aspects related to inducing inflammation is the presence of inflammatory cells at the injured site due interactions between the damaged tissue and circulating leukocytes. After stimulation of inflammation, the vascular endothelium begins to express adhesion molecules, which facilitates the migration of inflammatory cells into the injured tissue [32, 33].

Furthermore, inflammatory mediators such as prostaglandins (PGE_2), thromboxane (TXA2), leukotrienes (LTD4), nitric oxide (NO), cyclooxygenase (COX), tumor necrosis factor-α (TNF-α), and interleukins are released by nearby cells. These mediators enhance number of inflammatory cells (monocytes and neutrophils) at the site of injury, modulate vascular tone via vasodilatation and increased vascular permeability [34].

LLLT has promoted a decrease in intensity of exudate in both treated groups, demonstrating that in both periods the laser irradiation favored the decrease in serum fibrinous exudates; moreover, our results show that LLLT promoted a decrease in medullary hemorrhage. Honmura et al. [35] have previously employed an experimental model of induced inflammation and Ga-Al-As (780 nm) laser irradiation with 10 mW and 31.8 W/cm^2. The authors found that irradiation was effective in reducing the volume of the exudate decreasing vascular permeability. This suggests the existence of different mechanisms that may be involved in the inhibitory effects of LLLT on vascular permeability of electrolytes and plasma proteins [35]. Our outcomes also showed that treatment with LLLT is effective in reducing increased vascular permeability and edema, and as known, these are among the earliest events in the inflammatory response following by infiltration of leukocytes.

The intensity of hyperemia was significantly decreased in LLLT groups. However differences were found between these groups, showing that hyperemia in late RA+LLLT group was lower than in early RA+LLLT group. This result differs from previously found by other authors. Sandoval et al. [6] evaluated the effects of LLLT in clinical signs of inflammation and cellular composition of synovial fluid in inflamed knees, and they suggested that LLLT might have a positive effect on the vascular aspects of inflammatory process, mainly when treatment begins in early phases, promoting faster recovery. Therefore, further studies are needed to clarify this point.

As observed in our outcomes the levels of chondroblasts and osteoblasts decreased with LLLT, which is beneficial in synovial joints lubrication. The sinovial fluid (SF) is an ultrafiltrate of plasma with secreted contributions from cell populations lining and within the synovial joint space, including chondrocytes and synoviocytes. Maintenance of normal SF lubricant composition and function are important for joint homeostasis in disorders like osteoarthritis, rheumatoid arthritis, and joint injury. Changes in lubricant composition and function accompany alterations in the cytokine and growth factor environment and increased fluid and molecular transport through joint tissues [36]. Therefore, putting together all outcomes of this study, LLLT seems to be beneficial in treatment of collagen-induced RA both in early than in late stages of disease progression.

Conclusion

We can conclude that LLLT with parameters employed in this study is able to modulate inflammatory response in experimental model of collagen-induced rheumatoid arthritis. The therapy is effective both in early as well as in late progression stage of disease. However, further studies are needed to investigate effects of different parameters than employed in this study.

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