UNIVERSIDADE NOVE DE JULHO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA REABILITAÇÃO

MARTHA TRINDADE MANCHINI

PROPRIEDADES ANTINFLAMATÓRIAS DO LASER DE BAIXA INTENSIDADE APÓS O INFARTO DO MIOCÁRDIO: PARTICIPAÇÃO DOS PEPTÍDEOS VASOATIVOS E CITOCINAS NO REMODELAMENTO CARDÍACO.

São Paulo
2014

MARTHA TRINDADE MANCHINI

PROPRIEDADES ANTINFLAMATÓRIAS DO LASER DE BAIXA INTENSIDADE APÓS O INFARTO DO MIOCÁRDIO: PARTICIPAÇÃO DOS PEPTÍDEOS VASOATIVOS E CITOCINAS NO REMODELAMENTO CARDÍACO.

Tese apresentada à Universidade Nove de Julho, para obtenção do título de Doutor em Ciências da Reabilitação.

Orientador: Prof. Dr. José Antônio Silva Júnior
Co-orientador: Prof. Dr. Andrey Jorge Serra

São Paulo

2014

FICHA CATALÓGRAFICA

Manchini, Martha Trindade.
Propriedades anti-inflamatórias do laser de baixa intensidade após o infarto do miocárdio: participação dos peptídeos vasoativos e citocinas no remodelamento cardíaco. / Martha Trindade Manchini. 2014.
70 f.
Tese (doutorado) – Universidade Nove de Julho - UNINOVE, São Paulo, 2014.
Orientador (a): Prof. Dr. José Antônio Silva Júnior

1. Infarto do miocárdio. 2. Laserterapia. 3. Laser de baixa potência. I. Silva Júnior, José Antônio. II. Titulo.

CDU 615.831

São Paulo, 24 de fevereiro de 2014.

TERMO DE APROVAÇÃO

Aluno(a): MARTHA TRINDADE MANCHINI

Título da Dissertação: "EFEITO ANTI-INFLAMATÓRIAS DO LASER DE BAIXA INTENSIDADE NO INFARTO DO MIOCÁRDIO EM RATOS: PARTICIPAÇÃO DOS PEPTÍDEOS VASOATIVOS NA CARDIOPROTEÇÃO".

Presidente: PROF. DR. JOSÉ ANTONIO SILVA JUNIOR _____

Membro: PROFA. DRA. MARICILIA SILVA COSTA _____

Membro: PROF.DR. PAULO JOSE FERREIRA TUCCI _____

Membro: PROF. DR. ANDREY JORGE SERRA _____

Membro: PROF.DR. PAULO DE TARSO CAMILLO DE CARVALHO _____

DEDICATÓRIA

Esta longa caminhada deve-se exclusivamente ao apoio de grandes pessoas, meus pais. Por isso, dedico esta tese aos meus pais, ***Elcio*** *e* ***Cleuza*** *por todo incentivo, amor e por sempre me apoiarem nas minhas decisões. AMO VOCÊS!*

“Adiante! Pelos caminhos maus, se não houver outros; pelos bons, se for possível. Mas adiante, apesar de todos os obstáculos para conseguir o objetivo”.

Charles Dickens (1812-1870)

AGRADECIMENTOS

Agradeço a Deus, por sempre iluminar meus caminhos e conquistas.

Agradeço meu orientador Prof. Dr. José Antônio Silva Junior (Zé) pela oportunidade e por ter sido fonte de grande inspiração na área da pesquisa. Para mim foi satisfação de tê-lo como orientador e hoje acredito que nos tornamos amigos.

Agradeço ao Prof. Dr. Andrey Serra por toda a paciência em me orientar com as análises estatísticas da tese. A sua paixão pela pesquisa é um dos motivos de querer seguir em frente sempre. Serei eternamente agradecida por isso!

Agradeço a Profa. Dra. Regiane Albertini e ao Prof. Dr. Paulo de Tarso pelos ensinamentos do laser.

Agradeço ao Prof. Dr. Paulo Tucci por ter acolhido no seu laboratório de Fisiologia Cardíaca da Unifesp e ter permitido a oportunidade de participar das reuniões científicas, da realização do meu protocolo experimental, do dia a dia no seu laboratório. Professor, você é um exemplo para todos nós!!

Agradeço a Profa. Dra. Katia de Angelis pelos ensinamentos na aula de redação científica e ter contribuído com meu Doutoramento Sanduíche na Wright State University onde tive a felicidade de conhecer a Prof. Dr. Mariana Morris.

Agradeço a todo corpo docente do programa de Doutorado em Ciências da Reabilitação da Universidade Nove de Julho e a ajuda das secretárias Ligia e Camila.

Agradeço aos técnicos de laboratório da UNINOVE: Ângela e Luciana pelo apoio e incentivo!

Agradeço a UNINOVE pela oportunidade, acredito que sem o apoio dessa Universidade não teria concretizado meu sonho.

Agradeço aos novos amigos que fiz no laboratório de Fisiologia Translacional da Uninove.

Agradeço aos alunos de iniciação científica: Michel, Eduardo, Débora!

Agradeço em especial, a minha amiga de pós-graduação, Janaina de Oliveira Brito (Jana)! Realizamos o doutoramento sanduíche juntas na mesma Universidade e tivemos a oportunidade de nos tornarmos grandes amigas!

Agradeço aos meus amigos de laboratório em especial, Ednei, Flavio, Stella, Fabíola, Bruno, Eduardo Santana por terem me apoiado, me ajudado na coleta de material biológico e por termos compartilhado grandes momentos (risadas, conversas, pelos momentos de frustração e alegrias). Galera vocês estão no meu coração para sempre!

Agradeço aos meus amigos, familiares, pacientes e alunos que tiveram paciência e por acreditarem no meu sonho. Em especial a agradeço a minha irmã Claudia, que por vocês deixei de visita-la por conta dessa fase "maluca" – obrigada por compreender.

Agradeço em especial a uma amiga que conheço desde os tempos da graduação, Mariane Castiglione. Amiga obrigada pela força – estamos juntas!!!

Agradeço em especial a CAPES por ter concedido a bolsa de Doutoramento Sanduíche. Obrigada por permitir esse sonho que tornou realidade.

Não poderia deixar de agradecer a todos os meus professores, desde a época do colégio, aos professores da graduação da fisioterapia, principalmente o Prof. Antônio Carlos Costa Curta (Toninho) que orientou na minha primeira pesquisa com crioterapia e não poderia de deixar de mencionar ao meu orientador do mestrado, Prof. Dr. Jorge Leandro Nascimento. Sempre lembrarei de vocês! OBRIGADA!!

Obrigada UNINOVE por permitir a concretização dessa etapa!

Agradeço a CAPES e CNPq por todo apoio financeiro!

E por fim, agradeço a todas as pessoas que me ajudaram de forma direta ou indiretamente!!

Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.

***Charles Chaplin* (1889-1977)**

RESUMO

Propriedades antiinflamatórias do laser de baixa intensidade após o infarto do miocárdio: participação dos peptídeos vasoativos e citocinas no remodelamento cardíaco.

O infarto do miocárdio (IM) é problema de saúde mundial, atingindo milhões de pessoas no mundo. As suas repercussões pós-IM promovem no homem grandes índices de morbidade e mortalidade no mundo. Nesse sentido, essa doença é alvo de inúmeras pesquisas que visam estudar as disfunções ventriculares, remodelamento cardíaco e progressão para a insuficiência cardíaca. A busca de estratégias terapêuticas visam atenuar e/ou melhorar todos esses aspectos. Recentemente, o laser de baixa intensidade surge como ferramenta não farmacológica que visa modular ou atenuar os aspectos envolvidos no remodelamento cardíaco adverso pós-IM, atenuando a depressão funcional cardíaca e o tamanho do infarto. O objetivo do presente estudo foi avaliar os efeitos do laser de baixa intensidade (LBI) em modelo experimental de ratos que foram submetidos à oclusão da artéria coronária. Foram utilizados 82 ratos wistar que foram divididos em três grupos: grupo controle (Con=14), grupo infartado (IM=28) e grupo infartado + Laser (IM+Laser=30). Todos os animais foram submetidos a análise pelo ecocardiograma após três dias de infarto para se determinar o tamanho do infarto e função cardíaca. Posteriormente, analisamos a expressão proteica (IL-6, TNF-α, CINC-1 e IL-10) e gênica (IL-6 e IL-1β) de citocinas inflamatórias, expressão gênica (ECA, ECA2, cinina B1 e B2 e receptor *mas*) e expressão proteica de receptor *mas*. Analisados também a expressão gênica de fatores apoptóticos (Bax e Bcl-2) e cardiotrofina-1. Os resultados alcançados foram diminuição do tamanho do infarto no grupo IM+Laser (34±2,6) comparado com o grupo MI (42±2,7); e o grupo IM +Laser cursou com melhora da fração de encurtamento (35±1) em relação ao grupo MI (27±2). Ademais, avaliações funcionais não foram significantes entre os grupos. Além disso, o mRNA de ECA-2 do grupo MI+Laser mostraram-se aumentados após a LBI em comparação com MI ($p \leq 0,05$) assim como do receptor *mas* ($p \leq 0,05$). O tratamento com LBI aumentou significativamente os níveis de expressão de receptor B2 de cininas ($p \leq 0,05$). O tratamento com LBI aumentou

significantemente a expressão gênica de Bcl-2 no grupo MI+Laser quando comparado com o grupo IM ($p \leq 0,05$), no entanto não observamos diferença significante na expressão gênica de bax. A razão bcl2/bax sugere ativação menor de processos apoptóticos após a LBI. Verificamos que após a LBI, o grupo MI+laser apresentou maior expressão gênica de cardiotrofina 1 em relação ao grupo IM ($p \leq 0,05$). Concluímos que os parâmetros funcionais apresentaram melhora após irradiação com laser. Nossos dados sugerem que após a irradiação, o laser diminui a inflamação, facilitando a sobrevivência celular e cicatrização pós-infarto, o que atribui a contribuição do LBI no remodelamento cardíaco adverso após-IM.

Palavras-chave: infarto do miocárdio, laserterapia, laser de baixa potência

ABSTRACT

Anti-inflammatory properties of low level laser therapy after myocardial infarction: vasoactive peptides and cytokines in cardiac remodeling.

Myocardial infarction (MI) is a worldwide health problem affecting millions of people in the world. The repercussion post-MI effects can cause elevated morbidity and mortality in the worldwide. Therefore, myocardial infarction it is the aim of many research which study ventricular dysfunction, cardiac remodeling and progression to heart failure . The search for new therapeutic strategies aimed at reducing and / or improve all these aspects. Recently, the low lever laser therapy appears as a new non-pharmacological tool which could attenuated the infarcted myocardial into adverse cardiac remodeling after MI and even improved heart function and reduced infarct size. The aim of this study was to evaluate the effects of low-level laser therapy (LLLT) in experimental rats that underwent coronary artery occlusion . 82 Wistar rats were used which were divided into three groups: control group (Con = 14), infarcted group (MI = 28) and infarcted group + Laser (MI + Laser = 30) . All animals were subjected to analysis by echocardiography after three days to determine myocardial infarct size and cardiac function. Subsequently , we analyzed protein expression (IL - 6 , TNF - α , CINC - 1 and IL - 10) and gene (IL - 6 and IL - 1β) of inflammatory cytokines, gene expression (ACE , ACE2 , kinin B1 and B2 and but receptor) protein and expression of receptor *mas*. We also analyzed the gene expression of apoptotic factors (Bax and Bcl - 2) and cardiotrophin - 1 . The results were reduced infarct size in MI+Laser group (34 ± 2.6) when compared with MI group (42 ± 2.7); IM + Laser group studied showed improved in fractional shortening (35 ± 1) when compared to MI group (27 ± 2). Furthermore, others functional parameters analyzed weren't significant between groups. In addition, the ACE -2 mRNA in MI + Laser increased after the LLLT when compared with MI ($p \leq 0.05$) and with receptor *mas* too ($p \leq 0.05$). Treatment with LLLT significantly increased the expression levels of kinins B2 receptor ($p \leq 0.05$) . Treatment with LLLT significantly increased the gene expression of Bcl - 2 in group IM + Laser compared with IM ($p \leq 0.05$), however no significant difference in gene expression of bax. The ratio of bcl2/bax

suggests a lower activation of apoptotic processes after laser therapy. We found that after LLLT, IM + laser group showed higher gene expression of cardiotrophin 1 in relation to MI ($p \leq 0.05$). We conclude that functional parameters showed improvement after laser irradiation. Our data suggest that after irradiation, the laser decreases inflammation and facilitating cell survival post myocardial infarction, thus suggesting the contribution of the LLLT in adverse cardiac remodeling after MI.

Keywords: myocardial infarction, laser therapy, low level laser therapy

ÍNDICE

Resumo VII

Abstract IX

1.Contextualização 12

1.1. Infarto do Miocárdio 12

1.2. Fisiopatologia do Infarto do Miocárdio 12

1.3. Papel do Sistema Renina Angiotensina (SRA) e do Sistema Calicreína Cinina (SCC) no Infarto do Miocárdio 15

1.4. Laser de Baixa Intensidade (LBI) 17

2.Objetivo 20

2.1. Objetivo geral 20

2.2. Objetivo específico 20

3. Resultados 21

3.1. Artigo 1 - Amelioration of cardiac function and activation of anti-inflammatory vasoactive peptides expression in the rat myocardium by low level lasertherapy 21

3.2. Artigo 2 - Modulation of inflammatory response on Myocardial Infarction due to low level laser therapy (LLLT) 44

4. Discussão 60

5. Conclusão 66

6. Referência 67

1. Contextualização

1.1 Infarto do miocárdio

As doenças cardiovasculares (DCV) são compostas pelas doenças do coração, doenças cerebrovasculares e doenças dos vasos sanguíneos. As DCV correspondem a mais de 17,3 milhões de mortes ano em todo o mundo (World Health Organization, 2011).

Neste sentido, atualmente no Brasil, dentro do grande grupo das doenças cardiovasculares, a doença isquêmica do coração, mais especificamente o infarto do miocárdio (IM), corresponde à terceira principal causa de morte (Ministério da Saúde, 2013), tornando-se doença complexa e multifatorial, que leva a insuficiência cardíaca congestiva, bem como perda da qualidade de vida (Kannel, 2000; Lee *et al.*, 2004).

Apesar dos avanços consideráveis no diagnóstico, prevenção e tratamento da doença cardíaca isquêmica, a disfunção contrátil e insuficiência cardíaca consequente ao infarto continuam sendo problemas de saúde mundial e com grande ônus econômico (Kannel, 2000; Lee *et al.*, 2004). A fim de restabelecer o fluxo sanguíneo para região miocárdica acometida pela isquemia, o tratamento do infarto agudo do miocárdio é basicamente suportado pela desobstrução coronariana (farmacológica ou cirúrgica) ou composição de anastomoses arteriais. Todavia, essas técnicas podem apresentar menor grau de eficiência, cursando com reestenoses e outras complicações (Abbate *et al.*, 2007).

1.2 Fisiopatologia do Infarto do Miocárdio

O infarto do miocárdio é um dano irreversível resultante de isquemia prolongada, consequente à oclusão total ou parcial de uma ou mais artérias coronárias. A interrupção do fluxo sanguíneo para o tecido cardíaco dá início a eventos que culminam na morte dos cardiomiócitos que, gradualmente, são substituídos por fibrose de reparação (Fishbein *et al.*, 1978; Pfeffer *et al.*, 1979). Com a perda de miocárdio contrátil, se inicia ciclo vicioso de sobrecarga no ventrículo esquerdo (VE) com deterioração da função de bomba e consequente progressão para IC (Francis *et al.*, 2001). A oclusão cirúrgica da artéria coronária em animais de experimentação como no rato, é o modelo

bastante útil e muito frequente em estudos de fisiopatologia e terapêutica, pois sua evolução para IC se assemelha em muitos aspectos a do infarto observado no homem (Fishbein *et al.*, 1978; Hasenfuss, 1998; Francis *et al.*, 2001). Ademais, os ratos apresentam a vantagem de não possuírem numerosa circulação colateral, o que permite a ocorrência de infartos transmurais pela oclusão de um ramo coronário epicárdico (Johns & Olson, 1954; Spadaro *et al.*, 1980).

Devido à necrose isquêmica, na fase aguda do IM, tanto no homem quanto no animal de experimentação, ocorre resposta inflamatória aguda com infiltração leucocitária, fagocitose das células necróticas e reabsorção dos componentes celulares e da matriz extracelular comprometida, configurando o início do processo de reparação tecidual (Frangogiannis *et al.*, 2002). A partir desse período, incide a deposição dos componentes da nova matriz extracelular, principalmente o colágeno, no tecido infartado e na zona de transição para o tecido viável, culminando na formação da cicatriz do infarto (Anversa *et al.*, 1993; Sun *et al.*, 2000).

Com a sobrecarga do tecido remanescente, o processo inflamatório crônico e a participação de fatores neuro-humorais presentes no pós-IM, dá-se início ao remodelamento cardíaco. Este é definido como o conjunto de modificações gênicas, moleculares, celulares e intersticiais que ocorrem nos tecidos cardíacos e podem se expressar clinicamente por alterações do tamanho, forma e função deste órgão (Mittmann *et al.*, 1998; Swynghedauw, 1999). No que se refere ao remodelamento do miocárdio sobrevivente, ocorrem alterações na estrutura, forma e função dos cardiomiócitos (hipertrofia e depressão da contração e do relaxamento), da matriz extracelular (fibrose intersticial e perivascular) e dos vasos sanguíneos (redução da capilaridade, hipertrofia da musculatura lisa, disfunção vascular). Essas alterações, embora sejam algumas vezes consideradas como mecanismos compensatórios, na sua progressão tornam-se substrato para disfunção ventricular, dos sintomas de IC e das mortes relacionadas a essa doença (Mittmann *et al.*, 1998; Swynghedauw,1999).

Portanto, o processo de cicatrização no coração é dificultado pela baixa regeneração do miocárdio e pela substituição do músculo infartado por tecido cicatricial (Ertl et al, 2005). Essas alterações no tecido conjuntivo estão

presentes com 40 min após a oclusão coronária experimental e a degradação do colágeno é observado após 24h em ratos (Dixon et al, 2011; Ertl et al, 2005). A estrutura normal do colágeno praticamente desaparece durante a primeira semana após o infarto. A extensão da degradação do colágeno se correlaciona com o grau de expansão de infarto (Ertl et al, 2005). E as atividades das colagenases e proteinases estão aumentadas e têm sido relacionadas à rápida degradação do colagéno na matriz extracelular no IM. Além disso, as células inflamatórias liberam proteases que contribuem para a remoção do tecido necrosado e os miofibroblastos para a reconstrução de nova rede de colágeno (Ertl et al, 2005). Após várias semanas, cicatriz sólida é formada com uma estrutura de colagéno estável, em geral com pouca celularidade (Ertl et al, 2005).

Uma vez que o miocárdio infartado é substituído pelo tecido cicatricial sem haver regeneração muscular, estudos clínicos demonstraram que quando há prejuízo de 40% do ventrículo esquerdo, seja por meio de um infarto único e extenso ou combinação de infartos de menores dimensões, a bomba cardíaca está em risco de insuficiência (Holmes et al, 2005). Embora muitos atribuam esse achado simplesmente a redução da quantidade de miocárdio sadio contribuindo para a ejeção (Holmes et al, 2005; Bonilha et al, 2005), e vários estudos encontraram que o grau de deficiência sistólica está diretamente relacionada com a complacência do infarto. Assim, a rigidez do infarto poderia limitar a função diastólica do miocárdio saudável, pois limita utilizar o mecanismo de Frank-Starling para ajustar a ejeção ventricular (Holmes et al, 2005).

Quando as sobrecargas cardíacas perduram por período prolongado, é regra que a evolução do remodelamento miocárdico termine comprometendo o estado contrátil. Em avaliações tardias após-IM, a diminuição do inotropismo tem sido habitual. Os infartos do miocárdio de pequenas dimensões acarretam pouca repercussão para o miocárdio remoto e, portanto, geralmente, não afetam o inotropismo (Bonilha et al, 2005). O tempo decorrido desde a oclusão da coronária, o tamanho do infarto e o tipo de preparação utilizada para estudar o desempenho miocárdico são fatores capazes de modular as repercussões funcionais acarretadas para o miocárdio remoto após infarto.

Dentre os processos patológicos de remodelamento pelos quais o coração após IM passam incluem-se a hipertrofia e fibrose cardíaca, que resultam em insuficiência cardíaca e eventualmente morte. E a apoptose de cardiomiócitos ocorre na fase inicial do infarto agudo do miocárdio e é um importante processo de morte celular em resposta ao insulto.

A mudança mais importante nas propriedades mecânicas do infarto agudo do miocárdio é que, ao longo dos primeiros minutos de isquemia, o miocárdio gradualmente perde a sua capacidade de gerar força sistólica (Holmes et al 2005). Desta maneira, o tamanho e a propriedade mecânica da área infartada podem determinar o grau de comprometimento da função do ventrículo esquerdo (Holmes et al, 2005).

Nesse sentido, inúmeras abordagens terapêuticas foram desenvolvidas para modular ou interferir nas diversas fases e aspectos que envolvem o remodelamento e disfunção ventricular, com sucessos consideráveis na melhora da função cardíaca, redução dos sintomas, melhora da qualidade de vida e aumento da sobrevida. Entretanto, por não existirem medicamentos ou procedimentos capazes de reparar ou substituir a cicatriz fibrótica por tecido contrátil, esforços vêm ocorrendo nos últimos anos para se determinar tratamentos que atuem no campo da reparação e regeneração cardíacas.

1.3 Papel do Sistema Renina Angiotensina (SRA) e do Sistema Calicreína Cinina (SCC) no Infarto do Miocárdio

O sistema renina angiontensina possui papel importante na fisiopatologia do infarto do miocárdio e contribui para progressão da insuficiência cardíaca (Johnston 1994). Este sistema é composto pelo angiotensinogênio, renina, enzima conversora de angiotensina (ECA) e receptores de angiotensina II. O peptídeo efetor deste sistema, a angiotensina II, foi caracterizado como peptídeo com grande poder vasoconstrictor, mitogênico, neurotransmissor e estimulador de aldosterona (Dostal et al., 1996), tornando-se o principal responsável pelos efeitos patofisiológicos deste sistema (Bader 2013).

Há mais de duas décadas verificou-se que além do sistema renina-angiotensina circulante, os tecidos possuíam seu próprio SRA (Ganten et al., 1971, Ganten e Speck, 1978) pois todos os componentes deste sistema foram

encontrados em órgãos como cérebro, coração, rins e vasos (Unger et al., 1991; Dostal et al., 1992; Lee et al., 1993), locais onde a angiotensina II poderia ser gerada e exercer suas ações localmente. O aumento da angiotensina II no tecido cardíaco pós-IM está relacionado com o aumento do estresse oxidativo que ativaria as vias inflamatórias (Marchesi et al., 2008) e apoptóticas (Dimmeler et al., 2000).

Desta forma, o bloqueio do sistema renina angiontensina com os inibidores da ECA são observados na melhora de diversas condições patofisiológicas conforme analisado no modelo animal de hipertensão, através da administração de losartan e outros antagonistas do receptor AT1.

Esses achados demonstram a prevenção ou atenuação da pressão sanguínea elevada assim como a hipertrofia cardíaca associada (Zhu et al., 1997) e contribuiriam para a melhora do remodelamento cardíaco (Pfeffer et al., 1990; SOLVD 1991).

A angiotensina (1-7) [Ang-(1-7), Campagnole-Santos et al. 1989; Santos et al. 1990; para revisão: Passos-Silva et al., 2013) recentemente considerada hormônio biologicamente ativo do SRA, possui funções opostas àquelas atribuídas ao principal componente efetor do SRA, a angiotensina II. O homólogo da Enzima Conversora de Angiotensina (ECA), a ECA 2 (Fraga-Silva et al., 2013) pode formar Ang (1-7) a partir da Ang II ou menos eficientemente a partir da hidrólise de Ang I que subsequentemente forma Ang (1-7) (Arita et al., 2012). Este hormônio é peptídeo endógeno com propriedades vasodilatadoras e está relacionado à cardioproteção, sendo seus efeitos mediados pela ativação do receptor Mas (Passos-Silva et al. 2013).

Já o sistema calicreína cinina é considerado sistema antagônico ao sistema renina angiotensina, pois este sistema apresenta características vasopressoras e vasodilatadoras e a sua contribuição para o sistema cardiovascular resulta no papel regulatório na homeostase vascular (Davis et al., 1979; Levine et al., 1980).

E as cininas podem ser consideradas mediadores de mecanismos cardioprotetores. No entanto, esse sistema é afetado pelos inibidores da enzima conversora de angiotensina através da atenuação na formação de angiotensina II, e potenciam os efeitos da bradicinina, devido à inibição de sua degradação (Davis et al., 1979; Levine et al., 1980).

Desta forma, eles impedem as ações locais e sistêmicas da ANG II e potenciam os efeitos da bradicinina. As cininas exercem as suas ações farmacológicas através de dois receptores transmembranares acoplados à proteína G, os receptores de cinina B1 e B2 (Bortone F et al., 2008). Enquanto o receptor B2 de cininas é expressa em forma constitutiva na maioria das células e dos tecidos em mamíferos, o receptor de cinina B1 é um receptor induzível e expressa principalmente em estados inflamatórios (Bortone F et al., 2008).

1.4 Laser de Baixa Intensidade (LBI)

Recentemente, o laser de baixa intensidade (LBI) tornou-se alternativa terapêutica por modular vários processos biológicos e, dependendo do comprimento de onda, dose e condição do tecido irradiado, pode contribuir com um efeito anti-inflamatório (Lopes-Martins *et al.*, 2006; Albertini *et al.,* 2007; Aimbire *et al.,* 2008; Albertini, *et al.*, 2008; Bortone *et al.*, 2008; Lima *et al.*, 2009; Xavier *et al.,* 2010; Silva *et al.,*2011; Pires *et al,* 2011; Mesquita-Ferrari *et al.,* 201), reduzir a dor e acelerar a proliferação celular (Huang *et al.*, 2011; Peplow *et al*., 2012). Assim, ao atuar em nível celular, pode provocar modificações bioquímicas, bioelétricas e bioenergéticas, promovendo aumento do metabolismo, proliferação e maturação celular, quantidade de tecido de granulação e na diminuição dos mediadores inflamatórios, facilitando o processo de cicatrização (Lopes-Martins *et al.*, 2006; Albertini *et al.,* 2007; Aimbire *et al.,* 2008; Albertini, *et al.*, 2008; Bortone *et al.*, 2008; Lima *et al.*, 2009; Xavier *et al.,* 2010; Silva *et al.,*2011; Pires *et al,* 2011; Huang *et al.*, 2011; Mesquita-Ferrari *et al.,* 2011; Peplow *et al*., 2012).

A absorção molecular do laser causa aumento do metabolismo celular, caracterizado pela estimulação de fotorreceptores na cadeia respiratória mitocondrial, alterações nos níveis de ATP celular, na liberação de fatores de crescimento e na síntese de colágeno (Tuby *et al*., 2006; Huang *et al.*, 2011; Peplow *et al.,* 2012).

A modulação celular, ou seja, a ativação ou inibição de processos celulares de expressão gênica ou proteica, ainda é pouco estudada. Em trabalhos de nosso grupo (Bortone et al., 2008 e Silva et al.,2011) e em colaboração com outros grupos de pesquisa (Albertini *et al.,* 2007; Aimbire *et*

al., 2008; Albertini *et al.*, 2008; Lima *et al.*, 2009; Xavier *et al.,* 2010; Pires *et al,* 2011; Mesquita-Ferrari *et al.,* 2011) observou-se diminuição da expressão de mediadores inflamatórios pela laserterapia, levando a redução do processo inflamatório.

Entretanto, a ação do laser no IM ainda está para ser esclarecida e pouco se sabe sobre o comportamento do miocárdio remanescente ao infarto frente a esta terapia. Oron *et al.* (2001) analisaram o efeito da irradiação laser diodo (ƛ=810 nm) em modelos experimentais de oclusão da artéria coronária anterior para a produção do infarto do miocárdio em ratos e cães e observaram atenuação do tamanho do infarto. O laser terapêutico irradiado em coração de ratos pós-IM poderia reduzir a perda de tecido do miocárdio e esse fenômeno poderia ter um importante efeito benéfico em pacientes após infarto agudo do miocárdio ou isquemia cardíaca (Ad & Oron *et al.,* 2001).

Analisando o miocárdio de ratos infartados tratados com o laser de baixa potência (ƛ= 804 nm), Tuby *et al.*, em 2006, demonstraram o aumento significativo na expressão de VEGF (fator de crescimento vascular endotelial) e da enzima sintetase de óxido nítrico induzível (iNOS) em tecidos de animais irradiados.

Desta forma, independente da condição fisiopatológica, os benefícios da laserterapia abrangem a redução do processo inflamatório com redução de seus mediadores químicos. São demonstrados resultados de melhora da inflamação e edema de pata induzida por carragenina [redução de RNA mensageiro de Cox-2 (Albertini *et al.,* 2007); redução da expressão gênica de citocinas pró-inflamatórias (Albertini *et al.*, 2008); menor expressão de receptores de cininas (Bortone *et al.*, 2008); diminuição da expressão gênica de calicreínas (Silva *et al.,*2011)], da redução da inflamação de vias aéreas induzida por lipopolissacarídeo [diminuição da expressão de interleucinas (Aimbire *et al.,* 2008; Lima *et al.*, 2009)], na tendinite [diminuição da expressão de citocinas (Xavier *et al.,* 2010); diminuição da expressão gênica de citocinas pró-inflamatórias e aumento da expressão de citocina anti-inflamatória (Pires *et al,* 2011)], na cicatrização (Peplow *et al.,* 2012) e na reparação muscular [redução da expressão gênica de citocinas pró-inflamatórias (Mesquita-Ferrari *et al.,* 2011)] entre outros. Todavia, os dados acerca das repercussões desta terapia sobre o miocárdio e, sobretudo no miocárdio remanescente à área de

infarto, são incipientes, sendo necessários mais estudos sobre a influência da laserterapia de baixa intensidade sobre o infarto e o processo de remodelamento.

O estudo do miocárdio remanescente ao infarto é de suma importância ao entendimento dos processos de remodelamento e disfunção cardíaca. Assim como no homem, o remodelamento ocorrido no miocárdio remanescente ao IM em ratos implica em disfunção contrátil que consequentemente exacerba a disfunção ventricular e a ICC, renovando o ciclo vicioso desta cardiopatia (Francis *et al.*, 2001).

Assim, pretende-se avaliar em modelo de oclusão coronariana e laser de baixa intensidade o tamanho do infarto e a função ventricular por ecocardiograma e hemodinâmica. Especialmente, pretende-se avaliar as modificações da expressão gênica que ocorrem no miocárdio tratado com laser terapêutico induzido por oclusão coronária.

2. Objetivo

2.1 Objetivo geral

Analisar o efeito do laser de baixa intensidade (LBI) após três dias de infarto do miocárdio em animais submetidos à oclusão da artéria coronária descendente anterior esquerda.

2.2 Objetivos específicos

Artigo 1

Avaliar o efeito do laser de baixa intensidade nos componentes da expressão cardíaca calicreína cinina e sistema renina angiotensina em ratos com infarto do miocárdio assim como função do ventrículo esquerdo.

Artigo 2

Avaliar o perfil inflamatório no miocárdio infartado e no miocárdio não infartado que foram submetidos ao modelo de infarto do miocárdio e tratado com laser de baixa intensidade. Também foram analisados nesse estudo, a expressão gênica de cardiotrofina-1 e fatores anti-apoptose e pró-apoptóticos.

3. Resultados

3.1 – Artigo 1

Martha Trindade Manchini, Andrey Jorge Serra, Regiane dos Santos Feliciano, Eduardo Tadeu Santana, Ednei Luis Antônio, Regiane Albertini, Paulo de Tarso Camillo de Carvalho, Jairo Montemor, Paulo José Ferreira Tucci,José Antônio Silva Jr. **Amelioration of cardiac function and activation of anti-inflammatory vasoactive peptides expression in the rat myocardium by low level lasertherapy.** Submitted to **Plos One**, September 2013.

Nossos dados sugerem diminuição da resposta inflamatória gerada pelo laser de baixa intensidade (LBI) pós-infarto em ratos e concomitantemente a participação da via de geração de Ang 1-7 e do receptor B2 na cardioproteção gerada pela LBI pós-infarto em ratos.

Amelioration of cardiac function and activation of anti-inflammatory vasoactive peptides expression in the rat myocardium by low level lasertherapy.

Short title: Lasertherapy modulates cardioprotection

Martha Trindade Manchini[1], Andrey Jorge Serra[1], Regiane dos Santos Feliciano[1], Eduardo Tadeu Santana[1], Ednei Luis Antônio[2], Regiane Albertini[1], Paulo de Tarso Camillo de Carvalho[1], Jairo Montemor[2], Paulo José Ferreira Tucci[2] and José Antônio Silva Jr[1*].

[1]Universidade Nove de Julho, UNINOVE, Rua Vergueiro, 235 - Liberdade 01504-001 São Paulo, SP- Brasil

[2]Universidade Federal de São Paulo, UNIFESP, Rua Coronel Lisboa, 819 Vila Clementino 04020-041 - Sao Paulo, SP - Brasil

Author contribution

Martha Trindade Manchini, Eduardo Tadeu Santana, Regiane dos Santos Feliciano, and Ednei Luis Antônio performed major parts of the experiments, including animal surgeries and gene expression protocols; Jairo Montemor was responsible for echocardiogram analysis; Regiane Albertini and Paulo de Tarso Camillo de Carvalho were responsible for laser protocol and dosage; Paulo José Ferreira Tucci and Andrey Jorge Serra performed the statistical analyses, oversaw the design and performance of the experiments, and edited the final format of the paper; José Antônio Silva Jr. participated in the experimental design, data interpretation and critical discussion of the paper. All authors approved the final format of the manuscript.

*Address for correspondence: José Antônio Silva Jr, UNINOVE, Rua Vergueiro, 235 - Liberdade 01504-001 São Paulo, SP – Brasil – Phone/Fax +551133859222 - josejr@uninove.br

ABSTRACT

Low-level laser therapy (LLLT) has been used as an anti-inflammatory treatment in several disease conditions, even when inflammation is a secondary consequence, as in the myocardial infarction (MI). However, the action mechanism of protective LLLT on the remaining myocardium of rats with MI remains unclear. The relevance of the Renin-Angiotensin System (RAS) in cardiovascular diseases is based on the efficiency of angiotensin-converting enzyme (ACE) inhibition to improve survival and cardiac function in patients with heart failure. Interestingly, the ACE inhibition blockades the vasoconstrictor angiotensin II (Ang-II) generation and also avoids the degradation of the vasodilator peptide bradykinin (BK). The Kallikrein-Kinin system (KKS) is implicated with positive effects of ACEis therapy. ACE-2 is a membrane-associated carboxy-peptidase responsible for the conversion of AngII to the vasodilatory peptide Ang1–7. This heptapeptide, allied to BK, is shown to exert cardioprotective effects locally in the myocardium. Using an experimental rat myocardial infarct (MI) model, we analyzed the cardioprotective effect of low level laser therapy (LLLT) in cardiac remodeling, and the possible contribution of RAS and KKS vasoactive peptides expression in this milieu. LLLT treatment effectively reduced MI size and decreased the myocardial mRNA expression of interleukin-1beta and interleukin-6 in comparison to the non-irradiated rat tissue. Moreover, the ACE-2 and *mas* mRNA and protein levels were upregulated in LLLT treated group compared to non-irradiated rats. LLLT treatment significantly increased the expression levels of B2 kinin receptor in comparison to non-treated rats. Our data suggest that LLLT improved cardiac function and attenuated left ventricular remodeling post-MI. The protective effects of LLLT could to be mediated, at least in part, through vasodilators peptides expressed in the post-infarction myocardium.

INTRODUCTION

Renin–angiotensin system (RAS) plays a pivotal role in the pathophysiology of myocardial infarction (MI), and in the development of heart failure [1]. The angiotensin converting enzyme (ACE) converts Ang-I (angiotensin I) into the vasoconstrictor Ang-II, which is the major effector of this system and therefore responsible for most of the pathophysiological effects of the RAS [2]. Although found in the systemic circulation, Ang-II is also produced in the cardiac tissue by a local RAS [3]. Ang-II is shown to increase oxidative stress that could activate inflammatory [4] and apoptotic [5] pathways. In fact, RAS blockade with ACE inhibitors (ACEis) or angiotensin receptor

blockers (ARBs) are shown to ameliorate several cardiac pathological conditions. These molecular suppressions improves the cardiac remodeling and its outcome [6-9]. A vasoactive system, the kallikrein-kinin system (KKS), also is produced locally in the cardiac muscle. KKS is markedly affected by ACEis therapy. A diminished degradation of bradykinin (BK), a potent vasodilator kinin peptide, is reported after ACE inhibition [10,11]. Kinins exert their pharmacological actions through two transmembrane receptors coupled to G protein, the kinin B1 and B2 receptors [12]. While kinin B2 receptor is expressed in a constitutive way in most cells and tissues in mammals, the kinin B1 receptor is an inducible receptor expressed mostly in inflammatory states [12].

Until the discovery of the enzyme ACE2, the enzyme that cleaves Ang-II to generate the vasodilator and anti-fibrotic peptide Ang1–7 [13], BK was the most antagonistic physiological response to RAS activation. Bradykinin increased availability was rolled as an important component of the ACEis success therapy [10]. ACE2 expression and activation was noted to increase after experimental MI, possibly avoiding the deleterious effects of RAS. The peptide Ang1-7, the heptapeptide generated by ACE2, is found in heart and kidneys and most tissues related to cardiovascular system homeostasis [2,14]. Ang1-7 is implicated in the prevention of Ang-II-induced cardiovascular hypertrophy and remodeling [15-18].

Low level laser therapy (LLLT) has become an alternative therapy to modulate various biological processes and depending on the wavelength, dosage and condition of the irradiated tissue, may contribute an anti-inflammatory effect, reducing the pain and accelerate the cell proliferation [12,19-22]. We previously showed that lasertherapy was effective to modulate kinin B1 and B2 receptor in subplantar muscle of rat paw carrageenan-induced inflammation [12]. In addition, using the same model, we reported that both plasma and tissue pre-kallikrein expression were modulated after laser irradiation [22].

The use of LLLT to treat pathophysiological conditions started four decades ago [23]. To our best knowledge, the first report of lasertherapy usage on experimental MI was published in 2000 [24], suggesting that laser irradiation could attenuate infarct-associated remodeling. Myocardial infarction comprises the ischemic area of the myocardium subserved by an occluded coronary artery. Immediately after MI, tissue injury and death of cardiomyocytes trigger a synchronized acute inflammatory response that can last hours, days or weeks [For review: see 25]. Several pro-inflammatory cytokines and chemokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis

factor α (TNFα) participate of this inflammatory phase [26]. Although some studies used LLLT to treat cardiac dysfunctions and reduce the myocardial infarct size after laser irradiation [27-29], debate continues over the molecular action mechanisms of laser on the myocardium. To date, none of these studies assessed vasoactive peptides systems expression in a rat model of MI treated with lasertherapy. As inflammation appears secondary to myocardial hypoxia as MI consequence and lasertherapy could reduce cytokines expression [19,21], we hypothesized that laser could diminishes the acute inflammation in the myocardium after MI. This event could help to ameliorate cardiac function via vasoactive peptides, promoting a vasodilation to fulfill the lack of oxygen and nutrients caused by obstruction of the tissue´s blood supply. The aim of the current study was to examine the effect of the low level lasertherapy on expression of cardiac components of KKS and RAS in rats with MI. The effect of LLLT on left ventricular (LV) function was also assessed.

METHODS

Ethics Statement

All the experimental procedures were performed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The project research was approved by the Institutional Research Ethics Committee of the Nove de Julho University (No 0015/2012), São Paulo, Brazil. All surgery was performed under ketamine and xylazin anesthesia, and all efforts were made to minimize suffering.

Animals and MI surgical procedure

Female Wistar rats (n=82) with 10 weeks of age were subjected to thoracotomy and infarction by coronary occlusion. The surviving rats were randomly divided into infarcted non-treated group (MI, n=28) and infarcted laser-treated group (MI+Laser, n=30). Rats that received the same surgical procedure for thoracotomy without coronary ligation served as control group (Con, n=14). For MI induces, under ketamine (50 mg/kg) plus xylazin (10 mg/kg) anesthesia the coronary artery was occluded near its origin as previously described [30]. All parameters evaluated in this study were analyzed 3 days after MI.

Lasertherapy

After surgery, the animals were immediately randomized into two experimental group (with or without lasertherapy). The laser device used was a Aluminum Indium Gallium Phosphorus – AlGaInP (Twin Laser – M M Optics ®) with wavelength 660 nm, power 15mW, laser beam spot size 0,785 cm^2, energy density 22,5Jcm^2, irradiation time 60 sec, and energy delivered 1,1 Joules. The laser dose used in this study was similar to [27]. However, we chose the wavelength of 660nm due beneficial effects reported using rats with heart failure to achieve an inflammatory profile in this condition. All laser irradiation parameters used were summarized in Table 1. The laser beam was placed in contact with the myocardium surface corresponding to the infarcted area. After ligation as describe above, the heart was put in the chest to recover itself and then the heart was put out and random to receive or not the laser irradiation. The optical fiber was fixed with a delivery arm and precisely positioned with the fiber tip 3 cm above the myocardium. This allowed for a laser beam spot size of 0,785 cm^2.

Assessment of MI size, geometry and function of LV

Three days after coronary occlusion or sham surgical, animals were anesthetized as described above (K-X mixture) and LV measurements were performed using a 12-MHz transducer connected to a HP Sonos-5500 echocardiograph (Hewlett–Packard, California, USA). The MI size was evaluated on transversal 2-dimensional view of the LV and expressed as the proportion of the LV perimeter as previously described [30-33]. The prediction of infarction size by echocardiogram was similar to tetrazolium staining (data not shown) as observed by [31]. The MI was defined by echocardiography as any segment with increased echogenicity and/or change in myocardial thickening or systolic movement (hypokinesia, akinesia, or dyskinesia). The diastolic (DA) and systolic (SA) LV areas were measured by 2-dimensional images at the basal, midview, and apical view. The LV systolic function was determined by the fractional area change (FAC) as a function for following equation: FAC= DA-SA/DA x 100. Pulsed Doppler at the ventricular side of the mitral valve provided the flow velocity curve to analyze the diastolic function parameters (E and A waves and E/A ratio).

Biometric data and biological sample

After echocardiography study, the rats received a urethane overdose (4.8 g $kg^{-,1}$ i.p.) and heart as well as left lung was quickly removed. The LV weight was used as an

indicator of myocardial mass. The lung wet weight (WW) and dry weight (DW) were determined before and after samples were dried at 70°C until they achieved constant weight in order to determine lung water content (H_2O): H_2O (%) = [(WW-DW)/WW] x 100. LV fragments of remote area to MI were placed in 5% saline solution to remove excess blood. The myocardial tissue was stored in cryogenic tube and kept frozen in liquid nitrogen for molecular analysis.

Gene expression quantification

Total RNA was extracted from left ventricle (LV) samples and Real-time PCR assay was performed to access mRNA quantification. Thawed tissues were homogenized in 1 ml of TRIzol reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated accordingly to the manufacturer´s instructions.

One microgram of total RNA was used for cDNA synthesis and Real-Time PCR gene expression analysis. Initially, contaminating DNA was removed using DNase I (Invitrogen) at a concentration of 1 unit/µg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM $MgCl_2$ for 15 min at 37 °C, followed by incubation at 95°C for 5 min for enzyme inactivation. Then, the reverse transcription (RT) was carried out in a 200µl reaction in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen). The reactions conditions were: 20 °C for 10 min, 42°C for 45 min and 95°C for 5 min.

The reaction product was amplified by real time PCR on the 7500 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBR Green core reaction kit (Applied Biosystems). The thermal cycling conditions were: 50 ºC for 2 min, then 95ºC for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Experiments were performed in triplicates for each data point. Target gene mRNA abundance was quantified as a relative value compared with an internal reference, GAPDH, whose abundance was believed not to change between the varying experimental conditions. Primers used for real time PCR were: rat kinin B1 primers forward 5’-CCTTCCAGGCTTAAACGATTCTC-3’ and reverse 5’-GGTTGGAGG ATTGGAGCTCTAGA-3’ (GenBank accession number NM_030851.1); rat kinin B2 primers forward 5’-CCACCACGGCCTCTTTCAG-3’ and reverse 5’-CGAACAGCACCCAGAGGAA-3’ (GenBank accession number NM_001270713.1); rat interleukin-6 primers forward 5'-GAGGAGACTTCACAGAGGAT-3' and reverse 5'-

TCCTTAGCCACTCCTTCTGT-3' (GenBank accession number NM_012589.2); rat interleukin-1b primers forward 5'-CAGGAAGGCAGTGTCACTCA-3' and reverse 5'-GGGATTTTGTCGTTGCTTGT-3' (GenBank accession number M98820.1); To access ACE, ACE2 and Mas receptor mRNA quantification, the following primers were used: rat ACE forward 5'-CACCGGCAAGGTCTGCTT-3' and reverse 5'-CTTGGCATAGTTTCGTGAGGAA-3' (GenBank accession number NM_012544.1), rat ACE2 forward 5'- GCCAGGAGATGACCGGAAA-3' and reverse 5'-CTGAAGTCTCCATGTCCCAGATC-3' (GenBank accession number NM_001012006.1); rat Mas receptor primers forward 5′-CATCTCTCCTCTCGGCTTTGTG-3′ and reverse 5′- CCTCATCCGGAAGCAAAGG-3′ (GenBank accession number NM_012757.2). GAPDH primers were forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5_-GCCCCACGGCCATCA-3' (GenBank accession number NM_017008). One microliter of RT reaction was used for Real-Time PCR.

Western blot analysis

Frozen LV was homogenized in cell lysis buffer (100mM Tris, 50 mM NaCl, 10mM of ethylenediaminetetraacetic acid (EDTA) and 1% Triton X-100) supplied with a proteinase inhibitor cocktail (Sigma Chemical Corp., St Louis, MO, USA). Samples containing 30 μg of the homogenate were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels. Separated proteins were transferred onto hydrophobic polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Biosciences; Piscataway, J, USA), and the transfer efficiency was monitored with 0.5% Ponceau S staining of the blot membrane. Membranes were soaked in a blocking buffer (5% non-fat dry milk, 10mM Tris–HCl, pH7.6, 150mM NaCl and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4◦C using specific goat anti-rat mas receptor antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After overnight incubation, membranes were washed three times and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution; Zymed, San Franscisco, CA, USA). Detection was performed with enhanced chemiluminescence reagents (Amersham Biosciences).

Statistical analysis

Data were analyzed with GraphPad Prism software (La Jolla, CA, USA). The Shapiro-Wilk and Levene tests were used to verify normality and error variances, respectively. Results were evaluated using Student's *t*-test for comparisons of MI size for two groups and with Chi-squared test for analysis of mortality and frequency. One-way ANOVA complemented by Newman–Keuls test was used to detect differences between three groups at sample with normal distribution. However, Kruskal-Wallis followed by Dunn's multiple comparison tests was applied for no-normality data. A p value ≤ 0.05 was considered significant with two-tailed probability and results are expressed as mean ± SEM. Quantitative values for target gene and GAPDH mRNA transcription were obtained from the threshold cycle number, where the increase in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized on the basis of GAPDH expression as an endogenous RNA control. ΔCt values of the samples were determined by subtracting the average C_t value of target gene mRNA from the average C_t value of the internal control GAPDH. The $2^{-\Delta\Delta Ct}$ parameter was used to express the relative expression data. For Western blot analysis, although identical amounts of protein were loaded into each well, the GAPDH expression levels were used as a loading control and to normalize the data.

RESULTS

Cardiac function and infarction size

A total amount of 82 rats was subjected to MI surgical induction, 12 of them died immediately after coronary occlusion. Thus, 32 and 38 rats comprised the infarcted group without (MI) and with (MI+Laser) lasertherapy, respectively. One animal from treated group was excluded for not presenting MI. A small number of animals (4/32 *vs.* 7/37) died following three days post-MI between MI and MI+Laser groups, respectively, with no statistical difference ($p>0.05$, Chi-Squared test).

The Figure 1A shows the mean infarction size in the MI and MI+Laser groups. The size of infarction was notably smaller in the MI+Laser group compared with the MI group ($p=0.02$, Student's *t*-test). The size of infarction was categorized according to a cut-off point of 37% of LV (Figure 1B) as demonstrated by [28]. Interestingly, the number of large infarcts (> 40%) was significantly lower in the laser-treated group compared with the non-treated group ($p=0.004$, Chi-Squared test).

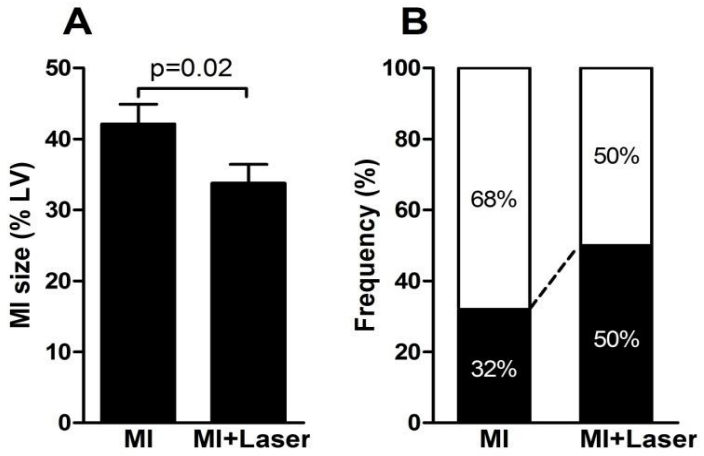

Figure 1. (A) Size of the infarct as a percentage of left ventricular (LV) perimeter in infarcted rats (MI) and infarcted rats treated with laser (MI+Laser) after three days of coronary occlusion. (B) Frequency of large (□) or small plus mild (■) infarctions (p value = 0.004, Chi-squared test).

Table 1 shows the biometric and echocardiographic data. The body weight values were similar among all groups and the MI did not induce changes in myocardial mass. Although the mean value of pulmonary water content was increased in the MI group, no significant difference among the three experimental groups was found.

Regarding the transthoracic echocardiography, MI induced a significant increase of LV systolic area, however, lasertherapy did not affect the LV alterations induced by MI. Myocardial infarction resulted in a remarkable reduction in fractional area change (FAC) in treated and non-treated rats compared with the Con group. Interestingly, our data suggests that lasertherapy attenuated the systolic dysfunction provoked by MI. On pulsed Doppler, although E and A waves were not altered post-MI, rats presented a restrictive LV filling pattern defined as an increased ratio of early (E) to late (A) filling velocities and rapid deceleration of the early filling wave. Lasertherapy did not change these parameters.

Table 1. Biometric and echocardiographic data 72 hours after coronary occlusion and laser therapy.

Variables	Experimental groups			*p* value
	Con (n=14)	MI (n=28)	MI+Laser (n=30)	
Biometric				
BW (g)	206 ± 6	200 ± 4	201 ± 5	= 0.7
LV (mg)	681± 39	693 ± 47	751 ± 28	= 0.3
LV/BW (mg/g)	3.6 ± 0.2	3 ± 0.4	3.8 ± 0.2	= 0.5
H_2O (%)	79 ± 0.3	81 ± 0.5	79 ± 0.8	= 0.06
Echocardiography				
LVDA (mm^2/BW)	0.01427± 0.0007	0.01611 ± 0.0011	0.01593 ± 0.0007	= 0.3
LVSA (mm^2/ BW)	0.0038 ± 0.0001*	0.0116 ± 0.0008	0.0103 ± 0.0003	< 0.0001
FAC (%)	72 ± 1*	27 ± 2	35 ± 1#	< 0.0001
E Wave (cm^2)	0.69 ± 0.03	0.68 ± 0.05	0.73 ± 0.03	= 0.4
A Wave (cm^2)	0.28 ± 0.01	032 ± 0.07	0.25 ± 0.08	= 0.055
E/A	2.6 ± 0.2*	4.5 ± 0.9	5 ± 0.7	= 0.02

BW, body weight; FAC, fractional area change; LV, left ventricular; H_2O, lung water content; LVDA, left ventricular diastolic area; LVSA, left ventricular systolic area; FAC, fraction area change; E, E wave; A, A wave. One-way ANOVA was applied in comparisons. Data are presented as mean (standard errors).

* Significant difference *vs.* MI and MI+Laser

Significant difference *vs.* MI

Anti-inflammatory response of LLLT

Gene expression of interleukin-1 beta and interleukin-6 strongly increased 3 days after MI. Laser irradiation after coronary occlusion was effective to reduce these cytokines mRNA amount to values similar to control rats (Figure 2A and 2B). MI resulted in distinct kinin receptors expression. While kinin B1 mRNA content was considerably increased after MI, kinin B2 mRNA expression did not change 3 days after

MI. Kinin B2 receptor expression showed a distinctly augmentation after MI and laser irradiation, whereas LLLT significantly decreased the kinin B1 receptor mRNA content after MI (Figure 3A and 3B).

ACE and ACE2 mRNA content were also quantified (Figure 4). Myocardial infarction markedly increased the ACE expression 3 days after coronary occlusion. The lasertherapy significantly reduced ACE expression in the myocardium after MI (Figure 4A). MI did not change ACE2 expression, however, after MI and laser irradiation, ACE2 gene expression showed an augmentation when compared to all experimental groups (Figure 4B).

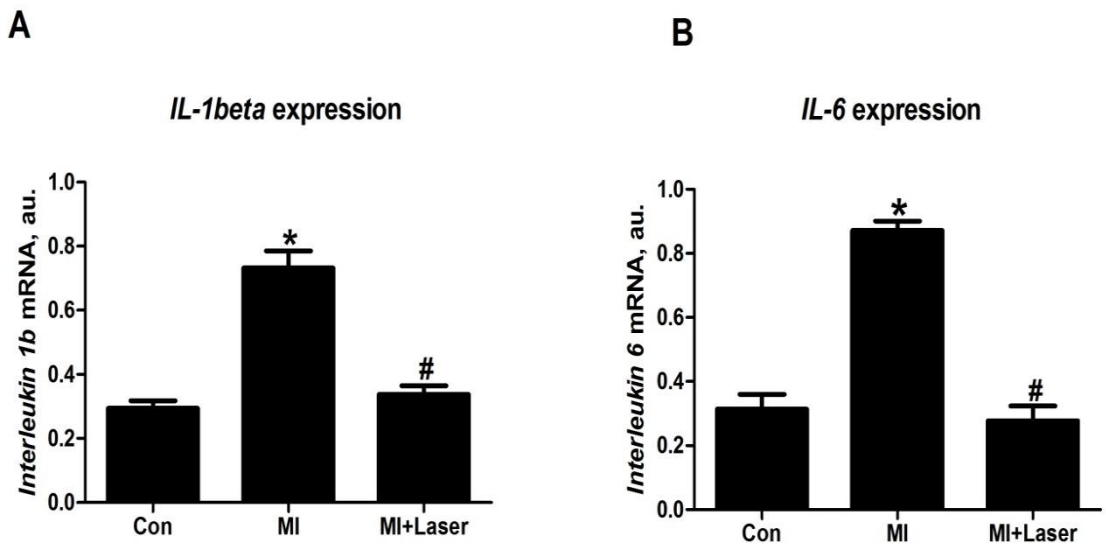

Figure 2. **Quantitative real-time RT-PCR of interleukins 1 (IL-1) and 6 (IL-6) on myocardial tissue.** Myocardial infarction presented an acute inflammation of the myocardium, as observed as an increased of cytokines IL1 and IL6 in the left ventricles (LV) of MI rats. A downregulation of interleukin 1 (**A**) and interleukin 6 (**B**) expression was detected in the group IM+Laser, explicating a anti-inflammatory effect of low level lasertherapy (LLLT). Data are mean±S.E.M. *$p<0.05$ vs Con group; #$p<0.05$ vs MI group.

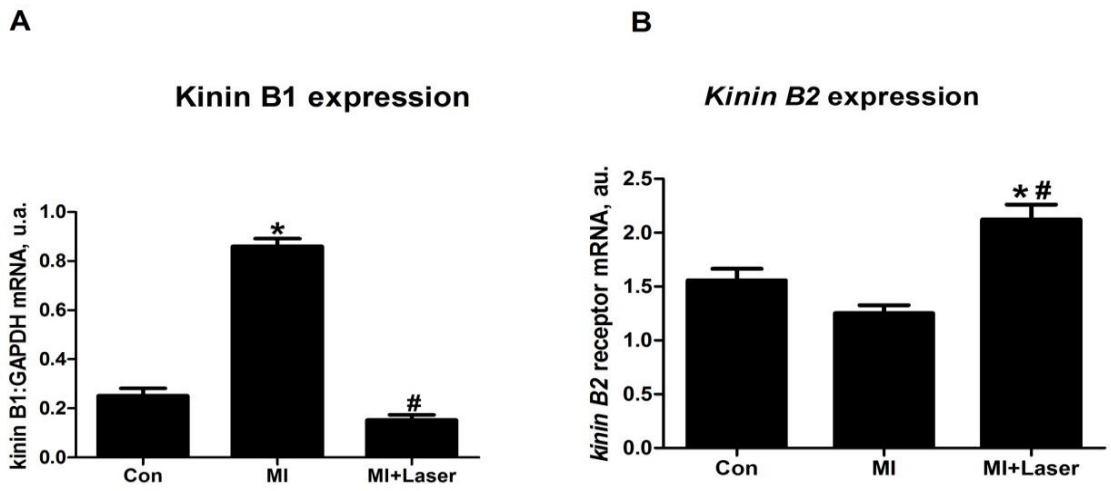

Figure 3. Quantitative real-time RT-PCR of kinin B1 and kinin B2 receptors on myocardial tissue. Distinct kinin receptors modulation was observed in the infarcted myocardium. An increased kinin B2 receptor expression (A) and a diminished kinin B1 expression (B) was observed in the left ventricles (LV) of MI+Laser rats. Data are mean±S.E.M. *p<0.05 vs Con group; #p<0.05 vs MI group.

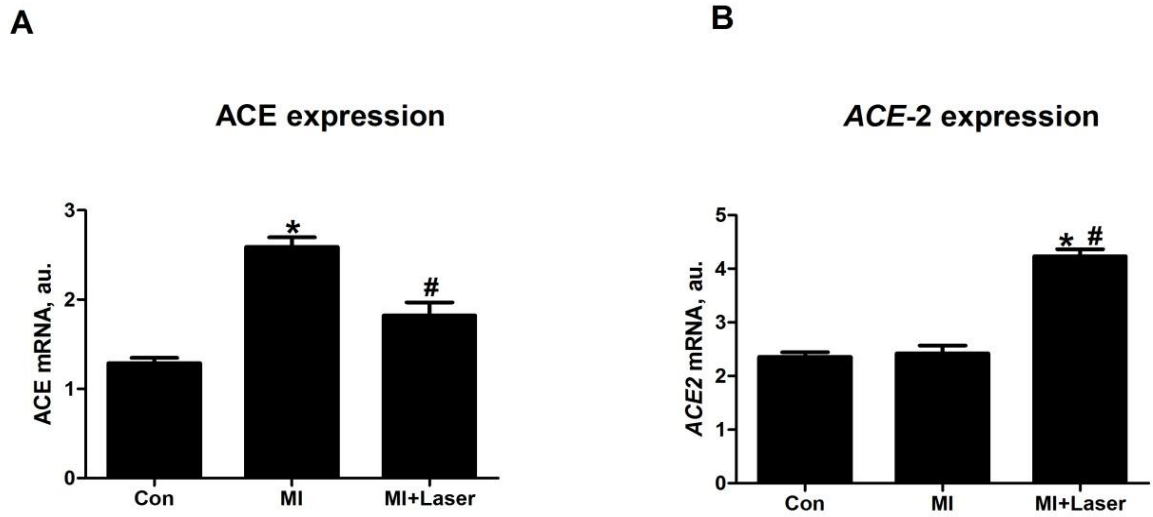

Figure 4. Quantitative real-time RT-PCR analysis of ACE and ACE2 in myocardial tissue. An up-regulation of ACE (**A**) was detected after MI. ACE2 (**B**) was strongly expressed in in the LV of MI+Laser rats. Data are mean±S.E.M. *p<0.05 vs Con group; #p<0.05 vs MI group.

The *mas* receptor expression behaved similarly to ACE2 expression, showing a significantly increase after MI and lasertherapy (Figure 5A). The Mas receptor protein corroborated the findings of *mas* mRNA quantification. The laser-irradiated myocardium presented a strongly increase of Mas receptor protein expression 3 days after MI (Figure 5B).

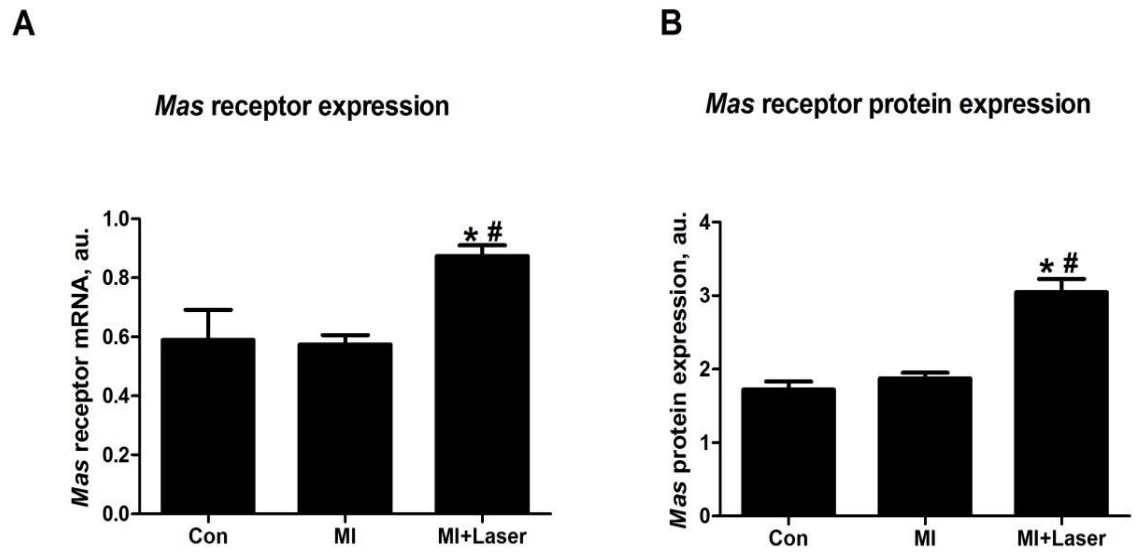

Figure 5. Quantitative real-time RT-PCR and western blotting analysis of mas receptor in myocardial tissue. An up-regulation of gene (**A**) and protein (**B**) mas expression was detected in IM+Laser rats. The augmented mas receptor expression might be an indicative of the ACE2/Ang1-7/mas receptor axis activation. This may result in a protective response of the myocardium after infarction and laser irradiation. Data are mean±S.E.M. *p<0.05 vs Con group; #p<0.05 vs MI group.

DISCUSSION

In the current study, the lasertherapy was capable to increase kinin B2 receptor expression in the myocardium. As kinins are continuously released during cardiac hypoxia and ischemia [34,35], the binding of kinins to endothelial B2 receptors may lead to the release of nitric oxide (NO) and prostacyclin (PGI2), exerting vasodilator effect and preserving myocardial stores of energy-rich phosphates and glycogen [34], eliciting a cardioprotection [37]. Therefore, an upregulated kinin B2 receptor expression on cardiac muscle after laser irradiation could contribute to tissue recovery after MI, modulating a vasodilation necessary to irrigate the border zone and remote area of

infarction. In fact, a reduced KKS activity in the myocardium facilitates the development of cardiac failure [37].

Kinin B1 receptor expression after inflammatory insult was observed by different authors [38,39]. However, to our knowledge, we were the first to show a diminished kinin B1 receptor expression after lasertheraphy in the subplantar muscle [12] and herein in the myocardium. Kinin B1 is expressed as an inducible receptor [40,41] whose expression depends on the strength of the inflammatory stimulus. As expected, the MI-related inflammation resulted in an augmented expression of B1 receptor. Interestingly, this event was blunted by lasertherapy. This observation, if analyzed together with the reduced levels of interleukins -1 and -6 after MI and laser irradiation, suggest that laser was effective to downregulate inflammatory mediators expression after MI [42].

Worthy of mention the current study brings new insight about laser actions, mainly by the suggestion of RAS participation in the anti-inflammatory LLLT response. The observation that ACE mRNA content was increased 3 days after MI corroborates several studies in experimental MI and humans with MI and cardiac failure [43,44]. Interestingly, we observed for the first time a diminished ACE expression by lasertherapy after MI, however, ACE expression did not reach the expression values found in control animals.

Several studies have reported that ACE2 expression is a protective counterbalance of RAS actions [43,44]. The increment of ACE2 content after MI has been reported in human and rodents. Patients with ischemic heart failure showed an increased ACE and ACE2 immunoreactivity in the myocardium when compared to normal subjects [43]. Myocardial infarction in Sprague-Dawley rats increased cardiac ACE and ACE2 mRNA compared to control [43]. Our study indicates that ACE2 mRNA content augmentation (after MI followed by LLLT) may contribute to cardioprotection.

Noteworthy is the augmentation of ACE2 expression coordinated to Mas receptor expression. This synchronized expression of both ACE2 and Mas receptor is observed by various studies [45-48]. These observations suggest a participation of the heptapeptide Ang1-7 in the lasertherapy action mechanism in the myocardium, once Mas receptor is activated exclusively by Ang1-7 [45]. In fact, the Ang1-7 is shown to exert several cardioprotective actions in the heart [for review, see 49]. *Mas* receptor gene ablation abolished binding and renal activity of Ang1–7 in mice. *Mas* transfection

increased Ang1–7 activity, which was blocked by the specific Ang1–7 antagonist, A-779 [50]. Some authors reported that loss of Ang1–7 immunoreactivity after MI within the infarcted area contrasted with an apparent increased expression of the peptide in the zones bordering the infarcted region of the left ventricle [51]. Intravenous infusion of Ang1–7 significantly diminished the left ventricular end-diastolic pressure, preserved coronary flow and endothelial function [52]. The *mas* knockout mice showed an impaired cardiac function and structure [53].

From the above, a picture is emerging in favour of the participation of the ACE2-Ang1-7-Mas receptor axis in the protective action of LLLT. Taking together, the improved cardiac function data we presented here might be, as least partially, related to the vasoactive increased expression in the MI+Laser group. For instance, another work reported an impaired heart function observed in mice with targeted disruption of ACE2 [54] as values of LV fractional shortening (FS) and velocity of circumferential fiber shortening that were severely decreased when compared to wild-type mice. Furthermore, selective blockade of B2 receptors by Hoe 140 reduces coronary blood flow and contractility, and increases left ventricular end diastolic pressure [55]. We presented that LV fractional shortening was diminished in the infarcted heart, and a FS augmentation was observed after laser irradiation in infarcted rats. The reduced infarction size after LLLT was described previously by [28,29]. Herein, using the same energy density from these previous studies, we provide more evidence that LLLT may result in a significant reduction of infarct size.

Considering the data presented and discussed in this manuscript and from the current state of knowledge regarding the anti-inflammatory efficacy of LLLT, our findings indicate that laser irradiation may exert beneficial effects to the myocardium after MI. The reduction of infarcted area and the improvement of cardiac function, together with the upregulation of protective kinin B2 and a diminished kinin B1 and interleukins mRNA expression are in consonance with the expected anti-inflammatory response of LLLT. Moreover, the increased expression of ACE2 and Mas receptor expression suggest the participation of cardioprotective Ang1-7 in the post-MI milieu after laser irradiation.

To conclude, these findings broaden our understanding of the cardioprotective lasertherapy on the cardiac tissue and the relevance of vasoactive systems in relation to the pathophysiology of myocardium infarction.

Clinical perspectives

Many studies have demonstrated the beneficial effects of LLLT, a non-pharmacological therapy, suggesting that it could be a potential therapy to reduce inflammation in several tissues in experimental protocol and humans. In fact, reduction of cytokines expression has been reported by many authors. Herein, we showed a counterbalance between the augmented expression of cardioprotective components of RAS and KKS and a decreased expression of inflammation mediators, together with reduction of infarction size and amelioration of cardiac function acutely after MI and laser irradiation. Further studies must be conducted to observe if these alterations remain chronically in the myocardium and their clinical repercussion.

References

1. Johnston, C. I. (1994) Tissue angiotensin converting enzyme in cardiac and vascular hypertrophy, repair, and remodeling. Hypertension 23: 258-268. doi: 10.11.61/01.HYP.23.2.258.

2. Bader, M. (2013) ACE2, angiotensin(1–7), and Mas: the other side of the coin. Pflugers Arch. 465: 79-85.

3. Mello, W.D. (2003) Effect of extracellular and intracellular angiotensins on heart cell function; on the cardiac renin-angiotensin system. Regul. Pept. 114:87–90. doi: 10.1016/S0167-0115(03)00121-6.

4. Marchesi, C., Paradis, P., Schiffrin, E.L. (2008) Role of the renin-angiotensin system in vascular inflammation. Trends Pharmacol. Sci. 29:367–374. doi: 10.1016/j.tips.2008.05.003.

5. Dimmeler, S., Zeiher, A.M. (2000) Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. Regul. Pept. 90: 19–25. doi: 10.1016/S0167-0115(00)00105-1.

6. Pfeffer, M. A. and Braunwald, E. (1990) Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 81:1161-1172. doi: 10.1161/01.CIR.81.4.1161.

7. Pfeffer, M. A., Braunwald, E., Moye, L. A., Basta, L., Brown, E. J., Jr., Cuddy, T. E., Davis, B. R., Geltman, E. M., Goldman, S., Flaker, G. C. et al. (1992) Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators. N. Engl. J. Med. 327:669–677. doi:

10.1056/NEJM199209033271001.

8. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS): The CONSENSUS trial study group. N. Engl. J. Med. 1987; 316: 1429–1435. doi: 10.1056/NEJM198706043162301.

9. Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure: The SOLVD investigators. N. Engl. J. Med. 1991; 325: 293–302. doi: 10.1056/NEJM199108013250501.

10. Araújo, R.C., Mori, M.A., Merino, V.F., Bascands, J.L., Schanstra, J.P., Zollner, R.L., Villela, C.A., Nakaie, C.R., Paiva, A.C., Pesquero, J.L., Bader, M., Pesquero, J.B. (2006) Role of the kinin B1 receptor in insulin homeostasis and pancreatic islet function. Biol. Chem. 387: 431-6. doi: 10.1515/BC.2006.057.

11. Meotti, F.C., Campos, R., da Silva, K., Paszcuk, A.F., Costa, R., Calixto, J.B. (2012) Inflammatory muscle pain is dependent on the activation of kinin B1 and B2 receptors and intracellular kinase pathways. Br. J. Pharmacol. 166:1127-39. doi: 10.1111/j.1476-5381.2012.01830.x.

12. Bortone, F., Santos, H.A., Albertini, R., Pesquero, J.B., Costa, M.S., Silva, J.A. Jr. (2008) Low level laser therapy modulates kinin receptors mRNA expression in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation. Int Immunopharmacol. 8:206-10. doi: 10.1016/j.intimp.2007.09.004.

13. Pereira, M.G., Ferreira, J.C., Bueno, C.R. Jr., Mattos, K.C., Rosa, K.T., Irigoyen, M.C., Oliveira, E.M., Krieger, J.E., Brum, P.C. (2009) Exercise training reduces cardiac angiotensin II levels and prevents cardiac dysfunction in a genetic model of sympathetic hyperactivity-induced heart failure in mice. Eur. J. Appl. Physiol. 105: 843-50. doi: 10.1007/s00421-008-0967-4.

14. Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K. and Jeyaseelan, R. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. Circ. Res. 87: E1-E9. doi:10.1161/01.RES.87.5.e1.

15. Nadu, A.P., Ferreira, A.J., Reudelhuber, T.L., Bader, M., Santos, R.A. (2008) Reduced isoproterenol-induced renin-angiotensin changes and extracellular matrix deposition in hearts of TGR(A1-7)3292 rats. J. Am. Soc. Hypertens. 2: 341-8. doi: 10.1016/j.jash.2008.04.012.

16. Kompa, A.R., See, F., Lewis, D.A., Adrahtas, A., Cantwell, D.M., Wang, B.H.,

Krum, H. (2008) Long-term but not short-term p38 mitogen-activated protein kinase inhibition improves cardiac function and reduces cardiac remodeling post-myocardial infarction. J. Pharmacol. Exp. Ther. 325: 741-50. doi: 10.1124/jpet.107.133546.

17. Mercure, C., Yogi, A., Callera, G.E., Aranha, A.B., Bader, M., Ferreira, A.J., Santos, R.A., Walther, T., Touyz, R.M., Reudelhuber, T.L. (2008) Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. Circ. Res. 103: 1319-26. doi: 10.1161/CIRCRESAHA.108.184911.

18. Al-Maghrebi, M., Benter, I.F., Diz, D.I. (2009) Endogenous angiotensin-(1-7) reduces cardiac ischemia- induced dysfunction in diabetic hypertensive rats. Pharmacol. Res. 59: 263-8. doi: 10.1016/j.phrs.2008.12.008.

19. Aimbire, F.; Albertini, R.; Correa, J.C.; Silva, J.A. Jr.; Costa, M. S. Low Level Laser Therapy (LLLT) Decreases Pulmonary Microvascular Leakage (2008) Neutrophil Influx and IL-1beta Levels in Airway and Lung from Rat Subjected to LPS-Induced Inflammation. Inflammation 31:189-97. doi: 10.1007/s10753-008-9064-4.

20. Albertini, R. ; Aimbire, F. ; Villaverde, ; Silva, J.A. Jr.; Costa, M. S. (2007) COX-2 mRNA expression decreases in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation after low level laser therapy. Inflammation Research 56: 228-29. doi: 10.1007/s00011-007-6211-6.

21. Albertini, R. ; Villaverde, A.B., Aimbire, F. ; Bjordal, J., Brugnera, A., Mittmann, J., Silva, J.A.; Costa, M. S. (2008) Cytokine mRNA Expression Is Decreased in the Subplantar Muscle of Rat Paw Subjected to Carrageenan-Induced Inflammation after Low-Level Laser Therapy. Photomed. Laser Surgery 26: 19-24.doi: 10.1089/pho.2007.2119.

22. Silva., M.P.; Bortone, F.; Silva, M.P., Araujo, T.R.; Costa, M.S., Junior, J. A. (2011) Inhibition of carrageenan-induced expression of tissue and plasma prekallikreins mRNA by low level laser therapy in rat paw edema. Revista Brasileira de Fisioterapia (print). 15:1-7.doi: org/10.1590/S1413-35552011005000005.

23. Mester, E., Spiry, T., Szende, B., Tota, J.G. (1971) Effect of laser rays on wound healing. Am. J. Surg. 122: 532–535. doi:10.1016/002-9610.

24. Whittaker P., Patterson M.J. (2000) Ventricular remodeling after acute myocardial infarction: effect of low-intensity laser irradiation. Lasers Surg Med. 2: 29-38.doi: 10.1002/1096-9101.

25. van Nieuwenhoven, F.A., Turner, N.A. (2013) The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. Vascular

Pharmacology 58: 182–188. doi: 10.1016/j.vph.2012.07.003.

26. Frangogiannis, N.G., Smith, C.W., Entman, M.L. (2002) The inflammatory response in myocardial infarction. Cardiovasc. Res. 53: 31-47. doi: 10.1016/S0008-6363(01)00434-5.

27. Oron, U., Yaakobi, T., Oron, A., Mordechovitz, D., Shofti, R., Hayam. G., Dror, U., Gepstein, L., Wolf, T., Haudenschild, C., Haim, S.B. (2001) Low-energy laser irradiation reduces formation of scar tissue after myocardial infarction in rats and dogs. Circulation 103: 296-301.doi:10.1161/01.CIR.103.2.296.

28. Oron, U., Yaakobi, T., Oron, A., Hayam, G., Gepstein, L., Wolf, T., Rubin, O., Ben-Haim, S.A. (2001) Attenuation of infarct size in rats and dogs after myocardial infarction by low-energy laser irradiation. Lasers Surg. Med. 28: 204–11.

29. Ad, N., Oron, U. (2001) Impact of low level laser irradiation on infarct size in the rat following myocardial infarction. Int. J. Cardiol. 80: 109–16.doi:10.1016/SO167-5273.

30. Nozawa E., Kanashiro R.M., Murad N., Carvalho A.C., Cravo S.L., Campos O., et al. (2006) Performance of two-dimensional Doppler echocardiography for the assessment of infarct size and left ventricular function in rats. Braz J Med Biol Res. 39: 687- 95. doi.org/10.1590/S0100-879X2006000500016.

31. dos Santos L., Mello A.F., Antonio E.L., Tucci P.J. (2008) Determination of myocardial infarction size in rats by echocardiography and tetrazolium staining: correlation, agreements, and simplifications. Braz J Med Biol Res. 41:199-201. doi.org/10.1590/S0100-879X2008005000007.

32. Santos, A.A., Helber, I., Flumignan, R.L., Antonio, E.L., Carvalho, A.C., Paola, A.A., Tucci, P.J., Moises, V.A. (2009) Doppler echocardiographic predictors of mortality in female rats after myocardial infarction. J. Card. Fail.15:163-8. doi: 10.1016/j.cardfail.2008.10.017.

33. Kanashiro, R.M., Saraiva, R.M., Alberta, A., Antonio, E.L., Moisés, V.A., Tucci, P.J. (2006) Immediate functional effects of left ventricular reduction: a Doppler echocardiographic study in the rat. J. Card. Fail.12: 163-9.doi:10.1016/j.cardfail.2005.09.007.

34. Linz, W., Wiemer, G., and Scholkens, B.A. (1993) Bradykinin prevents left ventricular hypertrophy in rats. J. Hypertens. 11(Suppl 5): S96–S97.

35. Scholkens, B.A. (1996) Kinins in the cardiovascular system. Immunopharmacology 33**:** 209–217.

36. Madeddu, P., Milia, A.F., Salis, M.B., Gaspa, L., Gross, W., Lippoldt, A., and Emanueli, G. (1998) Renovascular hypertension in bradykinin B2-receptor knockout mice. Hypertension 23**:** 305–509.doi:10.1161/01.HYP.32.3.503.

37. Sharma J.N. (2008) Cardiovascular Activities of the Bradykinin System. The Scientific World Journal 8: 384–393. doi: 10.1100/tsw.2008.53.

38. Gross, E.R., Gross, G.J. (2006) Ligand triggers of classical preconditioning and postconditioning. Cardiovasc. Res. 70:212–221. doi:10.1016/j.cardiores.2005.12.019.

39. Yin. H., Chao, J., Bader, M., Chao, L. (2007) Differential role of kinin B1 and B2 receptors in ischemia-induced apoptosis and ventricular remodeling. Peptides 28: 1383–1389.doi:10.1016/j.peptides.2007.05.010.

40. Potier, L., Waeckel, L., Vincent, M.P., Chollet, C., Gobeil, F. Jr., Marre, M., Bruneval, P., Richer, C., Roussel, R., Alhenc-Gelas, F., Bouby. N. (2013) Selective kinin receptor agonists as cardioprotective agents in myocardial ischemia and diabetes. J. Pharmacol. Exp. Ther. 346: 23-30. doi: 10.1124/jpet.113.203927.

41. Westermann, D., Walther, T., Savvatis, K., Escher, F., Sobirey, M., Riad, A., Bader, M., Schultheiss, H.P., Tschöpe, C. (2009) Gene deletion of the kinin receptor B1 attenuates cardiac inflammation and fibrosis during the development of experimental diabetic cardiomyopathy. Diabetes 58:1373-81. doi: 10.2337/db08-0329.

42. Yang, Z., Wu, Y., Zhang, H., Jin, P., Wang, W., Hou, J., Wei, Y., Hu, S. (2011) Low-level laser irradiation alters cardiac cytokine expression following acute myocardial infarction: a potential mechanism for laser therapy. Photomed. Laser Surg. 29: 391-8. doi: 10.1089/pho.2010.2866.

43. Zisman, L.S., Keller, R.S., Weaver, B., Lin, Q., Speth, R., Bristow, M.R., et al. (2003) Increased angiotensin-(1–7)-forming activity in failing human heart ventricles: evidence for upregulation of the angiotensin-converting enzyme Homologue ACE2. Circulation 108: 1707–1712.doi:10.1161/01.CIR.0000094734.67990.99.

44. Burrell, L.M., Risvanis, J., Kubota, E., Dean, R.G., MacDonald, P.S., Lu, S. et al. (2005) Myocardial infarction increases ACE2 expression in rat and humans. Eur. Heart. J. 26: 369–375. doi: 10.1093/eurheartj/ehi114.

45. Silveira, K.D., Barroso, L.C., Vieira, A.T., Cisalpino, D., Lima, C.X., Bader, M., Arantes, R.M., Dos Santos, R.A., Simões-E-Silva, A.C., Teixeira, M.M. (2013) Beneficial effects of the activation of the Angiotensin-(1-7) MAS receptor in a murine

model of adriamycin-induced nephropathy. PLoS One 8: e66082. doi: 10.1371/journal.pone.0066082.

46. Feltenberger, J.D., Andrade, J.M., Paraíso, A., Barros, L.O., Filho, A.B., Sinisterra, R.D., Sousa, F.B., Guimarães, A.L., de Paula, A.M., Campagnole-Santos. M.J., Qureshi, M., Dos Santos, R.A., Santos, S.H. (2013) Oral Formulation of Angiotensin-(1-7) Improves Lipid Metabolism and Prevents High-Fat Diet-Induced Hepatic Steatosis and Inflammation in Mice. Hypertension 62: 324-30. doi: 10.1161/HYPERTENSIONAHA.111.00919.

47. Souza, A.P., Sobrinho, D.B., Almeida, J.F., Alves, G.M., Macedo, L.M., Porto, J.E., Vêncio, E.F., Colugnati, D.B., Dos Santos, R.A., Ferreira, A.J., Mendes, E.P., Castro, C.H. (2013) Angiotensin II Type 1 receptor blockade restores angiotensin-(1-7)-induced coronary vasodilation in hypertrophic rat hearts. Clin. Sci. (Lond) 125: 449-59. doi: 10.1042/CS20120519.

48. Fraga-Silva, R.A., Costa-Fraga, F.P., Murça, T.M., Moraes, P.L., Martins Lima, A., Lautner, R.Q., Castro, C.H., Soares, C.M., Borges, C.L., Nadu, A.P., Oliveira, M.L., Shenoy, V., Katovich, M.J., Santos, R.A., Raizada, M.K., Ferreira, A.J. (2013) Angiotensin-converting enzyme 2 activation improves endothelial function. Hypertension 61: 1233-8. doi: 10.1161/HYPERTENSIONAHA.111.00627.

49. Passos-Silva, D.G., Verano-Braga, T., Santos, R.A. (2013) Angiotensin-(1-7): beyond the cardio-renal actions. Clin. Sci. (Lond). 124: 443-56. doi: 10.1042/CS20120461.

50. Keidar, S., Kaplan, M., Gamliel-Lazarovich, A. (2007) ACE2 of the heart: From angiotensin I to angiotensin (1-7). Cardiovasc. Res. 73:463-9. doi:10.1016/j.cardiores.2006.09.006.

51. Averill, D.B., Ishiyama. Y., Chappell, M.C., Ferrario, C.M. (2003) Cardiac angiotensin-(1–7) in ischemic cardiomyopathy. Circulation 108: 2141–2146.doi:10.1161/01.CIR.0000092888.63239.54.

52. Loot, A.E., Roks, A.J., Henning, R.H., Tio, R.A., Suurmeijer, A.J., Boomsma, F., et al. (2002) Angiotensin-(1–7) attenuates the development of heart failure after myocardial infarction in rats. Circulation 105:1548–1550. doi:10.1161/01.CIR.0000037125.61227.68.

53. Santos, R.A., Castro, C.H., Gava, E., Pinheiro, S.V., Almeida, A.P., Paula, R.D., et al. (2006) Impairment of in vitro and in vivo heart function in angiotensin-(1–7)

receptor MAS knockout mice. Hypertension 47: 996–1002. doi: 10.1161/01.HYP.0000215289.51180.5c

54. Crackower, M.A., Sarao, R., Oudit, G.Y., Yagil, C., Kozieradzki, I., Scanga, S.E., et al. (2002) Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature 417: 822–828. doi:10.1038/nature00786.

55. Koide, A., Zeitlin, I.J., and Parratt, J.R. (1993) Kinin formation in ischaemic heart and aorta of anaesthetized rats. J. Physiol. (Lond.) 467**:** 125P.

3.2 - Artigo 2

Martha Trindade Manchini, Andrey Jorge Serra, Regiane dos Santos Feliciano, Rodolfo de Paula Vieira, Eduardo Tadeu Santana, Ednei Luis Antônio, Regiane Albertini, Paulo de Tarso Camillo de Carvalho, Rodrigo Labat, Paulo José Ferreira Tucci and José Antônio Silva Jr. **Modulation of inflammatory response on Myocardial Infarction due to low level laser therapy (LLLT).**

Modulation of inflammatory response on Myocardial Infarction due to low level laser therapy (LLLT)

Martha Trindade Manchini[1], Andrey Jorge Serra[1], Regiane dos Santos Feliciano[1], Rodolfo de Paula Vieira[1], Eduardo Tadeu Santana[1], Ednei Luis Antônio[2], Regiane Albertini[1], Paulo de Tarso Camillo de Carvalho[1], Rodrigo Labat[1], Paulo José Ferreira Tucci[2] and José Antônio Silva Jr[1*].

[1]Universidade Nove de Julho, UNINOVE, Rua Vergueiro, 235 - Liberdade 01504-001 São Paulo, SP- Brasil

[2]Universidade Federal de São Paulo, UNIFESP, Rua Coronel Lisboa, 819 Vila Clementino 04020-041 - Sao Paulo, SP - Brasil

ABSTRACT

Myocardial infarction (MI) in rats promoted by occlusion of the left anterior descending coronary artery is the most common model in experimental research that elucidate the functional, structural and molecular changes associated with ischemic heart disease. Low lever laser therapy (LLLT) has become an alternative therapy to modulate various biological processes due its anti-inflammatory effect, reduction of pain and acceleration of cell proliferation. The objective of this study were to analyze the effect of LLLT on cytokine expression and apoptosis in the remote and infarcted myocardium areas. While TNF-alpha, IL-6 and CINC-1 expression did not alter after laser irradiation at infarcted area, our results demonstrate the cardioprotective effect of LLLT diminishing cytokines expression in the remote area of the myocardium. Laser irradiation increased anti-apoptotic bcl2 gene expression after MI. However, at infarcted area, gene expression of cardiotrophin-1 showed a strong augmentation after LLLT.

INTRODUCTION

Large myocardial infarction induce a process of cardiac remodeling which includes morphological, histological and molecular changes in the infarcted area and non-infarcted myocardium (Ertl et al, 2005). Remodeling is a strong prognostic determinant and is highly correlated with the incidence of arrhythmias and sudden cardiac death (Sun et al, 2000). Myocardial infarction

size is dynamic since the loss of viable myocardium is progressive after coronary artery occlusion during several hours (infarct extension). Furthermore, the infarcted region may expand or (Frangogiannis et al, 2002) contract during the first weeks of infarction, even after the loss of viable myocardium has finished. Infarct expansion itself is a critical determinant of remodeling and thus prognosis (Moses et al, 2000).

Acute myocardial infarction (MI) results in the activation of the inflammatory response (Frangogiannis et al, 2002; Ruparelia et al, 2013), mobilisation and recruitment of leukocytes to the infarcted area [Ruparelia et al, 2013; Nahrendorf M et al, 2007]. Moreover, myocardium remote has also been associated with activation of pro-inflammatory pathways and infiltration of leukocytes and these events are increasingly important in left ventricular remodeling after MI [Ruparelia et al, 2013].

The intense inflammatory process may have adverse effects on cardiac remodeling, such as on cardiac rupture or dilation progression to heart failure and death (Nian et al, 2004).

Leukocyte infiltration is a source of cytokines like IL-6, TNF-α and IL-1 which are involved in the acute inflammatory response, and fibrosis in the repair process and post-MI and correlated with adverse remodeling (Nah et al 2009).

Recently, researchers have demonstrated increased leukocyte recruitment, TNF-α and IL-6 in the border zone of the ischemic myocardium (Nah et al, 2009) as well as increased expression of IL-6 and the cytokine IL-6 receptor (RIL-6) in transgenic mice associated with cardiac hypertrophy (Hirota et al, 1995).

These cytokines were also found elevated in patients with unstable angina in the first days of hospitalization and related increase in coronary events (Biassuci et al, 1999). Therefore, the plasma levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha and interleukin-6 are increased in patients post-MI and this increase is gradual and observed in patients with heart failure (Deswal et al, 2001).

Cardiotrophin-1 has also been analyzed both in the first hours and weeks after MI. Cardioprotective effects are evident in the early periods of post-MI by promoting the proliferation of myofibroblasts and allow the survival of cardiomyocytes (Freed DH, et al 2005).

Low lever laser therapy (LLLT) has become an alternative to modulate various biological processes and depending on the wavelength, dosage and condition of the irradiated tissue can contribute to decreasing inflammation, reducing the pain and accelerating cell proliferation [14 - 19].

The use of LLLT to treat pathophysiological conditions began four decades ago [Mester et al 1971]. To our knowledge, the first report of the use of LLLT in experimental models of MI was in 2000 [Whittaker et al 2000], suggesting that laser irradiation attenuates post-MI remodeling. Although some studies have reported the LLLT attenuated cardiac dysfunction as well as reduced infarct size [22-24], the debate continues through the molecular mechanisms of the laser in the myocardium. Until now, it was not reported by any study the inflammatory profile in the infarcted and non-infarcted myocardium in an experimental model of MI treated with low level laser therapy as well as the expression of cardiotrophin-1 and apoptotic factors. The aim of this study was to evaluate the possible cardioprotective effects of LLLT in cardiac remodeling in rats with MI.

METHODS

Ethics Statement

All the experimental procedures were performed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The project research was approved by the Institutional Research Ethics Committee of the Nove de Julho University (No 0015/2012), São Paulo, Brazil. All surgery was performed under ketamine and xylazin anesthesia, and all efforts were made to minimize suffering.

Animals and MI surgical procedure

Female Wistar rats (n=82) with 10 weeks of age were subjected to thoracotomy and infarction by coronary occlusion. The surviving rats were randomly divided into infarcted non-treated group (MI, n=28) and infarcted laser-treated group (MI+Laser, n=30). Rats that received the same surgical procedure for thoracotomy without coronary ligation served as control group (Con, n=14). For MI induces, under ketamine (50 mg/kg) plus xylazin (10 mg/kg) anesthesia the coronary artery was occluded near its origin as previously described [30]. All parameters evaluated in this study were analyzed 3 days after MI.

Lasertherapy

After surgery, the animals were immediately randomized into two experimental group (with or without lasertherapy). The laser device used was a Aluminum Indium Gallium Phosphorus – AlGaInP (Twin Laser – M M Optics ®) with wavelength 660 nm, power 15mW, laser beam spot size 0,785 cm^2, energy density 22,5Jcm^2, irradiation time 60 sec, and energy delivered 1.1 Joules. The laser dose used in this study was similar to [25]. However, we chose the wavelength of 660nm due beneficial effects reported using rats with heart failure to achieve an inflammatory profile in this condition. The laser beam was placed in contact with the myocardium surface corresponding to the infarcted area. After ligation as describe above, the heart was put in the chest to recover itself and then the heart was put out and random to receive or not the laser irradiation. The optical fiber was fixed with a delivery arm and precisely positioned with the fiber tip 3 cm above the myocardium. This allowed for a laser beam spot size of 0,785 cm^2.

Histology

The hearts were removed three days after infarction by coronary occlusion and fixed in 4% buffered formaldehyde overnight. The fragments were washed with PBS, dehydrated through a graded series of ethanol, diaphonized with Xylol and embedded with Paraplast. Thereafter, the samples were cut into sections of 3 µm thick and stained with hematoxylin and eosin. A single pathologist, who was blinded to all groups, examined the pathological specimens and neutrophils.

Cytokine measurements

The levels of TNF-α, lL-6, GRO alpha/CINC-1 (cytokine-induced neutrophil chemoattractant) and IL-10 were measured on infarcted area and non infarcted area respectively using commercially available ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the instructions of the manufacturer. The optical density was measured at a wavelength of 450 nm in Microplate Reader (2020, Anthos, Eugendorf, Austria) (Vieira, RP et al, 2013).

Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) was measured as an index of the presence of neutrophils. infarcted area and non infarcted area samples were obtained from rats killed 3 days after myocardial infarction and LLLT. ~20 mg the tissues were homogenized in 0,4 ml of HTAB for 15 seconds. The homogenates were agitated in a vortex and centrifuged in and then the supernatants obtained were analyzed in MPO activity. Ten microliters of the supernatant were added 200 ml of o-dianisidine dihydrochloride using an ELISA plate. In each well were used to sample 100 ml of 2 ml o-dianisidine dihydrochloride. The absorbance was measure at wavelength of 460 nm and recorded at 15 second intervals for 2 min.

Gene expression quantification

Total RNA was extracted from left ventricle (LV) samples and Real-time PCR assay was performed to access mRNA quantification. Thawed tissues were homogenized in 1 ml of TRIzol reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated accordingly to the manufacturer´s instructions.

One microgram of total RNA was used for cDNA synthesis and Real-Time PCR gene expression analysis. Initially, contaminating DNA was removed using DNase I (Invitrogen) at a concentration of 1 unit/µg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM $MgCl_2$ for 15 min at 37 °C, followed by incubation at 95°C for 5 min for enzyme inactivation. Then, the reverse transcription (RT) was carried out in a 200µl reaction in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen). The reactions conditions were: 20 °C for 10 min, 42°C for 45 min and 95°C for 5 min.

The reaction product was amplified by real time PCR on the 7500 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBR Green core reaction kit (Applied Biosystems). The thermal cycling conditions were: 50 ºC for 2 min, then 95ºC for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Experiments were performed in triplicates for each data point. Target gene mRNA abundance was quantified as a relative

value compared with an internal reference, GAPDH, whose abundance was believed not to change between the varying experimental conditions. Primers used for real time PCR were: rat Bax primers forward (GenBank accession number M_017059.2) sense 5'- ACTCCCCCCGAGAGGTCTT-3', antisense 5'-AGTTGAAGTTGCCATCAGCAAA-3′ e Bcl-2 (GenBank accession number NM_016993.1) sense 5′-GCTACGAGTGGGATACTGG-3′, antisense 5′-GTGTGCAGATGCCGGTTCA-3; cardiotrofin 1 (sense 5`-ATGAGCCAGAGGGAGGGAAG-3`, antisense 5`-TCAGGCAACGCCCCC-3`).

Statistical analysis

Data were analyzed with GraphPad Prism software (La Jolla, CA, USA). The Shapiro-Wilk and Levene tests were used to verify normality and error variances, respectively. One-way ANOVA complemented by Newman–Keuls test was used to detect differences between three groups at sample with normal distribution. However, Kruskal-Wallis followed by Dunn's multiple comparison tests was applied for no-normality data. A p value ≤ 0.05 was considered significant with two-tailed probability and results are expressed as mean ± SEM. Quantitative values for target gene and GAPDH mRNA transcription were obtained from the threshold cycle number, where the increase in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized on the basis of GAPDH expression as an endogenous RNA control. ΔCt values of the samples were determined by subtracting the average C_t value of target gene mRNA from the average C_t value of the internal control GAPDH. The $2^{-\Delta\Delta Ct}$ parameter was used to express the relative expression data. For Western blot analysis, although identical amounts of protein were loaded into each well, the GAPDH expression levels were used as a loading control and to normalize the data.

Results

Anti-inflammatory response of LLLT

As known, cytokines are elevated after coronary occlusion and observe these values after three days of MI in the myocardium remote with increased protein expression of IL-6, CINC-1 and TNF-α. The laser irradiation after MI was able to reduce these values (Figure 1A), suggesting an important role of anti-inflammatory effect of LLLT. However protein expression of IL-10 was not changed.

The myocardium remote TNF-α/IL-10 and IL-6/IL-10 ratio in the MI group were significantly higher than in group MI + Laser demonstrating that laser modulated the pro-inflammatory profile post-MI (Figure 1A).

The increasing protein expression of IL-6, CINC-1 and TNF-α in the infarcted area were observed, but laser irradiation wasn't able to modulate these values (Figure 1B).

Myeloperoxidase (MPO) activity

The LLLT was not able to modulate the response of MPO activity in non infarcted area (Figure 3A).

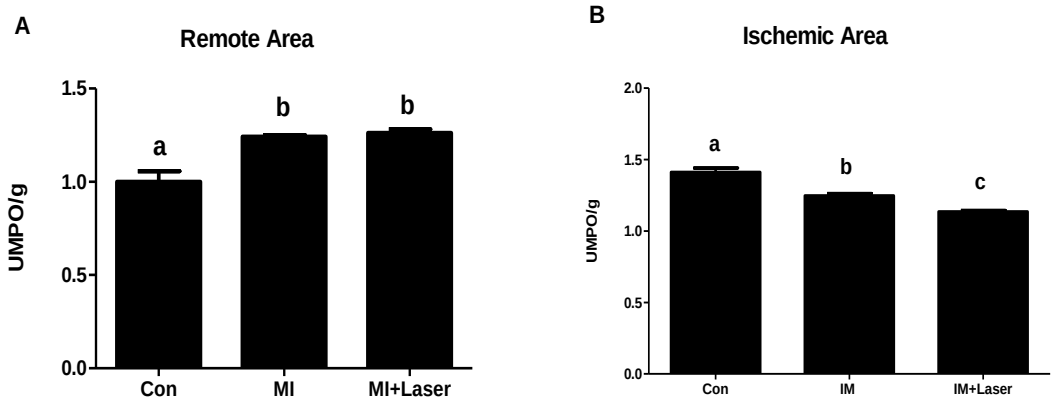

Figure 2. Myeloperoxidase (MPO) activity. The LLLT was not able to modulate the response of MPO activity non infarcted area three days after MI (A). The lower activity of MPO in the ischemic area of IM + Laser compared with IM and control (B). The same letter indicates no different values of ANOVA. Different letters indicate significant difference among different means.

A) Remote Area **B) Ischemic Area**

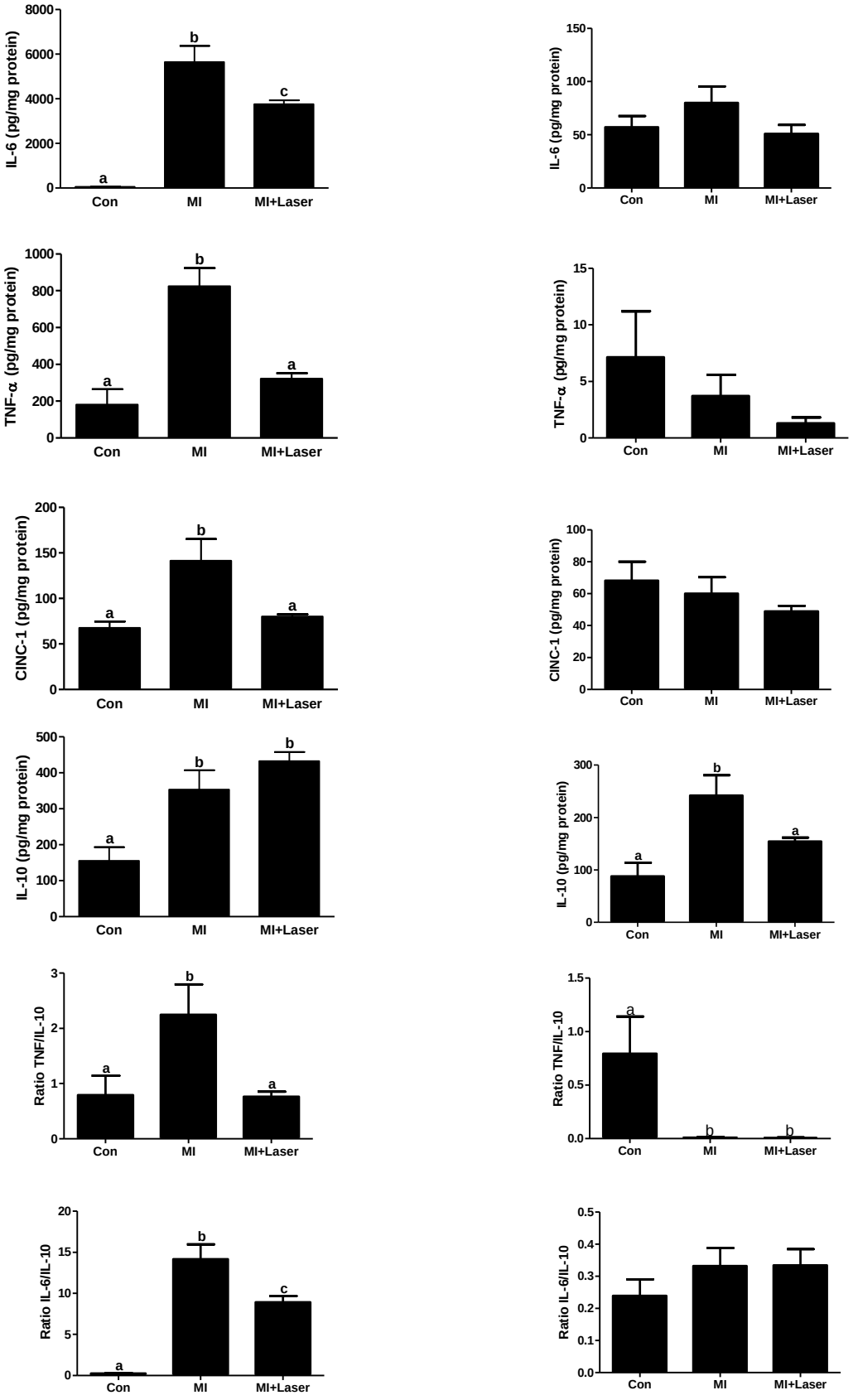

Figure 1. Quantitative analysis by ELISA of interleukin 6 (IL-6), CINC-1 (cytokine-induced neutrophil chemoattractant), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) in remote area (A) and ischemic area (B). The same letter indicates no different values of ANOVA. Different letters indicate significant difference among different means (n=7 per group).

Apoptotic factors and Cardiotrofin 1

Our data demonstrate increased bcl-2 gene expression in myocardium irradiated (Figure 6). This evidence suggests that the laser stimulates cell survival after infarction by inhibiting apoptosis of viable area of the infarcted heart. It could ameliorate the cardiac function after irradiated infarcted heart. The Bax mRNA expression not changed.

The gene expression of cardiotrophin-1 was also analyzed three days after coronary occlusion and observed decreased mRNA expression of CT-1 in the ischemic area and laser significantly increased these values (Figure 4), suggesting that LLLT stimulates the proliferation of myofibroblasts to generate scar, with replacement of muscle by connective tissue due to collagen deposition.

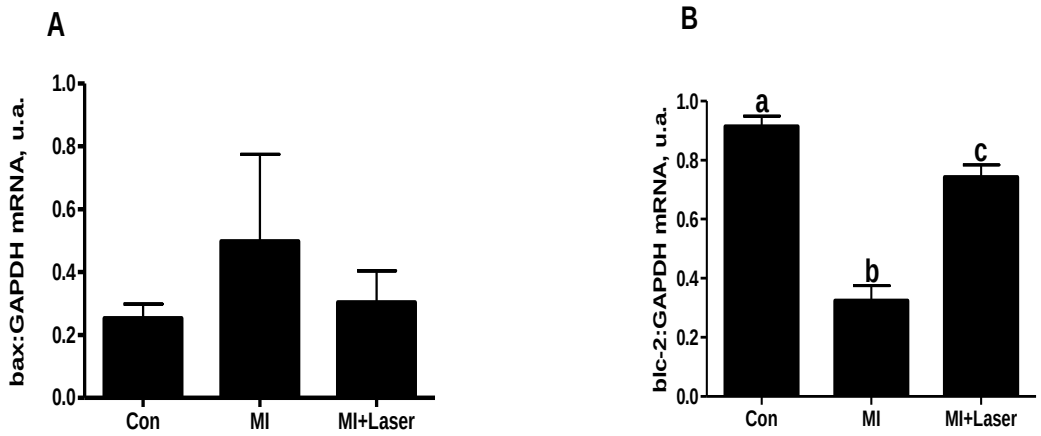

Figure 3. Quantitative real-time RT-PCR analysis of BAX (A) and Bcl-2 (B) in myocardial tissue. The same letter indicates no different values of ANOVA. Different letters indicate significant difference among different means.

In figure 4, our data show the increased of cardiotrofin 1 expression in MI+Laser group. This evidence suggests that the laser stimulates proliferation of myofibroblasts for the generation of scar.

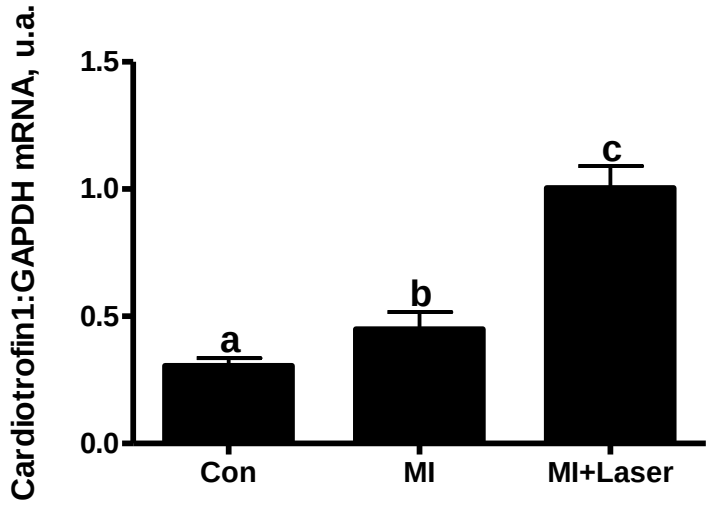

Figure 4. Quantitative real-time RT-PCR analysis of cardiotrofin 1 myocardial tissue. The same letter indicates no different values of ANOVA. Different letters indicate significant difference among different means.

Discussion

In the present study, the low level laser therapy was able to decrease proinflammatory cytokines and apoptosis as well as improves the repair response through increasing cardiotrophin-1. These effects could diminish the progressive changes that occur after MI and also adverse myocardial remodeling. These findings indicate that the laser can exert cardioprotective effect by modulating the progression of adverse remodeling after MI.

After the first hours of myocardial infarction an inflammatory response with marked leukocyte infiltration and release of inflammatory cytokines including TNF-α, IL-6 and IL-1β occurs [Frangogiannis et al 2002; Nian M 2004; Nah DY 2009].

The increase of cytokines have crucial role in myocardial fibrosis and also in the pathological progression in ventricular remodeling by inducing the inflammatory response via NF-kB. (Li Y; Frangogiannis et al 2002].

Recently, IL-6 is associated with increased myocardial hypertrophy [Frangogiannis et al 2002] and in the development of heart failure [Deten et al, Tao Z et al 2011].

Our data are the first to show that low level laser therapy was capable to modulate this inflammatory response and contribute to recovery after MI and may reduce the effects of heart failure. This observation may be seen by reducing the protein expression levels of TNF-α, IL-6, CINC-1 after treatment suggesting that the LLLT could exert anti-inflammatory effects in myocardial ischemia generated by MI. Although we did not observe significance in IL-10, TNF-α/IL-10 ratio showed to be significantly lower in MI + Laser, demonstrating the anti-inflammatory profile of LLLT in myocardial ischemia.

Our results are in agreement with the Deten et al which observed that the increasing of expression of IL-6 in the infarcted myocardium might be involved in early compensatory hypertrophy after-IM and this effect could be attenuated by the laser.

The anti-inflammatory effects of LLLT through decreasing the expression of inflammatory mediators is reported by several authors [14-17] and corroborates our findings.

In the acute phase of MI, the increase of inflammatory cytokines such as TNF-α and IL-1β stimulates apoptosis [Frangogiannis et al 2002; Pengsheng et al 2013]. And interestingly, we observed in our study the decreasing TNF-α and the increasing of blc-2 indicating that the LLLT could exert anti-apoptotic effects promoting the survival of viable cardiomyocytes in the MI area.

The knowledge of the inflammatory response in ischemic myocardium and the role of cytokines after MI would allow us to understand the healing and remodeling after MI. Therefore the immunomodulatory therapies like low level laser therapy could be promising for attenuating inflammatory response, improve cardiac remodeling and possibly improve cardiac function after MI.

References

1. Ertl G, Frantz S. Healing after myocardial infarction. Cardiovasc Res.2005; 66(1): 22– 32.

2. Sun Y, Weber KT. Infarct scar: a dynamic tissue. Cardiovasc Res.2000;46(2):250–6.

3. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovascular Research.2002; 53:31–47.

4. Moisés VA, Ferreira RL, Nozawa E, Kanashiro RM, Campos Fº O, Andrade JLd, Carvalho ACC, Tucci PJF. Structural and functional characteristics of rat hearts with and without myocardial infarct. Initial experience with Doppler echocardiography. Arq Bras Cardiol. 2000;75:131-6.

5. Ruparelia N, Digby JE, Jefferson A, Medway DJ, Neubauer S, Lygate CA, Choudhury RP. Myocardial infarction causes inflammation and leukocyte recruitment at remote sites in the myocardium and the renal glomerulus. Inflamm Res 2013;62(5):515-25.

6. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med. 2007 Nov 26;204(12):3037-47.

7. Nian M, Lee P, Khaper N, Liu P. Inflammatory Cytokines and Postmyocardial Infarction Remodeling. Circ Res. 2004;94:1543-1553.

8. Nah DY, Rhee MY. Korean Circ J. The inflammatory response and cardiac repair after myocardial infarction. 2009. Oct;39(10):393-8.

9. Hirota H, Yoshida K, Kishimoto T, Taga T. Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. Proc Natl Acad Sci U S A. 1995 May 23;92(11):4862-6.

10. Biasucci LM, Liuzzo G, Fantuzzi G, Caligiuri G, Rebuzzi AG, Ginnetti F, Dinarello CA, Maseri A. Increasing levels of interleukin (IL)-1Ra and IL-6 during the first 2 days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events. Circulation. 1999 Apr 27;99(16):2079-84.

11. Deswal A, Petersen NJ, Feldman AM, Young JB, White BG, Mann DL. Cytokines and cytokine receptors in advanced heart failure: an analysis of thecytokine database from the Vesnarinone trial (VEST). Circulation. 2001 Apr 24;103(16):2055-9.

12. Freed DH, Cunnington RH, Dangerfield AL, Sutton JS, Dixon IM. Emerging evidence for the role of cardiotrophin-1 in cardiac repair in the infarcted heart. Cardiovasc Res. 2005 Mar 1;65(4):782-92.

13. Swynghedauw B. Molecular mechanisms of myocardial remodeling. Physiol. Rev. 1999;79(1):215-62.

14. Lopes-Martins RA, Marcos RL, Leonardo PS, Prianti AC Jr, Muscará MN, Aimbire F, Frigo L, Iversen VV, Bjordal JM. Effect of low-level laser (Ga-Al-As 655 nm) on skeletal muscle fatigue induced by electrical stimulation in rats. J Appl Physiol. 2006;101:283-8.

15. Albertini, R. ; Aimbire, F. ; Villaverde, ; Silva Ja Jr ; Costa, M. S. . Cytokine mRNA Expression Is Decreased in the Subplantar Muscle of Rat Paw Subjected to Carrageenan-Induced Inflammation after Low-Level Laser Therapy. Photomedicine & Laser Surgery. 2008;26:19-24.

16. Bortone F ; Henrique Alves dos Santos ; ALBERTINI, R. ; Pesquero JB ; COSTA, M. S. ; SILVA JA Jr . Low level laser therapy modulates kinin receptors mRNA expression in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation. International Immunopharmacology.2008; 8:206-210.

17. Xavier, Murilo; David, Débora Rodrigues ; de Souza, Renato Aparecido ; Arrieiro, Arthur Nascimento ; Miranda, Humberto ;Santana, Eduardo Tadeu ; Silva, José Antonio ; Salgado, Miguel Angel Castillo ; Aimbire, Flávio ; Albertini, Regiane . Anti-inflammatory effects of low-level light emitting diode therapy onachilles tendinitis in rats. Lasers in Surgery and Medicine (Print).2010;42: 553-558.

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

19. Peplow PV, Chung TY, Baxter GD. Photodynamic modulation of wound healing: a review of human and animal studies. Photomed Laser Surg. 2012 Mar;30(3):118-48.

20. Mester, E., Spiry, T., Szende, B., Tota, J.G. (1971) Effect of laser rays on wound healing. Am. J. Surg. 122: 532–535. doi:10.1016/002-9610.

21. Whittaker P., Patterson M.J. (2000) Ventricular remodeling after acute myocardial infarction: effect of low-intensity laser irradiation. Lasers Surg Med. 2: 29-38.doi: 10.1002/1096-9101.

22. Tuby H, Maltz L, Oron U. Modulations of VEGF and iNOS in the rat heart by low level laser therapy are associated with cardioprotection and enhanced angiogenesis. Lasers Surg Med. 2006;38:682–88.

23. Oron U, Yaakobi T, Oron A, Hayam G, Gepstein L, Wolf T, Rubin O, Ben-Haim SA. Attenuation of infarct size in rats and dogs after myocardial infarction by low-energy laser irradiation. Lasers Surg Med. 2001;28:204–11.

24. Ad N, Oron U. Impact of low level laser irradiation on infarct size in the rat following myocardial infarction. Int J Cardiol.2001; 80(2-3):109–16.

25. Oron, U., Yaakobi, T., Oron, A., Mordechovitz, D., Shofti, R., Hayam. G., Dror, U., Gepstein, L., Wolf, T., Haudenschild, C., Haim, S.B. (2001) Low-energy laser irradiation reduces formation of scar tissue after myocardial infarction in rats and dogs. Circulation 103: 296-301.doi:10.1161/01.CIR.103.2.296.

26.Vieira RP, Silva RA, Oliveira-Junior MC, Greiffo FR, Ligeiro-Oliveira AP, Martins MA, Carvalho CRF. Exercise Deactives Leukocytes in Astha. Int J Sports Med. Doi: http//dx.doi.org/10.1055/5-0033-1358

27. Li Y, Takemura G, Okada H, Miyata S, Maruyama R, et al. (2006) Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure. Cardiovasc Res 71: 684–694.

28. Deten A, Volz HC, Briest W, Zimmer HG. C ardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats. Cardiovascular Research 2002; 55: 329–340

29. Tao Z, Chen B, Tan X, Zhao Y, Wang L, et al. (2011) Coexpression of VEGF and angiopoietin-1 promotes angiogenesis and cardiomyocyte proliferation reduces apoptosis in porcine myocardial infarction (MI) heart. Proc Natl Acad Sci U S A 108: 2064–2069.

30. Chen P, Pang S, Yang N, Meng H, Liu J, Zhou N, Zhang M, Xu Z, Gao W, Chen B, Tao Z, Wang L, Yang Z. Beneficial Effects of Schisandrin B on the Cardiac Function in Mice Model of Myocardial Infarction. PLoS One. 2013 Nov 8;8(11):e79418

4. Discussão

Estudos que utilizam o modelo de infarto do miocárdio no rato visam estudar a fisiopatologia do IM assim como a sua progressão para a insuficiência cardíaca. O modelo experimental do IM no rato é sem dúvida o modelo mais utilizado e suficientemente consolidado na literatura desde os tempos de 1954 por Johns & Olson até os mais recentes trabalhos que caracterizam as repercussões cardíacas e sistêmicas que são semelhantes às dos humanos.

Para escolha da linhagem e do sexo dos animais, utilizamos a linhagem wistar, pois já é bastante frequente o seu uso no nosso grupo e que reproduziu todas as alterações morfológicas e funcionais que estão presentes no pós-IM. Embora, alguns estudos utilizam ratos machos, nós escolhemos ratas fêmeas e conforme observamos na tabela 1 (*artigo 1*), o modelo foi capaz de reproduzir todas as manifestações pós-infarto, além de apresentarem baixa mortalidade após a cirurgia de oclusão coronariana (Antonio EL, 2008).

Nesse sentido, a oclusão da artéria coronária causa isquemia prolongada ocorrendo lesão e morte de células do miocárdio. Todos os infartos têm área central de necrose (infarto), cercada por área potencialmente viável de lesão por hipóxia, que pode ser recuperada ou pode progredir para necrose. A área da lesão, por sua vez, é cercada por tecido isquêmico viável.

Com a perda de miocárdio contrátil, se inicia ciclo vicioso de sobrecarga no ventrículo esquerdo (VE) com deterioração da função de bomba cardíaca e consequente progressão para insuficiência cardíaca (Francis *et al.*, 2001).

Nesse sentido, inúmeras abordagens são desenvolvidas para modular ou atenuar as diversas fases e aspectos que envolvem o remodelamento e disfunção ventricular decorrente do pós-IM.

Frente a esta hipótese, é reconhecido na literatura que o laser de baixa intensidade (LBI) vem sendo empregado na prática clínica e experimental por modular vários processos biológicos tais como: efeito anti-inflamatório (Lopes-Martins *et al.*, 2006; Albertini *et al.,* 2007; Aimbire *et al.,* 2008; Albertini, *et al.*, 2008; Bortone *et al.*, 2008; Lima *et al.*, 2009; Xavier *et al.,* 2010; Silva *et al.,*2011; Pires *et al,* 2011; Mesquita-Ferrari *et al.,* 201), reduzir a dor e acelerar a proliferação celular (Huang *et al.*, 2011; Peplow *et al*., 2012).

Ao longo dos anos, Oron e colaboradores vem medindo esforços para estudar os possíveis efeitos do laser de baixa intensidade no modelo experimental de IM. Seu início foi a partir de 2001 (N Ad; Oron; Oron et al) que o grupo evidenciou que o LBI reduzia o tamanho do infarto assim como o aumento da expressão do fator de crescimento vascular endotelial (VEGF) e oxido nítrico sintase (iNOS) (Tuby H & Oron, 2006). Todos esses efeitos foram associados com os efeitos cardioprotetores do LBI.

Embora Oron et al tenham encontrado resultados positivos com os efeitos da irradiação laser em músculo cardíaco infartado, não havia se estudado até o momento, as vias de sinalização celular que o laser poderia atuar no remodelamento do miocárdio remoto pós-infarto assim como na função cardíaca.

Com as descobertas do efeito do LBI no infarto, avanços foram feitos e o grupo Yang et al (2011) estudaram o efeito do LBI nas citocinas inflamatórias um dia e duas semanas após o IM e foram o primeiro grupo a analisar a função cardíaca através da analise por ecocardiograma. Observaram atenuação do tamanho do infarto no grupo que recebeu a irradiação laser e o aumento do fator de estimulação de colônias de granulócitos / macrófagos (GM-CSF) e redução da *fractalkine* e foram associados com o efeito protetor do LBI. Entretanto não encontraram resultados animadores na função cardíaca.

Recentemente, Yang et al (2013) avaliaram os efeitos do LBI na expressão de radicais livres de oxigênio no remodelamento miocárdico pós IM. Os efeitos do LBI atenuaram o tamanho do infarto, aumentou a espessura da parede ventricular e na atenuação da formação de fibras de colágeno via redução da expressão superóxido dismutase (SOD) e aumento da expressão malondialdeído (MDA). Estes efeitos sugerem a melhora do remodelamento cardíaco após o IM.

Frente a este cenário, o nosso grupo traz nova visão sobre o efeito da irradiação do laser de baixa intensidade tanto no remodelamento, função cardíaca e possível via de sinalização celular que esta terapia poderia modular ou atenuar após o IM.

Seguindo alguns aspectos metodológicos (Oron et al, 2001), a dose do laser utilizada foi de 1,1 Joules e as avaliações ocorreram três dias após IM. Para isso, utilizamos 82 animais wistar, devido a variabilidade do tamanho dos

infartos (Antonio EL et al, 2009) e para a analise da função cardíaca e analise molecular consideramos animais com área de infarto igual ou superior a 37% do ventrículo esquerdo. Esta dimensão dos infartos grandes está associado com severo remodelamento ventricular esquerdo e piora no prognóstico da doença (Francis J et al, 2001; Pfeffer MA et al, 1979; dos Santos A et al, 2012). Deste modo, o que difere nosso trabalho dos demais é que nos valemos da randomização dos infartos para as analises tanto de função cardíaca e moleculares.

Nesse sentido, obtemos resultados positivos no que se refere a redução do tamanho do infarto no grupo MI+Laser (*artigo 1 – figura 1A*) o que corrobora com outros estudos (Oron et al, 2001; Oron et al;2006; Yang et al;2011). E um achado inédito dos nossos resultados é a frequência do tamanho do infarto. Interessantemente, o número de infartos grandes (>37%) foi significantemente menor no grupo que recebeu a terapia laser comparado com o grupo que não recebeu a terapia laser (*artigo 1 – figura 1B*).

Estudos associam a piora da disfunção ventricular após infarto com níveis elevados de citocinas inflamatórias. Esse acentuado processo inflamatório pode trazer consequências adversas no remodelamento cardíaco auxiliando na ruptura cardíaca ou dilatação progredindo para insuficiência cardíaca e óbito (Nian et al,2004). Até mesmo no processo de reparo e fibrose após-IM e correlacionados com remodelamento adverso (Nah et al 2009).

Nossos dados sugerem que o laser de baixa intensidade é capaz de modular o perfil inflamatório após o infarto do miocárdio através da diminuição da expressão gênica de IL-6 e IL-1β (*figura 2 - artigo 1*) e diminuição da expressão proteica de IL-6, TNF-α e CINC-1 (*figura 1A- artigo 2*) na área remota do infarto. Outra descoberta nesse estudo, é que o laser modulou o perfil inflamatório na área remota ao infarto e não alterou o perfil inflamatório na área isquêmica.

Sendo relatado por Ruparelia e colaboradores (2013) que o miocárdio remoto ao infarto também tem sido associado com a ativação de vias pró-inflamatórias e infiltrado leucocitário e esses eventos são cada vez mais importantes no remodelamento ventricular esquerdo pós-IM. Portanto, sugere-se que o laser modula a resposta inflamatória podendo contribuir para a

recuperação pós-infarto e dessa maneira diminuir as consequências do remodelamento cardíaco adverso.

Outra hipótese que a irradiação laser exerceria efeito antiinflamatório no miocárdio infartado é através do sistema calicreína cinina. Conforme demonstramos no artigo 1, o laser diminuiu a expressão do receptor de cinina B1 no qual depende de estímulo inflamatório ao passo que houve aumento da expressão do receptor de cinina B2 que permite a liberação de oxido nítrico (NO) e prostaciclina, exercendo efeito vasodilatador, sugerindo contribuir na recuperação do tecido pós-infarto. Esta observação corrobora com a redução dos níveis de citocinas pró-inflamatórias após a irradiação laser no grupo MI+Laser (*Artigo 1 e artigo 2*).

Este estudo traz nova possibilidade da ação do laser no miocárdio infartado através da participação do sistema renina angiotensina. O aumento da expressão da ECA em modelos experimentais de infarto são relacionados com insuficiência cardíaca. Interessantemente os nossos resultados apresentaram diminuição desses valores no grupo MI+Laser. Ao passo que houve aumento da expressão da ECA2 no grupo irradiado quando comparado com os demais grupos. E o receptor *mas* se comportou similarmente a expressão de ECA2 (*artigo 1*). Deste modo, especulamos a participação do eixo ECA2-Ang 1-7- receptor *mas* na cardioproteção gerada pela LBI pós-infarto em ratos (*artigo 1*).

Os dados da função cardíaca (artigo 1) melhoraram significantemente e isto pode ser, em parte devido ao aumento dos peptídeos vasoativos no grupo MI+laser. A função cardíaca foi analisada em camundongos que apresentaram ausência da ECA2, e os valores de fração de encurtamento do ventrículo esquerdo foi severamente diminuída quando comparados com os *wild-type*. Conforme observamos no nosso estudo (*artigo 1*), a fração de encurtamento do ventrículo esquerdo apresentou-se diminuída no grupo IM, no entanto observamos o aumento dessa variável no grupo IM+Laser.

Outro aspecto que deve ser analisado no pós-infarto é a apoptose, pois esta contribui para o desenvolvimento da insuficiência cardíaca e a perda de cardiomiócitos.

Como os cardiomiócitos possuem baixo potencial para divisão celular, a prevenção da morte celular tem importantes implicações no tratamento das

doenças cardiovasculares. Nesse sentido, a apoptose pode ser ativada através de duas vias, intrínseca e extrínseca. Os membros das proteínas da família Bcl-2 são os principais reguladores da via apoptótica intrínseca e desempenham papel importante na regulação da apoptose de cardiomiócitos (Wang et al, 2014) . E o aumento da expressão de Bcl-2 protege os cardiomiócitos e a morte celular induzida por hipóxia. Este efeito anti-apoptótico foi observado no grupo MI+Laser (*artigo 2*).

Embora os resultados da expressão do Bax não serem significantes, sabe-se que o seu aumento desencadeia serie de eventos pro-apoptóticos (Wang et al, 2014). Nesse sentido, esta evidência sugere que o laser estimula a sobrevivência celular após o infarto, inibindo a apoptose da área viável do coração possivelmente para manter boa função cardíaca após o infarto (*artigo 2*).

Sabe-se que a terapia laser modula o processo inflamatório e isto foi envidenciado por nós conforme bem documentado no texto acima, a partir deste ponto analisamos a possível contribuição desta terapia frente ao processo de cicatrização. Para isto, analisamos a expressão gênica de cardiotrofina 1 na área infartada (*artigo2*).

A cardiotrofina 1 pertence a família da interleucina 6, e no coração está relacionada a cardioproteção. Estudos demonstram esse efeito protetor tando modelo experimental e em humanos (Liao Z et al, 2002; Ghosh S et al; 2000). Freed et al (2003) relatam que a cardiotrofina-1 no pós-IM, pode exercer importante papel na formação da cicatriz e no curso do remodelamento da cicatriz. E nossos resultados, o laser aumentou a expressão gênica de cardiotrofina-1 no grupo IM+Laser sugerindo que o laser estimula a proliferação de miofibroblastos para a geração da cicatriz, com substituição de músculo por tecido conjuntivo devido à deposição de colágeno.

Todos os resultados encontrados nesse trabalho sugerem que o laser foi capaz de modular ou atenuar o remodelamento cardíaco adverso pós-infarto, assim como, reduziu o tamanho do infarto e melhora da função cardíaca. Esses efeitos positivos do laser foram capazes de diminuir a inflamação, sem alterar o perfil inflamatório da área isquêmica do infarto.

Embora muitos estudos demonstrem o efeito benéfico da laserterapia como nova estratégia não farmacológica sugerindo como terapia para a

redução da inflamação em muitos tecidos tanto em humanos quanto em experimentação animal, se faz necessária analise a longo prazo e se esses benefícios da irradiação laser encontrados na fase aguda do infarto se estende para estágios mais tardios da doença isquêmica (progressão da insuficiência cardíaca).

Deste modo, novas estratégias terapêuticas do laser de baixa intensidade devem ser estudadas nesse modelo de oclusão coronária, tais como dose e comprimento de onda cuja principal meta é atingir um efeito terapêutico positivo em humanos.

5. Conclusões

1. O modelo experimental de oclusão coronária em ratos reproduziu as alterações funcionais, aumentou cintocinas teciduais (IL-6, TNF-alpha e IL-1β), somado ao aumento de fatores apoptóticos assim como enzima conversora de angiotensina.
2. O laser de baixa intensidade diminuiu a resposta inflamatória através das citocinas inflamatórias e notamos também que o receptor de cininas B1 apresentou-se reduzidos, sugerindo o efeito antiinflamatório do LBI.
3. A participação da via de geração de Ang 1-7 e do receptor de cininas B2 na cardioproteção gerada pela LBI pós-infarto em ratos podendo ser atribuída com melhora da função cardíaca e diminuição do tamanho do infarto.
4. A terapia laser de baixa intensidade facilitaria o processo de cicatrização.

6. Referências

1. WHO. World Health Organization. Global Atlas on cardiovascular disease prevention and control. 2011.

2. Ministério da Saúde. www.datasus.gov.br. Acesso realizado em 08 de junho de 2013.

3. Kannel WB. Incidence and epidemiology of heart failure. Heart Fail Rev. 2000; 5(2):167-73.

4. Lee WC, Chavez YE, Baker T, Luce BR. Economic burden of heart failure: A summary of recent literature. Heart Lung. 2004; 33(6):362-71.

5. Abbate A, Biondi-Zoccai GGL, Agostoni P, Lipinski MJ, Vetrovec GW. Recurrent angina after coronary revascularization: A clinical challenge. Eur Heart J. 2007; 28(9):1057-65.

6. Fishbein M, Maclean D, Maroko P. Experimental myocardial infarction in the rat: Qualitative and quantitative changes during pathologic evolution. Am J Pathol. 1978; 90(1):57-70.

7. Pfeffer M, Pfeffer J, Fishbein M, Fletcher P, Spadaro J, Kloner R, Braunwald E. Myocardial infarct size and ventricular function in rats. Circ Res. 1979; 44(4):503-12.

8. Francis J, Weiss RM, Wei SG, Johnson AK, Felder RB. Progression of heart failure aftermyocardial infarction in the rat. Am J Physiol Regul Integr Comp Physiol. 2001;281(5):R1734-45.

9. Hasenfuss G. Animal models of human cardiovascular disease, heart failure and hypertrophy. Cardiovasc Res. 1998; 39(1):60-76.

10. Johns T, Olson B. Experimental myocardial infarction I. A method of coronary occlusion in small animals. Ann Surg. 1954; 140(5):675-82.

11.Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res. 2002; 53(1):31-47.

12. Anversa P, Li P, Zhang X, Olivetti G, Capasso JM. Ischaemic myocardial injury and ventricular remodelling. Cardiovasc Res. 1993; 27(2):145-57.

13. Sun Y, Zhang JQ, Zhang J, Lamparter S. Cardiac remodeling by fibrous tissue after infarction in rats. J Lab Clin Med. 2000; 135(4):316-23.

14. Mittmann C, Eschenhagen T, Scholz H. Cellular and molecular aspects of contractile dysfunction in heart failure. Cardiovasc Res. 1998; 39(2):267-75.

15. Swynghedauw B. Molecular mechanisms of myocardial remodeling. Physiol. Rev. 1999;79(1):215-62.

16. Dixon JA, Spinale FG. Myocardial remodeling: cellular and extracellular events and targets. Annu Rev Physiol. 2011;73:47-68.

17. Ertl G, Frantz S. Healing after myocardial infarction. Cardiovasc Res.2005; 66(1): 22– 32.

18. Holmes JW, Borg TK, Covel JW. Structure and mechanics of healing myocardial infarcts. Annu. Rev. Biomed. Eng. 2005;7:223–53.

19. Bonilha AMM, Saraiva RM, Kanashiro RM, Portes LA, Antonio EL, Tucci PJF. A routine electrocardiogram cannot be used to determine the size of myocardial infarction in the rat. Braz J Med Biol Res. 2005; 38:615-9.

20. Zhang Y, Köhler K, Xu J, Lua D, Braun T, Schlitt A, Buerke M, Müller-Werdan U, l Werdan K, Ebelt H. Inhibition of p53 after acute myocardial infarction: Reduction of apoptosis is counteracted by disturbed scar formation and cardiac rupture. J Mol Cell Cardiol. 2011; 50:471–8.

21. Bader M, Ganten D. Update on tissue renin-angiotensin systems. J Mol Med.; 86(6):615-21, 2008.

22. Campagnole-Santos MJ, Diz DI, Santos RA, Khosla MC, Brosnihan KB, Ferrario CM. Cardiovascular effects of angiotensin-(1-7) injected into the dorsal medulla of rats. Am J Physiol. 1989; 257(1 Pt 2):H324-9.

23. Fraga-Silva RA, Costa-Fraga FP, Murça TM, Moraes PL, Martins Lima A, Lautner RQ, Castro CH, Soares CM, Borges CL, Nadu AP, Oliveira ML, Shenoy V, Katovich MJ, Santos RA, Raizada MK, Ferreira AJ. Angiotensin-converting enzyme 2 activation improves endothelial function. Hypertension. 2013; 61(6):1233-8.

24. Arita DY, Cunha TS, Perez JD, Colucci JA, Ronchi FA, Nogueira MD, Arita LS, Aragão DS, Teixeira Vde P, Casarini DE. Overexpression of urinary N-domain ACE in chronic kidney dysfunction in Wistar rats. Clin Exp Hypertens. 2012; 34(6):389-96.

25. Passos-Silva DG, Verano-Braga T, Santos RA. Angiotensin-(1-7): beyond the cardio-renal actions. Clin Sci (Lond). 2013; 124(7):443-56.

26. Davis R, Ribner HS, Keung E, Sonnenblick EH, Lejemtel TH. Treatment of chronic congestive heart failure with captopril, an oral inhibitor of angiotensin-converting enzyme. *N. Engl. J. Med*. 301 (3): 117-121, 1979.

27. Albertini, R. ; Aimbire, F. ; Villaverde, ; Silva Ja Jr ; Costa, M. S. . COX-2 mRNA expression decreases in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation after low level laser therapy. Inflammation Research.2007; 56: 228-229.

28. Aimbire, F.; Albertini, R.; Correa, Jc; Silva Ja Jr ; Costa, M. S. . Low Level Laser Therapy (LLLT) Decreases Pulmonary Microvascular Leakage, Neutrophil Influx and IL-1beta Levels in Airway and Lung from Rat Subjected to LPS-Induced Inflammation.. Inflammation.2008;31:189-197.

29. Albertini, R. ; Aimbire, F. ; Villaverde, ; Silva Ja Jr ; Costa, M. S. . Cytokine mRNA Expression Is Decreased in the Subplantar Muscle of Rat Paw Subjected to Carrageenan-Induced Inflammation after Low-Level Laser Therapy. Photomedicine & Laser Surgery.2008;26:19-24.

30. Bortone F ; Henrique Alves dos Santos ; ALBERTINI, R. ; Pesquero JB ; COSTA, M. S. ; SILVA JA Jr . Low level laser therapy modulates kinin receptors mRNA expression in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation. International Immunopharmacology.2008; 8:206-210.

31. Mafra de Lima, F; Albertini, R. ; Silva Ja Jr ; Silva Ms ; Aimbire, F. . Low level laser therapy (LLLT): attenuation of cholinergic hyperreactivity, beta(2)-adrenergic hyporesponsiveness and TNF-alpha mRNA expression in rat bronchi segments in E. coli lipopolysaccharide-induced airway inflammation by a NF-kappaB dependent mechanism.. Lasers in Surgery and Medicine.2009; 41:68-74.

32. Xavier, Murilo; David, Débora Rodrigues ; de Souza, Renato Aparecido ; Arrieiro, Arthur Nascimento ; Miranda, Humberto ;Santana, Eduardo Tadeu ; Silva, José Antonio ; Salgado, Miguel Angel Castillo ; Aimbire, Flávio ; Albertini, Regiane . Anti-inflammatory effects of low-level light emitting diode therapy on achilles tendinitis in rats. Lasers in Surgery and Medicine (Print).2010;42: 553-558.

33. Silva, MP; Bortone F ; Araujo, TR ; Silva, Marcelo de Paula ; Costa, M. S.; Junior, J. A. S. . Inhibition of carrageenan-induced expression of tissue and plasma prekallikreins mRNA by low level laser therapy in rat paw edema. Revista Brasileira de Fisioterapia (Impresso).2011;15:1-7.

34. Pires, D; Xavier, M ; Araujo, TR; Junior, J. A. S.; Aimbire, F.; Albertini, R. . Low-level laser therapy (LLLT; 780 nm) acts differently on mRNA expression of anti- and pro-inflammatory mediators in an experimental model of collagenase-induced tendinitis in rat. Lasers in Medical Science.2011;26:85-94.

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

36. Mesquita-Ferrari, Raquel Agnelli; Martins, Manoela Domingues; Silva, Tatiana Dia ; Piovesan, Roberto Farin; Junior, J. A. S; Pavesi, Vanessa Christina Santos; Bussadori, Sandra Kalil ; Fernandes, Kristianne Porta Santos. Effects of low-level laser therapy on expression of TNF-? and TGF-? in skeletal muscle during the repair process. Lasers in Medical Science.2011;26:335-340.

37. Peplow PV, Chung TY, Baxter GD. Photodynamic modulation of wound healing: a review of human and animal studies. Photomed Laser Surg. 2012 Mar;30(3):118-48.

38. Tuby H, Maltz L, Oron U. Modulations of VEGF and iNOS in the rat heart by low level laser therapy are associated with cardioprotection and enhanced angiogenesis. Lasers Surg Med. 2006;38:682–88.

39. Ad N, Oron U. Impact of low level laser irradiation on infarct size in the rat following myocardial infarction. Int J Cardiol.2001; 80(2-3):109–16.

40. Oron U, Yaakobi T, Oron A, Hayam G, Gepstein L, Wolf T, Rubin O, Ben-Haim SA. Attenuation of infarct size in rats and dogs after myocardial infarction by low-energy laser irradiation. Lasers Surg Med. 2001;28:204–11.

41. Yang Z, Wu Y, Zhang H, Jin P, Wang W, Hou J, Wei Y, Hu S. Low-level laser irradiation alters cardiac cytokine expression following acute myocar dial infarction:apotential mechanism for laser therapy.Photomed Laser Surg. 20 11 Jun;29(6):391-8.

42. Yang J, Huang Z, Zhou Y, Sai S, Zhu F, Lv R, Fa X. Effect of low-level laser irradiation on oxygen free radicals and ventricular remodeling in the infarcted rat heart. Photomed Laser Surg. 2013 Sep;31(9):447-52