ESTEFANNY RUIZ GARCIA

FRACTION L17 OF *Lachesis muta muta* VENOM PROMOTES THE CELLULAR PROLIFERATION *IN VITRO* AND ACCELERATES THE WOUND HEALING IN MICE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Animal, para obtenção do título de *Magister Scientiae*.

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APROVADA: 05 de abril de 2017.

Mariaurea Matias Sarandy Souza

Edvaldo Barros

Reggiani Vilela Gonçalve

(Orientadora)

Nuestra recompensa se encuentra en el esfuerzo y no en el resultado,

un esfuerzo total, es una victoria completa

Mahatma Gandhi

¡Dedicado al esfuerzo de mi madre, quien con seguridad obtuvo su victoria!

Te Amamos.

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ABSTRACT

RUIZ-GARCIA, Estefanny, M.Sc., Universidade Federal de Viçosa, April, 2017. **Fraction L17 of *Lachesis muta muta* venom promotes the cellular proliferation *in vitro* and accelerates the wound healing in mice**. Adviser: Reggiani Vilela Gonçalves.

Lachesis muta muta is a Viperid snake distributed throughout South America and its bite has been reported to cause hypotension, bradycardia, vomiting, diarrhea and affects the coagulation cascade. Studies have shown that proteins isolated from the *L. m. muta* venom have pharmacological activity against carcinogenic cell lines, coagulation disorders and hypertension control. The present work reported the proliferating activity *in vitro* and *in vivo* of the fraction 17 of *L. m. muta* (L17) on the wound healing. We tested the activity of L 17 at different concentrations in 3T3 fibroblasts and HaCaT Keratinocytes, as well as their activity on the healing process in Swiss-Webster mice. L17 did not affect the survival of fibroblasts and keratinocytes in cell culture and further with 10 μg/ml promoted the proliferation of keratinocytes after 72 h of treatment. In vivo experiment, L17 accelerated the healing process, increasing the cellularity at day 8, and the blood vessel production at day 4 and 8. Likewise, it stimulated the deposition of type I and type III collagen and favored the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) as well as the reduction of the markers of oxidative stress malondialdehyde (MDA) and carbonyl protein (CP). The results suggest that L 17 could be effective in the healing treatment, since it favors the cellularity and the angiogenesis; in addition, it promotes the deposition of collagen for the formation of Extra Cellular Matrix (ECM). L17 possibly has a proliferative and protective effect against oxidative stress in the wound healing, been a prospective treatment.

RESUMO

Ruiz-Garcia, Estefanny, M.Sc., Universidade Federal de Viçosa, abril de 2017. **Fração L17 do veneno de *Lachesis muta muta* promove a proliferação celular *in vitro* e acelera a cicatrização em camundongo.** Orientadora: Reggiani Vilela Gonçalves.

Lachesis muta muta é uma serpente da família viperidae distribuída por América do Sul e tem sido reportado que sua mordedura causa hipotensão, bradicardia, vomito, diarreia e efeitos na cascada de coagulação. Vários estudos têm mostrado que proteínas isoladas do veneno de *L. m. muta* tem apresentados atividades farmacológicas contra linhas celulares de câncer, distúrbios da coagulação e controle sobre a hipertensão. O presente trabalho reporta a atividade proliferativa *in vitro* e *in* vivo da fração L17 isolada do veneno de *L. m. muta* (L17) sobre o processo cicatricial. Nos testamos a atividade de L17 a diferentes concentrações em fibroblastos 3T3 e em queratinócitos HaCaT, assim como sua atividade sobre o processo de cicatrização em camundongos suíços. L17 não afeito a sobrevivência de fibroblastos e queratinócitos em cultura celular e além de isso com 10 µg / ml promoveu a proliferação de queratinócitos HaCaT depois de 72 horas de tratamento. O experimente *in vivo* mostro que L17 acelerou significativamente o processo cicatricial com um incremente na celularidade ao dia 8 e na produção de vasos sanguíneos nos dias 4 e 8. Além disso, L17 estimulou deposição de colágeno tipo I e colágeno tipo III e favoreceu a atividade das enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT), assim como a redução dos marcadores de estresse oxidativo malondialdeido (MDA) e proteínas carboniladas (PC). Estes resultados sugerem que L17 poderia ser efetivo nos tratamentos de cicatrização, pelo favorecimento na celularidade e a angiogenêse, importantes para a nutrição do tecido, assim como promovendo a deposição de colágeno para a formação da Matriz Extracelular (MEC). L17 provavelmente tem um efeito proliferativo e protetivo sobre o estresse oxidativo no processo cicatricial, sendo um tratamento prospetivo na aceleração da cicatrização.

SUMARIO

INTRODUÇÃO GERAL

Cicatrização

A cicatrização é um processo complexo que deve ocorrer de forma organizada, a fim de se obter um fechamento tecidual adequado (Tang et al., 2014). Este processo é mediado por diferentes fatores de crescimento, citocinas e células mesenquimais e precedido por 3 etapas: inflamação, proliferação e remodelação (Mendoça e Netto, 2009). Na etapa inflamatória células da medula óssea migram para a área da lesão, diferenciando-se em neutrófilos e linfócitos para prover uma proteção inicial ao tecido lesado, com o fim de atrair outras células como monócitos e macrófagos (Xing et al., 2015). Este processo é mediado por citocinas pro inflamatórias e fatores de crescimento, como IL-1 (interleucina 1), IL-6, IL-8 e TNF-α (fator de necroses tumoral alfa), atraindo macrófagos e outras células ao tecido lesado (Hsu & Mustoe, 2010). Moléculas como TGF-β (Fator de crescimento transformante β) atuam ativando a expressão de outros fatores de crescimento como VEGF (fator de crescimento endotelial vascular) e, ao final do processo inflamatório, estes mediadores vão estimular a migração de queratinócitos, fibroblastos, e a formação de novos vasos no tecido lesado (Puolakkainen et al., 1995; Stojadinovic et al., 2007). Simultaneamente acontece o processo de hemostasia mediado pela ativação de PDGF (fator de crescimento derivado de plaquetas) iniciando a fase inflamatória (Park et al., 2014). Durante esta etapa ocorre a degranulação do tecido e a formação de fibrina com formação do tampão plaquetário (Guo & DiPietro, 2010; Tang et al., 2014).

Na segunda fase do processo cicatricial ocorre a proliferação de células do tecido que são responsáveis pela regeneração da pele, neste momento ocorre a reepitelização e a síntese dos componentes da matriz extracelular (MEC) como o colágeno, os glicosaminoglicanos e os proteoglicanos (Branco-Neto et al., 2006; Gonçalves et al., 2014). Além disto, ocorre a liberação de citocinas anti-inflamatória como IL-10 e IL-13 que vão controlar o processo inflamatório evitando o desenvolvimento de uma inflamação crônica e consequentemente o retardo no processo cicatricial (Ram et al, 2015). Os fatores de crescimento TGF-β, FGF (Fator de crescimento fibroblasto), e VEGF são expressos e vão ativar os processos de proliferação de fibroblastos e ao mesmo tempo o processo angiogênico (Jonson & Wigeus, 2012). A angiogenêse ocorre no início da fase proliferativa, onde células mesenquimais hematopoiéticas migram para a área da ferida promovendo a produção de vasos sanguíneos com o fim de prover oxigenação e nutrição ao tecido novo favorecendo assim a proliferação celular (Ram et al., 2015; Galiano et al., 2016). Na fase proliferativa o ferro (Fe), a vitamina C e arginina atuam como co-fatores da síntese de colágeno, além disto, aminoácidos como a glutamina estimulam a proliferação de fibroblastos e células endoteliais aumentando a produção de tecido de granulação (Gainza, 2015).

A última etapa do processo cicatricial é a remodelação tecidual (Guo & Dipreto, 2010). Nesta fase ocorre diminuição do número de capilares, fibroblastos e substituição de colágeno do tipo III por colágeno do tipo I aumentando a força de tensão da matriz (Guo & DiPietro, 2010; Ram et al., 2015), além disso, a ação de enzimas como a hyaluronidase e as metaloproteinases (MMPs) degradam componentes da MEC e promovem a remodelação do tecido neo-formado (Armstrom & jude, 2002).

Nos últimos anos tem aumentado o interesse por estudos sobre o efeito de compostos de origem animal na regeneração cutânea, embora as diferentes atividades destes biocompostos ainda não sejam bem conhecidas (Utkin, 2015). Tang et al., (2014) relatou o efeito antimicrobiano das secreções cutâneas do anuro *Fejervarya cancrivora*, atuando também na proliferação de fibroblastos e queratinócitos, a ativação de células imunológicas e a expressão de fatores de crescimento e citocinas. No trabalho feito por Sammy et al., (2015) observou-se o uso de uma phospholipase A2 extraída De *Python reticulatus* sobre o processo cicatricial, a qual modulou a ativação da via NF-kβ acelerando o reparo cutâneo. Estes estudos mostram o interesse crescente nas proteínas de origem animal, que estão proporcionando uma ampla informação na área farmacêutica, sugerindo um alto potencial farmacológico (Utkin, 2015).

Toxinologia: desenvolvimentos e avanços

A toxinologia teve início na época da colônia, quando Von Humboldt em 1805 decidiu estudar os componentes das setas usadas pelas tribos americanas para a caça, as quais eram impregnadas por uma sustância tóxica, sendo identificada como um alcaloide de uma planta do gênero *Strychnos* (família Loganiácea) que provocava um bloqueio neuromuscular (De Lima et al., 2010). Esta nova linha de investigação começou a ser desenvolvida na América Latina no final do século XIX e início do século XX com os trabalhos realizados por Vital Brazil em 1895, que descreveu pela primeira vez os casos de envenenamento por *Bothrops jararaca* e *Crotalus durissius terrificus* mostrando à comunidade científica a importância da pesquisa de antivenenos. Vital Brazil, ainda desenvolveu o primeiro soro antiofídico contra *Crotalus durissius terrificus* em 1898, e logo em seguida Albert Calmette descreveu o primeiro soro antiofídico contra *Naja* sp. Joao Batista Lá Cerda, que em 1984 conseguiu coagular leite, dissolver fibrina e gema de ovo com o uso do veneno de *Bothrops sp.*, além de provocar a lises de células com veneno de *Lachesis sp.* (Lima et al., 2010). Para a época de 1931, Clodomiro Picado seguindo os estudos brasileiros, iniciou o estudo das serpentes venenosas da Costa Rica e suas características toxicológicas (Gutierrez, 2002), expandindo os estudos de toxinologia a outras partes da América.

A partir destes estudos, se iniciaram os isolamentos dos diferentes componentes destes venenos, bem como bioquímicos destas proteínas tóxicas, pretendendo-se elucidar o grande potencial farmacológico dos mesmos (Gutierrez, 2002). Em 1938 Karl H. Slotta junto a seu colega Fraenkel Conrat realizaram o primeiro isolamento de uma toxina de *C. d. terrificus*, Crotoxina (De Lima et al., 2010). Em 1948 Mauricio Oscar da Rocha Silva descreveu a Bradiquinina de *Bothrops jararaca* e seu potencial farmacológico no estudo da regulação da pressão arterial (Rocha et al.,1974). Em 1987 Vital Brasil demonstrou as bases fisiológicas dos venenos de *Micrurus sp.* e sua ação neuromotora *in vitro* (Brazil, 1987).

A partir destes trabalhos iniciais tem-se desenvolvido uma grande quantidade de investigações sobre componentes dos venenos de serpentes e sua ação fisiopatológica para ser utilizados nos tratamentos a diferentes tipos de doenças. Estudos têm demostrado a ação de diferentes atuações de proteínas dos venenos, como por exemplo, atividade antitumoral (Klein et al., 2011), antibacteriana (Wang et al., 2011) e antinoceceptivas (Leite dos Santos et al, 2012), apresentando assim uma ampla variedade sobre as vias de atuação fisiológica.

Venenos das serpentes em farmacologia

Desde o uso do primeiro medicamento baseado em uma proteína dos venenos de uma serpente, Captopril, a mais de 30 anos, tem-se ampliado a investigação do potencial farmacológico dos venenos, testando a bioatividade de suas proteínas na pesquisa de novos medicamentos (Koh & Kimi, 2012). Podemos relatar uma ampla variedade de proteínas caracterizadas e testadas tanto fisiopatologicamente, como farmacologicamente na primeira década do século XXI. Em 2001 foi isolado de *B. pirajai*, 2 PLA_2S homólogas com diferença em um aminoácido Lys 49 para PrTXI (piratoxina I) e Asp 49 para PrTXIII (piratoxina III), determinando sua atividade neurotóxica, hemolítica e anticoagulante respectivamente (Soares et al., 2001). Em 2003 uma proteína anticoagulante Lectina tipo C, TSV-FIX-BP foi isolada de *Trimeresurus stejnegeri* (Lee et al., 2003) e em 2004 foi estudada a atividade hipotensiva de uma proteína do venenos de *B. erythromela*, SVVEGF, que apresentou efeitos no aumento da permeabilidade vascular (Junqueira de Azevedo et al., 2004). Em 2005 foi caracterizada a atividade pró-coagulante de 3 metaloproteinases, PIEoVMP1, PIIIEoVMP2 e EoVMP3 isoladas de *Echis ocellatus* (Howes et al., 2005). Em 2010 foram identificadas 6 novas 3FTXs de *Micrurus frontalis*, todas com uma atividade de bloqueio de receptores nicotínicos de acetilcolina (Moreira et al., 2010). Wang e colaboradores apresentaram em 2011 um novo peptídeo antimicrobiano, ativador de citocinas pró- inflamatórias caracterizado como Cathelicidin-BF isolado de *Bungarus fasciatus*. Em 2013 foram isolados 2 serine protease de *B. pirajai*, BPirSP27 e BPirSP41 as quais mostraram efeitos inibitórios da atividade hemolítica e atividade anti-inflamatória, deixando aberta a porta para novos estudos das atividade dessas proteínas (Menaldo et al., 2013). Leonardi et al., (2015) descreveu uma proteína VMP α-fibrinogenolítica de *Vipera ammodytes*, Va F1 com uma atividade anticoagulante por hidrólises de fator X, protombinas e plasminogênio, e Ache et al., (2015) identificou outra VMP de *B. pauloensis* que inibe a adesão celular e angiogênese, podendo ser utilizada como alternativa para inibição de vascularização tumoral. Neste trabalho foi identificada a atividade de uma fração isolada da serpente *Lachesis muta muta* como uma possível alternativa farmacológica para o tratamento cicatricial de feridas cutâneas.

Lachesis muta muta

Lachesis sp. pertencente à família Viperidae, subfamília Crotalinae, é o maior viperido encontrado e o segundo maior entre as serpentes venenosas do mundo (Zamundio & greene, 1997, Campbell & Lamar 1989), sendo descritos espécimenes que excedem os 2.80 metros no Brasil (De Souza et al., 2007). O gênero está composto por três espécies: *Lachesis melanocephala*, distribuída pela costa do Caribe e Centro América, *L. stenophrys* reportada em Costa rica e panamá, e *L. muta* distribuída na Colômbia, Venezuela, Trinidade, Guayana, Suriname, equador, Peru e Brasil, esta última subdividida em dois sub espécies, *L. muta muta* distribuída na região amazónica do Peru, Equador, Colômbia, Venezuela e Brasil, e *L. muta rhombeata* encontrada na região da mata atlântica brasileira (Madrigal et al., 2012).

Embora os casos de acidentes por *Lachesis muta muta* não são muito frequentes, o mecanismo de envenenamento tem sido estudado e sabe-se que sua mordedura provoca edema, hemorragia, necroses, náuseas, coagulopatia, hipotensão, shock e nefropatías (Cardoso et al., 2003). Além disso, tem-se observado o bloqueio neuromuscular pela alteração de receptores de acetilcolina e miotoxicidade (Damico et al., 2005). Casos de envenenamento na Amazônia brasileira relataram edema e hemorragia local, hipotensão, diarreia e vômito depois de 30 minutos da mordedura (De Souza et al., 2007). Dias et al., (2016) reportou que 3 mg/kg do veneno inoculado provoca morte em ratos wistar em um período de 10 a 60 minutos, precedida por hipotensão e bradicardia, depressão respiratória e hemorragia pulmonar. Além de isso, a dose letal média (DL50) do veneno de *L. muta* foi reportado por Brown et al. (1973), com 1,5 mg/kg (i.v), 1,6-6,2 mg/ml (i.p) e 6,0 mg/ml (sc) provocando a morte na metade dos animais inoculados. Estes relatos tem ampliado o interesse por estudar os mecanismos moleculares que causam estas complicações e os componentes do veneno que mediam estes processos patológicos.

O estudo do transcriptoma das glândulas de *L. muta muta* revelou uma ampla variedade de proteínas encontradas no veneno do viperido, entre as quais foram identificadas as famílias proteicas svMPPs, BPPs, CNPs, serine proteasas, lectinas tipo C, PLA_2S, LAAO (Junqueira de Azevedo et al., 2006). Além disso, Junqueira identificou VEGF e proteínas da familia das 3FTX, proteínas com atividade neurotóxica encontradas principalmente em venenos de elapidos (Castro et al., 2015). Estes dados foram contrastados com o estudo de proteomica realizado por Sanz et al., (2008) que identificou de 7 a 8 famílias protéicas com aproximadamente 40 proteínas. SvMPPs foram as mais abundantes (32-38%), seguidas das serin proteases (25-31%), BPPs e CNPs (15 %). Sanz também identificou proteínas CRISP, no veneno de *L. muta*, não sendo encontrada nas outras espécies do gênero, e não reportou a identificação de VEGF reportado por Junqueira de Azevedo et al., (2006).

Sánchez et al., (1991 e 1995) caracterizou o fator hemorrágico LHF-I e LHF-II no veneno de *L. m. muta.* Várias fosfolipases tem sido identificadas e caracterizadas em seu veneno, como por exemplo LMPLA$_2$S-II de 18 KDa que exibiu uma atividade hemolítica e inibiu a agregação plaquetária (Fuly et al., 2002). Damico et al., (2005a) caracterizou a atividade enzimática de 2 PLA$_2$S, LmTX-I com 14,2 KDa e LmTX-II com 14,18 kDa. LmTX-I foi citotóxica em músculo esquelético e células do rim (Damico et al., 2006) além de provocar uma resposta inflamatória mediada pela ativação de mastócitos e o incremento da permeabilidade microvascular *in vivo* (n), enquanto LmTX-II provocou um bloqueio neuromuscular em camundongos (Damico et al., 2005b). LM-PLA$_2$-I de *L. m. muta* demostrou ação hemolítica e na agregação plaquetária, induzindo edema e miotoxicidade, além de promover proliferação em células dos gânglios retinais (da Silva Cunha et al., 2011). Outra PLA$_2$S caracterizada por Damico et al., (2012), identificada como LmrTX com 14 KDa exibiu uma atividade antitrombótica.

Duas metaloproteinases foram identificadas no veneno de *L. m. muta* e provocaram alterações do sistema de coagulação, Mutalysin II de 22 KDa exibiu uma atividade hemorrágica (Machado de Avila et al., 2011), enquanto LMR-47 Rhombeobin de 47 KDa exibiu una atividade pro-coagulante (Torres Huaco, 2013). Foi identificada uma L-aminooxidase no veneno de *L. m. muta* com um peso de 120 KDa exibindo uma ação hemorrágica e mio necrótica, além de promover apoptoses quando testada em linhagens celulares de câncer (Bregge Silva et al., 2012). Outras proteínas no veneno de *L. m. muta,* tais como BPPs de 21 KDa, tem atividade hipotensiva e NPs apresentou uma atividade osmorreguladora (Soares et al., 2005). Outra serin protease foi identificada como

Akallikrein-like protease de 33 KDa com atividade hipotensiva (Felicori et al., 2003). Estes trabalhos exibem a diversidade proteica dos venenos elucidando seu papel durante o processo de envenenamento.

OBJETIVOS

Objetivo geral

Avaliar o efeito da fração 17 (L17) isolada do veneno total de *L. m. muta* sobre a proliferação celular *in vitro* e seu efeito proliferativo, angiogênico e antioxidante sobre o processo cicatricial *in vivo*.

Objetivos específicos

Medir a atividade de L17 sobre o metabolismo e proliferação celular de fibroblastos e queratinócitos *in vitro.*

Analisar *in vivo,* a velocidade de fechamento das feridas de segunda intenção em camundongos Swiss-Webster, tratadas com L17.

Avaliar o efeito proliferativo e angiogênico em feridas cutâneas de segunda intensão em camundongos Swiss-Webster, tratadas com L17.

Identificar a ação de L17 sobre a proliferação e a degranulação de mastócitos no tecido em formação, com o fim de determinar seus efeitos sobre a ativação de células imunológicas no processo inflamatório.

Identificar os efeitos de L17 sobre a sínteses de colágeno tipo III e I, e seu efeito sobre a reorganização do tecido cicatricial em formação.

Determinar o efeito de L17 sobre o estresse oxidativo no tecido de granulação, através da análise das enzimas antioxidantes Superóxido dismutase (SOD) e Catalase (CAT) e sua ação sobre os marcadores de estresse oxidativo malondialdeido (MDA) e proteinas carboniladas (PC), com o fim de identificar as implicações sobre a resposta inflamatória e a diferenciação celular no tecido cicatricial.

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FRACTION L17 OF *Lachesis muta muta* VENOM PROMOTES THE CELLULAR PROLIFERATION *IN VITRO* AND ACCELERATES THE WOUND HEALING IN MICE

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ABSTRACT

Lachesis muta muta is a Viperid snake distributed throughout South America and its bite has been reported to cause hypotension, bradycardia, vomiting, diarrhea and affects the coagulation cascade. Studies have shown that proteins isolated from the *L. m. muta* venom have pharmacological activity against carcinogenic cell lines, coagulation disorders and hypertension control. The present work reported the proliferating activity *in vitro* and *in vivo* of the fraction 17 of *L. m. muta* (L17) on the wound healing. We tested the activity of L 17 at different concentrations in 3T3 fibroblasts and HaCaT Keratinocytes, as well as their activity on the healing process in Swiss-Webster mice. L17 did not affect the survival of fibroblasts and keratinocytes in cell culture and further with 10 μg / ml promoted the proliferation of keratinocytes after 72 h of treatment. I*n vivo* experiment, L17 showed significant acceleration in the improvement of the healing process, with an increase in cellularity at day 8 of the experiment, and increased blood vessel production at day 4 and 8 in animals treated with L17. Likewise, it stimulated the deposition of type I and type III collagen and favored the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) as well as the reduction of the markers of oxidative stress malondialdehyde (MDA) and carbonyl protein (CP). The results suggest that L 17 could be effective in the healing treatment, since it favors the cellularity and the angiogenesis important for the nutrition of the tissue, in addition it promotes the deposition of collagen for the formation of Extra Cellular Matrix (ECM). L17 possibly has a proliferative and protective effect against oxidative stress in the wound healing, been a prospective treatment.

KEY WORDS: *Lachesis muta muta*, venom, proliferation, oxidative stress, skin, keratinocytes, fibroblast.

HIGHLIGHTS

L17 active the proliferation process of fibroblast and keratinocytes in cell culture.

L17 improve the wound healing process by increase of cellularity, blood vessel and collagen deposition reducing the wound area.

L17 has an antioxidant effect over the wound healing process.

INTRODUCTION

Healing of cutaneous wounds is a complex process that occurs after tissue damage being preceded by three stages: inflammation, proliferation and remodeling (Mendoça e Netto, 2009). In each of these stages, different cells and mediators act for tissue regeneration. Immunological cells migrate to the wound area in order to provide protection and mediate the expression of pro and anti-inflammatory proteins (Zpaderska & DiPietro, 2005, Strbo et al., 2014). Neutrophils are the first to arrive of the scar area and these send signals for the migration of macrophages in the tissue that mediate expression of cytokines and growth factors (Koh & DiPietro, 2011; Wilgus et al., 2013). Simultaneously, hemostasis occurs, where the coagulation cascade is activated, in order to avoid the bleeding process (Sabino et al., 2015). During the proliferative phase fibroblasts, keratinocytes and endothelial cells proliferate in tissue in order to synthesize components of the Extra Cellular Matrix (ECM) such as elastic fibers and collagen, and stimulate the production of blood vessels, important for oxygenation and nutrition of the new tissue (Elçin et al., 2001, Na et al., 2017). In the final stages of the process, several proteins, such as metalloproteinase and hyaluronidase, works in the degradation of matrix components, controlling vascular density and ceases the proliferative process (Fronza et al., 2014; Rohani & Parks, 2015). Several growth factors play an important role in all phases of the scar process (Briquez et al., 2015). VEGF (vascular endothelial growth factor) is the main actor in the angiogenesis process, as well as TGF-β (Transforming growth factor β) in the cellular differentiation process, stimulating the migration of fibroblasts and keratinocytes (Elçin et al., 2001; Lichtman et al., 2016). Cytokines such as IL-8 (Interleukin 8) and IL-1 (Interleukin 1) mediate pro-inflammatory processes and their expression is controlled by other anti-inflammatory cytokines such as IL-10 to avoid excessive inflammation (King et al., 2014).

Compounds of animal origin has been studied to find an effective treatment to accelerate the healing process and improve the quality of life of patients (Utkin, 2015). It has been reported the wound healing effect of small peptides secreted by anurans (Tang et al., 2014), and proteins isolated from snake venom with antibacterial and healing activity (Wang et al., 2011), however, it is not yet well known pharmacological potential of the proteins isolates of the venom. Viperids snake venom have been extensively studied as a resource for biologically active compounds (Kini & Evans, 1989; Fatima & Fatah, 2014; Izidoro et al., 2014). Proteins with fibrinolytic activity were isolated of *Bothrops jarraca* venom (Sugiki et al., 1995). Other with anti-inflammatory activity such as serine proteinase was identified in the *B. pirajai* venom (Menaldo et al., 2013) and coagulant and pro-inflammatory activity was found in proteins isolated from *Daboia russeli siamensis* venom (Guo et al., 2013), as well as anti-parasitic activity of L-amino-oxidase was found in *Lachesis muta* venom (Breggel-Silva et al., 2012). However, few studies have showed the effect of proteins isolated from snake venoms on the healing process.

L. m. muta it is Snakes belonging to pit vipers specific to South America distributed in the Amazon region of Peru, Ecuador, Colombia, Venezuela and Brazil (Zamundio & greene, 1997, Madrigal et al., 2012). Has been widely studied due to the toxicity of its venom, since its bite causes muscular paralysis, acute respiratory, cardiovascular alteration, hemorrhage, edema and coagulation disorders (Cardoso et al., 2003; Damico et al., 2005). Transcriptional studies of *L. m. muta* showed the wide variety of protein families in its venom, studied for their physiological and pharmacological action (Junqueira et al., 2006). Approximately 30 to 40 proteins have been identified in the *L. muta* venom among them, Metalloproteinase, Phospholipases A2 (PLA_2S), serine proteinases and C-type lectin, Bradykinin-potentiating peptides (BPPs), L-amino acid oxidases (LAAOs) (Sanz et al., 2008), exhibiting a possible pharmacological potential. Based on this, we investigated the protective and proliferative effect of the L17 fraction of *L. m. muta* on wound healing and its implication on the cell proliferative and angiogenesis process, as well as the antioxidant action.

MATERIALS AND METHODS

Lachesis muta muta venom and fraction

L17 fraction (L17) purified of the venom of the snake *L. m. muta* was donated by the Immunochemistry of proteins Laboratory of the Federal University of Minas Gerais (UFMG). The chosen of the fraction it was made after a pilot experiment *in vitro* of several fraction of *Lachesis muta muta* and *Crotalus d. terrificus* over the cellular metabolist (data not showed). The sample was transported in eppendorf, in solution, at 4°C and kept in the laboratory of experimental pathology, in the Department of Animal Biology of the Federal University of Viçosa (UFV) at -20°C.

SDS-PAGE and protein dosage

L17 concentration was determined by Lowry method (Lowry *et al.*, 1951) using BSA pattern (Bovine Serum Albumin) and the identification of the protein was made by SDS-PAGE gel in a electrophoretic process. L17 and the crude venom (40 µg) were diluted in sample buffer and separated in 15 % polyacrylamide gel, according to Laemmli (Laemmli, 1970). The gel ran for 1 hour at 200 V. The molecular weights of protein bands were determined using standard makers (Bio- Rad California, USA).

Cell viability of 3T3 fibroblast and HaCaT keratinocytes cell culture

3T3 fibroblasts and HaCaT keratinocytes were donated by the Laboratory of Immunochemistry of Proteins of the UFMG and used to evaluate the cytotoxicity of L17. The cells were cultured in cell culture flasks T-75 (Thermo) with Dulbecco's Modified Eagle's medium (DMEM, Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich), 0.2% gentamycin (Gilbco by Life Technologies) and maintained at 37°C with 10% CO_2. 3T3 and HaCaT cells were counted in Neubauer chamber (10^4 cells /well) and seeded in 96 well plates for 24 h at 37°C and 10% CO_2, then were incubate in different concentrations of the L17 or the crude venom (20-1.25 µg / ml). After the incubation period, the cells were tested with Alamar Blue® to assess cell viability (Damico et al., 2007, with modification). Alamar blue was diluted in DMEM with 10% v/v concentration and added to the wells, then incubated for 3 hours and values were measured on Synergy 2 (Bio-tek) fluorome, 540 nm for excitation and 590 nm for emission.

HaCaT keratinocytes proliferation

HaCaT keratinocytes were Cultured in cell culture flasks T-75 (Thermo) with Dulbecco's Modified Eagle's medium (DMEM, Sigmae Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich), 0.2% gentamycin (Gilbco by Life Technologies) and maintained at 37°C with 10% CO_2. After cell counting with Neubauer chamber (10^4 cells /well), HaCaT keratinocytes were planted in 96-well plates for 24 h at 37 °C and 10% CO_2. Then the cells were incubated with two different concentration of the crude venom or L17 (20-10 µg/ml) for 24, 48 or 72 hours. After the incubation period, the cells were tested with Alamar Blue® to assess cell viability (Damico et al., 2007, with modification). Alamar blue was diluted in DMEM with 10% v/v concentration and added to the wells, then incubated for 3 hours and values were measured on Synergy 2 (Bio-tek) fluorome, 540 nm for excitation and 590 nm for emission.

Animals

Mice Swiss-Webster (n=36), males, 7 week ,weighing 25 ± 30 g, were obtained from the Central Animal House of the Health and Biological Sciences Center, Federal University of Viçosa, Brazil. Mice were maintained for 12 h under light/dark conditions (temperature: 22±2°C, humidity: 60–70%), and were given appropriate diet and water *ad libitum*. All animals were maintained in individual cages, cleaned daily, under controlled environmental conditions. The University Animals Ethics Committee approved all procedures (registration No. 365/2015).

Experimental designed

Mice were anesthetized by intra peritoneal injection of Pentobarbital (70 mg/kg). Dorsolateral shaving of the animals was performed, and the area was degreased with ethyl ether (Merck®, Rio de Janeiro, Brazil), followed by using 70% ethanol and 10% povidone-iodine for antisepsis (Johnson Diversey ®,Rio de Janeiro, Brazil). A wound of 10 mm in diameter, for secondary intention wound closure, were made by surgical incision in the skin and subcutaneous tissue with a scalpel, until the dorsal muscular fascia was exposed. The mice were randomized into four groups with nine animals in each: C group (vehicle control): saline solution 0.9 %; SS group (positive control): 0.6g of silver sulfadiazine cream (1%), L1 group: 5 µl of L17 diluted in water Mili Q (100 µl/ml); L2 group: 5 µl of L17 diluted in water Mili Q (200 µl/ml). In all groups, wounds were cleaned daily with 0.9% saline solution before each treatment. Wounds were topically treated daily for the 12 days. The wound tissue was collected each 4 days of three animals per group and then the animals were euthanized by cervical dislocation.

Calculation of the rate of wound contraction

The healing response of wound closure was evaluated by measuring the wound area every 4 days in digitized images with the dimensions of 320×240 pixels (24 bits/pixel) obtained using a digital video camera (W320, Sony, Tokyo, Japan). The wound areas were calculated by computerized planimetry using the Image Pro-Plus program version 4.5 (Media Cybernetics, Silver Spring, USA), previously calibrated. Wound Contraction Index (WCI) was calculated using the following ratio: (initial area of the wound (Ao) - area measured on a given day (Ai)/initial area of the wound (Ao)) ×100 (Gonçalves et al. 2016). The wounds were used to obtain measurements for contraction rate after 4, 8 and 12 days. The wound of the animals of the 12 days was selected for analysis of the progress of the healing process because it was from this wound that tissue was collected on the final day of the experiment.

Histologic and stereological analysis

Sections of skin were fixed in Karnovsky's solution for 24 h before embedding into paraffin (Leica Multicut 2045®, Reichert- Jung Products, Jena, Germany). Thin sections (5 mm) were cut and processed in a xilol and alcohol series. Section (5µm) was stained with H&E for analysis of fibroblast and blood vessel (Fisher et al., 2008). Sirius red (Sirius red

F3B, Mobay Chemical Co.,Union,N.J.,USA) was used for differentiating collagen fibers, according to the differential property of birefringence since thick. Collagen fibers (type I) appear in shade so bright colors ranging from red to yellow, and thin reticular fibers (collagen type III) appear bright green, under polarization. (Sigma, St. Louis, Mo, USA) (Junqueira et al., 1979; Lattouf et al., 2014).

Sections were analyzed through image examination. Images were captured using a BX-60® bright-field microscope (Olympus, Tokyo, Japan) connected to QColor-3® digital camera (Olympus). A total tissue area of $6.21 \times 10^6 \mu m^2$ was examined. Ten histological fields were randomly sampled in each skin section using a 20 objective lens. A grid containing 500 points within a standard test area (AT) of 73×10^3 μm^2 a tissue level was super imposed over each image. Stereological parameters of volumetric density (Vv) were calculated by counting the points over fibroblasts, blood vessels and type I and type III collagen, using the ratio: Vv=PP/PT, in which PP is the number of points occurring over the structure of interest, and PT is the total number of points on the test system (Novaes et al., 2015). Scar tissue sections stained with toluidine blue were used for mast cells identification (Leclere et al., 2006). Using a 20 objective lens, 10 histological fields were randomly analyzed in each histological section to obtain a total area of 6.21×10^6 μm^2. The number of mast cells per unit histological area was calculated according to the relation QA= Σ mast cells / AT (De La Cerda, 2003).

Biochemical analysis

Fragments of tissue were collected from each wound, rapidly frozen in liquid nitrogen and stored in freezer –80°C. Samples of these fragments were homogenized in phosphate buffered saline (PBS) and centrifuged at 5°C. The supernatant was used for analysis of Superoxide dismutase (SOD) measured according with the protocol described by Siddiqui et al. (2005). Catalase (CAT) measured according with the protocol described by Aebi's method (Aebi 1984) and modified by Pieper et al. (1995) (Gonçalves et al. 2016). Malondialdehyde (MDA) was measured in accordance with the protocol described by Nozaki et al., (2004) and Carbonyl proteins analyzed according with the protocol described by Dalle- Donne et al., (2014). The biochemical data were normalized in relation to total protein levels in the supernatant, quantified according to the Bradford method (Bradford, 1976).

Statistical analysis

Results obtained are expressed as mean ± SEM. Comparisons among groups with parametric distribution (Newman–Keuls) were performed using one-way ANOVA (SOD, CAT, MDA, and Carbonyl proteins). For non-parametric distributed data, a Kruskal–Wallis test followed by one-way Analysis of Variance on Ranks was performed (collagen fibers, blood vessels, cellularity and mast cells). Statistical significance was set at $p<0.05$. The analyses were performed using the GraphPad Prism 5.0® (GraphPad Software Inc., San Diego, Calif., USA).

RESULTS

L17 of *Lachesis muta muta*

The fraction L17 had a concentration of 2.92 µg/µl. The identification of the molecular mass by SDS-PAGE band identification allowed us to observe a band at the wheight between 12 and 17 KDa (Figure 1A and 1B).

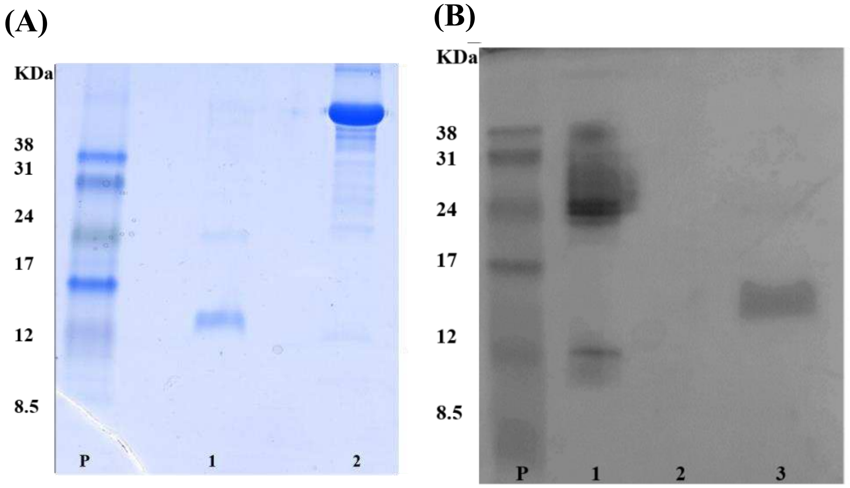

Figure 1. SDS-PAGE crude venom and the fraction 17 *of L. m. muta* samples (40µg) analyzed in 15% polyacrylamide gel. Stained with Colloidal Coomassie Brilliant blue (A): P: Molecular weight pattern, 1: L17 (40µg), 2:BSA concentration pattern (40 µg) and silver (B): P: Molecular weight pattern 1: Crude Venom; 2: L17 not reduced condition and 3: L17 reduced condition.

Cell viability and cell proliferation

In 3T3 fibroblasts, all concentrations of the crude venom exhibited cytotoxic activity, whereas L17 did not affect cell viability ($p<0.0001$). However, in HaCaT keratinocytes with a higher concentration (20µg/ml) of the crude venom a viability rate was of 59% ($p<0.001$), on the other hand HaCaT keratinocytes treated with L 17 had a viability rate of 100 % in all concentrations tested (Figure 2A and 2B).

In relation HaCaT keratinocytes cell proliferation in the concentration of 10 µg/ml of L17, the cells showed progressive increased in the cell proliferation when compared to crude venom ($p<0.0001$). In contrast the cells treated with L17 at 20 µg/ml did not present a progressive increase in cellularity, but maintained the cell viability ($P<0.0001$). Cells treated with crude venom showed only an increase in cell density at 72 hours with 10 µg/ml of the venom (Figure 2C).

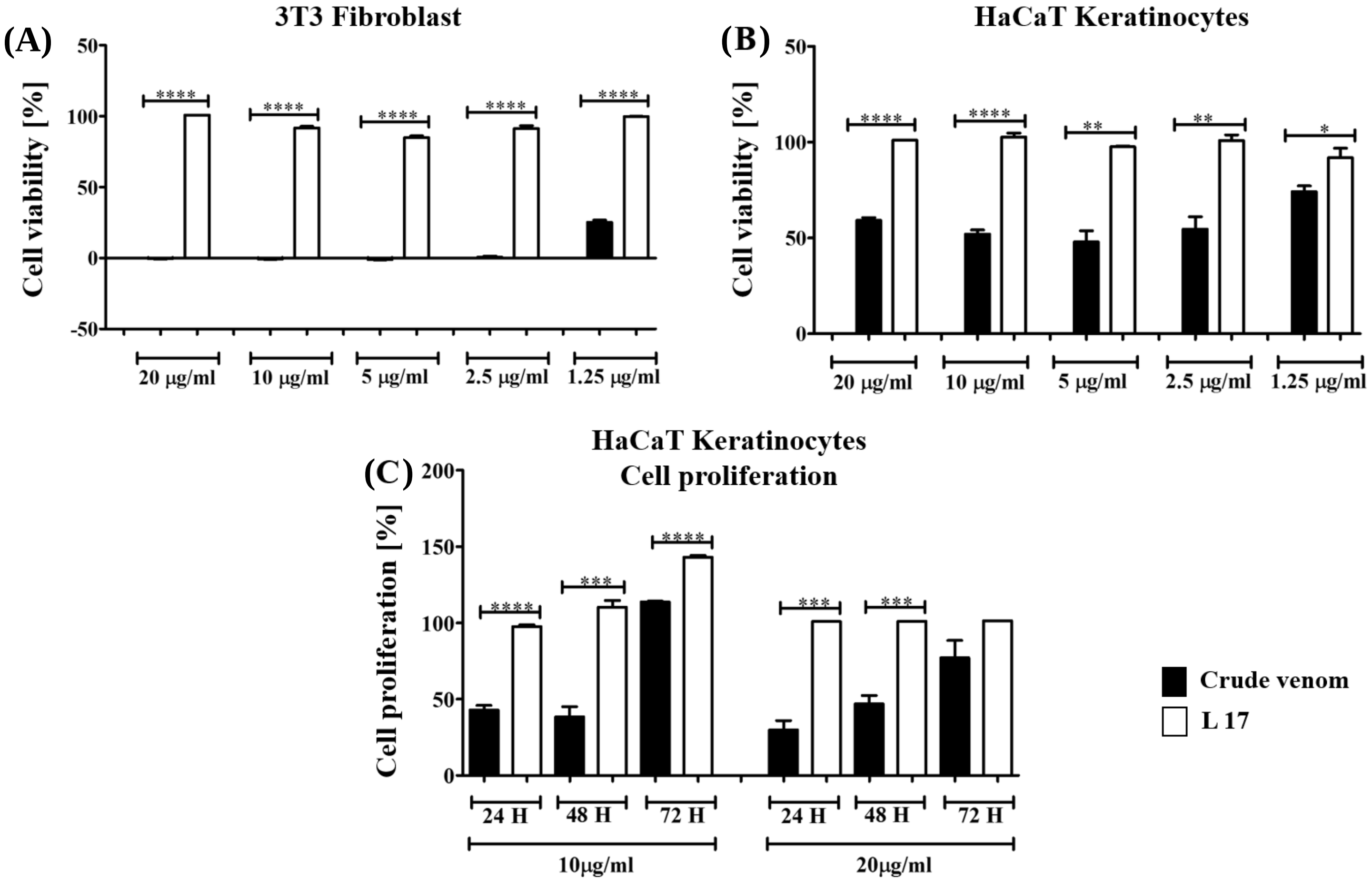

Figure 2. Cell survival percentage of 3T3 fibroblast (1x10^4 cells) (A) and HaCaT keratinocytes (1x10^4 cells) (B), after 24 h with treatment of crude venom or L17 fraction of *L. m. muta* at different concentrations. Percentage of cell proliferation of HaCaT keratinocytes (C) after treatment of crude venom or L17 of *L. m. muta* at 24, 48 and 72 h. Cell viability and proliferative profile was analyzed by Alamar Blue® assay. Data were represented as mean ± SEM and statistical differences between Groups ($P<0.05$ *) ($P<0.01$ **) ($p<0.001$ ***) ($p<0.0001$ ****) by Tukey test.

In vivo experiments

Area and contraction of the wound

Wound area was significantly reduce in the L1 group in the days 8 and day 12 ($p<0.01$) compared with the C group. While the WCI was significantly higher in the L1 group, compared with the C group in the days 8 and 12 ($p<0.05$).

Table 1. Area (mm^2) and wound contraction rate (mm^2 / day) in mice treated with L17.

DAY	Area/Contraction	C	SS	L1 (100 µg/ml)	L2 (200 µg/ml)
0	Area (mm^2)	110.00±0.00	100.00±0.00	105.00±0.00	108.00±0.00
	WCI (%)	00.00±00.00	00.00±00.00	00.00±00.00	00.00±00.00
4	Area (mm^2)	76,66±9,42	83,33±9,42	60,0±21,60	80,00±8,16
	WCI (%)	30,30±10,49	16,66±11,54	42,85±25,19	25,92±9,25
8	Area (mm^2)	70,0±16,32**	53,33±12,47	23,33±4,71**	46,66±12,47
	WCI (%)	36,36±18,18*	46,66±15,27	77,77±5,49*	56,79±14,14
12	Area (mm^2)	20±00.00**	13,33±4,71	6,66±4,71**	11,66±2,35
	WCI %)	81,81±00.00*	86,66±5,77	93,65±5,49*	89,19±2,67

Wound area and wound contraction index (WCI) for 12 days of treatment. C group: saline solution (0.9%), SS group: Silver sulfadiazine (1%), L1 group: L17 (100 µg/ml), L2 group (200 µg/ml).Data were represented as mean ± SEM and statistical differences between Groups (P<0.05*) (P<0.01**) (p<0.001***) Tukey test.

Cellularity and blood vessel

At day 4, L17 treatment presented a significantly higher number of cells compared to the SS group (group L1 $p<0.05$ and L2 group ($p<0.01$).At the 8 day the L2 group significantly increase it cellularity when compared with C and SS group ($p<0.001$) and with the L1 group ($p< 0.05$). On day 12 the cellularity in the treatment groups decreased significantly compared to the control group ($p<0.001$), L2 Group was significantly lower that the SS group ($p<0.001$). (Figure 3A).

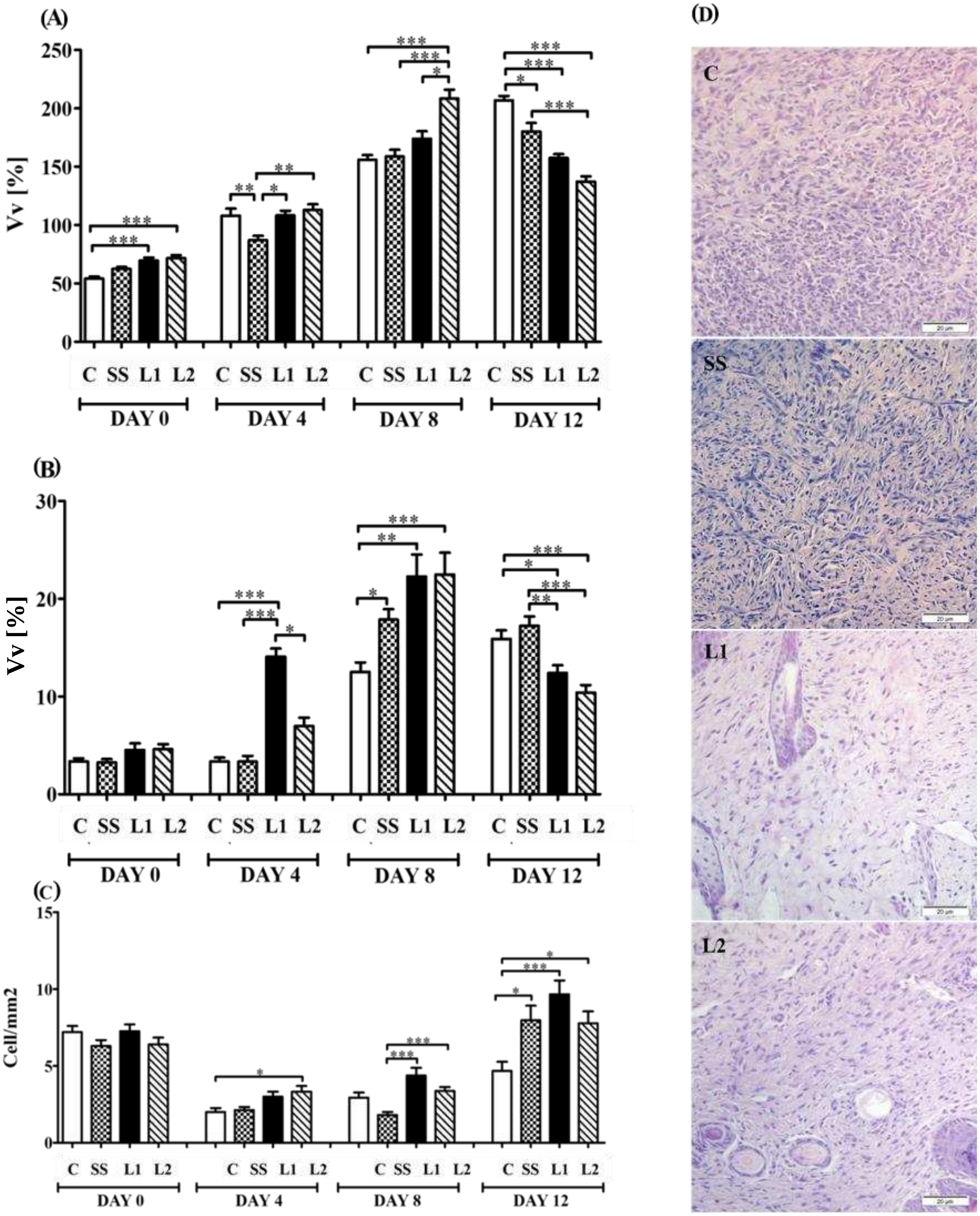

Figure 3. Proportion of cells **(**A), Proportion of blood vessels (B) and Proportion of mast cells (C) in the scar tissue in different days. Tissue fragments were collected every 4 days. C group: saline solution (0.9%), SS group: Silver sulfadiazine (1%), L1 group: L17 (100 µg/ml), L2 group (200 µg/ml). Data were represented as mean ± SEM and statistical differences between Groups (P<0.05 *) (P<0.01 **) (p<0.001 ***) Dunn´s multiple comparison test. Representative photomicrographs of showing the cells and blood vessel distribution in the day 12 in the scar tissue of the mice (D).

In relation of vascularization, after 4 days, L1 group had an increase in blood vessel density compared to C ($p<0.01$), SS ($p<0.01$) and L2 ($p<0.05$) groups. At day 8, blood vessels increased significantly in L1 group ($p <0.01$) and L2 group ($p<0.001$) and the SS group ($P<0.05$), in relation to the control group. Whereas on day 12 the density of blood vessels increased significantly in the C group compared with L1 ($P<0.05$) and L2 ($P<0.001$) groups and SS group also increased compared to L1 ($p<0.001$) and L2 ($p<0.0001$) groups (Figure 3B). The date showed in the figure 3 were demonstrated for Representative photomicrographs (Figure 3D).

Mast cells in the tissue

At day 4 there was a significant increase of mast cells in L2 group compared to control group ($p<005$). L1 and L2 groups also increased significantly at day 8 compared to the SS group ($p<0.001$). At day 12, the number of mast cells for the SS, L1 and L2 groups remained high in the L17 treated groups when compared to control (Figure 3C).

Collagen deposition in the tissue

L17 proved been effective in increasing type III collagen deposition. At day 4, L1 and L2 groups were significantly higher than C group ($p<0.001$), likewise SS was higher than C group ($p<0.05$). At day 8, the SS group had greater deposition of collagen, than the C ($p<0.001$), L1 ($p<0.001$) and L2 ($p<0.01$) groups. At day 12, L1 group was significantly larger compared to C group ($P<0.001$) and L2 ($p<0.05$), but did not have significant differences compared to SS group (Figure 4A).

Type I collagen deposition also was greater in the L17 groups. On day 8 of treatment L1 group presented a significant increase compared to C ($p<0.05$) and SS ($p<0.001$) groups. While L2 group was significantly higher compared to C and SS ($p<0.001$) groups. At day 12, L1 group presented higher type I collagen deposition when compared to C and SS groups ($p<0.001$). On the same day L2 group was significantly higher than the SS ($p<0.01$) and C ($p<0.001$) groups (Figure 4B). The date showed in the figure 4 were demonstrated for Representative photomicrographs (Figure 4C).

Antioxidant activity

The SOD levels increased on the 4 day in L1 Group than L2, SS ($p<0.001$) and C ($p<0.001$) groups, while C group was significantly higher than SS ($p<0.001$) and L2 ($p<0.05$), and L2 group increased SOD levels on day 8, compared with the other group ($p<0.001$). On day 12, SOD levels were not significantly different between groups (Figure 5A).

The CAT levels increased on the 8 day in L2 compared to L1 ($p<0.001$), SS ($p< 0.01$) and C ($p<0.05$) groups, however, C group was higher compared to L1 group ($p<0.05$). On day 12, CAT levels of L2 group remained significantly higher compared to the SS group ($p<0.05$) and L1 group ($p<0.05$) (Figure 5B). MDA levels increased On day 4 in SS group in relation to L1, L2 and C ($p<0.001$) groups, that presented low levels. At day 12, C group increased their MDA levels compared to L1, L2 (p<0.01) and SS (p<0.05) groups (Figure 5C).

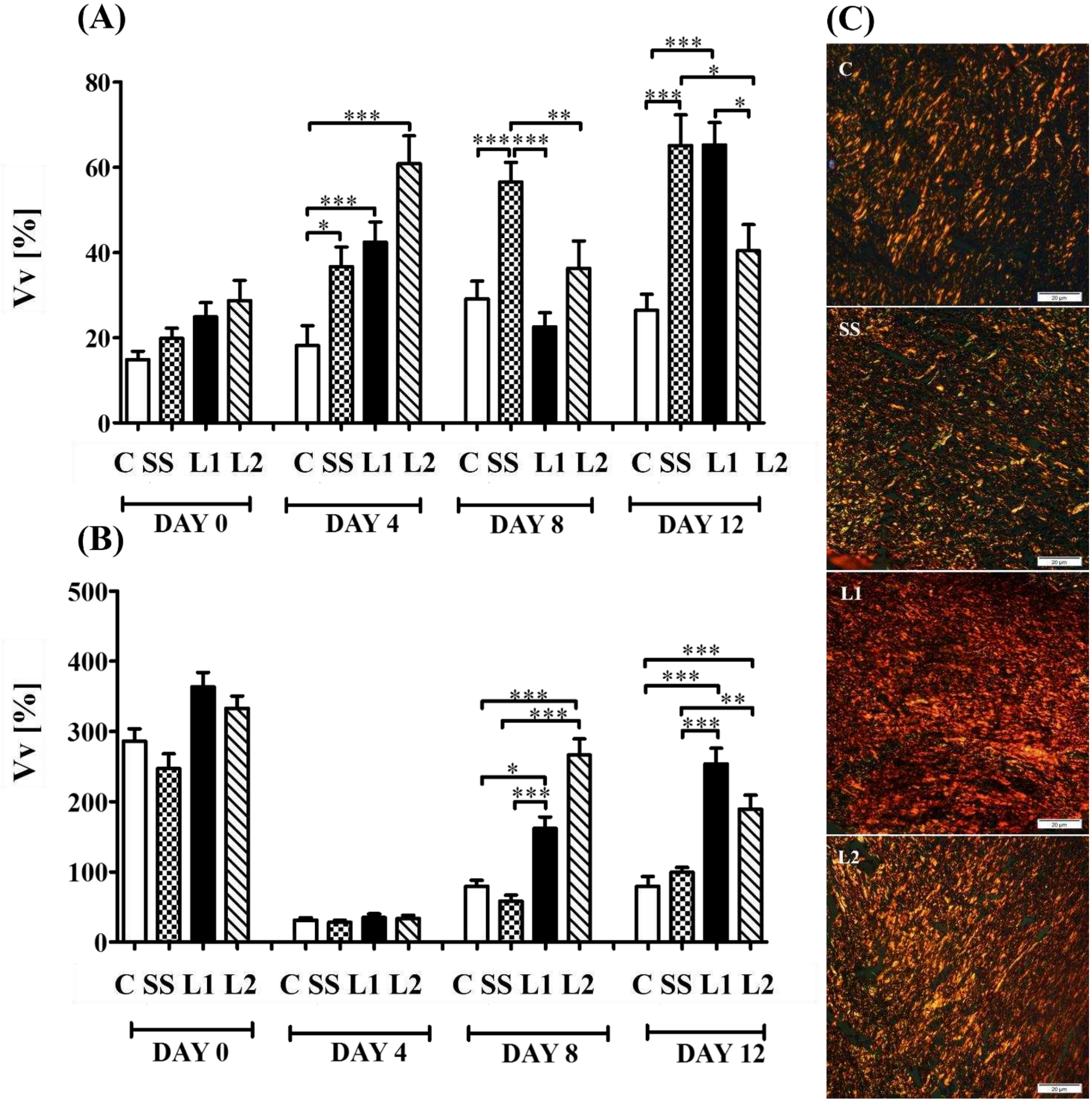

Figure 4. Proportion of type III collagen fibers (A) and type I collagen fibers (B). Tissue fragments were collected every 4 days. C group: saline solution (0.9%), SS group: Silver sulfadiazine (1%), L1 group: L17 (100 µg/ml), L2 group: (200 µg/ml). Data were represented as mean ± SEM and statistical differences between Groups (P<0.05 *) (P<0.01 **) (p<0.001 ***) Dunn´s multiple comparison teste comparison test. Representative photomicrographs of collagen distribution in the day 12 of6 scar tissue of the mice (C). Collagen fibers (type I) appear in shades of bright colors ranging from red to yellow and thin reticular fibers (collagen type III) appear bright green.

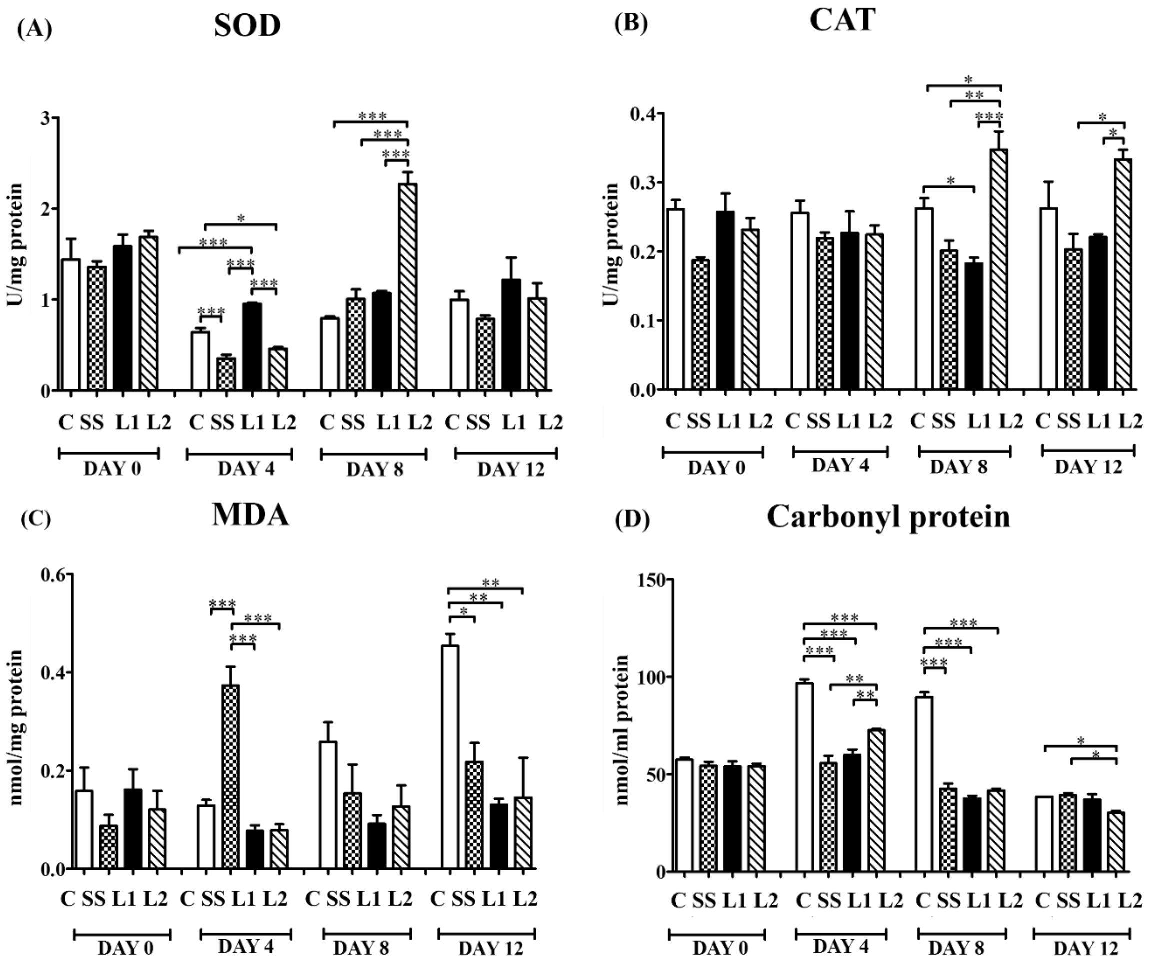

Figure 5. Levels of Superoxide dismutase (SOD) (A), Catalase (CAT) (B), malondialdehyde (MDA) (C) and carbonyl proteins (CP) (D) in the scar tissue of mice. Tissue fragments were collected every 4 days. C group: saline solution (0.9%), SS group: Silver sulfadiazine (1%), L1 group: L17 (100 µg/ml), L2 group (200 µg/ml). Data were represented as mean ± SEM and statistical differences between Groups ($P<0.05$ *) ($P<0.01$ **) ($p<0.001$ ***) Tukey test.

On the other hand, the levels of carbonylated protein (CP) in the groups treated with fraction L17 were lower than in the control groups. At day 4, the C group had the highest CP levels compared with the L1, L2 and SS ($p<0.001$) groups and the L2 group was significantly higher than L1 and SS ($p<0.01$) groups. On day 8, CP levels remained significantly higher in C group compared with the others groups (p<0.001) and on day 12, L2 group showed a significant decrease in CP compared to C and SS group (p<0.05) (Figure 5D).

DISCUSSION

The skin is the first barrier of protection of the body against external stimulus, however it can be affected by various factors such as burns, wounds, bacteria, viruses and different diseases that affect the homeostasis of the tissue and cause damage (Guo & DiPrieto, 2010). For this reason, recent research has focused on finding compounds that improve the repair process. In the present study, we investigated the effect of fraction 17 of *L. m. muta* on tissue in healing. Our work showed that L17 does not affect the viability of fibroblasts and keratinocytes; on the contrary, it promotes cell proliferation in keratinocytes. In addition, we found a decrease in wound area, an increase in cellularity and angiogenesis in the first days as well as a proliferation of non-degranulated mast cells at the end of treatment. L17 also stimulated the production of type I and type III collagen during the healing process and showed to have an antioxidant effect on the tissue by the increase of the levels of SOD and CAT, simultaneously diminishing the markers of oxidative stress MDA and CP, protecting the tissue of oxidative damage.

Fibroblasts are one of the main cells of the skin and are responsible for the synthesis of collagen and the reorganization of ECM (Darby et al., 2014). The increase of fibroblasts during the inflammatory and proliferative phase of the wound healing, increases the synthesis of collagen and accelerates the production of granulation tissue (Sriram et al., 2015) improving the wound healing process. Other cells that act in the process are keratinocytes, being the principal cells of the epidermis (Bazzoni & Dejana, 2002; Oshio et al., 2017). Studies report their activity in the neovascularization by the VEGF expression (Limat et al. 1991; Bao et al., 2009). Our *in vitro* assay showed that after 24 hours of treatment, cell metabolism of fibroblasts and keratinocytes was not inhibited with any of the concentrations of L17. We reported an increased numbers of keratinocytes in the culture after 24, 48 And 72 hours when treated with 10 µg of the fraction. These results are similar to those reported by Ramos et al. (2007) where, an alternagin-C extracted from the viperid *B. alternatus* promoted proliferation of endothelial cells. A phospholipase A2 (PLA_2S) from *L. m. muta* also presented proliferating activity in retinal ganglion cells culture (da Silva et al., 2011) suggesting proliferating activity in proteins isolated from viperid venoms.

Crude venom of viperids has been shown to have a cytotoxic effect on different cell lines (Zhang & Wei, 2007). *Lachesis sp*. venom was strongly cytotoxic on breast, pancreas and melanoma carcinogenic cell lines at low concentrations (Crecenti et al., 2002) while the total venom of *Vipera lebetina* provoked cytotoxicity in Human Umbilical Vein Endothelial Cells (Kakanj et al., 2015). Our work confirmed the cytotoxicity of the crude venom of *L. m. muta*, since in 3T3 fibroblast cells culture, 2.5 µg/ml of the crude venom provoked cytotoxicity in 100% of the cells, whereas, in HaCaT keratinocytes it was observed that with 2.5µg/ ml the cell viability was reduced only to 50%, showed a resistant against the venom. Other works reported that HaCaT keratinocytes showed a 70% a cell viability whit 60 µg/ml of *Montivipera bornmuelleri* venom (Sawan et al., 2017); Similarly with 20 µg/ml of the spider venom of *Loxoceles intermedia,* the viability was of 40% (Paixão-Cavalcante et al., 2006). Simultaneously the results found in our work at a concentration of 20 µg/ml over

HaCaT keratinocytes suggest that HaCaT cells lines present a resistance of different crude venoms. These results show the possible application of the isolated venom proteins of viperids in the proliferative processes, as well as, confirming the cytotoxic activity of this venom on different cell lineages, being a possible alternative as pharmacological treatment for several pathological processes.

Increased cellularity during progression of healing in mice Swiss-Webster treated with L17 confirms our *in vitro* results. In inflammatory phase, endothelial and immunological cells migrate to the wound area to produce cytokines and protect the tissue from invading agents, while fibroblasts and keratinocytes increase during the proliferative phase of the process and decrease during the remodeling phase to give way to the reorganization of the ECM (Guo & DiPrieto, 2010; Yates et al., 2012). Histological observations showed that L17 promoted cell proliferation on days 4 and 8 of the experiment, returning to normal levels on day 12. In control groups, we observed a greater inflammatory infiltrate during day 4 and the increase of cellularity in the Day 12. These results suggest that L17 promoted the migration and proliferation of cells to the lesion area during the inflammatory and proliferative stage, contributing in the production of ECM components and the migration of cells responsible for the chemiotaxic processes in the tissue. Other work confirmed our findings that the increased of the cellularity in the healing tissue enhance re-epithelialization accelerate wound healing (Kazemi- Darabadi et al., 2014; Jin et al., 2016) and regulate the process of remodeling of the tissue neo formatted by modulation of degradation proteins like metalloproteinases (Gill & Park, 2008). The reduction of cellularity in the animals treated with L17 in the final of the experiment, suggests a modulation on the proliferative and inflammatory processes in the late stage. Possibly by the activation of proteins in charge of the degradation of components of the ECM and control of cell, contributing to the remodeling of the new tissue (Toriseva et al., 2012).

The angiogenesis process is the main modulator of the nutrition and oxygenation of the tissues by the blood vessels production, allowing the mobilization of nutrients and the migration of immunological cytokines and chemokine on the wound (Zhang et al., 2016). L17 stimulated the production of blood vessels during days 4 and 8 of the experiment. The levels returned in the day 12, whereas the control groups showed only an increase in vascularization on the last day of the experiment. Mukherjee et al. (2014) showed the pro-angiogenesis activity of the isolated proteins snake venom with a peptide isolated of *Daboia russelii russelii* venom in *in vitro* assay alternagin-C of *B. alternatus* also induced vascularization increased *in vitro* and *in vivo* (Ramos et al., 2007). These results suggest L17 promotes the nutrition of scar tissue and accelerates the process by increasing vascularization.

Like fibroblasts and keratinocytes, other cells act in cutaneous tissue, such as mast cells (Wilgus, 2008). Although their role in the healing process is, still not well-defined, studies have suggested that these cells have an implication in neovascularization by promoting VEGF production (Puxeddu et al., 2003) and are the principal producer of VEGF in mice skin (Tellechea et al., 2016). Other authors suggest that increase of mast cell degranulation, may increase the inflammatory process (Kho & DiPietro, 2011; Wei et al., 2013). In our study, a high proportion of mast cells in the tissue was founded in normal tissue, this number decreased during the healing process, but increased again on day 12. During the late phase of healing, high number of mast cells on the edge of the wound stimulate the organization of collagen, participant in the remodeling of the tissue (Iba et al., 2004). Mast cell also promote the recruitment of neutrophils after an injury, and its absent are related with low number of neutrophils and delayed in the healing process (Shiota et al., 2010). Our results show that L17 promoted the proliferation of mast cells over the edge of the wound, contributing to the production of blood vessels and the reorganization of collagen in the final phases of the healing process in mice, and possibly, it is implicated in the recruitment of neutrophils, improving the remodeling process.

Another important component of the skin is collagen, the main component of ECM and responsible for providing tensile strength in tissue, also to modulate the migration of keratinocytes and fibroblasts (Fuentes-Calvo et al., 2012). Our histological analysis showed that L17 increased type III collagen deposition during days 4 and 12 of the scar tissue, while type I collagen increased during days 8 and 12. Normal tissue presents a high amount of type I collagen, but during the healing process this is almost absent in the early stages of wound healing, being replaced by type III collagen and restoring its level at the end of the healing process (Jiang et al., 2016). These data agree with the fact that type I collagen is considered the mature collagen of the skin, provided strength to the tissue, while the type III collagen is an immature collagen and does not provide enough force to remodel the tissue, been replaced at the end of the healing process (Broder et al., 2013). Other viperid proteins have promoted the production of collagen and the reorganization of scar tissue as the reported for Sant'Ana et al. (2011), showing an increase in the deposition of type I collagen at a concentration of 60 ng of C-alternus of *Bothrops alternatus*. L17 of *L. m. muta* accelerates the process of maturation of collagen, providing strength to the tissue, contributing to a better remodeling process.

During pathological processes, there is an increase in oxidative stress due to the production of free radicals (Monga et al., 2013). At the same time, the organism has developed mechanisms to control the damages caused by different oxidizing agents (Lu et al., 2010). However, this balance is affected when ROS levels are not controlled, causing macromolecule damage and Deregulating cellular processes (Deavall et al., 2012). Several enzymes act to maintain control over these stress processes. SOD and CAT are antioxidant enzymes responsible for oxidative maintenance in tissues. The increase in SOD synthesis accelerates the skin healing process (Shukla., 1999) and promotes fibroblast migration and proliferation due to H_2O_2 catalysis, increasing the tensile strength of the tissue in formation (Fukai & Ushio-Fukai, 2011). The fibroblast activity may be affected by ROS increase (Trachootham et al., 2010), that can causing delays in the wound healing processes due to disruption in cell migration and proliferation processes (Schäfer & Werner, 2008). We measured the antioxidant effect of L17 *in vivo.* Animals treated with 200 µg of L17 increased levels of SOD during days 4 and 8 and of CAT during day 12, which was also activated by 100 µg of L17. Parallel levels of MDA and CP were low in the L17 treated groups. Several studies have reported the oxidative effect of snake venoms on different tissues (Al-Quraishy et al., 2014; Asmari et al., 2015). Thoamy et al. (2014) reported that the total venom of *Naja ahaje* increases the production of free radicals in hepatic and renal tissue. In contrast, studies by Das et al., (2011) reported the antioxidant activity of the purified NN-32 fraction of *Naja naja* on the production of lipid peroxidation by the reduction of MDA and the increase of

SOD and CAT activity. Our results suggest that the increase of SOD and CAT in the animals treated with L17 possibly promoted the protection against the action of ROS and consequently promoted decreased the levels of MDA and CP on the tissue, suggests having antioxidant activity in the healing process, thus contributing to a tissue protection and promoting the acceleration of the healing process.

CONCLUSION

Our work showed increased cellularity *in vitro* and *in vivo* experiment, L17 increased angiogenesis in the early days of the healing process and the synthesis of collagen, mainly type I. L17 promoted the migration of mast cells at the wound edge possibly and also promoted antioxidant protection to the tissue by promoting increase in the levels of SOD and CAT and decrease in the variables of MDA and CP. L17 of *L. m. muta* is a promising resource in the search for active compounds of animal origin as healing treatment. Other studies must be performed to elucidate the molecular mechanisms of its activity.

Conflict of interest statement

The authors have no conflict of interest with the publication of this work.

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