UNIVERSIDADE NOVE DE JULHO
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EFEITO DO LASER DE BAIXA POTÊNCIA SOBRE CÉLULAS MUSCULARES C2C12 SUBMETIDAS À LESÃO POR VENENO DA SERPENTE *BOTHROPS JARARACUSSU.*

CAMILA APARECIDA ALVES DA SILVA

São Paulo - SP
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Dissertação apresentada à Universidade Nove de Julho, para obtenção do título de Mestre em Ciências da Reabilitação.

Orientadora: Profa. Dra. Stella Regina Zamuner.

Coorientadora: Profa. Dra. Raquel Agnelli Mesquita Ferrari.

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RESUMO

O veneno das serpentes do gênero *Bothrops*, induz uma intensa reação inflamatória local podendo evoluir para necrose tecidual. A soroterapia apresenta eficácia em neutralizar os efeitos sistêmicos, porém sua ação não se estende as manifestações locais. O laser de baixa potência (LBP) é usado em situações de lesão muscular, pois apresenta efeitos biológicos, tais como analgésicos, antiinflamatórios e cicatrizantes. O objetivo deste trabalho foi analisar o efeito do LBP em células musculares C2C12 submetidas à lesão por veneno da serpente *Bothrops jararacussu* quanto a: viabilidade, descolamento celular e liberação das enzimas CK e LDH. As células receberam o veneno (12.5 µg/mL) e foram imediatamente irradiadas com LBP Índio Gálio Alumínio Fósforo e Arseneto de Gálio Alumínio, nos comprimentos de onda (λ) 685nm e 830nm, densidade de energia 4J/cm^2, potência de 100mW, energia total 1.3J, tempo de aplicação de 13 segundos por ponto e as células foram incubadas por 15, 30 e 60 minutos.Os resultados demonstraram que o veneno afetou a viabilidade e a integridade das células de forma dose-dependente, sendo escolhida a concentração 12.5 µg/mL para a realização dos experimentos com o LBP. O LBP causou aumento significativo na viabilidade celular, nos tempos analisados no λ 830 nm, entretanto o LBP no λ 685 nm foi efetivo somente nos tempos de 15 e 30 min. O LBP diminuiu a liberação de CK e LDH em todos os tempos analisados e com os dois λ utilizados. No entanto, o LBP não foi capaz de proteger as células contra o descolamento celular causado pelo veneno. Desta forma, verificou-se que o LBP foi capaz de proteger as células musculares C2C12 contra o potente efeito miotóxico do veneno *B. jararacussu* e que esta proteção está relacionada ao efeito protetor da membrana celular. Ainda, os resultados obtidos sugerem que o LBP pode ser considerado uma ferramenta terapêutica eficaz em pacientes picados por serpentes.

Palavras-chave: células musculares, mionecrose, laser, viabilidade e diferenciação celular

ABSTRACT

The venom of snakes of the genus *Bothrops*, induces an intense inflammatory reaction that leads to develop local tissue necrosis. The serum therapy is efficacious in neutralizing the systemic effects, but its action does not extend the local manifestations. The low power laser (LPB) is used in situations of muscles damage, due to its biological effects, such as analgesics, anti-inflammatory and healing. Thus, the objective of this study was to analyse the effect of LPB in C2C12 muscle cells subjected to injury by *Bothrops jararacussu* venom by: cell viability, monolayer integrity and release of CK and LDH enzymes. The cells received the venom (12.5 µl/mL) and were irradiated immediately with LBP Aluminum Gallium Indium phosphorus and Aluminium gallium arsenide, wavelength (λ) 685 nm and 830 nm, energy density of 4Jcm^2, potency of 100 mW, total energy 1.3 J, exposure time of 13 sec point and the cells were incubated for 15, 30 and 60 minutes. The results showed that the venom affected the viability and integrity of muscle cells in a dose-dependent manner, being chosen the dose of 12.5 µl/mL for the realization of experiments with the LBP. The LBP caused a significantly increased in cell viability at all times analyzed at λ 830 nm, however the LBP in the λ at 685 nm was effective only at 15 and 30 min. The LBP decreased the release of CK and LDH in all time analyzed and with two wavelengths used. However, the LBP was unable to protect cells against cell detachment caused by venom. Thus, this study has shown that the LBP was able to protect the C2C12 muscle cells against the myotoxic effect of *B. jararacussu* venom and this protection is related to the protective effect of cell membrane. Furthermore, the results obtained suggest that the LBP can be considered a therapeutic tool in patients bitten by bothropic snakes.

Key words: muscle cells, myonecrosis, laser, viability, and cell differentiation.

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LISTA DE ABREVIATURAS E SÍMBOLOS

AsGa – Arsento de Gálio

ATP – Adenosina trifosfato

BThtx I – Bothropstoxina I

BThtx II – Bothropstoxina II

CK – Creatina quinase

DMEM – Meio Eagle Modificado por Dulbecco

DNA – Ácido desoxirribonucleico

HeNe – Hélio Neônio

InGaAlP – Índio Gálio Alumínio Fósforo

J/cm^2 – Joules por centímetro ao quadrado

LBP – Laser de baixa potência

mL – mililitros

MEF2 – Fator estimulante miócito 2

MHC – Miosina de cadeia pesada

MRFs – Fatores de regulação miogênica

MTT - (3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue)

nm – nanômetros

PLA2 – Fosfolipases A2

rpm – Rotações por minuto

SFB – Soro fetal bovino

TLBP – Terapia Laser de Baixa Potência

VBj – Veneno da serpente *B. jararacussu*

µL – microlitros

λ – Comprimento de onda

1. CONTEXTUALIZAÇÃO

Os acidentes causados por serpentes peçonhentas constituem ainda, um problema de Saúde Pública em regiões tropicais do mundo. As serpentes responsáveis pelo maior número de acidentes ofídicos na América Latina pertencem ao gênero *Bothrops*, da família *Viperidae*. No Brasil, aproximadamente 90% dos acidentes ofídicos são provocados por serpentes desse gênero, sendo também consideradas as mais agressivas (BARRAVIERA, 1993, MINISTÉRIO DA SAÚDE 2009). Os acidentes causados pelas serpentes botrópicas não apresentam alta letalidade (0,31 %), porém devido à alta incidência, são consideradas de grande importância epidemiológica no país (BRASIL. MINISTÉRIO DA SAÚDE, 2001).

O envenenamento por serpentes do gênero *Bothrops* leva a manifestações sistêmicas caracterizadas por náuseas, vômitos, sudorese, hemorragia, hipotensão arterial, insuficiência renal e raramente choque, além de manifestações locais como dor, edema de instalação precoce e caráter progressivo, esquimoses, hemorragia, hipóxia, normalmente acompanhada por necrose tecidual (DOIN-SILVA *et al.,* 2009; MILANI *et al.,* 1997; CARNEIRO *et al.,* 2002).

A mionecrose local é uma consequência comum nos envenenamentos causados pelas serpentes do gênero *Bothrops*. A literatura indica que a mionecrose é causada por uma família de proteínas denominadas miotoxinas, componentes dos venenos botrópicos, as quais possuem características de fosfolipases A2 (PLA2) e atuam diretamente sobre a membrana da célula muscular, por se ligarem e alterarem a membrana plasmática (GUTIÉRREZ *et al.,* 1984; BRENES *et al.,* 1987). A miotoxicidade pode, ainda, ser consequência da isquemia dos vasos da microcirculação e de artérias intramusculares (GUTIÉRREZ, 1984; QUEIROZ & PETTA, 1984).

Nas situações de lesão muscular, o processo de formação do novo tecido requer que células precursoras mononucleadas quiescentes (células satélites) sejam ativadas, tornam-se mioblastos, se proliferam e se diferenciam em miotubos para se fundir às fibras lesadas ou formar uma nova fibra muscular funcional, para assim reparar as miofibrilas danificadas (BISCHOFF &

HEINTZ, 1994; DOMINOV *et al.,* 1998; GOUNDS, *et.al.,* 2002; SAKUMA *et al*.,2003; KUMAR *et al.,*2005; DOGRA *et al.,* 2007).

O reparo muscular é similar a miogênese, um processo regulamentado por fatores de transcrição miogênico básico, tais como MyoD, Myf5, miogenina e MRF4 chamados de fatores regulatórios miogênicos (MRFs) e fator estimulante miócito 2 (MEF2) que regulam a expressão de vários genes músculo-específicos, como a miosina de cadeia pesada (MHC) e creatina quinase (LASSAR *et al.,* 1994; OLSON *et al.,* 1995; YEN *et al.,* 2010).

Durante esse processo as células precursoras miogênicas expressam esses MRFs que estão envolvidos na proliferação e diferenciação. Os MRFs primários, MyoD e Myf5, são necessários para a ativação e proliferação das células satélites, enquanto os MRFs secundários, como a miogenina e MRF4 são necessários para a diferenciação em miotubos (CHARGÈ & RUDNICKI, 2004; RELAIX *et al*., 2006; ZAMMIT *et al*., 2006).

A célula muscular de linhagem C2C12 é um subclone da linhagem de células musculares C2, isoladas de células-satélites de camundongos (YAFFE & SAXEL, 1997). Essas células são consideradas um bom modelo para o estudo do crescimento e diferenciação celular, pois exibem a maioria das características de células musculares normais (LEE *et al.,* 2005; BARON *et al.,* 2000; GEORGES *et al.,* 2003). Além disso, o uso de mioblastos/miotúbulos de músculo esquelético, como alvos para venenos/toxinas da serpentes, tem sido sugerido como um modelo in vitro viável para estudar o mecanismo miotóxico(s), uma vez que ele se correlaciona com a miotoxicidade do veneno *in vivo* (LOMONTE *et al.,* 1999).

1.1 *Bothrops jararacussu*

A serpente *B. jararacussu* está distribuída na América do Sul, habitando a mata Atlântica. Essa serpente causa 0,8 a 10% dos acidentes ofídicos registrados no Brasil (MILANI *et al.,* 1997). O envenenamento causado por esta serpente tem grande mortalidade quando comparada com outras serpentes do mesmo gênero (SANCHEZ *et al.,* 1992), sendo que o grande nível de letalidade está atribuída aos 25 % de miotoxinas encontrada no veneno (HOMSI-BRANDERBURG *et al.,* 1988). Os estudos experimentais mostram que o

veneno dessa serpente causa necrose muscular a partir das miotoxinas encontradas no veneno que atuam diretamente sobre a membrana da célula muscular, causando dano tecidual proeminente (GUTIÉRREZ & LOMONTE, 1995).

1.2 Soroterapia

Atualmente, o tratamento preconizado para o acidente botrópico é a soroterapia com antiveneno botrópico poliespecífico. Devido à eficiência dos antivenenos, os coeficientes de letalidade decorrentes desses acidentes, têm revelado tendência decrescente ao longo do tempo. Os estudos experimentais têm sugerido que existe uma significante, embora parcial, neutralização da hemorragia, edema e mionecrose apenas quando o antiveneno é administrado rapidamente após o envenenamento (BATTELINO *et al.*, 1988).

1.3 Terapia com laser de baixa potência

A palavra *laser* é uma sigla que corresponde "*Light* A*mplification by Stimulated Emission of Radiation*, a qual significa "Amplificação da Luz por Emissão Estimulada de Radiação" (MALUF *et al.,* 2006; UCHOA *et al.,* 2010). A ação do laser consiste na absorção da luz pelos tecidos, resultando em modificações no metabolismo celular. Quando o laser é aplicado nos tecidos a luz é absorvida por fotorreceptores localizados nas células, sendo capaz de modular as reações bioquímicas específicas dentro da célula e estimular uma série de reações em cadeia mitocondrial, resultando em síntese de ATP (DORTBUDAK, 2000; STEIN *et al.,* 2005; RENNÓ *et al.,* 2011).

A terapia a laser de baixa potência (LBP) é considerada como um recurso bioestimulante em tecidos, por meio de seus efeitos biológicos, tais como analgésicos, antiinflamatórios e cicatrizantes (UCHOA *et al.,* 2010).

A irradiação do LBP estimula a proliferação de células satélites musculares, a angiogênese e expressão de fatores de crescimento, desempenhando assim uma função importante na regeneração muscular (NAKANO *et al.,* 2009). Além disso, o uso do LBP modula vários processos em diferentes sistemas biológicos, assim como modula de forma complexa as respostas celulares (NAKANO *et al.,* 2009; PETRI *et al.,* 2010).

1.4 Laser de baixa potência e acidentes ofídicos

Até o momento, poucos trabalhos foram publicados utilizando o LBP para a avaliação do efeito local causado por veneno ofídico. Dourado, *et al.,* (2011), avaliaram ação do LBP HeNe (λ 632,8 nm) e AsGa (λ 904 nm), densidade de energia 4 J/cm^2 no músculo gastrocnêmio submetido à lesão por veneno da serpente *Bothrops moojeni* e verificaram um aumento na angiogênese, diminuição de neutrófilos e aumento na proliferação de células reparadoras havendo a ativação de células satélites.

Em outros estudos *in vivo* demonstrou-se a eficácia do laser na resposta inflamatória local e em mionecrose local, havendo diminuição na concentração de creatina quinase e em áreas de mionecrose. Esse achado foi encontrado em estudos prévios realizados em que verificou também uma diminuição na mionecrose local induzidas pelo veneno de *B. jararacussu* e duas miotoxinas isoladas deste veneno a Bothropstoxina I (BthTX I) e Bothropstoxina II (BThtx II), sugerindo que o uso do LBP seja uma abordagem terapêutica local eficaz em casos de envenenamento botrópico. (DOURADO *et al.,* 2003; BARBOSA *et al.,* 2008; DOIN-SILVA *et al*., 2009; BARBOSA *et al.,* 2009; BARBOSA *et al.,* 2010).

1.5 Laser e células em cultura

A literatura demonstra que o LBP em cultura de células causa um aumento no número de células, síntese de DNA e RNA e aumento na taxa de ATP em células-tronco e em outras linhagens celular (ALGHMDI *et al.,* 2011).

No entanto, determinar parâmetros como comprimento de onda, densidade de energia, potência e tempo de aplicação do laser é importante para se obter uma resposta celular adequada ao tratamento. Hawkins & Abrahamse (2006), relataram que a aplicação do LBP (HeNe) utilizando baixas densidade de energia, como dose única de 5,0 J/cm^2 ou duas exposições de 2,5 J/cm^2 apresentou um efeito estimulante na resposta celular de fibroblastos “feridos”, resultando no aumento de migração celular, viabilidade e proliferação celular e atividade de ATP.

1.6 Justificativa

A literatura demonstra que a terapia laser de baixa potência (TLBP) reduz a mionecrose induzida por venenos botrópicos, no entanto o mecanismo para essa proteção não está esclarecido. Pouco se sabe a respeito dos mecanismos envolvidos na resposta intracelular de células musculares de linhagem C2C12 à laserterapia submetidos à lesão por veneno de *B. jararacussu*.

2. OBJETIVOS

2.1 Geral:

Este estudo teve como objetivo geral analisar as ações do laser de baixa potência sobre células musculares C2C12 submetidas à lesão por veneno de *B. jararacussu*.

2.2 Específicos

Através de ensaios *in vitro*, foram avaliados os efeitos da irradiação laser de baixa potência sobre células musculares C2C12, após lesão com o veneno de *B. jararacussu* quanto a:

I- Viabilidade das células.

II- Integridade das monocamadas.

III- Marcadores de lesão celular.

IV- Diferenciação das células.

3. MATERIAL E MÉTODOS

Este estudo foi realizado no laboratório de cultivo celular do curso de Mestrado e Doutorado em Ciências da Reabilitação da Universidade Nove de Julho (UNINOVE).

3.1 Veneno de serpente *B. jararacussu* (VBj):

Foram utilizados venenos extraídos de vários exemplares adultos de serpentes VBj, provenientes do serpentário da Universidade do Vale do Paraíba, fornecidos pelo prof. Dr. José Carlos Cogo. Os venenos foram reunidos em um mesmo tubo, homogeneizados, submetidos a liofilização e mantidos a – 20ºC até o momento de sua utilização.

3.2 Células musculares C2C12:

As células, provenientes da linhagem de mioblastos C2C12, foram gentilmente doadas pelo professor José Ernesto Belizário, do Instituto de Ciências Biomédicas - USP/SP. As células foram cultivadas no meio de cultura de Eagle modificado por Dulbecco (DMEM, Vitrocell, Campinas, SP, Brasil) contendo 10% de soro fetal bovino (SFB), (Cultilab, Campinas, SP, Brasil) e 1% de solução antibiótica-antimicótica (Cultilab).

3.3 Cultivo Celular:

Os mioblastos foram mantidos em estufa (HEPA class 3110, Thermo Electron Corporation, Marietta, OH, EUA) a 37°C, numa atmosfera úmida contendo 5% de CO_2. O monitoramento do crescimento celular foi realizado a cada 24 horas, utilizando microscópio invertido de fase (Eclipse TE 2000U, Nikon, Melville, NY, EUA).

O subcultivo foi realizado quando a monocamada celular se tornou subconfluente para a perpetuação da linhagem celular, sempre em fluxo laminar (Linha 400, Pachane, Piracicaba, SP, Brasil). Para tanto, sobrenadante

foi removido, as células lavadas com tampão PBS 1X (NaCl 140mM; KCl 2,5mM; Na2HPO4 8mM; KH2PO 1,4mM; pH 7,4) e tratadas com solução de tripsina 0,25% durante 3 minutos a 37°C. Após a incubação, foi realizada nova lavagem com meio, centrifugação a 1200 rpm a 10°C por 5 minutos (Centrífuga Excelsa 4-280R, Fanem, São Paulo, SP, Brasil) e posteriormente a ressuspensão em 1ml de meio DMEM. A viabilidade das células foi avaliada por contagem com corante vital azul de Trypan (0,4%) e foram utilizadas nos ensaios as culturas com viabilidade maior que 95%. A seguir, foram feitas diluições necessárias à semeadura das células, em placas de cultura de 96 poços.

3.4 Preparação de monocamadas de células musculares para ensaios com o veneno:

A partir das culturas celulares obtidas como descrito no item 3.3, foram feitas as diluições necessárias para a semeadura das células em placas de 96 poços ou lamínulas de vidro. Assim, $1x10^4$ célula/poço foi semeada em placas de 96 poços e colocadas em estufa numa atmosfera úmida contendo 5% de CO_2, a 37ºC, por 24 horas. Após esse período as células foram incubadas com o veneno, diluídos em meio DMEM, nas concentrações de 1, 6, 12,5, 25 ou 50 µg/mL, ou meio DMEM somente (controle) e incubadas por 15, 30 e 60 minutos.

3.5 Ensaio para a avaliação da viabilidade da monocamada de células musculares:

O ensaio de viabilidade celular foi realizado pelo método MTT. Após cada período de incubação, como descrito no item 3.4 o sobrenadante das culturas foi removido e as células foram lavadas com 100 µL de PBS 1X. Em seguida, adicionado 50 µL de MTT (3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) e incubadas por 3 horas a 37ºC. Terminado o tempo de incubação foi adicionado 100 µL de isopropanol para ressuspender e solubilizar o precipitado. Por fim, foi realizada a leitura da absorbância a 620nm com auxílio de um leitor de Elisa (2020, Anthos, Eugendorf, Áustria).

3.6 Ensaio para a avaliação da integridade da monocamada de células musculares:

Após cada período de incubação, como descrito no item 3.4, os sobrenadantes das culturas foram removidos e as células foram lavadas com 100 µl PBS 1X. Em seguida, foram adicionados 40 µL de cristal violeta (0,5%) em ácido acético (30%) por poço. Decorridos 15 minutos, a placas foram lavada e colocada para secar. A seguir, 100 µL de metanol absoluto (MERCK) foram adicionados em cada poço e a leitura da densidade óptica (D.O.) realizada em leitor de ELISA a 620 nm. A lesão causada foi definida como a porcentagem de diminuição da D.O, observada na monocamada submetida à ação do veneno em estudo, em relação à monocamada controle.

3.7 Irradiação laser de baixa potência

O dispositivo laser utilizado foi Laser da marca DMC® modelo Thera Lase (Fig. 1) e os parâmetros utilizados estão descritos na tabela 1.

Tabela 1. Parâmetros dosimétricos do Laser.

Meio ativo	InGaAlP e AsGaAl
Comprimento de onda	685 nm e 830 nm
Densidade de energia	4 J/cm^2
Energia total	1.3 J
Potência de saída	100 mW
Densidade de potência	0.33 W/cm^2
Área irradiada	0.3 cm^2
Área do feixe	0,028 cm^2
Área do feixe com espaçador	0,28 cm^2
Modo de aplicação	Pontual
Tempo	13 s

As células foram irradiadas imediatamente após a adição do veneno na cultura, e foi aplicada de forma pontual diretamente no poço pela parte inferior da placa. Os experimentos foram realizados em um ambiente com obscuridade parcial para não sofrer interferência da luz externa.

A cultura de mioblastos foi dividida em quatro grupos: Grupo 1: controle, as células não receberam veneno e não foram irradiadas; Grupo 2: células receberam veneno de VBj; Grupo 3: células que receberam VBj e tratamento com laser (λ 685 nm); Grupo 4: células que receberam VBj e tratamento com laser (λ 830 nm), (Fig. 2).

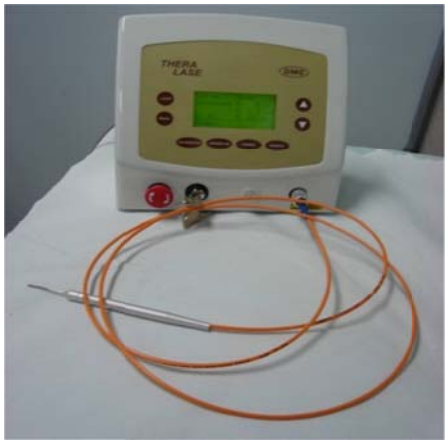

Figura 1: Aparelho Laser de Baixa Potência

B	CONT		VBj		VBj + 685nm		VBj + 830nm	
B								
B	CONT		VBj		VBj + 685nm		VBj + 830nm	
B								
B	CONT		VBj		VBj + 685nm		VBj + 830nm	
B								
B	CONT		VBj		VBj + 685nm		VBj + 830nm	
B								

Figura 2: Desenho da placa de culturas com os respectivos grupos experimentais. Primeira coluna refere-se ao Branco (B); Grupo 1: (CONT) células controle; Grupo 2: (VBj) as células receberam apenas veneno; Grupo 3: (VBj + 685 nm) as células receberam o veneno e foram irradiadas no comprimento de onda 685 nm; Grupo 4: (VBj + 830 nm) as células receberam o veneno e foram irradiadas no comprimento de onda 830 nm.

3.8 Análise do LBP sobre a integridade da monocamada de células musculares após incubação com o veneno de *B. jararacussu*.

As células C2C12 foram plaqueadas $1x10^4$ células poço em placas de 96 poços e incubadas por 24 horas para adesão celular. Após esse período as células receberam o veneno na concentração de 12.5 µg/mL, em meio de cultura (controle) e imediatamente foram irradiadas com laser, em seguida as células foram incubadas por 15, 30 e 60 minutos e o ensaio para a avaliação da integridade da monocamada foi realizado conforme descrito no item 3.6.

3.9 Análise do LBP sobre a citotoxicidade induzida pelo veneno de *B. jararacussu* em células musculares:

3.9.1 Ensaio da viabilidade celular MTT (brometo de 3, 4,5-dimetiltiazol – 2il 2,5- difenil tetrazol).

A viabilidade das células musculares C2C12 foi avaliada pelo método MTT. As células C2C12 foram plaqueadas $1x10^4$ célula/poço em placas de 96 poços e incubadas por 24 horas para adesão celular. Após esse período as células receberam o veneno na concentração de 12.5µg/mL, em meio de cultura (controle) e imediatamente foram irradiadas com laser, em seguida as células foram incubadas por 15, 30 e 60 minutos. A seguir, o ensaio da atividade mitocondrial foi realizado conforme descrito no item 3.5.

3.9.2 Análise da liberação da enzima Creatina Quinase (CK).

A atividade enzimática da enzima CK, presente no sobrenadante das culturas, foi considerada como parâmetro da lesão celular. As células musculares C2C12 foram plaqueadas $1x10^4$ célula/poço em placas de 96 poços e incubadas por 24 horas para adesão celular. Após esse período as células receberam o veneno na concentração de 12.5 µg/mL, em meio de cultura (controle) e imediatamente foram irradiadas com laser, em seguida, as células foram incubadas por 15, 30 e 60 minutos. Após a incubação, os sobrenadantes das culturas de cada grupo foram removidos e armazenados

em freezer – 80ºC. A dosagem de CK foi realizada por meio do kit CK NAC (Larborlab, São Paulo, Brasil), usando método cinético, em espectrofotômetro 340 nm a 37ºC. A atividade da CK foi calculada (unidades/mg de proteína) após a correção para proteína total (YEN *et al.,* 2010).

3.9.3 Análise da liberação da enzima lactato desidrogenase (LDH)

A atividade enzimática da LDH, presente no sobrenadante das culturas, foi considerada como parâmetro da lesão celular. As células C2C12 foram plaqueadas $1x10^4$ célula/poço em placas de 96 poços e incubadas por 24 horas para adesão celular. Após esse período as células receberam o veneno na concentração de 12.5 µg/mL, em meio de cultura (controle) e imediatamente foram irradiadas com laser em seguida as células foram incubadas por 15, 30 e 60 minutos. Em seguida os sobrenadantes das culturas de cada grupo foram removidos e armazenados em freezer – 80ºC, a dosagem de LDH foi realizada por meio do kit LDH Liquiform (Labtest, Minas Gerais,Brasil), usando método cinético, espectrofotômetro 340 nm a 37ºC. Como controle para 100 ou 0% de citotoxicidade as células foram incubadas com de Triton X-100 0.1% ou somente meio sem detergente, respectivamente. Cada amostra foi analisada em quadruplicata, e três experimentos independentes foram realizadas. Os resultados foram expressos pelo decréscimo da D.O., resultante da oxidação do NADH, na presença de piruvato, em relação ao tempo zero.

3.10 Diferenciação celular

Para a realização do ensaio de diferenciação celular as células foram divididas em tubos de ensaios de acordo com cada grupo experimental. As células receberam o veneno (12.5 µg/mL) ou apenas meio de cultura (controle) e centrifugadas por 2 minutos para a formação do preciptado celular e foram irradiadas de forma pontual na parte inferior do tubo. Posteriormente as células foram plaqueadas em lamínulas de 13 mm em placa de 24 poços e incubadas por 15, 30 e 60 minutos. Após esse período o sobrenadante das células de cada grupo experimental foi removido e imediatamente foi adicionando aos mioblastos meio de cultura Eagle modificado por Dulbecco (DMEM, Vitrocell,

Campinas, SP, Brasil), contendo 2 % de soro de cavalo para induzir a diferenciação, sendo incubados em estufa a 37ºC com 5% CO_2 por 4 dias. A diferenciação de mioblastos foi determinada pela análise morfológica de formação de miotubos multinucleados. As células foram analisadas, morfologicamente, por coloração de hematoxilina e eosina (H & E), dando uma boa indicação de progressão miogênica global (YEN *et al.,* 2010).

3.11 Análise Estatística

Para a análise dos dados foram utilizados média, desvio padrão e análise de variância (ANOVA) com auxílio do software “GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, E.U.A.). Nos experimentos de adesão celular e efeito do veneno da serpente *B. jararacussu* na viabilidade e descolamento celular foi utilizado o pós teste Dunnet e a significância estatística aceitável quando $p \leq 0.05$. Para os demais experimentos foram realizados pós teste Tukey e considerada aceitável quando $p \leq 0.05$. Todas as amostras foram feitas em quadruplicatas e três experimentos independentes foram realizados.

4. RESULTADOS

4.1 Artigo Publicado na Revista *Clinical & Experimental Medical Letter,* 53(1-2): 7-10. 2012.

Myotoxic effect of *Bothrops jararacussu* snake venom on C2C12 muscle cells.

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ABSTRACT:

Background: Viperid snakebite envenoming is characterized by prominent local tissue damage, including muscle necrosis. A frequent outcome of such local pathology is deficient skeletal muscle regeneration, which causes muscle dysfunction, muscle loss and fibrosis, thus provoking permanent sequelae that greatly affect the quality of life of patients. The causes of such poor regenerative outcome of skeletal muscle after viperid snakebites are not fully understood. The aim of this study was to analyze the effect of *B. jararacussu* snake venom on the viability and detachment of C2C12 muscle cells.

Material and Methods: C2C12 muscle cell line was used. The cells were grown in culture medium DMEM supplemented with 10% fetal bovine serum, incubated at 37 °C with 5% CO_2 for 24 hours for cell attachment, after that, the cells received the snake venom *B. jararacussu* in the respective concentrations 1, 6, 12.5, 25 and 50 µg/mL and incubated for 15, 30 and 60 minutes. The cell viability and detachment were analyzed by MTT and crystal violet assay, respectively, using the ELISA reader (620nm) and the results were subjected to statistical analysis ($p \leq 0.05$ - ANOVA/ Dunnet).

Conclusions: It is concluded that *Bothrops jararacussu* venom are myotoxic for muscle cells and this effect is dose and time dependent.

Key words: *Bothrops jararacussu*, myonecrosis, muscles cells, cell viability, detachment

Introduction

Accidents caused by venomous snakes constitute a public health problem in the world. Approximately 90% of snakebites in Brazil are caused by *Bothrops* snakes belonging to *Viperidae* family, they are also considered the most aggressive [1]. These accidents are of great medical importance because of its high incidence and severity, and usually are related to individuals that work in rural area [2].

Envenomings induced by Bothrops snakes are characterized by complex pathological and pathophysiological profiles that include prominent local tissue damage, i.e. necrosis, hemorrhage, blistering and edema, and systemic alterations, i.e. bleeding, coagulopathy, cardiovascular shock and renal failure [3]. Among these effects, local tissue damage leading to necrosis is particularly relevant, since it is frequently followed by poor tissue regeneration, with the occurrence of permanent sequelae associated with tissue loss and dysfunction, and their consequent social and psychological implications [4].

Many snakes produce venoms that include myotoxins as major determinants of their pathogenic action; these protein toxins damage the plasma membrane of muscle cells, causing myonecrosis [5]. The venom of the snake *Bothrops jararacussu* causes symptoms similar to other Bothrops species being myonecrosis the most notable local effect caused by this envenomation [6–9].

The knowledge of the venom actions as well as their molecular mechanisms is of importance [10]. It Is still scarce in the literature reporting the toxicity of *B. jararacussu* snake venom crude venom on C2C12 muscle cell line. The aim of this study was to analyze the effect of *B. jararacussu* snake venom on the viability and detachment of C2C12 muscle cells.

Material and Methods

Venom

B. jararacussu venom was supplied by the Serpentarium of the Center of Studies of the Nature at UNIVAP. The venom was lyophilized, kept refrigerated at 48C, and diluted in sterile saline solution (0.9%) immediately before use.

Cell culture

The C2C12 myoblasts cells were used. The cells were grown in culture medium DMEM containing 10% fetal bovine serum and 1% antibiotic-antimycotic and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cell growth monitoring was performed every 24 hours, using the inverted microscope stage (Eclipse TE 2000U, Nikon, Melville, NY, USA).

Cell viability

C2C12 cells were seeded at about 1 x 10^4 cells per well in 96-well plates, after 24 h incubation, the medium was replaced with the same medium containing the venom of *B. jararacussu* in the concentrations of 1, 6, 12.5, 25 e 50 µg/mL. The control group received the same volume of culture media without venom. After incubation for 15, 30 or 60 min, the medium was discarded and cell viability was determined. In brief, after each period of incubation with the venom, the cells were washed with 100 µL of 1X PBS. Then, added 50 µl of MTT (0.5 ug/ml in buffer) (3 - [4,5-dimethylthiazol-2YL] -2.5 diphenyltetrazolium-bromide; Thiazolyl blue-SIGMA) and incubated for 3 hours at 37°C. After the incubation time, were added 100 µl of isopropanol to resuspend and solubilize the precipitate. The absorbance of the supernatant was then measured spectrophotometrically in a ELISA reader at 620 nm.

Cell detachment assay

C2C12 cells were seeded at about 1 x 10^4 cells per well in 96-well plates, after 24 h incubation, the medium was replaced with the same medium containing the venom of *B. jararacussu* in the concentrations of 1, 6, 12.5, 25 e 50 µg/mL, and incubated for 15, 30 or 60 min, the control group received the same volume of culture medium without venom. After each incubation period

with venom, the supernatants of the cultures were removed and the cells washed with 100 µL 1X PBS and then added to 40 µL of crystal violet (0.5%) in acetic acid (30%) per well. After 15 minutes, the plates were washed and placed to dry. Next, 100 µL of absolute methanol (Merck) were added to each well and the optical density (OD) was read on ELISA reader at 620 nm. The injury caused was defined as the percentage decrease of DO observed in the monolayer subjected to the action of venom in relation to unstimulated myoblasts monolayer by the venom.

Statistics analysis

For the analysis of data were used, standard deviations, and analysis of variance (ANOVA). Statistical significance was checked by the Dunnet test and considered acceptable when $p \leq 0.05$.

Results

Effect of *B. jararacussu* snake venom on muscle cells viability.

Exposure of muscle cells to *B. jararacussu* venom, in monolayer culture, significantly decrease cell viability at 12.5, 25 and 50 µg/mL, in a dose-dependent manner in all period of time studied, when compared with control group (Fig. 1).

The dose of 6 µg/mL caused a statistically significant reduction in cell viability at 30 and 60 min (Fig. 1B,C) and a dose of 1 µg/mL had no effect on cell viability (Fig. 1).

Effect of *B. jararacussu* snake venom on muscle cells detachment.

Results showed that there was a statistically significant increase in the percentage of cell detachment in a dose-dependent manner in the concentration of 6, 12.5, 25 and 50 µg/mL compared to the control group, this effect being more pronounced at 25 and 50 µg/mL promoting 100% of cell detachment at 30 and 60 minutes after the venom incubation (Fig. 2).

Discussion

Snakebite envenomation is a major, although neglected, health problem in many parts of the world [5]. In addition to lethality, one of the most serious consequences of these envenomations, particularly in the case of viperid and some elapid snakes, is associated with prominent tissue damage leading to permanent sequelae such as tissue loss and dysfunction, [1, 11].

Myonecrosis is a prominent effect of envenomation induced by *B. jararacussu* snake venom that provokes muscular mass loss with a difficult regeneration [12]. The present study evaluated the toxicity of the venom of *B. jararacussu* through in vitro assays, being established in the literature as one of the first bioassay methods to evaluate the toxicity of substances to various tissues [12, 13], thus providing an alternative assessment of toxicity of snake venom. Our results demonstrated that the crude venom of *B. jararacussu* in the dosages tested showed toxic effects resulting in cell death in a dose-dependent manner, in C2C12 muscle cell line, confirming the effect of myotoxic venom. Our results are in agreement with data showing a cytotoxic activity in myoblasts and especially in myotubes in culture incubated with *B. asper* venom [14, 15]. Also, Collares-Buzato et al., [16] demonstrated a a direct cytotoxic effect of *B. moojeni* snake crude venom on a cultured renal tubule derived cell line.

B. jararacussu venom contain a concentration of 30% of crude venom PLA2 myotoxins [17-21]. *In vivo* study shows that the venom of this snake causes muscle necrosis from this myotoxins that act directly on the muscle cell membrane, causing prominent tissue damage [22]. The literature showas that once bound to muscle cells, the damage induced by myotoxic PLA2s to the plasma membrane might be of two main types: (1) a perturbation in the integrity of the bilayer by a mechanism independent of phospholipid hydrolysis, and (2) a membrane disruption based on enzymatic phospholipid degradation [23].

In our work, after exposure to the *B. jararacussu* crude venom, the C2C12 cells lose their contats with substratum starting with a concentration of 6 µg/mL. Like the majority of the bothropic venoms, *B. jararacussu* venom contains numerous components including metalloproteinases[24] and myotoxic phospholipases A_2[25, 26]. Phospholipases, metalloproteinases of high molecular weight belonging to the metalloproteinase/disintegrin-like/cysteine

rich proteins, and disintegrins are important components of the venom of several *Bothrops* species [26]. These proteins have been proven to display potent proteolytic effects on the extracellular matrix proteins and to interfere with integrin mediated cell functions [27-30]. In this context, a BjussuMP-I a RGD-P-III class hemorrhagic metalloprotease purified from *B. jararacussu* venom [24] may account for the cell-to-matrix detachment phenomenon observed here.

From these investigations, *Bothrops jararacussu* showed miotoxic effect against C2C12 cells. In addition, *B. jararacussu* also caused the detachment of these cells, an action in which myotoxins and hemorragins may participate. Thus we conclude that the venom of *Bothrops jararacussu* venom has toxic effect in C2C12 muscle cell line.

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Figure 1. Effect of *B. jararacussu* venom on cell viability in C2C12 muscle cells. C2C12 muscle cells were seeded and allowed to adhere in 96-well plates for 24 h. After this period the venom was added and incubated with venom in concentrations of 1, 6, 12.5, 25 and 50 µg/mL or medium alone (control) for 15, 30 and 60 min. Cell viability was determined by MTT assay. Each value represents the mean ± SEM of three independent experiments, ANOVA/Dunnet * $p<0.05$ compared with control. ** $P<0.01$ compared with control.

Figure 2. **Effect of *B. jararacussu* venom on detachment of C2C12 cells**. C2C12 muscle cells were seeded and allowed to adhere in 96-well plates for 24 h. After this period the venom was added and incubated with venom in concentrations of 1, 6, 12.5, 25 and 50 µg/mL or medium alone (control) for 15, 30 and 60 min. Cell detchament was determined by crystal violet assay. Each value represents the mean ± SEM of three independent experiments, ANOVA/Dunnet, * $p<0.05$ compared with control and ** $p<0.01$ compared with control.

Figure 1

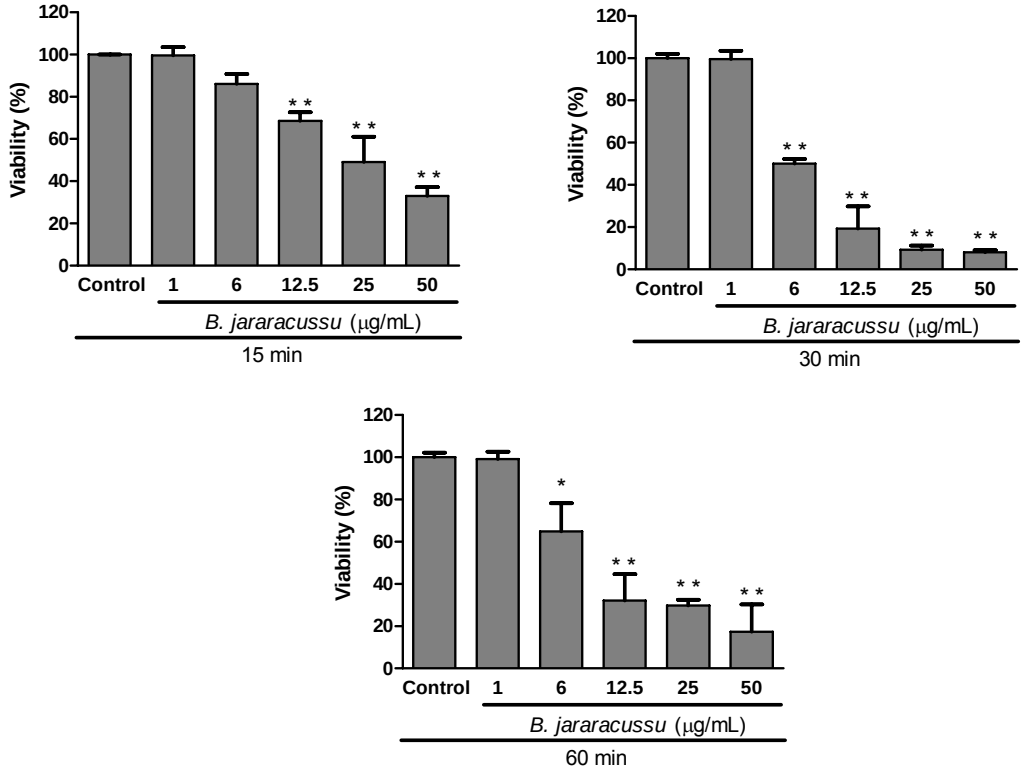
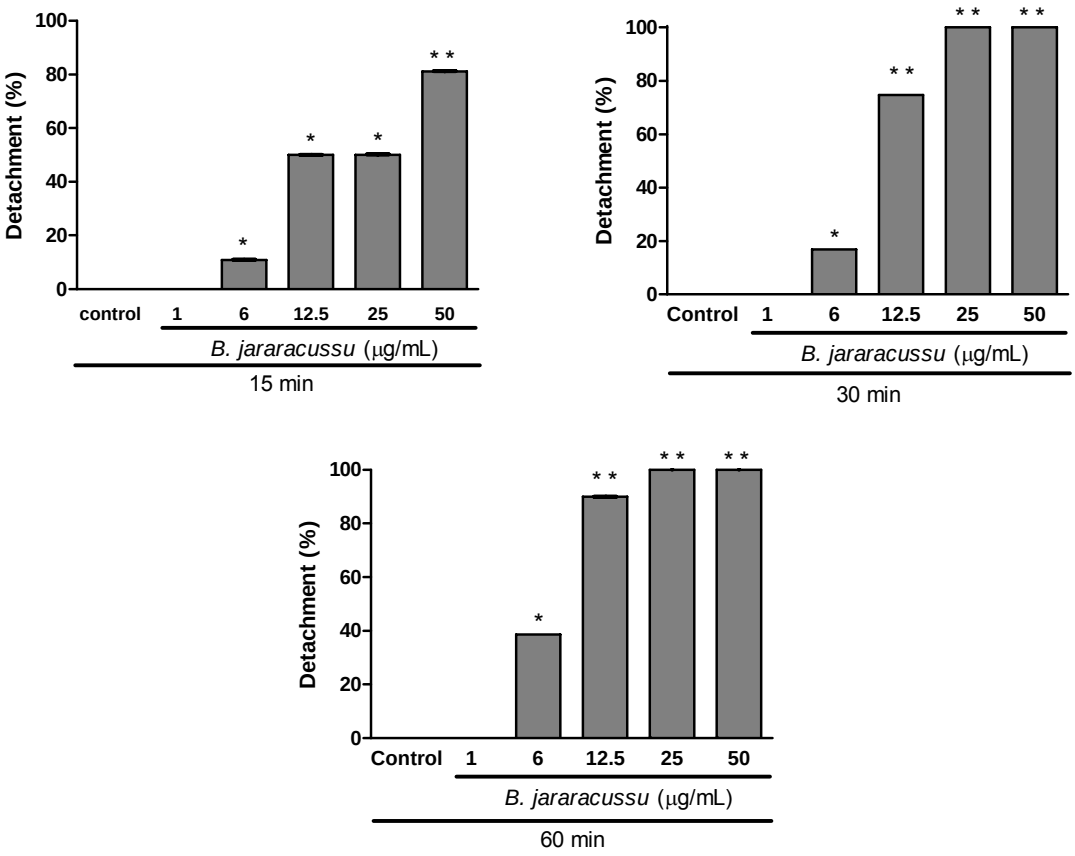

Viability (%)
120
100
80
60
40
20
0
Control
1
6
12.5
25
50
B. jararacussu (μg/mL)
15 min
30 min
60 min
* *
*

Figure 2


Detachment (%)
100
80
60
40
20
0
control
1
6
12.5
25
50
B. jararacussu (μg/mL)
15 min
Control
30 min
60 min
*
* *

4.2 Artigo Submetido à Revista *Toxicon.*

Effect of low level laser therapy (LLLT) on *Bothrops jararacussu* venom-induced myotoxicityin muscle cells.

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Abstract

Given the serious local pathophysiological effects caused by *Bothrops* snake venom mainly induced by muscle degeneration and also by the poor neutralization by the antivenom, this in vitro study aimed to analyze the effect of low level laser therapy against myotoxicity induced by *B.jararacussu* venom on myoblast in culture. The LLLT was able to increase cell viability by wavelengths 685 and 830 nm in a dose at 4 Jcm^2. Furthermore, there was a decreased of the release of the enzymes creatine kinase and lactate dehydrogenase in both wavelengths used and in all periods of time analyzed. LLLT did not show any effect on cell detachment with these laser parameters towards the myoblast cell line. The data reported herein suggest that the LLLT has cytoprotection against bothrops snake venom by protecting the cell membrane.

Key words: snake venom;myonecrosis; low level lase therapy; cell viability;myoblast.

1. INTRODUCTION

Acute skeletal muscle injury is a common manifestation in envenomations caused by *Bothrops jararacussu* snake venom leading to necrosis and loss of muscle mass and represents the main sequela caused by this snake (Milani et al., 1997; da Silva et al., 2003; Santo neto et al., 2004; Vomero et al., 2009). This skeletal muscle injury is due to a large amount of myotoxins present in this venom, which represents about 30% of the crude venom (Rodrigues-Simioni et al., 1983; Homsi-Brandenburgo et al., 1988;). This venom component has phospholipase A2 (PLA2) structure and act directly on the muscle cell membrane by binding and altering the plasma membrane (Gutiérrez et al., 1984; Brenes et al., 1987).

The recommended treatment for Bothrops snakebites accidents is the serum therapy that is efficient to minimize the systemic effects when administered rapidly after the bite, in contrast, serum therapy is not effective in neutralizing the local effects and consequent disabilities caused by Botropic venom (Cardoso et al., 1993; Zamuner et al., 2004). Thus, the searches for therapies that prevent or even counteract the local effects caused by bothropic venom are of great importance.

It has been described that irradiation of cells at certain wavelengths can trigger specific biochemical reactions as well as alter cellular metabolism (AlGhamdi et al., 2012). Additionally, laser irradiation has the potential to stimulate the proliferation and migration of cell types that are essential for reepithelialization, angiogenesis, and granulation tissue formation and could be used to stimulate wound repair (Peplow et al., 2010). In this way, low-level laser therapy (LLLT) has been shown to induce biological activities associated to

tissue recovery (Nakano et al., 2009). This photobiomodulation has been extensively applied in the treatment of skeletal muscle regeneration (Shefer et al., 2003) wound healing (Hawkins et al., 2005) and skin wound care (de Araujo et al., 2007). In this regard, previous study from our group showed that LLLT causes reduction of myonecrosis (Barbosa et al., 2009) and local inflammation (Barbosa et al., 2008) caused by *Bothrops jararacussu* venom. Furthermore, the reduction of myonecrosis caused *Bothrops moojeni* venom has been also shown after LLLT (Dourado et al., 2003). However, the mechanism involved in LLLT protection in muscle cell after bothropic venoms injection has not yet been determined.

Investigate the actions of the venom snakes and their molecular mechanisms are of great importance, as well as, the establishment of an effective therapeutic resource to minimize the local venom-induced myonecrosis. Thus, in the current study we investigated some mechanisms involved in the ability of LLLT to protect the muscle cells against *Bothrops jararacussu* venom.

2. MATERIAL AND METHODS

2.1*Bothrops jararacussu* venom

B. jararacussu venom was supplied from the Center of Studies of the Nature at UNIVAP. The venom was lyophilized and kept under refrigeration at 4°C, being diluted in culture medium immediately before use.

2.2 Cell Culture

The murine skeletal muscle C2C12 cell line was used as the venom target. C2C12 cells were maintained at subconfluent levels in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were maintained at subconfluence densities and passage every two days. C2C12 muscle cells were plated $1x10^4$ cell / well in 96 well plates and incubated for 24 hours. After this period the cells were incubated with venom concentration of 12.5 µg/mL in culture medium and the cells were immediately irradiated with laser, then the cells were incubated for 15, 30 and 60 minutes. The venom dose was chosen on the basis of previous study from our group which showed that a dose of 12.5 µg/mL decrease 50-60 % cell viability in the period of 15 to 60 min (Silva et al., 2012).

2.3 Laser irradiation

A low level laser, Thera lase D.M.C. (São Carlos, SP, Brazil), operating continuous wave either in 685 nm or 830 nm wavelengths was used through the whole experiment to irradiate the cells. The laser parameters for both

wavelengths were 100 mW of power, 13 sec irradiation time with spacer beam irradiated area of 0.28 cm^2 which corresponded to a laser dose of 4 J/cm^2. The optical power of the laser was calibrated using a Newport multifunction optical meter (model 1835C, Newport Corp., Irvine, CA). That laser dose, low enough to avoid any thermal effect, was chosen on the basis of studies reported in the literature that had shown a beneficial effect of the low-level laser in cultured cells (Huang et al., 2009). Cells were irradiated immediately upon addition of the venom in the culture and were applied directly into the well from the bottom plate. The experiments were conducted in an environment with partial obscurity to not suffer interference from external light.

2.4. Irradiated venom

The purpose of this experiment was to clarify if the laser irradiation can modify the biological activity of the venom. For that, the venom of *B. jararacussu* was diluted and irradiated before the incubation with C2C12 cells, using the same laser parameters.

2.5 Experimental groups

The experimental groups were, as follows:

Control: Cells grown in fresh medium and non-irradiated.

Venom: cells incubated with venom (12.5 µg/mL).

Venom + 685 nm: cells incubated with venom and irradiate with 685 nm, 4 J/cm²

Venom + 830 nm: cells incubated with venom and irradiate with 830 nm, 4 J/cm²

iVenom + 685 nm: cells incubated with irradiate venom with 685 nm, 4 J/cm²

iVenom + 830 nm: cells incubated with irradiate venom with 830 nm, 4 J/cm²

2.6 Cell viability assay

The analysis of cell viability was performed by 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After incubation with venom for 15, 30 and 60 min, the cells were washed with 100 µL of PBS and MTT was added to cell culture to a final 50 µl and the cells were incubated for 3 hours at 37°C. After the incubation time, 100 µl of isopropanol was added to each well to dissolve the formazan crystals. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 620 nm.

2.7 Cytotoxicity assay

After incubation times (15, 30 and 60 min), the supernatants collected. Lactate dehydrogenase (LDH) activity was determined in 50 µl of supernatant using a commercial colorimetric assay as described by the manufacturer (Labtest, Minas Gerais, Brazil). As controls for 100 and 0% cytotoxicity, cells were incubated with 0.1% Triton X-100 containing medium, or medium without detergent, respectively. Each sample was assayed in triplicate wells, in at least three independent experiments.

2.8 Creatine kinase (CK) activity

After incubation times (15, 30 and 60 min), the supernatants collected. Creatine kinase (CK) activity was determined in 50 µl of supernatant using a commercial colorimetric assay, kit CK NAC, as described by the manufacturer

(Larborlab, São Paulo, Brazil). Each sample was assayed in triplicate wells, in at least three independent experiments. The activity of CK was calculated.

2.9 Cell detachment

After incubation with venom (15, 30 and 60 min), the culture supernatants were removed and the monolayers washed with 100 µl of PBS and then added 40 µl of Crystal Violet (0.5%) in acetic acid (30%) per well. After 15 minutes, the plates were washed and laid to dry. Next, 100 µl of absolute methanol (Merck) were added to each well and reading the optical density (OD) was read in ELISA reader at 620 nm. The cell detachment caused was defined as the percentage decrease of DO observed in the monolayer subjected to the action of venom in relation to unstimulated myoblasts.

2.10 Statistical analysis

The statistical significance among the control and venom-treated groups was determined by one-way analysis of variance (ANOVA) followed by the Tukey test. A value of $P<0.05$ indicated statistical significance.

3. RESULTS

3.1 Effect of LLLT on viability

To investigate the mechanism of LLLT on the reduction of myonecrosis observed in the muscle after injection of botropic venom in vivo (Ana e Dourado), cultures of differentiated C2C12 cells (myotubes) were incubated with *B. jararacussu* venom (12.5 µg/ml) and immediately irradiate with low level laser for 13 sec. The results show that LLLT increases cell viability by 90 and 100% of C2C12 myoblasts at wavelengths of 685 nm and 830 nm within 15 min incubation and 86% and 92% at both wavelengths, respectively, during 30 minutes after the addition of the venom (Fig. 1 A and B). However, within 60 minutes, there was an increase in cell viability by 54% only at the wavelength 830 nm compared with the control group. (Fig. 1C).

3.2 Effect of LLLT on the CK release

To verify whether the LLLT protection of the myotoxic activity is related to its direct effect on muscle cells viability, we studied the effect of the *B. jararacussu* venom incubation on the myoblast C2C12 cell line on the basis of CK activity. The results showed a reduction in the CK levels in the groups treated with laser (685nm and 830nm) in all periods of time analyzed (Fig. 2 A, B, C), when compared to the group that received only venom.

3.3 Effect of LLLT on LDH release

The loss of membrane integrity after venom incubation and laser treatment was monitored by the analysis of the release in the medium of the cytoplasm enzyme LDH. Cells treated with venom presented increased levels of

LDH activity in their supernatant compared with cell that received only medium. LLLT caused an inhibition of the LDH release, in all period of time studied, and by both wavelengths (Fig. 3 A, B, C).

3.4 Effect of LLLT on cell adhesion

As shown in Fig. 4, incubation with crude venom of *B. jararacussu* at 12.5 µg/mL induced a significant increase in cellular detachment at 15, 30 and 60 min. LLLT did not induce any significant change in cellular detachment of myoblast by both analyzed wavelengths (685 nm and 830 nm) (Fig. 4 A, B and C).

3.5. Effect of irradiated *B.jararacussu* snake venom

To test if the laser is able to induce changes in the venom components, venom was irradiated before the incubation with the cells. The laser was applied in the same parameters used to irradiate C2C12 cells. Our results showed that both irradiated and non-irradiated venom caused the same effect in the cell viability and detachment (Tab 1 and 2).

4. Discussion

Literature indicates that, in humans, the main invalidating effect of *B. jararacussu* venom is the disruption of muscle tissue and consequent myonecrosis (Vomero et al., 2009). Muscle necrosis caused by this venom is mainly attributed to the action of its PLA2s myotoxins (Honsi-Brandeburgo, 1988). The plasma membrane has been proposed to be the primary site of action for the PLA2 myotoxins, which alter its permeability (Rangel et al, 2011). Furthermore, reports shows that muscle necrosis caused by botropic venoms result mostly from the combined action of myotoxic phospholipases A2 (PLA2) and metalloproteinases (SVMPs) (Hernandez et al., 2011) Myotoxic PLA2s disrupt the integrity of the plasma membrane of skeletal muscle fibers (Gutiérrez and Ownby, 2003) while SVMPs are able to degrade extracellular matrix components (Gutiérrez et al., 2005; Moraes and Selistre-Araujo., 2006).

In the present study the myoblast C2C12 cell line was used to examine the direct effect of *B. jararacussu* venom on muscle cell. The use of skeletal muscle myoblasts/myotubes as targets for snake venom/toxins has been suggested as a viable in vitro model to study myotoxic mechanism(s), as it correlates with in vivo myotoxicity (Lomonte et al., 1999). Our results have demonstrated a cytotoxic effect of *B. jararacussu* snake crude venom on a cultured myoblast cell line. These results are in agreement with cytotoxic activities from crude venom and venoms components, such as myotoxins and metalloproteinases, studied on C2C12 cell line (Silva et al., 2012; Bustillo et al., 2012; Rangel et al., 2011; dos Santos et al., 2011; Lomonte et al., 1999). In order to further characterize *B. jararacussu* venom cytotoxicity, we examined plasma membrane integrity by LDH and CK release in the supernatant. Both

test showed a large increase in the release of these enzymes compared to control cells, confirming the venom toxicity.

The literature suggests the LLLT as an alternative therapy for the treatment of bothrops snakebite accidents by its ability to decrease inflammation, hemorrhage and myonecrosis after experimental bothropic envenomation (Dourado et al., 2003; Barbosa et al., 2009; Nadur-Andrade et al., 2012). However, the biological mechanisms related to the local protection by the laser irradiation against botropic venom-induced local effects are not understood. Here, we investigated some mechanisms involved in the ability of LLLT to protect the muscle cells against *B. jararacussu* venom. In order to verify whether the laser is capable of altering the venom components and thus decrease its cytotoxicity, the venom was irradiated using the same laser parameters used to irradiate the C2C12 cell. Irradiate *B. jararacussu* venom showed the same cytotoxicity observed with the non-irradiated venom. This result indicates that the laser irradiation does not modify the venom components but acts in the cellular response. Barbosa *et al.,* (2008), using an in vivo model, have shown that irradiated venom induced the same level of edema as the non-irradiated venom.

In this study the effect of LLLT on the cytotoxicity caused by *B. jararacussu* venom was evaluated using the laser density of 4 J/cm^2 in two wavelengths; a red wavelength at 685 nm and an infrared wavelength at 830 nm, after the venom incubation with C2C12 muscle cells. The laser dose was chosen based on the literature showing, in cultured cells, a beneficial effect of red or infrared laser as low as 3 or 5 J/cm^2 and a larger dose, over 16 J/cm^2 lose the beneficial effect and may even become harmful (Huang et al., 2009).

The results obtained in our study have shown that LLLT in a dose of 4 J/cm^2 reduced considerably the cytotoxicity in muscle cell culture. In agreement with our study, Dourado *et al.,* (2003) and Barbosa *et al.,* (2009) in an in vivo study found that the LLLT in wavelength at 904 and 685 nm and energy density of 4 J/cm^2 and 4.2 J/cm^2, respectively, was able to significantly decrease venom-induced myonecrosis demonstrated by histology and confirming by reduced levels of CK release. The main venom component that causes muscle cell damage is PLA2 myotoxin (Gutiérrez and Ownby, 2003). It has been proposed two main types of damage induced by myotoxic PLA2s on plasma membrane of muscle cells: (1) a perturbation in the integrity of the bilayer by a mechanism independent of phospholipid hydrolysis, and (2) a membrane disruption based on enzymatic phospholipid degradation (Gutierrez and Ownby, 2003). It is possible that, in our experimental model, the laser is protecting the cell membrane by a mechanism independent of phospholipid hydrolysis; this hypothesis is based on the fact that the laser does not protected against cell detachment observed in our study. It is well established that low level light is absorbed by components of the respiratory chain which leads to changes in both mitochondria and the cytoplasm initiating a signaling cascade that promotes cellular cytoprotection. It is possible that the cytoprotection occurred in our study is due an intracellular mechanism induced by LLLT. Therefore, further studies are needed to investigate this hypothesis.

Based on the results of this study, the use of phototherapy with low level laser caused protection on muscle cell, and could be considered a therapeutically tool for snakebites patients. However, there is a need for further

in vitro studies to improve knowledge about the mechanisms involved in laser effect in the local damage caused by bothropic venoms.

The authors declare that there are no conflicts of interest

Acknowledgments

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Legends

Figure 1: Effect of LLLT on the viability of C2C12 cells *after B. jararacussu* venom incubation. C2C12 muscle cells were plated into 96 well plates and incubated for 24 hours for cellular adhesion. After this period the venom was added (12.5 µg/mL) and cells were immediately irradiated with laser (4J/cm^2) in wavelengths of 685 nm and 830 nm or cells received only irradiation with laser and were incubated for 15, 30 and 60 minutes. Cell viability was determined by MTT assay. Each value represents the mean ± SEM of three independent experiments. # $p < 0.05$ compared to control and * $p < 0.05$; ** $p < 0.01$ compared to venom.

Figure 2: Effect of LLLT on the CK activity of C2C12 cells after*B.jararacussu* venom incubation. C2C12 muscle cells were plated into 96 well plates and incubated for 24 hours for cellular adhesion. After this period the venom was added (12.5 µg/mL) and cells were immediately irradiated with laser (4 J/cm^2) in wavelengths of 685 nm and 830 nm or cells received only irradiation with laser and were incubated for 15, 30 and 60 minutes. Determination of CK activity was determined using the kit CK NAC (Laborlab). Each value represents the mean ± SEM of three independent experiments $p < 0.05$ compared to control and * $p < 0.05$; ** $p < 0.01$ compared to venom.

Figure 3: Effect of LLLT on LDH activity of C2C12 cells after *B. jararacussu* venom incubation. C2C12 muscle cells were plated into 96 well plates and incubated for 24 hours for cellular adhesion. After this period the venom was added (12.5 µg/mL) and cells were immediately irradiated with laser

(4J/cm^2) in wavelengths of 685 nm and 830 nm or cells received only irradiation with laser and were incubated for 15, 30 and 60 minutes. Determination of LDH activity was determined using the kit LDH Liquiform (Labtest). Each value represents the mean ± SEM of three independent experiments.# p< 0.05 compared to control and * p< 0.05; ** p< 0.01 compared to venom.

Figure 4: Effect of LLLT on detachment of C2C12 cells after *B. jararacussu* venom incubation. C2C12 muscle cells were plated into 96 well plates and incubated for 24 hours for cellular adhesion. After this period the venom was added (12.5µg/mL) and cells were immediately irradiated with laser (4J/cm2) in wavelengths of 685 nm and 830 nm or cells received only irradiation with laser and were incubated for 15, 30 and 60 minutes. The cellular detachment was determined by the Crystal Violet assay. Each value represents the mean ± SEM of three independent experiments.

Table 1- Effect of LLLT on cell viability of C2C12 cells after irradiated *B. jararacussu* venom incubation.

	Cell viability (%)		
Time (min)	**Venom**	**iVenom 685nm**	**iVenom 830nm**
15	43.2 ± 3.1	48.1 ± 1.6	52.3 ± 2.9
30	52.4 ± 2.3	49.4 ± 1.6	54.3 ± 3.1
60	37.2 ± 4.3	48.1 ± 1.3	50.8 ± 1.9

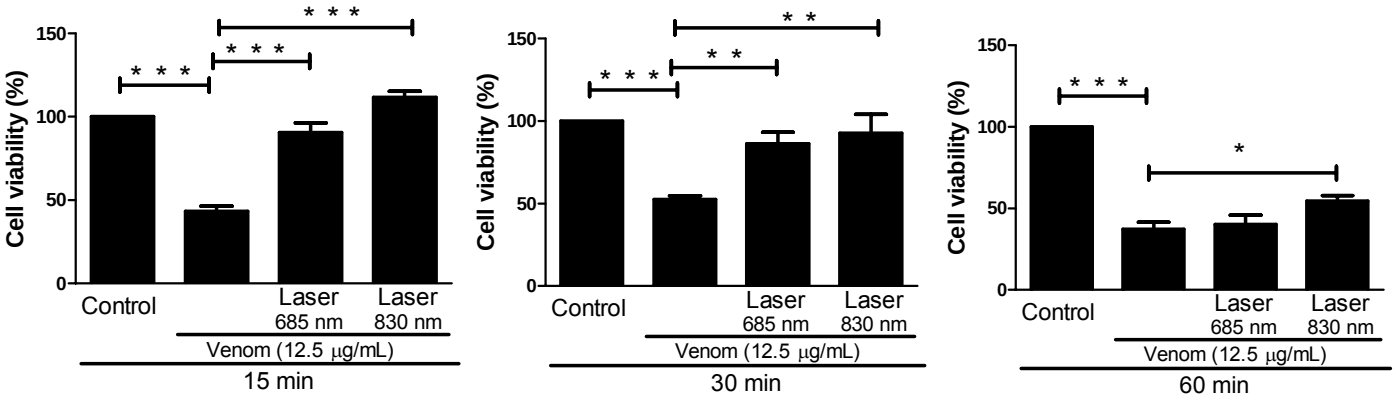
C2C12 muscle cells were plated into 96 well plates and incubated for 24 hours for cellular adhesion. After this period the irradiated venom was added (12.5µg/mL) and was incubated for 15, 30 and 60 minutes. Cells incubated with medium were used as control. Cell viability was determined by MTT assay.

Table 2-Effect of LLLT on cell detachment of C2C12 cells after irradiated *B. jararacussu* venom incubation.

	Detachment (%)		
Time (min)	**Venom**	**iVenom 685nm**	**iVenom 830nm**
15	64.0 ± 14.1	63.8 ± 5.9	66.7 ± 2.8
30	52.8 ± 8.2	54.0 ± 3.4	51.0 ± 2.7
60	67.9 ± 12.9	68.7 ± 3.5	71.8 ± 4.1

C2C12 muscle cells were plated into 96 well plates and incubated for 24 hours for cellular adhesion. After this period the irradiated venom was added (12.5µg/mL) and was incubated for 15, 30 and 60 minutes. Cells incubated with medium were used as control. The cellular detachment was determined by the Crystal Violet assay.

Figure 1


Cell viability (%)
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Control
Laser 685 nm
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Venom (12.5 μg/mL)
15 min
Cell viability (%)
150
100
50
0
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Control
Laser 685 nm
Laser 830 nm
Venom (12.5 μg/mL)
30 min
Cell viability (%)
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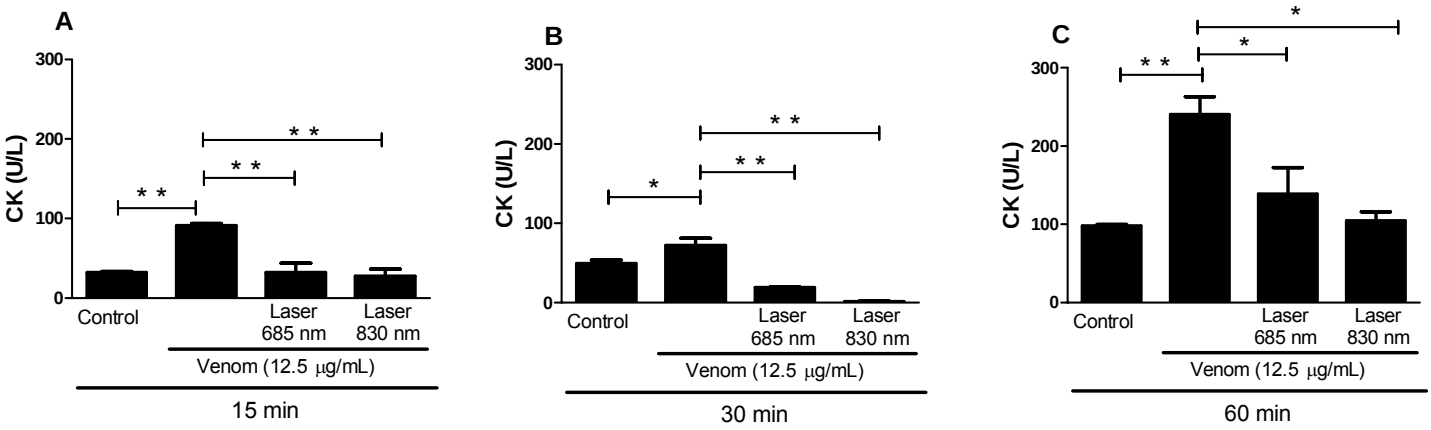
Control
Laser 685 nm
Laser 830 nm
Venom (12.5 μg/mL)
60 min

Figure 2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CK (U/L)
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Control
Laser 685 nm
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Venom (12.5 μg/mL)
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Control
Laser 685 nm
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Venom (12.5 μg/mL)
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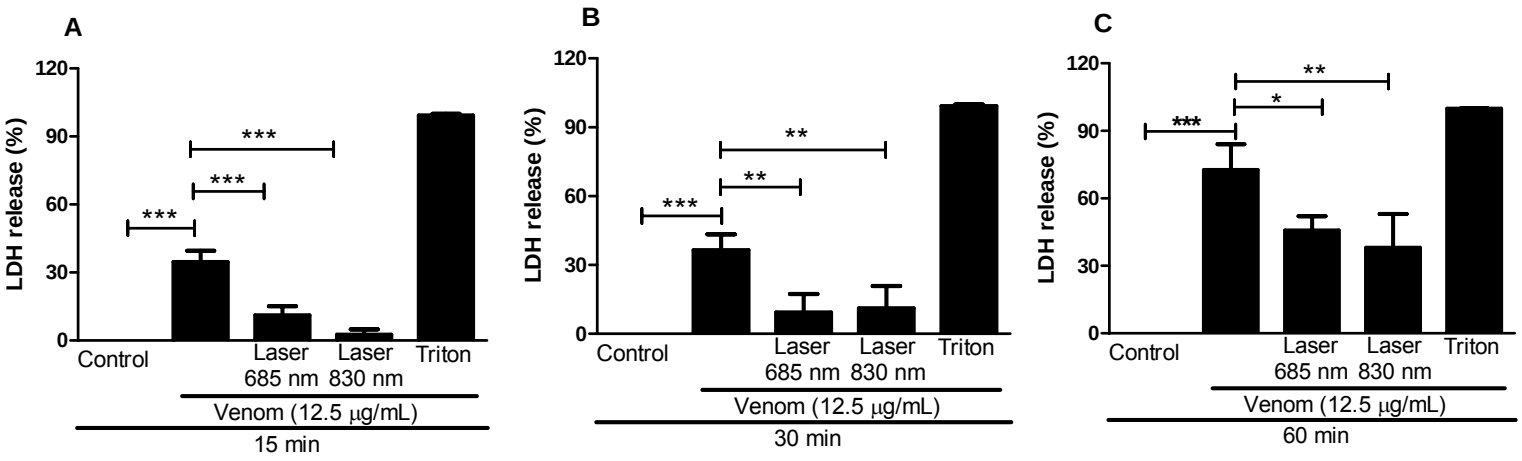
Control
Laser 685 nm
Laser 830 nm
Venom (12.5 μg/mL)
60 min

Figure 3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LDH release (%)
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90
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30
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Control
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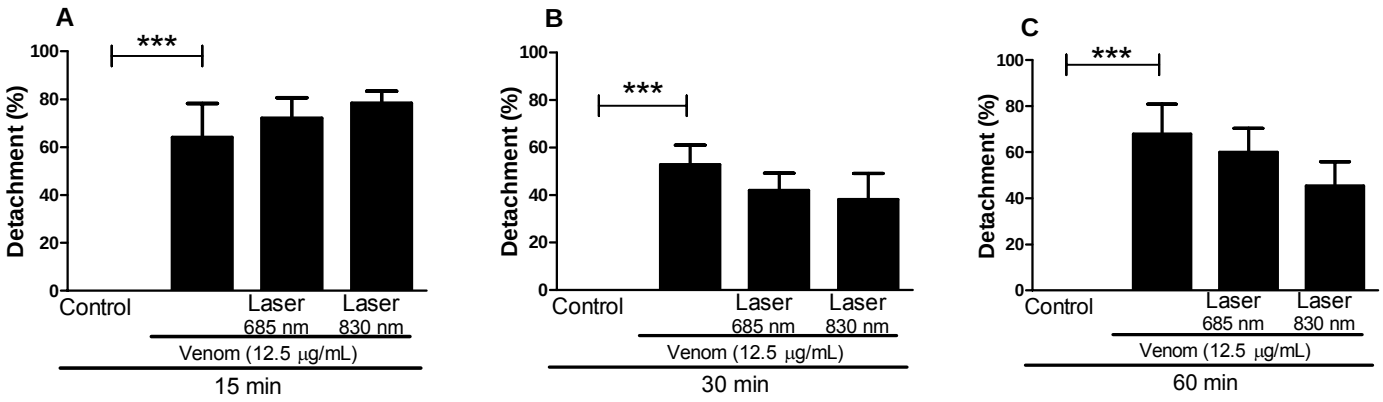
Laser 830 nm
Triton
Venom (12.5 μg/mL)
15 min
30 min
60 min

Figure 4


A
B
C
***
Detachment (%)
100
80
60
40
20
0
Control
Laser
685 nm
Laser
830 nm
Venom (12.5 μg/mL)
15 min
30 min
60 min

4.3 Resultados ainda não publicados

4.3.1 Efeito do veneno da serpente *B. jararacussu* na adesão celular.

Os resultados demonstraram uma diminuição estatisticamente significativa na adesão celular em todas as concentrações testadas (6, 12.5, 25 e 50 µg/mL) quando comparadas com o grupo controle nos períodos de 15, 30 e 60 minutos (Fig. 1 A, B e C), esse efeito foi mais pronunciado na concentração de 25 e 50 µg/mL, nos períodos de 30 e 60 min, após a incubação com o veneno.

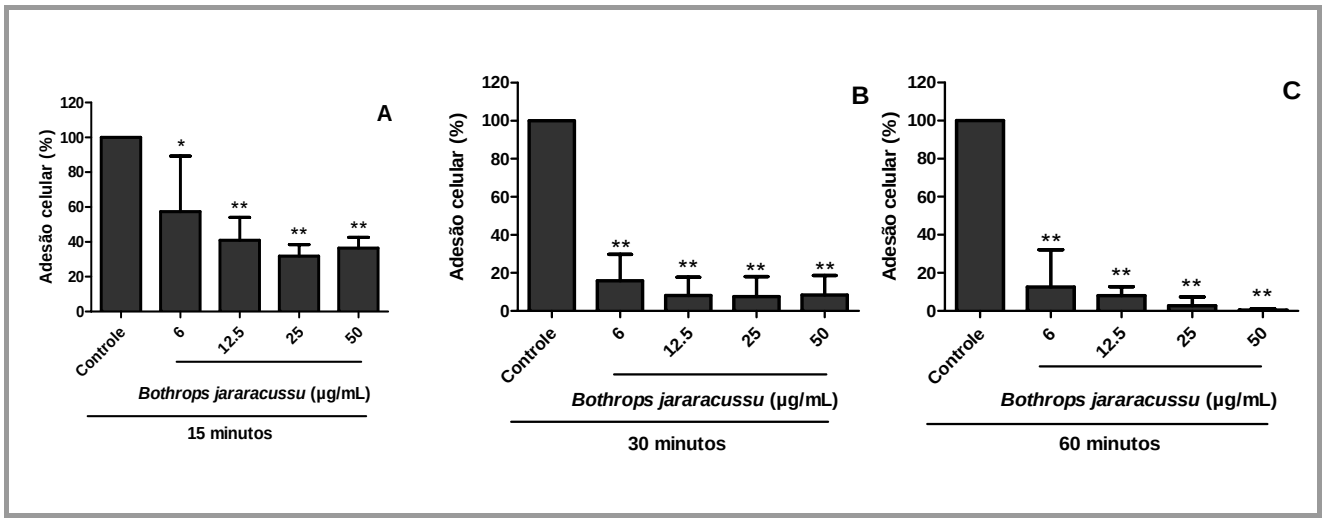


Figura 1. Efeito do veneno *B. jararacussu* na adesão celular: Células musculares C2C12 foram plaqueadas em placas de 96 poços e receberam imediatamente o veneno nas concentrações 6, 12.5, 25 e 50 µg/mL ou somente meio de cultura (controle) e incubadas por 15, 30 e 60 minutos. A adesão celular foi determinada pelo método MTT. Cada valor representa a média ± EPM de três experimentos independentes, Anova DUNNET * $p \leq 0,05$ e ** $p \leq 0,01$.

4.3.2 Diferenciação Celular

Para verificar a ação do laser na proteção das células musculares C2C12, na proliferação e formação de miotubos, as células receberam o veneno *B. jararacussu,* foram irradiadas e após cada período de incubação (15, 30 e 60 min) o sobrenadante foi removido e as células receberam meio de cultura contendo 2% de soro de cavalo para induzir a diferenciação e permaneceram em estufa por 4 dias. Os resultados mostraram que células não tratadas com o veneno e incubadas com soro de cavalo 2% exibiram um formato alongado e fino, observado no microscópio óptico (Fig. 1 A, B, C). Células incubadas com o veneno morreram, isso ocorreu em todos os períodos de incubação (Fig. 1 D, G e F). No entanto, células que receberam o veneno e foram irradiadas com o laser apresentaram uma morfologia similar a do controle, apresentando células alongadas e finas, o que caracteriza a diferenciação das células musculares C2C12 (Fig. 1 E, F, H, I, K e L).

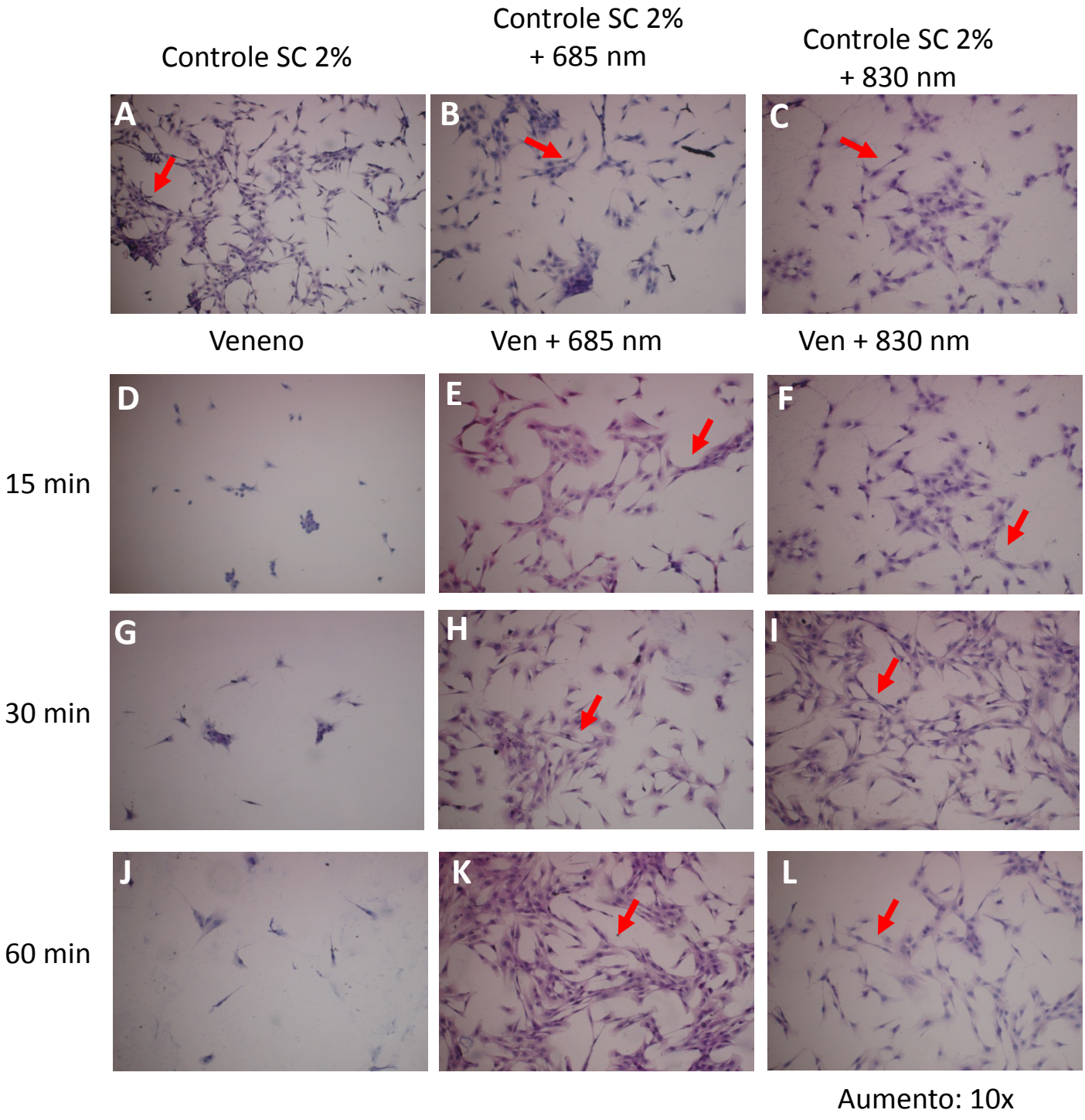


Figura 1: Diferenciação das células musculares C2C12. As células C2C12 foram divididas em tubos de ensaios, receberam o veneno e centrifugadas para a formação do preciptado celular. Em seguida foram irradiadas de forma pontual na parte inferior do tudo. Posteriormente as células foram plaqueadas em lamínulas de 13 mm em placa 24 poços e incubadas por 15, 30 e 60 minutos. Após cada período experimental o sobrenadante das células foi removido e substituído por meio DMEM suplementado com 2% de soro de cavalo e incubadas por 4 dias. Para determinação da diferenciação celular, foi realizada a análise morfológica das células por meio da coloração de Eosina&Hematoxilina.

5. CONSIDERAÇÕES FINAIS

O presente estudo permitiu com concluir que o veneno da serpente *B. jararacussu* apresenta um potente efeito miotóxico de maneira dose-depende nos períodos analisados para esta linhagem celular, demonstrando a importância do estabelecimento de um recurso terapêutico que atue de forma coadjuvante à soroterapia.

Baseado nos resultados encontrados com o uso do laser de baixa potência podemos concluir que o uso da fototerapia neste modelo experimental foi capaz de proteger a célula muscular contra a ação do veneno da serpente *B. jararacussu*, corroborando com resultado de estudos *in vivo* que demonstram a eficácia deste recurso em casos de lesão por veneno botrópico. Desta forma, o laser de baixa potência vem sendo estabelecido na literatura como uma terapêutica eficaz em casos de acidentes por serpentes peçonhentas. No entanto, mais estudos devem ser realizados para um melhor entendimento do mecanismo de proteção do laser de baixa potência neste tipo celular.

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7. APÊNDICE

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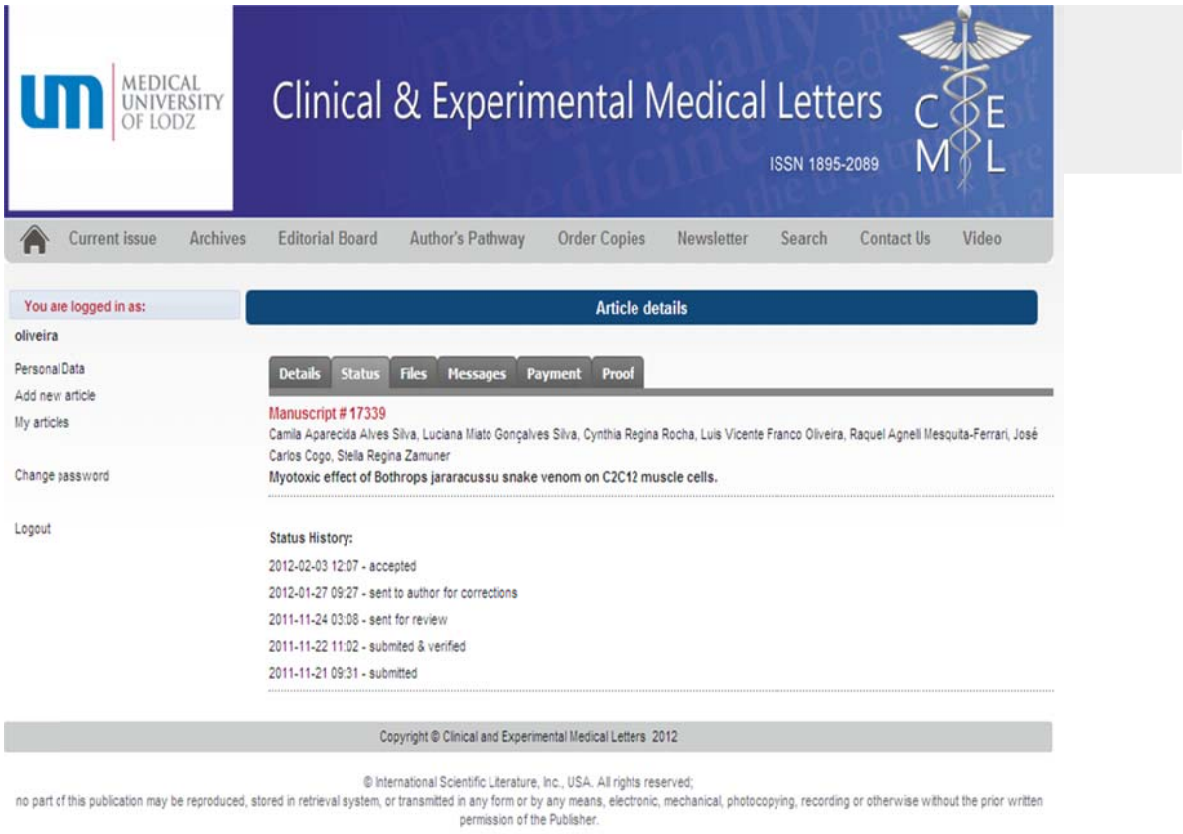

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Myotoxic effect of *Bothrops jararacussu* snake venom on C2C12 muscle cells

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