Núbia Braga Pereira

Efeitos do veneno de *Loxosceles similis* no modelo de implante subcutâneo de esponja em camundongos *Swiss*

Faculdade de Medicina

Tese de doutorado

Efeitos do veneno de *Loxosceles similis* no modelo de implante subcutâneo de esponja em camundongos Swiss

Tese apresentada ao curso de Pós-graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Patologia.

Área de concentração: Patologia investigativa

Orientadora:
Profa. Dra. Luciana Moro
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Fevereiro de 2014

ATA DA DEFESA DE TESE DA ALUNA

NUBIA BRAGA PEREIRA

Realizou-se, no dia 17 de fevereiro de 2014, às 13:30 horas, Sala Prof. Nello Rangel K3 - 163 / ICB - UFMG, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *Efeito do veneno bruto da Loxosceles similis no modelo de implante subcutâneo de esponja em camundongos Swiss*, apresentada por NUBIA BRAGA PEREIRA, número de registro 2010675562, graduada no curso de C. BIOLÓGICAS, como requisito parcial para a obtenção do grau de Doutor em PATOLOGIA, à seguinte Comissão Examinadora: Prof(a). Luciana Moro - Orientador (UFMG - Faculdade de Medicina), Prof(a). Roselene Ecco (UNIVERSIDADE FEDERAL DE MINAS GERAIS), Prof(a). Milene Alvarenga Rachid (UFMG), Prof(a). Mary Suzan Varaschin (Universidade Federal de Lavras, Departamento de Me), Prof(a). Paulo Eduardo Alencar de Souza (Pontifícia Universidade Católica de Minas Gerais).

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.
Belo Horizonte, 17 de fevereiro de 2014.
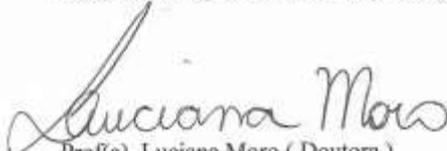
Prof(a). Luciana Moro (Doutora)

Prof(a). Roselene Ecco (Doutora)

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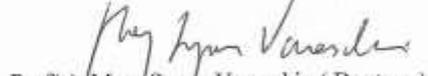
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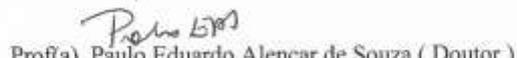
Prof(a). Paulo Eduardo Alencar de Souza (Doutor)

Ainda que eu falasse as línguas dos homens e dos anjos, e não tivesse amor, eu nada seria....

1 coríntios 1:14

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RESUMO

CAPÍTULO 1

O envenenamento por aranhas do gênero *Loxosceles* provoca um conjunto de sinais e sintomas, chamado de loxoscelismo, que na maioria dos casos manifesta-se através do quadro dermonecrótico. O modelo mais utilizado para o estudo do loxoscelismo cutâneo é o coelho. No entanto, o desenvolvimento de um modelo animal de menor porte, fácil manuseio, menor custo e manutenção são necessários para o estudo da patogênese do loxoscelismo. A inflamação provocada pelo veneno bruto de *Loxosceles similis*, foi avaliada considerando a ativação de neutrófilos e macrófagos, vasodilatação, hiperemia, edema, hemorragia e a produção do TNFα e VEGF utilizando o modelo murino de implante de esponjas. Trinta e dois camundongos Swiss, machos (6-8 semanas) foram implantados subcutaneamente com discos de esponjas de poliéster-poliuretano. Quatorze dias após a implantação os animais foram separados em dois grupos: (1) grupo controle: 16 camundongos que receberam intra-implante 30 µL de solução salina; (2) grupo tratado: 16 camundongos que receberam a injeção intra-implante de 30 µL (0.5 µg) do veneno bruto de *L. similis*. Os animais foram eutanaziados com xilazina/quetamina 1h e 4h pós-injeção intra-implante. Microscopicamente, os implantes dos grupos tratados apresentaram inflamação aguda caracterizada por: infiltrado neutrofílico, edema, vasodilatação, hiperemia e hemorragia intensa. Alguns vasos apresentaram-se com as suas paredes rompidas. Através da análise morfométrica foi possível observar que a área de vasos foi significativamente maior nos grupos tratados. Os parâmetros bioquímicos como: conteúdo de hemoglobina, níveis das citocinas *fator de necrose tumoral* (TNFα), *fator de crescimento endotelial* (VEGF), atividade das enzimas n-acetil- β-glucosaminidase (NAG) e mieloperoxidase (MPO) foram significativamente maiores nos grupos tratados. Os efeitos do veneno de *Loxosceles* no tecido de granulação do implante em camundongos foram similares ao que se observa no loxoscelismo cutâneo em outras espécies (humanos ou coelhos). Consequentemente, o modelo murino de implante de esponjas promove um novo método para investigação de mecanismos celulares e moleculares associados com o loxoscelismo cutâneo.

Palavras chaves: Loxoscelismo, implante de esponja, *Loxosceles, Loxosceles similis*

ABSTRACT

CHAPTER 1

Envenomation by *Loxosceles* spider bite leads to a set of signs and symptoms, called loxoscelism, which in most cases manifests through the dermonecrotic frame. The development of a smaller size animal model, of easy handling and maintenance, and lower cost is needed to study the loxoscelism pathogenesis. The inflammatory effects of the *Loxosceles similis* crude venom was evaluated considering neutrophil and macrophage activation, vasodilatation, hyperhaemia, edema, hemorrhage and TNF-α and VEGF production using the murine sponge implant model. Thirty two, male, Swiss mice (6–8 weeks old) were implanted subcutaneously with polyether–polyurethane sponge discs. Fourteen days post implantation, animals were separated into two groups: (1) control group–16 mice received 30 µL of saline intra-implant; (2) treated group-sixteen mice injected with 0.5 µg/30 µL of *L. similis* crude venom intra-implant. The animals were euthanized with xylazine/ketamine after 1 and 4 h post-injection. Microscopically, implants of the treated groups presented an acute inflammation characterized by: neutrophilic infiltrate, edema, vasodilatation, hyperhaemia, and severe hemorrhage. Some vessels presented ruptured walls. Under morphometric analysis, vessel area was bigger in the treated groups compared with the control ones. The biochemical parameters, hemoglobin content, levels of the cytokines *tumor necrosis factor* (TNFα) and vascular endothelial growth factor (VEGF), inflammatory enzyme activities n-acethyl-β-glucosaminidase (NAG) and myeloperoxidadase (MPO) were also significantly higher in the venom-treated groups. The effects of *Loxosceles* venom in the granulation tissue of the implant in mice were similar to those observed in cutaneous loxoscelism in other species (human and rabbits). Consequently, the murine sponge implant model provides a new method to investigate cellular/molecular mechanisms associated with cutaneous loxoscelism.

Keywords: Loxoscelism, sponge implants, Loxosceles, *Loxosceles similis*.

RESUMO

Capítulo 2

O envenenamento por aranhas *Loxosceles* conduz a um conjunto de sinais e sintomas, denominado loxoscelismo, que na maioria dos casos se manifesta sob o quadro dermonecrótico. Neste estudo, utilizou-se o modelo de implante subcutâneo de esponja em camundongo para descrever os efeitos do veneno de *Loxosceles similis*, com ênfase em 24 h após a injeção intraimplante e para avaliar a patogenia do loxoscelismo 1, 4 e 24 horas após a injeção do veneno. O presente trabalho tem como objetivos: (1) caracterizar histologicamente os efeitos do veneno bruto de *L. similis* no implante subcutâneo de esponjas; (2) quantificar os mastócitos do implante e mensurar sua atividade de desgranulação; (3) quantificar os subtipos de colágeno tipo I e III; (4) verificar, quantificar e avaliar a importância da apoptose no implante 1, 4 e 24 horas após a injeção do veneno na patogenia do loxoscelismo. Trinta camundongos *Swiss* (6-8 semanas, machos) foram implantados subcutaneamente com esponjas de poliéster-poliuretano. Após o 14° dia de implante, os animais foram divididos em seis grupos (n=5 para cada grupo): três grupos controles (C1h, C4h e C24 h), no qual receberam a injeção intraimplante de 30μL de solução salina e três grupos tratados (T1h, T4h e T24 h), no qual receberam a injeção intraimplante de 30μL (0,5 μg) do veneno de *L. similis*. Após os intervalos de 1, 4 e 24 horas os animais foram eutanaziados, os implantes foram colhidos e processados para microscopia de luz e eletrônica. À microscopia de luz observou-se, nos implantes injetados com veneno, inflamação aguda com intenso edema, presença de trombos ocludentes, vasculite, aumento dos índices de mastócitos e de desgranulação de mastócitos e de apoptose de células gigantes. Além disso, observou-se degradação de colágeno dos tipos I e III. As análises das ultraestruturas evidenciaram-se vários tipos celulares em apoptose. Os presentes resultados sugerem que a apoptose, a desgranulação de mastócitos e a degradação do colágeno são importantes na patogenia do loxoscelismo e sua ocorrência em alguns tipos celulares pode explicar a dificuldade no reparo da úlcera que comumente se observa no loxoscelismo.

Palavras chaves: Loxoscelismo, apoptose, implante de esponjas, desgranulação de mastócitos.

ABSTRACT 2

CHAPTER 2

Envenomation by the *Loxosceles* spider causes loxoscelism, a pattern of signs and symptoms that primarily manifests in the dermonecrotic form. Our studies have shown that a mouse subcutaneous sponge implantation model may be useful in evaluating the effects of *Loxosceles similis* venom. This model provides an ideal microenvironment in which to study loxoscelism; however, it is still important to evaluate its pathogenesis and to observe the effects of *L. similis* venom for longer time periods than those in previous studies of this model. The aims of this study are: (1) to histologically characterize the effects of *L. similis* crude venom in a subcutaneous sponge implant; (2) to quantify the mast cells present in the implant and to measure their degranulation activity; (3) to quantify collagen subtypes I and III; and (4) to verify, quantify, and evaluate the effects of apoptosis in the implant on the pathogenesis of loxoscelism at 1h, 4h, and 24 h after injecting the venom. Thirty *Swiss* mice (6-8 weeks, male) were subcutaneously implanted with polyester-polyurethane sponge discs. Fourteen days post-implantation, the animals were divided into six groups (5 animals per group): three control groups (C1h, C4h, and C24h), in which the mice received 30 µl injections of intra-implant saline, and three treated groups (T1h, T4h, and T24h), in which the mice received 30 µL (0.5 µg) injections of *L. similis* crude venom at 1h, 4h, and 24 h intervals. After each time interval, the animals were euthanized, and the implants were harvested and processed for light and electron microscopic analyses. The following results were observed in the implants harvested from the treated groups: acute inflammation with marked edema, thrombus, and vasculitis, as well as increased levels of mast cells and mast cell degranulation, and apoptosis in giant cells. Furthermore, degradation of collagen types I and III was observed. An analysis of the ultrastructure revealed apoptosis in various cell types. The present results suggest that apoptosis in some cell types associated with an increase in mast cell degranulation and the degradation of collagen fibers are important in the pathogenesis of loxoscelism therefore may explain the difficulty in repairing the ulcer is commonly observed in severe cases of loxoscelism cutaneous in humans.

Key words: Loxosceles, Loxoscelism, Sponge implants, apoptosis

LISTA DE TABELAS E FIGURAS

LISTA DE ABREVIATURAS E SÍMBOLOS

L. similis	*Loxosceles similis*
L. laeta	*Loxosceles laeta*
L. anômala	*Loxosceles anômala*
L. gaucho	*Loxosceles gaucho*
Ml	Mililitro
MEC	Matriz extracelular
V	Voltagem
Kg	Quilogramas
µL	Microlitro
µm-	Micrometros
µg-	Micrograma
mM-	Milimolar
M –	Molar
HE-	Hematoxilina-eosina
PBS-	Do inglês *Phosphate buffered saline*
TBS –	Do inglês *Tris buffered saline*
TUNEL	Do inglês *Terminal deoxynucleotidyltransferase-mediated dUPT nick end-labelling* – Marcação *in situ* da fragmentação do genoma com terminal transferase de deoxinucleotídeo.

MPO	Mieloperoxidase
NAG	N-acetilβ-glucosaminidase
TNFα	Fator de Necrose Tumoral
VEGF	Fator de crescimento endotelial
CAPI –	Centro de Aquisição e processamento de imagens
UFMG –	Universidade Federal de Minas Gerais
ICB-	Instituto de Ciências Biológicas
SINAN –	Sistema Nacional de Agravos Notificáveis
FAPEMIG-	Fundação de Amparo à Pesquisa de Minas Gerais
BCL 2-	Do inglês *B cell lymphoma 2* – Gene do Linfoma de células B
P53-	Do inglês *Protein 53* – Proteína 53
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico

SUMÁRIO

Capítulo 3

Capitulo 4

Apresentação

Essa tese de doutorado será apresentada no formato alternativo de artigos, de acordo com os critérios estabelecidos pela pós-graduação (Manual de Padronização dos Volumes Físicos e Eletrônicos das Dissertações e Teses) do Programa de Pós-graduação em Patologia. Segundo esses critérios, o formato alternativo deve conter: (1) introdução e justificativa aos dois artigos (texto redigido em português segundo as normas da ABNT com o máximo de 15 páginas); (2) cópia dos dois artigos; (3) considerações finais e (4) anexos - comprovantes de aceite, documento de aprovação no Comitê de Ética em Pesquisa da área e certificado de qualificação. Para facilitar a compreensão e a organização, a presente tese foi subdividida em quatro capítulos.

O capítulo 1 contém um artigo intitulado "Modelo de implante subcutâneo de esponjas para o estudo do loxoscelismo cutâneo experimental", que foi publicado em 2012, na Revista *Toxicon*.

O Capitulo 2 contém um artigo intitulado "Apoptosis, mast cell degranulation and collagen breakdown in the pathogenesis of loxoscelism in subcutaneously implanted sponges", que foi publicado na revista *Toxicon* em Junho de 2014.

O Capitulo 3 contém outros artigos que foram publicados durante o meu doutorado, sobre o loxoscelismo, mas que não estão diretamente ligados ao projeto de tese.

O Capitulo 4 possui alguns anexos como: comprovante de qualificação e aprovação pelo comitê de ética

Capítulo 1

Implante de esponja de poliéster-poliuretano em camundongos *Swiss* como um modelo de estudo para o Loxoscelismo

1. Justificativa

1 JUSTIFICATIVA

O termo Loxoscelismo é utilizado para descrever o conjunto de sinais e sintomas decorrentes dos acidentes provocados pela picada de aranhas do gênero *Loxosceles*. Essas aranhas são conhecidas popularmente como "aranhas-marrons" ou aranhas "violino". Tais acidentes podem induzir dois tipos de quadros clínicos distintos: o quadro cutâneo ou dermonecrótico e o quadro cutâneo-visceral ou sistêmico (GAJARDO-TOBAR, 1966; SCHENONE *et. al.,*1989; BARBARO *et al.,* 1994; DA SILVA *et al.*, 2004).

O loxoscelismo no Brasil tornou-se um problema de saúde publica e relevância médica. Entre o período de 2001-2009 foram notificados 51.865 acidentes (PEREIRA *et al*., 2011; Ministério da Saúde, 2012). Em Minas Gerais, entre os anos de 2001 e 2009 foram notificados 921 acidentes loxoscelicos (PEREIRA *et al*., 2011, Ministério da Saúde, 2012). Somente em 2007 foram registradas 269 notificações no interior de MG (PEREIRA *et al.,* 2011). Provavelmente, acidentes com aranhas-marrons são ainda mais frequentes, mas não são devidamente diagnosticados e/ou registrados (MACHADO *et al.,* 2005).

Em Minas Gerais, há relatos de três espécies de *Loxosceles*: *L. similis, L. laeta* e *L. anomala* (GERTSCH, 1967; ÁLVARES *et al.,* 2004). Em Belo Horizonte, Machado *et al.* (2005) fizeram o primeiro registro de *Loxosceles similis* em residências.

Sabe-se que a *L. similis*, espécie já encontrada em residências de Belo Horizonte (MACHADO *et al.,* 2005) induz experimentalmente, lesão cutânea semelhante àquela relatada para outras espécies de importância médica (PEREIRA *et al*., 2012b; CHATZAKI *et al*., 2012). Pereira *et al.* (2012b) analisaram a pele de coelhos que foram injetados intradermicamente com 0,5 µg (30 µL) do veneno de *L. similis* em intervalos de 2, 4, 6 e 8 horas pós a injeção. Esses autores observaram que a apoptose de células endoteliais está presente a partir de 2 horas após a injeção do veneno, além de trombos nos plexos dérmicos. Xu *et al.* (2009) mostraram que há forte relação entre a apoptose endotelial, o desnudamento do endotélio e a ocorrência de trombose. Adicionalmente, Bombeli *et al.* (1997) sugeriram que a apoptose de células endoteliais contribuem para o desenvolvimento de um estado pró-trombogênico. Dessa forma, Pereira *et al*. (2012b) afirmam que a apoptose de células endoteliais está envolvida com a formação dos trombos e a consequente necrose e ulceração observadas no loxoscelismo. No entanto, falta explicar o mecanismo pelo qual ocorre a apoptose.

O modelo experimental comumente utilizado para o estudo do loxoscelismo cutâneo tem sido o coelho. Porém, pesquisas acerca da patogênese do loxoscelismo podem ser

facilitadas com o uso de um modelo de menor porte, baixo custo e de fácil manuseio e manutenção. Estudos mostram que o implante de esponja em camundongo induz a formação de um tecido de granulação altamente vascularizado que tem sido utilizado em outros estudos (CAMPOS *et al*., 2011; CASTRO *et al*., 2012).

Consequentemente decidiu-se explorar o modelo de implante de esponjas em camundongo, considerando a grande quantidade de vasos sanguíneos que nelas se desenvolvem. Desta forma, obtém-se uma espécie de cultivo de células endoteliais *in vivo* que pode ser submetido à ação do veneno. No momento em que a esponja apresenta uma grande densidade de células endoteliais, inocula-se o veneno de *Loxosceles,* o qual lesa as células endoteliais e a matriz-extracelular.

2. Introdução

2 Introdução

2.1 Aranhas

As aranhas pertencem a Classe Arachnida, Filo Arthropoda, subfilo Chelicerata e Ordem Araneae. Anatomicamente o que as diferenciam dos demais artrópodes são: (1) os quatro pares de patas, (2) a ausência de antenas e asas, (3) os pedipalpos que as auxiliam na manipulação e envolvimento da presa e (4) o par de quelíceras, que contém um canal para injetar o veneno (FOELIX, 2011). O corpo das aranhas é constituído por duas partes: uma porção anterior, chamada de prossoma ou cefalotórax e uma porção posterior, conhecida como opstossoma ou abdômen. O prossoma suporta uma carapaça convexa distinta, que na maioria das aranhas é composta por 8 ocelos anteriormente (FOELIX, 2011).

Todas as aranhas são predadoras e, com exceção da família Uloboridae, todas possuem glândula de veneno e este veneno é utilizado para matar ou paralisar a presa. As aranhas não são capazes de ingerir a presa inteira, sendo assim, sua digestão é externa, com a prévia liquefação da presa por enzimas digestivas (FOELIX, 2011).

A diversidade de aranhas é imensa, existem cerca de 40.000 descritas até o momento (PLATNICK, 2013). No entanto, poucas espécies estão relacionadas com acidentes humanos. Dentre as aranhas que possuem importância médica estão as do gênero *Latrodectus*, conhecidas popularmente como viúva negra e as do gênero *Loxosceles*, conhecidas popularmente como aranhas marrons ou aranhas violino. Ambas estão geograficamente bem distribuídas em quase todo o mundo. Outras aranhas que também são consideradas de importância médica, porém com uma distribuição geográfica menor, são as do gênero *Phoneutria* conhecidas popularmente como armadeira, endêmicas do Brasil, e as do gênero *Atrax* e *Hadronyche*, que são endêmicas na Austrália (ISBISTER & WHITE, 2004).

2.2. Gênero *Loxosceles*

O gênero *Loxosceles* é composto por aranhas de coloração uniforme, que variam entre o marrom claro e marrom escuro, dependendo da espécie. A *Loxosceles gaucho*, por exemplo, apresenta mancha clara, já a *Loxosceles laeta* apresenta mancha escura no cefalotórax. Seu comprimento corporal e das patas variam de 8 a 15 mm e de 8 a 30 mm, respectivamente. Os machos têm o corpo menor e patas mais longas que as fêmeas (**Figura. 1A**).

A característica desse gênero é o formato do cefalotórax, que lembra um violino (**Figura 1C**), o qual apresenta seis ocelos dispostos em pares, formando uma linha curva em forma de U (**Figura 1C**). Além disso, tais aranhas possuem glândulas apócrinas e aparelho inoculador de veneno, composto por um par de quelíceras (**Figura 1D**), que são diminutas (0,3 mm) e geralmente deixam no local da picada dois sinais justapostos, o que sugere ser a injeção do veneno intradérmica (FOIL & NORMENT, 1979; TRENTINI, 2005). O padrão dos ocelos é um importante critério para a identificação dessas aranhas, já que a maioria das aranhas tem 8 ocelos, mas ele por si só não pode ser o único critério já que outras aranhas possuem 6 ocelos como as *Scytodes e Sicarius* (FUTRELL, 1992; SWANSON & VETTER, 2006).

As aranhas marrons são artrópodes sedentários, que podem viver entre 3-7 anos e geralmente se alimentam de pequenos insetos tais como traças, baratas, moscas e cupins (TRENTINI, 2005). Elas são lucífugas e por isso possuem hábitos noturnos, preferindo lugares escuros, secos e quentes tais como: pedras, tijolos, telhas empilhadas, troncos de árvores, fendas de muros e cavernas. Elas têm sido encontradas também em construções civis e no interior de domicílios, abrigando-se em frestas de gavetas, armários, embaixo de móveis, dentro de galpões contendo entulhos e em garagens, onde encontram um ambiente ideal para a sua sobrevivência (GERTSCH, 1967; FUTRELL, 1992; MACHADO *et al*., 2005). A aranha marrom normalmente não é agressiva, a maior parte dos acidentes loxoscélicos ocorre quando este artrópode é pressionado contra a pele ou se sente ameaçado de alguma forma.

Figura 1- Espécimes de *Loxosceles* - (A) Fêmea (à esquerda) e macho (à direita); (B) Observar maiores detalhes; (C) cefalotórax em formato de violino; em maior aumento detalhes dos 3 pares de ocelos posicionados em U; (D) Detalhe do par de quelíceras. Os espécimes foram coletados no Parque Estadual do Itacolomi, em Ouro Preto/MG (fêmea) e em Nova Lima/MG (macho) e pertencem à Coleção taxonômica do Laboratório de Aracnologia da UFMG, cujo curador é o prof. Adalberto J. Santos. Eles estão catalogados como UFMG 3582 e UFMG 3612. As aranhas foram fotografadas por Bárbara Teixeira Faleiro do Laboratório de Aracnologia, especialmente para o presente trabalho.

2.3 Distribuição geográfica

O gênero *Loxosceles* é composto por 107 espécies, descritas até o momento, que podem ser encontradas em regiões temperadas e tropicais (**Figura 2**) da América do Norte, Central e do Sul bem como, na África, Austrália, Europa e Ásia (PLATNICK, 2013; SWANSON & VETTER, 2006; HOGAN *et al*., 2004; SOUZA *et al.,* 2008; FISCHER *et al*., 2009; PLATINICK, 2014).

Na América do Sul há 36 espécies, das quais 12 já foram relatadas no Brasil (**Figura 3**): *L. laeta* NICOLET, 1849, *L. similis* MOENKHAUS, 1898, *L. anomala* MELLO-LEITÃO, 1917, *L. hirsuta* MELLO-LEITÃO, 1931, *L. immodesta* MELLO-LEITÃO, 1917, *L. intermedia* MELLO-LEITÃO, 1934, *L. adelaida* GERTSCH, 1967, *L. amazonica* GERTSCH, 1967, *L. gaucho* GERTSCH, 1967, *L. puortoi* MARTINS *et al.*, 2002, *L. chapadensis* BERTANI *et al.*, 2010 *e L. niedeguidonae* GONÇALVES-DE-ANDRADE *et al.,* 2012 (FIG. 2).

Em Minas Gerais, há relatos de três espécies de *Loxosceles*, *L. similis, L. laeta* e *L. anomala* (GERTSCH, 1967; ÁLVARES *et al.,* 2004), que são encontradas em vários municípios : Belo Horizonte, Ubá, Viçosa, Ouro Preto, Minas da Serrinha, Diamantina, Matozinhos, Pedro Leopoldo, Santana do Riacho e Juiz de Fora (ÁLVARES *et al.*, 2004; MACHADO *et al.,* 2005; BRUGIOLO *et al.*, 2011).
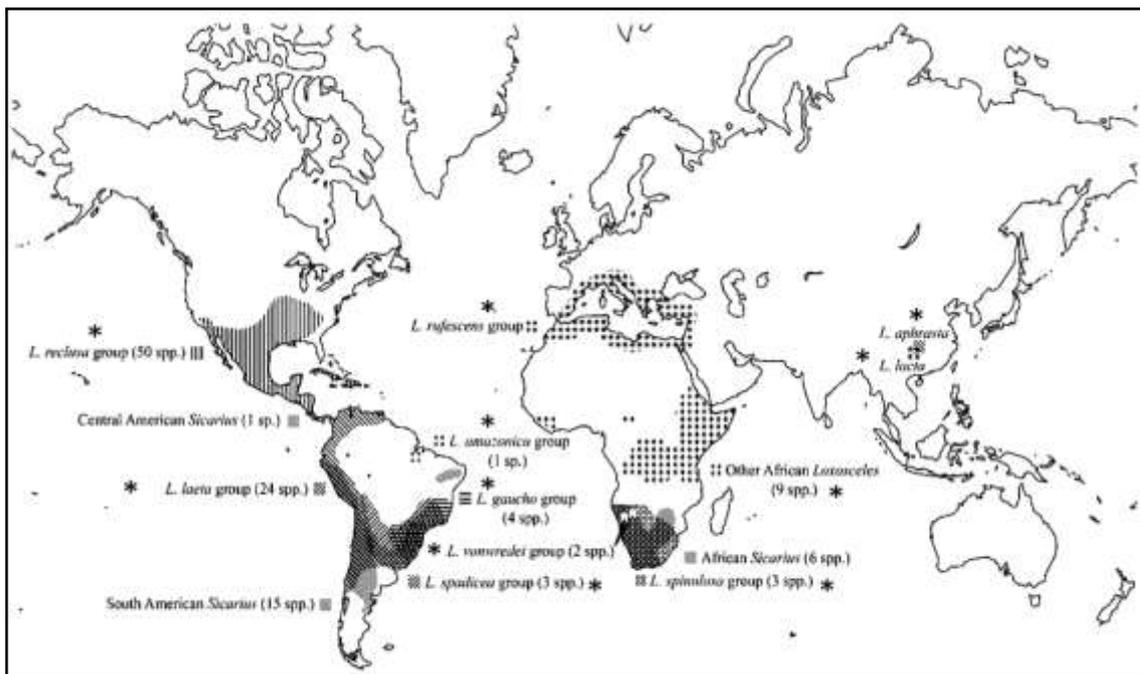
Figura 2- Distribuição geográfica dos gêneros *Loxosceles* (asteriscos) e Sicarius, ambos da família Sicariidae. Os grupos definidos de espécies *Loxosceles* estão indicados, com o número de espécies entre parênteses. A *L. similis*, espécie de interesse, pertence ao grupo gaucho. Adaptado de Binford *et al*., 2008.
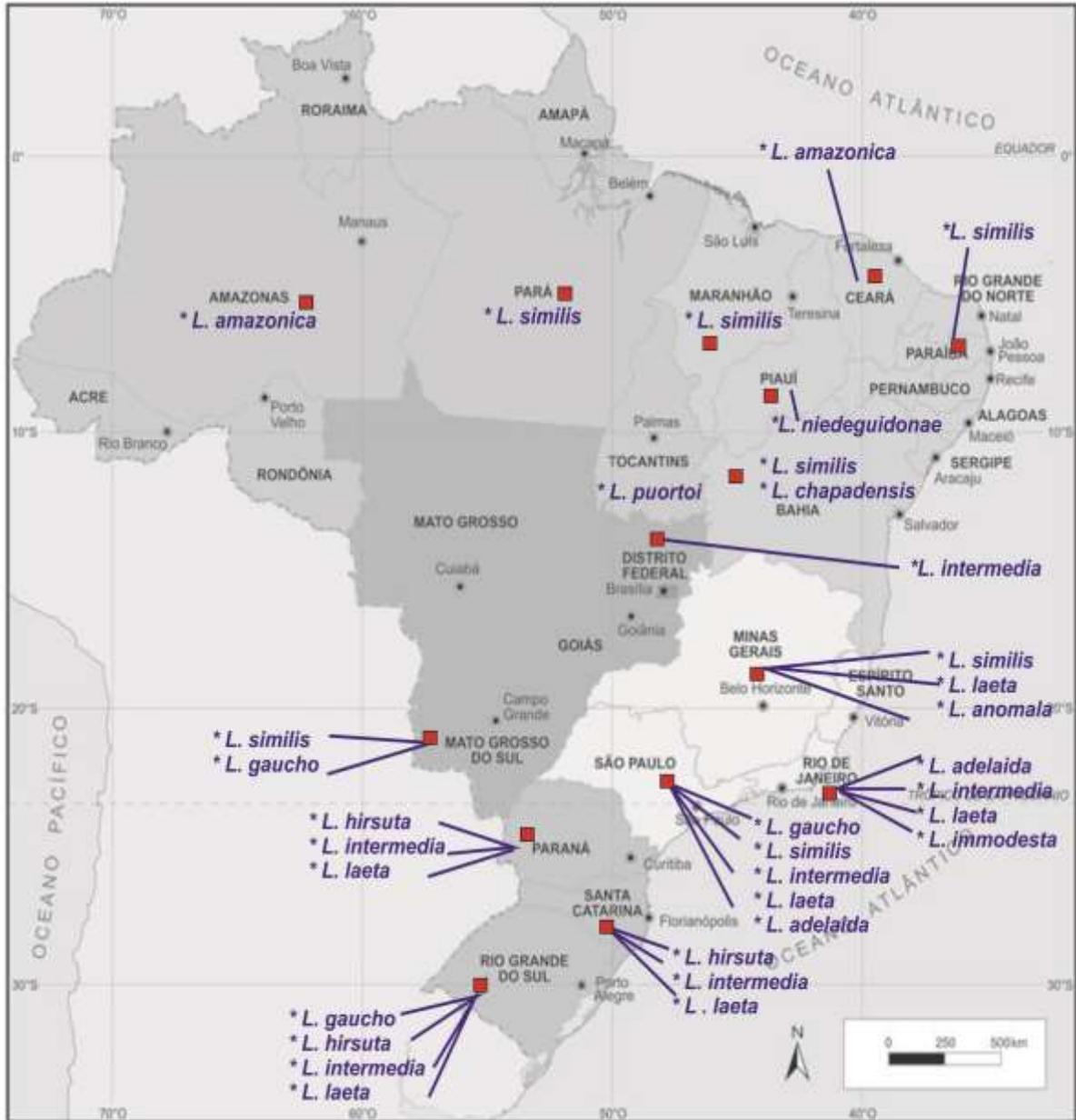
Figura 3- Distribuição geográfica das 12 espécies de *Loxosceles* nos Estados Brasileiros (grafadas em azul marinho). Observar também os Estados Brasileiros onde ocorrem os acidentes loxoscélicos (quadrado vermelho). Adaptado de IBGE, 2012.

2.4 Epidemiologia dos acidentes loxoscélicos

Os acidentes com *Loxosceles* são um problema de relevância médica no Brasil (HOGAN *et al.,* 2004). O número de acidentes cresceu bastante nos últimos anos, principalmente a partir de 1998 (BOCHNER & STRUCHINER, 2002) visto o aumento da divulgação e do conhecimento sobre esse tipo de acidente e a iniciativa do Ministério da Saúde de tornar obrigatória a notificação dos casos de araneísmo (BRASIL, 1999).

Entre os anos de 2001 e 2009 foram notificados no Brasil 68419 acidentes por *Loxosceles* (**Gráfico 1**). A maior parte dos acidentes ocorreu nas regiões Sul e Sudeste, principalmente em alguns Estados do Sul. O Estado do Paraná é o que notifica o maior número de acidentes (MARQUES-DA-SILVA; 2005; FISCHER, 2009; MINISTÉRIO DA SAÚDE, 2012).
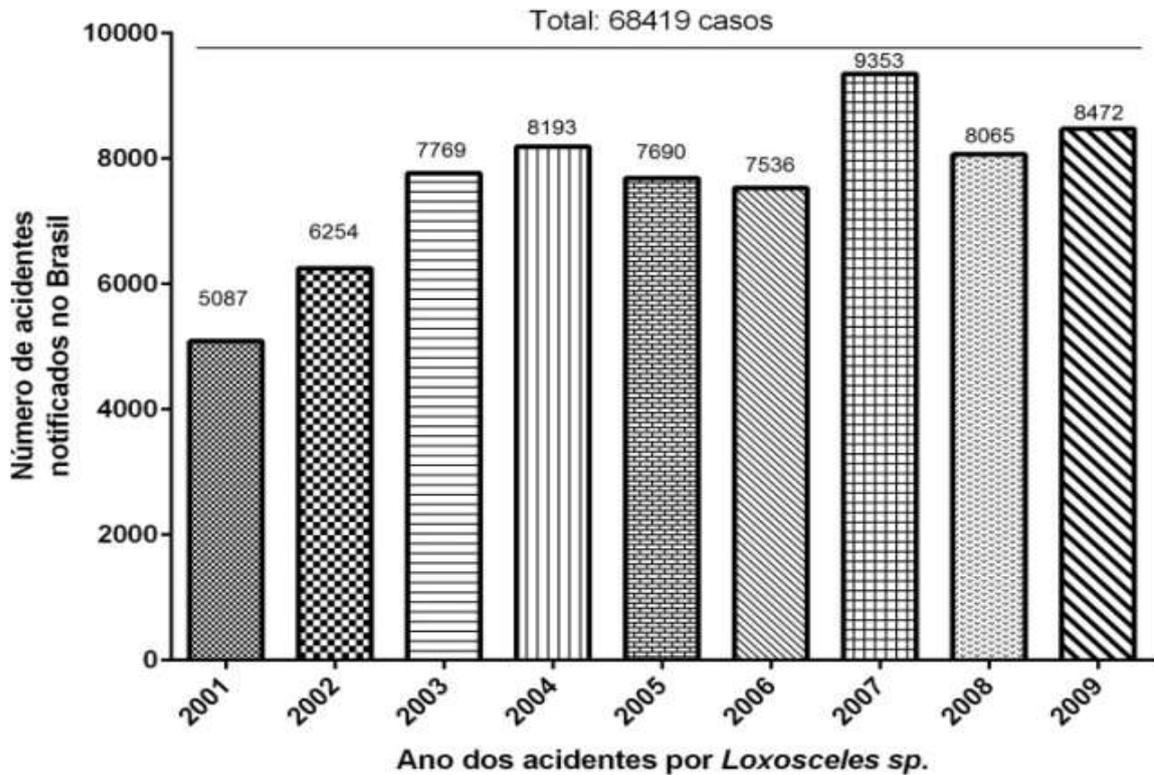
Gráfico 1- Notificação anual dos acidentes por *Loxosceles* no Brasil entre os anos de 2001-2009. Fonte: tabulação de dados do ministério da saúde.

Apesar de Minas Gerais não ser o Estado brasileiro com o maior número de notificações, o número de acidentes têm crescido consideravelmente ao longo dos anos. Entre os anos de 2001 e 2008 foram notificados 1077 acidentes por *Loxoscele*s (Gráfico 2). Somente em 2007 foram registradas 269 notificações (PEREIRA *et al.* 2011).
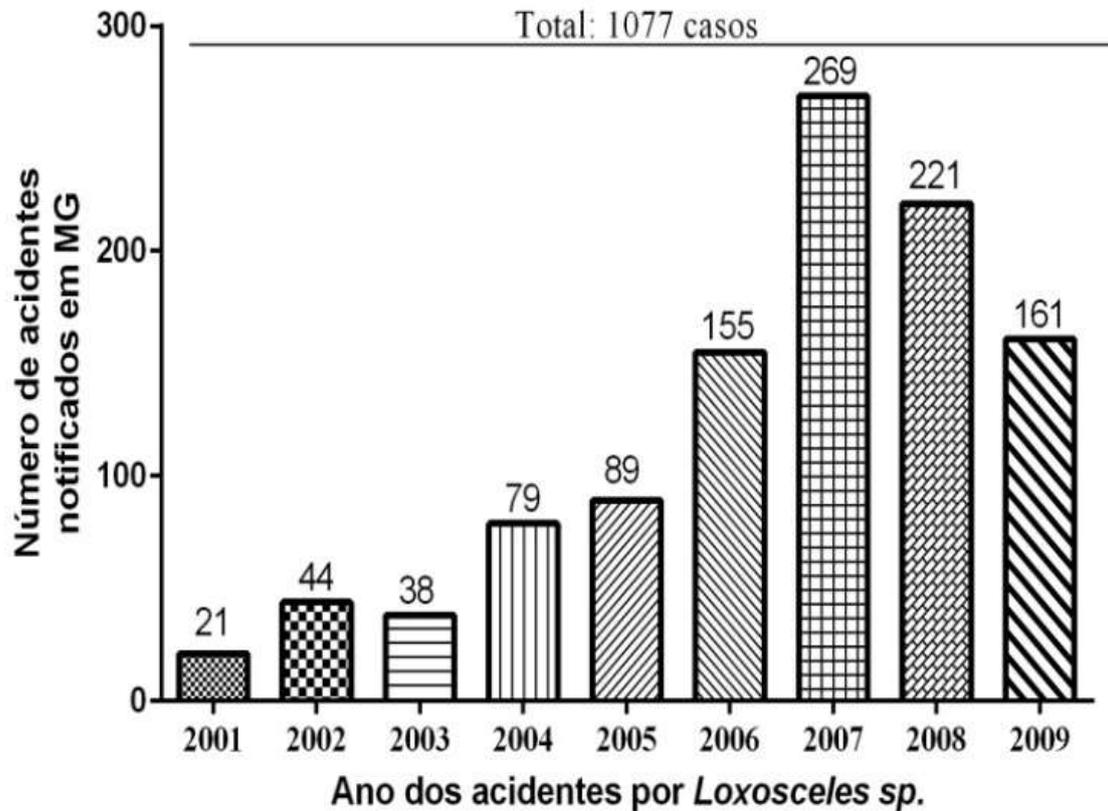
Gráfico 2- Notificação anual dos acidentes por *Loxosceles* em Minas Gerais entre os anos de 2001-2009. Fontes: tabulação de dados do ministério da saúde.

Mesmo havendo um crescimento considerável no número de acidentes loxoscélicos, a incidência real ainda é desconhecida, pois a picada da *Loxosceles* é indolor, os efeitos iniciais do envenenamento só são observados 2 a 8 horas após a picada e a aranha raramente é identificada. De acordo com o trabalho de Wright *et al.* (1997), apenas 12% dos pacientes (n= 111) levam a aranha para a identificação. Além disso, nem todos os acidentes loxoscélicos resultam em lesões dermonecróticas.

Provavelmente, acidentes com aranhas-marrons são ainda mais frequentes, mas não são devidamente diagnosticados e/ou registrados (MACHADO *et al.,* 2005).

2.5 Loxoscelismo: sinais clínicos, sintomas, diagnóstico e tratamento

A picada da *Loxosceles* conduz a um conjunto de sinais e sintomas, denominado loxoscelismo, que pode se manifestar sob dois quadros clínicos distintos: o quadro cutâneo ou dermonecrótico e o quadro cutâneo-visceral ou sistêmico. O desenvolvimento de quadro cutâneo e/ou sistêmico vai depender de alguns fatores relacionados com: o estado nutricional

e a idade do indivíduo que foi picado, o local da picada, a susceptibilidade individual ao veneno, o tempo decorrido entre o acidente e a procura por atendimento médico e o tratamento adequado (GAJARDO-TOBAR, 1966; SCHENONE *et al.,* 1989; BARBARO *et al.,* 1994; da SILVA *et al.*, 2004). Fatores relacionados com a biologia da aranha também podem contribuir com a gravidade dos acidentes, tais como: quantidade de veneno injetada, a espécie da aranha, o sexo e idade da aranha (HOGAN *et al*., 2004).

2.5.1 Loxoscelismo cutâneo

O quadro cutâneo é o mais frequente (84 a 97%) (BARBARO *et al.,* 1992; CHAIM, 2005) e se caracteriza por dermonecrose no local da picada (REES, *et al.,* 1984).

A picada pela *Loxosceles* nos casos menos graves causa uma reação local leve e indolor, que geralmente passa despercebida pelo paciente. Nos casos mais graves, a picada inicial é indolor e, após 2-8 horas a dor se intensifica e os pacientes podem em alguns casos, apresentar febre e cefaleia. Na porção central da lesão observa-se uma área avermelhada e edemaciada, em torno da qual se observa uma área brancacenta. Esse conjunto de sinais é denominado placa marmórea. Além disso, nas primeiras horas já se observa uma difusão radial do veneno (RODRIGUES *et al.,* 1986; FUTRELL, 1992; Da SILVA *et al.,* 2004; SWANSON & VETTER, 2006). Após 12-24 horas, observa-se a formação de uma bolha, que pode se tornar hemorrágica. Após alguns dias, a região da picada fica com uma coloração violeta e, na porção central, observa-se uma consistência endurecida (**Figura 4 A-D**).

A cicatrização ocorre por segunda intenção após 6 a 8 semanas (**FIG. 3 E-F**). Em casos mais graves forma-se uma úlcera de difícil cicatrização, que às vezes pode chegar a 40 cm de diâmetro e atingir a camada muscular. Dependendo de sua extensão pode haver a necessidade de excisão e posterior enxerto da pele (Da SILVA *et al.,* 2004; SWANSON *&* VETTER, 2006).

Figure 4- Casos clínicos de loxoscelismo cutâneo: (a) lesão inicial provocada pela picada da *L. reclusa* - Área central contendo uma área vermelho-arroxeada (seta azul) em torno da qual se observa uma área brancacenta (seta preta) formando assim a placa marmórea. É possível observar a difusão radial do veneno (seta branca); (B) Bolha (seta azul) com área hemorrágica (seta preta), no pé direito de um bombeiro de 31 anos, 12 horas após a picada, observar a difusão radial do veneno (seta branca); (C) Braço direito de uma paciente que procurou atendimento 48 horas após a picada, observar o edema e o eritema com áreas de equimose (seta branca); (D) Braço direito de um paciente que procurou atendimento médico 26 dias após picada de *Loxosceles*, observar extensa área ulcerada; (E) Cicatriz resultante da lesão D 113 dias e (F) 3 anos após a picada. Fonte: (A) e (B) casos clínicos atendidos no Hospital João XXIII/ MG (MAGALHÃES & SCHUTZE, 2010); (C-F) casos clínicos atendidos no Hospital Vital Brazil, Instituto Butantan, São Paulo; Reprodução autorizada pelo autor principal (HOGAN *et al*., 2004). Houve alteração nas fotos com acréscimos de setas, para fins didáticos.

2.5.2 Loxoscelismo sistêmico

O loxoscelismo cutâneo-visceral ou sistêmico ocorre com menor frequência (3 – 16% dos casos) e é observado apenas nos casos mais graves, no entanto, tem uma alta incidência em crianças (SAMS *et al.*, 2001). As manifestações clínicas iniciam 24-72 horas após o acidente (WASSERMAN *et al.*, 1983; WILLIAMS *et al.*, 1995; HOGAN *et al.*, 2004; STOECKER *et al.*, 2006) e incluem fraqueza, febre, vômito, alterações sensoriais, cefaléia, insônia, prurido generalizado e petéquias e, nos casos mais graves, convulsão e coma (MARTINEZ-VARGAS, 1987; SCHENONE *et al.*, 1989; FUTRELL, 1992; BRAVO et al., 1993). Adicionalmente, pode-se observar oligúria e urina escura em decorrência de hemólise intravascular e rabdomiólise, que segundo Sezerino *et al.* (1998) e França *et al.* (2002), podem causar anemia intensa e insuficiência renal, respectivamente.

2.5.3   Diagnóstico e tratamento do loxoscelismo

O diagnóstico do loxoscelismo geralmente é baseado: (1) na evolução do quadro clínico e no desenvolvimento da lesão dermonecrótica característica do loxoscelismo, (2) em informações epidemiológicas e (2) no reconhecimento do acidente pelo próprio paciente, quando o mesmo consegue captura e levar a *Loxosceles* ao centro médico, o que não ocorre na maioria dos casos, visto que os pacientes geralmente não percebem que foram picados (MÁLAQUE *et al.*, 2002). Adicionalmente, a biopsia da pele dos pacientes acidentados poderá ser utilizada no diagnóstico (Da SILVA *et al.,* 2004).

Estudos com ênfase no diagnóstico vêm sendo conduzidos por imunoensaios ELISA (*Enzyme Linked Immuno Sorbent Assay*), para identificação, quantificação e detecção das proteínas do veneno no tecido dermonecrótico, nos pêlos e no soro dos pacientes acidentados (CARDOSO *et al.*, 1988; CHÁVEZ-OLORTEGUI *et al.*, 1998; MILLER *et al.*, 2000; KRYWKO & GÓMEZ, 2002; GOMEZ *et al.*,2002). Ainda não existem exames laboratoriais específicos para diagnóstico de loxoscelismo disponíveis comercialmente.

No entanto, alguns parâmetros laboratoriais são analisados como forma de avaliação da gravidade do envenenamento tais como: hemoglobinúria, hemoglobina sérica, creatina quinase, bilirrubina indireta, lactato desidrogenase (HOGAN *et al.*, 2004).

Quanto ao tratamento dos pacientes, não existe um critério universal para a escolha da melhor terapia. Diversos tratamentos têm sido propostos e utilizados: heparina, analgésicos, corticoide, aspirina, vasodilatadores, anti-histamínico, antibióticos, oxigênioterapia e excisão

cirúrgica (MOLD & THOMPSOM, 2004; PAULI *et al.,* 2006). No Brasil, a soroterapia associada com corticoides constitui uma intervenção bastante utilizada na pratica clinica (GUILHERME *et al,,* 2001). Os soros anti-venenos específicos são citados na literatura como os que possuem melhor potencial terapêutico, principalmente se administrado nas primeiras horas após o acidente loxoscélico (PAULI *et al.,* 2006; CHATZAKI, *et al*., 2012).

2.6 Composição bioquímica do veneno

O veneno de *Loxosceles* é um líquido transparente, composto por uma mistura complexa de vários compostos e proteínas, produzidos pelas glândulas situadas no cefalotórax do animal e que se comunica com o exterior através de um aparelho inoculador (MARTINEZ–VARGAS, 1987). Essa mistura complexa é formada por: moléculas orgânicas de baixa massa molecular (<100 KDa), por polipepitídeos (3000-10000 KDa) e por proteínas de alta massa molecular (>10000 KDa). As proteínas de baixo peso molecular são formadas por ácidos nucléicos, aminoácidos livres, poliaminas neurotóxicas, monoaminas e sais inorgânicos (DA SILVA *et al*., 2004). Os polipeptídios e as poliaminas são as principais classes de compostos responsáveis pela toxicidade do veneno das aranhas. Além disso, lipases, hialuronidases, colagenases, fosfatases e esfingomielases também são encontradas no veneno (FUTRELL, 1992).

A fração dermonecrótica têm sido encontrada e descrita em diversas espécies do gênero *Loxosceles.* Geralmente são enzimas do tipo fosfolipase D e muitas delas estão envolvidas com a formação da lesão dermonecrótica, com a indução de resposta inflamatória, hemólise, agregação plaquetária, citotoxicidade, aumento da permeabilidade vascular, nefrotoxicidade e pela letalidade observada no loxoscelismo (CHAVES-MOREIRA *et al.*, 2009).

Outras proteínas de baixo peso molecular também tem sido descritas, como as metaloproteases (32 kDa), dentre elas as com ação fibrinogenolítica (20-28 kDa) e gelatinolítica (32-35 kDa), cujas atividades estão relacionadas com distúrbios hemostáticos decorrentes de hemorragia da derme, injúria de vasos sanguíneos e como um fator de difusão radial do veneno (característico do loxoscelismo) e sistêmico, facilitando a penetração dos outros componentes do veneno (DA SILVEIRA *et al*., 2007).

As hialuronidases (41 -43 kDa), descritas por Da Silveira *et al.,* (2007), são capazes de degradar o ácido hialurônico e sulfato de condroitina presentes na matriz extracelular,

funcionando como um fator de difusão radial do veneno, pelo fato dessa enzima possuir atividade de hidrolase.

Ainda não há um conhecimento completo sobre os componentes presentes no veneno da *Loxosceles*. No entanto, devido à utilização de técnicas de biologia molecular, diferentes toxinas recombinantes, também têm sido amplamente descritas (KALAPOTHAKIS *et al*., 2002; KALAPOTHAKIS *et al*., 2007; SADE *et al.,* 2012). A **tabela 1** mostra as principais enzimas constituintes do veneno de *Loxosceles* que já foram descritas.

Tabela 1-Principais atividades enzimáticas do veneno de *Loxosceles.* Adaptado de Da Silva et al., 2004 e de Chaim et al., 2011

Atividade enzimática	*Loxosceles* sp	Atividade	Referências
Esfingomielinase D/Fosfolipase D	*L. gaucho* *L. laeta* *L. intermedia* *L. similis* *L. adelaida* *L. reclusa* *L. deserta* *L. rufecens* *L. boneti* *L.. arizona*	Agregação plaquetária Hemólise Dermonecrose Infiltração de células inflamatórias	Mota & Barbaro, 1995, Tambourgi *et al.,* 1995; Kalapothakis et al., 2002; De Santi Ferrara et al., 2009; De Giuseppe et al., 2011, Gomes et al., 2011, Chaim et al., 2011; Chatzaki et al., 2012, Van Den Berg et al., 2012; Horta et al., 2012.
Hialuronidase	Várias	Facilita a dispersão de vários componentes tóxicos do veneno (espalhamento radiall)	Futrell, 1992; Young & Pincus (2001); Da Silveira et al., 2007; Ferrer et a., 2013; Chaim et al., 2011;
Fosfatase alcalina	*L. reclusa*	Não descrito	Norment *et al.,* 1979; Futrell, 1992;
Metaloproteases	*L. rufecens*	Caseinolítica, "*Gelatinolytic*", fibrinogenolítica	Young & Pincus, 2001, Da Silveira et al., 2002, Barbaro et al., 2005,
Proteases	*L. intermedia*	Hidrolise de entactina, proteoglicano, sulfato de heparina e membrana basal	Veiga *et al.,* 2000b; 2001a,b, Da Silveira et al., 2002.
Serino protease	*L. intermedia*	Gelatinolítica	Veiga *et al.,* 2000[b], Fernandes-Pedrosa et al., 2008
Esterase	*L. reclusa*		Norment *et al.,* 1979
5' Ribonucleotide Phosphohydrolas	*L. reclusa*	Não descrito	Futrell, 1992

e

Loxolisina A	*L. intermedia*	Fibronectinolítico, Fibrinogenolítico	Feitosa *et al.,* 1998
Loxolisina B	*L. intermedia*	*"Gelatinolytic"*	Feitosa *et al.*, 1998
Loxnecrogin A	*L. gaucho*	Dermonecrose em coelhos	Cunha *et al.* (2003)
Loxnecrogin B	*L. gaucho*	Dermonecrose em coelhos	Cunha *et al.* (2003)
Loxtox		Dermonecrose	Kalapothakis et al., 2007
LiRecTCTP	*L. intermedia*	Edema e aumento na permeabilidade vascular	Sade *et al.,* 2012

* Superfamília TCTP (Translationally Controlled Tumor Protein).

2.7 O veneno da *Loxosceles similis*

As principais espécies de *Loxosceles* consideradas como de importância médica no Brasil são *L. intermedia*, *L. gaucho* e *L. laeta*, embora haja outras espécies pouco estudadas, que podem representar um risco potencial para a população humana. Duas espécies, que inicialmente não eram citadas como de importância médica, *L. anomala* e a *L. similis,* já foram encontradas em Minas Gerais. A picada de *L. anomala*, de acordo com Bucaretchi *et al.* (2010), causa lesão dermonecrótica no homem. A *L. similis*, inicialmente pouco estudada, têm sido bem caracterizada pelo nosso grupo de pesquisa (SILVESTRE *et al.*, 2005; CHATZAKI *et al.*, 2012, PEREIRA *et al*, 2012 a e b).

Silvestre *et al.*, (2005) compararam o veneno bruto de *L. similis* com o veneno bruto das outras 3 espécies de *Loxosceles* consideradas de importância médica no Brasil. O perfil eletroforético do veneno de *L. similis* (SDS-PAGE) mostrou proteínas com perfis similares às encontradas no veneno de *L. intermedia* (5-140 KDa). Adicionalmente, neste trabalho foi observado que o veneno de *L. similis* é mais tóxico em camundongos que o da *L. intermédia.*

Estudos de PEREIRA *et al.*, 2012b, corroboram com os achados citados na literatura pois, utilizando 0,5 μg do veneno de *L. similis* (30μL de solução salina), injetada subcutaneamente em coelhos, foi possível observar edema, vasodilatação, hiperemia, infiltrado inflamatório intenso (heterofílico), multifocal, inclusive perivascular, caracterizando uma dermatite aguda multifocal intensa. Além disso, observou-se deposição de fibrina, trombose na derme, degeneração da parede de alguns vasos e rabdomiólise, caracterizando

uma miosite aguda necrosante. A concentração utilizada no estudo foi a menor já relatada em coelhos. Isso mostra que mesmo concentrações pequenas do veneno de *L. similis* são capazes de induzir lesões semelhantes àquelas produzidas pelo veneno de outras espécies descritas como sendo de importância médica no Brasil (ELSTON *et al*., 2000; OSPEDAL *et al*., 2002).

2.8 Modelo de Implante subcutâneo de esponjas para o estudo do loxoscelismo

Os biomateriais têm sido amplamente utilizados tanto pela engenharia de biomateriais quanto pelas pesquisas científicas (Anderson *et al*., 2008). Esta ampla utilização, do ponto de vista da engenharia de biomateriais, se dá pela tentativa de projetar um biomaterial de longa duração e que não provoque uma reação imunológica intensa no organismo. Os biomateriais no ponto de vista científico têm sido bastante utilizados para uma maior compreensão de diversos aspectos relacionados com o processo inflamatório no hospedeiro, com as relações complexas entre o biomaterial e o hospedeiro, com a angiogênese durante o processo de cicatrização de feridas e com varias doenças, tais como diabetes, lúpus eritematoso e neoplasias (TEIXEIRA *et al.,* 1999; CAMPOS *et al*., 2011).

O modelo de implante de esponjas de poliéster poliuretano é um tipo de biomaterial que têm sido utilizado em vários estudos (ANDRADE et al.; 1987; ANDRADE et al., 2001; CASTRO *et al.,* 2012; GUABIRABA *et al.,* 2013). O modelo consiste na implantação de uma matriz esponjosa no tecido subcutâneo de camundongos. A matriz implantada induz uma reação inflamatória que inclui lesão, interação do biomaterial com o sangue, formação de uma matriz, inflamação aguda, inflamação crônica, desenvolvimento de um tecido de granulação altamente vascularizado, reação do tipo corpo estranho e o desenvolvimento de uma cápsula fibrosa (**Figura 5**).
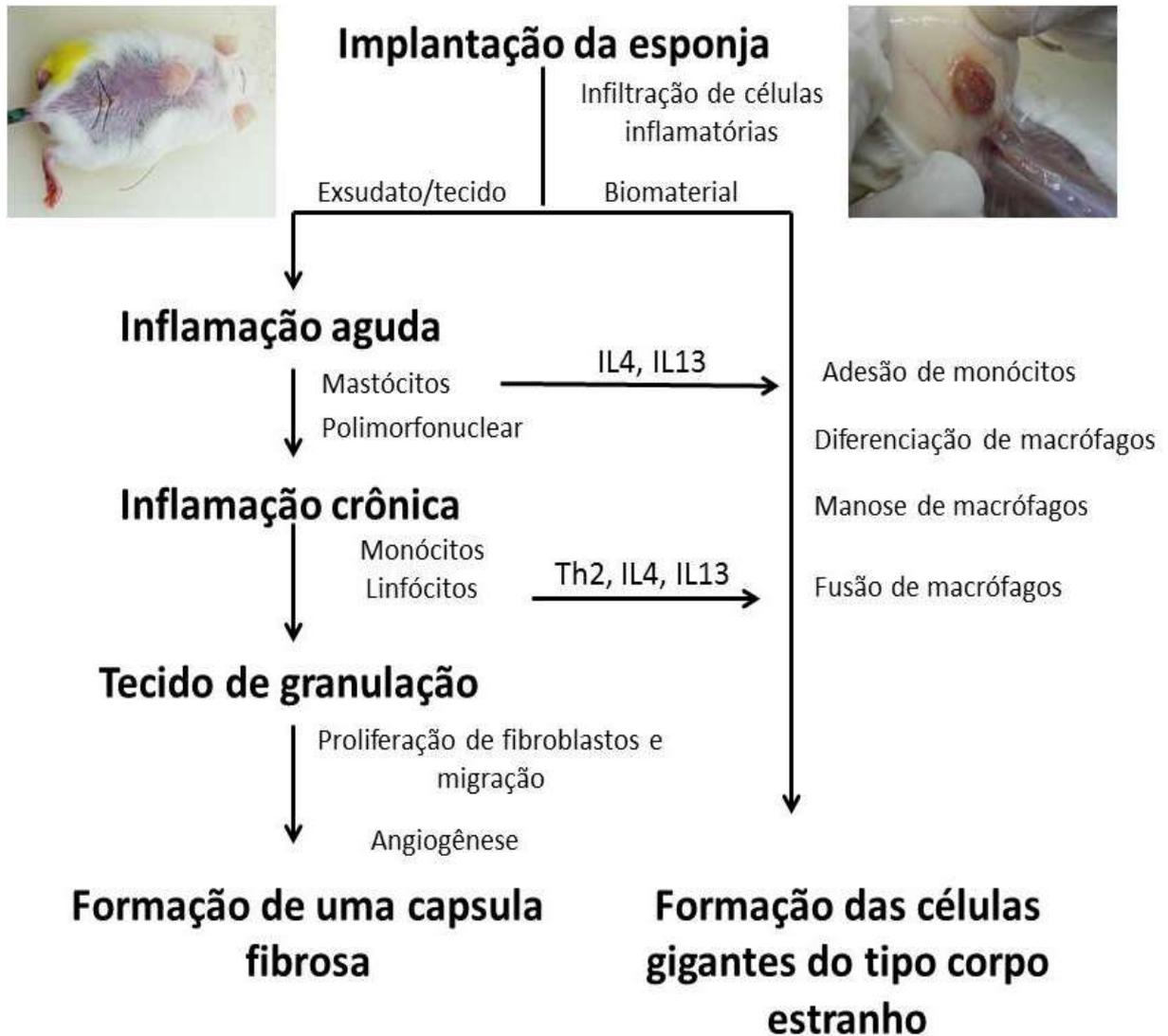
Figura 5: Resposta do sistema imunológico ao implante de esponja: Sequência de eventos envolvidos na inflamação e processo de cicatrização de feridas levando a formação de células gigantes do tipo corpo estranho. A figura também mostra a importância dos mastócitos na inflamação aguda e dos linfócitos Th2 na inflamação crônica, com a produção de IL4 e IL13, levando a indução da fusão de monócitos/macrófagos para formar as células gigantes do tipo corpo estranho. Adaptado de Anderson *et al*., 2008.

Esse modelo de implante subcutâneo de esponja possibilita o estudo e a modulação de processos inflamatórios, cicatrização e angiogênese. Além de permitir também que se investiguem os efeitos e os mecanismos de ação de drogas pró-inflamatórias (BAYLEY, 1988), anti-inflamatórias (HORI *et al*, 1996), agentes vasoativos (ANDRADE *et al*., 1992 e 1996) e atividades pró e anti-angiogênica de inúmeros compostos. O modelo também já foi utilizado para o estudo do angiogênese tumoral (ANDRADE *et al*., 1992) e em camundongos suscetíveis ao Lúpus (CAMPOS, 2008).

O modelo experimental comumente utilizado para o estudo do loxoscelismo cutâneo tem sido o coelho. Porém, pesquisas acerca da patogênese do loxoscelismo podem ser facilitadas com o uso de um modelo de menor porte, baixo custo, fácil manuseio, manutenção e utilização, o que permite o acompanhamento dos processos por longos períodos. Estudos mostram que o implante de esponja em camundongo induz a formação de um tecido de granulação altamente vascularizado (CASTRO *et al*., 2012; CAMPOS *et al*., 2011).

Além disso, o modelo de implante permite o estudo temporal do infiltrado inflamatório, a análise bioquímica dos fluidos coletados, o efeito das drogas sobre o processo, além de estudos morfológicos e morfométricos (ANDRADE *et al.,* 1987; BAYLEY, 1998; BARCELOS *et al.,* 2004; CAMPOS, 2008). A avaliação do desenvolvimento de estruturas vasculares na esponja pode ser feita a partir da dosagem do conteúdo de hemoglobina (índice indireto de vascularização) (PLUNKETT & HAILEY *et al*., 1990). A análise histológica associada a estudos morfométricos e bioquímicos apresentam-se, também, como aliados importantes para a avaliação da angiogênese. Como o processo inflamatório participa da reação do hospedeiro ao implante, Bradley *et. al.* (1982) desenvolveram um ensaio enzimático para a detecção do acúmulo de neutrófilos utilizando a enzima mieloperoxidase (MPO) como marcador quantitativo de neutrófilos.

Outra técnica bastante utilizada para a avaliação de células inflamatórias é a atividade da enzima lisossômica N-acetil-β-glucosaminidase (NAG), que se encontra presente em macrófagos ativados (BAILEY, 1988; CAMPOS, 2008). Esses ensaios têm sido utilizados com sucesso nos implantes subcutâneos (ANDRADE *et al.,* 1992; BAILEY, 1998; BARCELOS *et al.,* 2004; CAMPOS *et al.,* 2008). Algumas citocinas que têm sido dosadas nos implantes de esponjas (CAMPOS *et al*., 2008; CASTRO *et al*., 2012) e podem ser importantes para o estudo da patogenia do loxoscelismo são o VEGF (fator de crescimento do endotélio), que está relacionado com aumento de permeabilidade vascular e trombogenicidade (ROBBINSON & STRINGER, 2001; NAKAMURA *et al*., 2002) e o TNF-α (fator de necrose tumoral-α) que é um potente ativador de neutrófilos, mediando a aderência e quimiotaxia (SEDGWICK *et al*., 2000).

Ainda não havia na literatura estudos que avaliassem a viabilidade desse modelo para o estudo dos efeitos do veneno de *Loxosceles*. Sabe-se que as esponjas, de 10 e 14 dias após a implantação, apresentam grande concentração de células endoteliais e vasos bem formados (CAMPOS, 2008). Consequentemente, esse modelo pode permitir o desenvolvimento de um modelo murino como ferramenta de estudo para o loxoscelismo.

Assim, decidiu-se por explorar o modelo de implante de esponjas em camundongo, considerando a grande quantidade de vasos sanguíneos que nelas se desenvolvem. Dessa forma, obtém-se uma espécie de cultivo de células endoteliais *in vivo* que pode ser submetido à ação do veneno no momento em que a esponja apresentar uma grande densidade de células endoteliais.

3. Objetivos

3 OBJETIVOS

3.1 Objetivo geral

Desenvolver um modelo murino para o estudo loxoscelismo utilizando o modelo de implante subcutâneo de esponja.

3.2 Objetivos específicos

(1) Descrever as alterações histológicas induzidas 1 e 4 horas após a injeção intra-implante de 0,5 µg (30µL) do veneno de *L. similis* em implantes de 14 dias;

(2) Dosar a quantidade de hemoglobina, de NAG, MPO, TNFα e VEGF nos implantes pós injeção do veneno de *L. similis*;

(3) Mensurar a área de vasos nos implantes de esponjas.

4. Referências bibliográficas

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5. Artigo

Sponge implant in Swiss mice as a model for studying loxoscelism

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ABSTRACT

Envenomation by Loxosceles spider bite leads to a set of signs and symptoms, called loxoscelism, which in most cases manifests through the dermonecrotic frame. The development of a smaller size animal model, of easy handling and maintenance, and lower cost is needed to study the loxoscelism pathogenesis. The inflammatory effects of the Loxosceles similis crude venom was evaluated considering neutrophil and macrophage activation, vasodilatation, hyperhaemia, edema and hemorrhage and TNF-α and VEGF production using the murine sponge implant model. Thirty two male Swiss mice (6–8 weeks old) were implanted subcutaneously with polyether–polyurethane sponge discs. Fourteen days post implantation, animals were separated into two groups: (1) control group – 16 mice received 30 μL of saline intra-implant; (2) treated group-sixteen mice injected with 0.5 μg/30 μL of L. similis crude venom intra-implant. The animals were euthanized with xylazine/ketamine after 1 and 4 h post-injection. Microscopically, implants of the treated groups presented an acute inflammation characterized by: neutrophilic infiltrate, edema, vasodilatation hyperhaemia, and severe hemorrhage. Some vessels presented ruptured walls. Under morphometric analysis, vessel area was bigger in the treated groups compared with the control ones. The biochemical parameters, hemoglobin content, inflammatory enzyme activities (myeloperoxidase and n-acethyl-β-ο glucosaminidase) and levels of the cytokines, TNF-α and VEGF, were also significantly higher in the venom-treated groups. The effects of Loxosceles venom in the granulation tissue of the implant in mice were similar to those observed in cutaneous loxoscelism in other species (human and rabbits). Consequently, the murine sponge implant model provides a new method to investigate cellular/molecular mechanisms associated with cutaneous loxoscelism.

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1. Introduction

Loxoscelism is a set of signs and symptoms caused by the bite of spiders of the genus Loxosceles (Da Silva et al., 2004). Loxosceles (Araneae, Sicariidae) can be found in temperate and tropical regions of America, Oceania, Asia, Africa and Europe (Swanson and Vetter, 2006; Hogan et al., 2004; Souza et al., 2008). This genus represents a public health problem in Brazil, mainly in South and Southeast regions, with more than 3000 cases reported annually by the Ministry of Health (Hogan et al., 2004). Usually, the clinical manifestations of loxoscelism are characterized by necroulcerative dermatitis at the site of the bite (83.3% of the cases). However the envenoming can also cause systemic effects (16% of the victims) leading to acute renal failure, which may be lethal

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(Málaque et al., 2002; Hogan et al., 2004; Abdulkader et al., 2008). Locally, lesions caused by *Loxosceles* venom present edema, hemorrhage, inflammation with predominance of neutrophils, rhabdomyolisis, damage to the vessels wall, thrombosis, and dermonecrosis (Futrell, 1992; Ospedal et al., 2002; Pereira et al., 2010). In addition, according to some studies, *Loxosceles* venom causes cytoplasmic vacuolization, loss of adhesion (Hogan et al., 2004; Veiga et al., 2000; Veiga et al., 2001) and apoptosis of endothelial cells (Pereira et al., 2010). The family of *Loxtox* proteins (Kalapothakis et al., 2007), such as: sphingomyelinase-D, SMA protein, phospholipase-D dermonecrotic protein (DP) and dermonecrotic factors (DNF) were found and characterized in the venom of *Loxosceles* and were associated with local and systemic loxoscelism (Barbaro et al., 2005; Felicori et al., 2006; Da Silveira et al., 2007). The systemic and local effects of the venom are well described in human, rabbit, and guinea pig cutaneous tissue. The use of the murine model in loxoscelism study is restrained to inflammatory events analysis, since the dermonecrotic lesion does not develop in mouse following intradermal injection of the venom (Sunderkötter et al., 2001; Barbaro et al., 2010). Further understanding of intracellular and molecular mechanisms of loxoscelism is limited because rabbit model is far more expensive. Additionally, only few scientific probes are available for investigation of intracellular and molecular events of the envenoming in this specie. Thus, an animal model that would allow the investigation of these events is highly advantageous. The subcutaneous implantation of sponges have been used in several studies, because it is a model that resembles a cell culture *in vivo* by inducing an amplified inflammatory foreign body reaction that progresses to the formation of a highly vascular granulation tissue in which various components of subcutaneous tissue can be analyzed by biochemical, functional and histological parameters (Campos et al., 2008; Parrilha et al., 2011). Previously, we have investigated the effects of *Bothrops* venom on blood flow of the fibrovascular tissue induced by synthetic matrix implanted subcutaneously in mice (Vieira et al., 1992). We reasoned that this model could be used to study the actions of *Loxosceles* venom in mice thus, providing a new tool to investigate not only the inflammatory effects of the venom, but also the mechanisms of the injury. In this study, we set up a methodology based on subcutaneous implantation of sponge matrix to evaluate the inflammation pattern (neutrophil and macrophage infiltration, vasodilatation, hyperhaemia, edema and hemorrhage) induced by *Loxosceles* venom in mice.

2. Materials and methods

2.1. Venom extraction

The venom was extracted from the venom glands of adult animals by maceration and centrifugation according to Silvestre et al. (2005), and frozen at −80 °C until use.

2.2. Animals

Thirty two 6–8 weeks old male Swiss mice were housed individually and provided with chow pellets and water *ad libitum*. The light/dark cycle was 12:12 h with lights on at 7:00 a.m. and lights off at 7:00 p.m. Housing, anesthesia, and postoperative care concurred with the guidelines established by our local Institutional Animal Welfare Committee. The present study was approved by the Ethics Committee in Animal Experimentation (CETEA) of Universidade Federal de Minas Gerais (UFMG) process number 229/09 approved in June 9, 2010.

2.3. Preparation of sponge discs and implantation

Discs of Polyether–polyurethane sponge (Vitafoam Ltd., Manchester, UK), 6 mm thick, and 11 mm diameter (Fig. 1A) were soaked overnight in 70% v/v ethanol and boiled in distilled water for 15 min before implantation. Animals were anesthetized with xilasin/ketamin (1 mg/kg, Syntec of Brazil), the dorsal fur was shaved and the skin antissepsy was made with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, through a 1 cm long dorsal mid-line incision. Post-operatively, animals were monitored for any sign of infection at the operative site, discomfort or distress.

2.4. Venom injection and implant removal

Fourteen days post implantation, animals were separated into two groups: (1) control group – sixteen mice that injected with 30 μL of saline intra-implant; (2) treated group – sixteen mice injected with 0.5 μg/30 μL of *Loxosceles similis* crude venom intra-implant. Sixteen animals (8 control and 8 treated) were euthanized at 1 h and the remaining sixteen mice (8 control and 8 treated) 4 h post-injection. Five implants of each group were removed, weighed and frozen for biochemical analysis. Three sets of implants from each group were kept for histological analysis.

2.5. Histological staining and morphometric analysis

For each time interval 3 implants from both groups (control and treated) were fixed in 10% buffered formalin, pH 7.4 and processed for the paraffin embedding. Sections 5 μm thick were stained by hematoxylin/eosin (HE) for histological and morphometrical analysis. The vasodilatation induced by the venom was measured morphometrically. For that, images of 25 fields per slide by means of a planapochromatic objective (20×) in light microscopy (Olympus BX-640) were obtained. The images were digitalized through a JVC TK-1270/JGB microcamera and analyzed using the software Kontron Electronic, Carl Zeiss – KS300, version 2.

2.6. Hemoglobin (Hb) extraction and dosage

Blood content intra-implant was assessed by the amount of Hb detected in the tissue using the Drabkin method (Drabkin and Austin, 1932; Campos et al., 2008). Each implant was homogenized (Tekmar TR-10, Cincinnati, OH) in 5 mL of Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at 12,000 rpm for 20 min. The supernatants were filtered through a cellulose ester membrane (0.22 μm, Millipore, São Paulo, Brazil). The Hb concentration in the
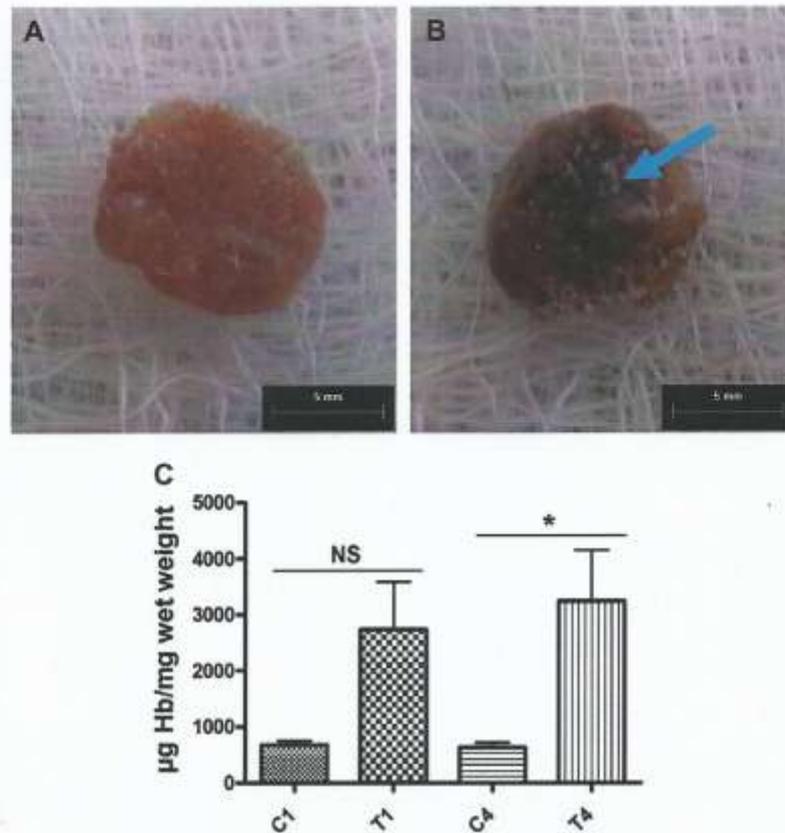
Fig. 1. Effects of intra-implant injection of the *L. similis* crude venom on vascular parameters. (A) Control group – implant 4 hour post injection of 30 μl of saline and (B) treated group – implant 4 hour post injection of 30 μl of *L. similis* crude venom (0.5 μg) – note area of hemorrhage (blue arrow); (C) the amount of hemoglobin (Hb) is higher in the venom treated group 4 h after inoculation The results are expressed as mean ± standard error of the mean of five animals for each group (P < 0.05); Kruskal–Wallis test and Dunn's post test. (C1 and C4 – control groups 1 h and 4 h post injection of saline, respectively) (T1 and T4 = treated groups 1 h and 4 h post injection of venom, respectively) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

samples was determined spectrophotometrically by measuring absorbance at 540 nm using an enzyme linked immunosorbent assay (ELISA) plate reader and compared against a standard curve of Hb. The content of Hb in the implant was expressed as mgHb/mg of wet tissue.

2.7. Tissue extraction and determination of MPO (myeloperoxidase) and NAG (N-acetylglucosaminidase) activities

The extent of neutrophil accumulation in the implants was measured by assaying MPO activity as previously described (Campos et al., 2008). The implants were weighed, homogenized in 2 mL of phosphate buffer (0.1 M NaCl, 0.02 M Na_3PO_4, 0.015 M NaEDTA, pH 4.7), centrifuged at 12,000 rpm for 10 min. The pellets were then resuspended in 2 mL of phosphate buffer (0.05 M Na_3PO_4, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) followed by three freeze-thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density, OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 50 μL of H_2SO_4 (4M). Results were expressed as change in OD/g of wet tissue. The infiltration of mononuclear cells into the implants was quantitated by measuring the levels of the lysosomal enzyme NAG present in high levels in activated macrophages. The implants were homogenized in 2 mL NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega, Madison, WI) and centrifuged (3000 rpm; 10 min at 4 °C). The resulting supernatant (100 μL) was incubated for 10 min with 100 μL of p-nitrophenyl-N-acetyl-b-ᴅ-glucosaminide (Sigma, Saint Louis, MO) prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na2HPO₄; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of (100 μL) of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring

the absorption at 400 nm. Results were expressed as change in OD/g of wet tissue.

2.8. Measurement of VEGF (Vascular Endothelial Growth Factor) and TNF-α (tumor necrosis factor-α)

The measurement of VEGF and TNF-α in the implants was carried out spinning (10,000 rpm for 30 min) 100 μL of the supernatant prepared for hemoglobin dosage (item 2.6). The analysis was made with Immunoassay Kits (R & D Systems, USA) following the manufacturer's protocol. Briefly, dilutions of cell-free supernatants were added in duplicate to ELISA plates coated with a specific murine monoclonal antibody against VEGF and TNF-α, followed by the addition of a secondary horseradish-peroxidase-conjugated polyclonal antibody (goat anti-mouse VEGF and goat anti-mouse TNFα). After washing to remove any unbound antibody-enzyme reagent, a substrate solution (50 μL of a 1:1 solution of hydrogen peroxide and tetra-methylbenzidine (10 mg/mL) in DMSO) was added to the wells. The color development was stopped, after 20 min of incubation, with 2N sulphuric acid (50 mL) and the intensity of the color was measured at 540 nm on a spectro-photometer (E max, Molecular Devices). Standards were 0.5 log10 dilutions of recombinant murine chemokines from 7.5 to 1000 pg mL (100 μL). The results were expressed as picogram of cytokine/mg of wet tissue.

2.9. Statistical analysis

Results are presented as mean ± standard deviation. Comparisons between two groups were carried out using Student's t-test for unpaired data. Comparisons between three or more groups were carried out using one-way analysis of variance (ANOVA) and differences between groups were assessed using Newman–Keuls (parametric data). When the groups distribution showed no normal distribution (nonparametric) Kruscal Wallis test and Dunn post test were applied. A $P < 0.05$ was considered significant.

3. Results

3.1. Macroscopy and hemoglobin content of the implants

At the 14th day post implantation, the sponge discs became enveloped by a fibrous connective tissue (Fig. 1A) containing visible blood vessels. Intra-implant venom injection resulted in intense hemorrhage more pronounced at 4 h after injection (Fig. 1B). Hemorrhage and hyperhaemia were confirmed by the amount of hemoglobin extracted from the venom-treated implants (Fig. 1C). The treated group presented mean hemoglobin values of 4.1 ± 1.2 μg/mg wet tissue at one hour post-injection and 4.7 ± 0.9 μg/mg wet tissue at 4 h post-injection. These values were higher than those of the control groups (1.4 ± 0.14 μg/mg wet tissue at 1 hour; and 1.3 ± 0.3 μg/mg wet tissue at 4 h).

3.2. Microscopical analysis

Under light microscopy, the implant of the control group contained an organized granulation tissue composed by fibroblasts and blood vessels and an inflammatory infiltrate of neutrophils and macrophages (Fig. 2A). In the venom-treated group implant, an intense neutrophilic inflammatory infiltrate, vasodilatation, hyperhaemia and edema were present at both time points (Fig. 2B) characterizing an acute inflammation. In addition, an intense hemorrhage and the rupture of some vessel walls, was noted in implants four hour after injection (Fig. 3A–F). Moreover, the average vessel area was higher in the venom-treated groups at both time points studied (Fig. 2C). The average vascular area of the control groups was 1.190 ± 1.420 μm² (1 hour post saline injection) and 1.595 ± 1.769 μm² (4 h post saline injection). In the treated-groups the mean vascular area was 2.027 ± 1.769 μm² and 5.480 ± 7.134 μm², at 1 and 4 hour post venom injection, respectively ($p < 0.0001$).

3.3. Action of Loxosceles venom in implants levels of MPO (myeloperoxidase) and NAG (N-acetyl-b-ᴅ-glucosaminidase)

The levels of MPO activity (a marker for activated neutrophils) in the treated group (4 h post injection) were higher compared with that of control groups (Fig. 4A). The MPO values of the treated groups were 0.27 ± 0.05 and 0.32 ± 0.14 while control groups were 0.13 ± 0.02 and 0.16 ± 0.07 for the intervals of 1 and 4 h, respectively. The levels of NAG activity (the marker for monocytes/macrophages) were also significantly higher in the treated group (4 h after injection) than that in control group (Fig. 4B). The NAG values of the treated group were 4771 ± 5521 and 5325 ± 676 while control groups were 3337 ± 4479 and 3154 ± 3791 or the intervals of 1 and 4 hours, respectively.

3.4. Measurement of VEGF (Vascular Endothelial Growth Factor) and TNF- (tumor necrosis factor-α)

The venom treated group showed higher levels of intra-implant VEGF (Fig. 5A) than the control one. The average values of the treated group were 1.5 ± 1.1 and 0.97 ± 0.7 pg/mg of tissue 1 and 4 hours after inoculation, respectively versus 0.09 ± 0.13 and 0.12 ± 0.05 pg/mg of tissue (1 and 4 hours after injection, respectively) of the control group. The inflammatory cytokine TNF-α (Fig. 5B) was also higher in the treated group compared with the saline treated implants. The average values of the treated group were 396 ± 1245 and 408 ± 8778 pg/mg of tissue 1 and 4 hours after inoculation, respectively versus 1474 ± 2236 and 2026 ± 3015 pg/mg of tissue (1 and 4 hours after injection, respectively) of the control group.

4. Discussion

Loxoscelic accidents can induce clinical manifestations: locally (dermonecrotic skin lesions) and/or systemically. The development of one or another will depend on several factors related to individuals, such as nutritional status, age, site of the bite, amount of injected venom, susceptibility to the venom and the time passed between the accident and treatment (Gajardo-Tobar, 1966; Schenone et al., 1989; Barbaro et al., 1994; Da Silva et al., 2004). Loxosceles bites can cause dermonecrosis in humans, guinea pigs, and
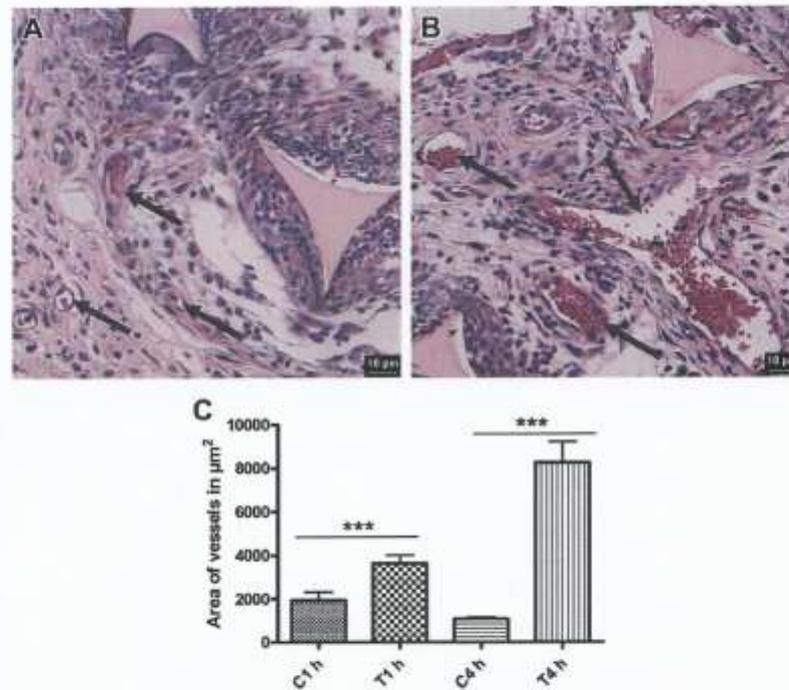
Fig. 2. Histological sections of subcutaneous implants. (A) Implant of control group at 4 h post injection – granulation tissue at day 14, observe the presence of newly formed blood vessels (black arrows) containing red blood cells; (B) implant of the venom-treated group at 4 h post injection – vasodilation and hyperaemia are observed (black arrows) (stain = HE); (C) blood vessels area (μm^2) of control group and treated group. The results are expressed as mean ± standard deviation of the mean, ***$P < 0.0001$; Kruskal–Wallis test and Dunn's post test. (C1 and C4 = control groups 1 h and 4 h post injection of saline, respectively); (T1 and T4 = treated groups 1 h and 4 h post injection of venom, respectively).

rabbits but not in mice and rats (Da Silva et al., 2004), thereby showing differential mammalian toxicity due to unknown reason. The rabbit is the animal model used for the study of loxoscelism, however, the maintenance of these animals is very expensive and their handling is cumbersome for routine laboratory work. Thus, we decided to work with an animal model of smaller size (mice), easy handling and maintenance, lower cost, besides allowing a better control of the experiment. The present study aimed to evaluate the viability of the sponge implant model in mice, considering the need of further investigation of the body's reaction to the venom. In this model, a fibrovascular tissue induced by subcutaneous implantation of a synthetic matrix mimics the events of cutaneous wound healing (inflammatory infiltrate cells, angiogenesis, fibrogenesis) as assessed by biochemical, histological, cellular and functional parameters (Marques et al., 2011; Campos et al., 2011; Andrade and Ferreira, 2009). Indeed, using this model we have previously shown that intra-implant injection of Bothrops jararaca venom resulted in decreased blood flow detected by slow [133]Xenom washout (Vieira et al., 1992). In the present study, it was possible to observe biochemical and histological changes induced by intra-implant injection of 0.5 μg of L. similis crude venom. The alterations included an inflammatory infiltrate predominantly neutrophilic at the injection site,

vasodilatation, hyperaemia, and edema, characterizing an acute inflammation. Besides, hemorrhage, and rupture of the vascular wall were also observed. Interestingly, these events are similar to those observed by several authors in rabbit, guinea pig and human but not in mice skin (Smith and Micks, 1970; Patel et al., 1994; Ospedal et al., 2002; Pereira et al., 2010; Sunderkötter et al., 2001; Ospedal et al., 2002; Barbaro et al., 2010). The difference in sensitivity between different species to spider venoms has been attributed to many factors (tissue damage, secondary vascular injury, release of inflammatory mediators) and to insufficient membrane lipid components such as sphingomyelin and products (He et al., 2001; Domingos et al., 2003). It has been demonstrated that co-administration of Loxosceles gaucho venom with sphingomyelin intradermally in mice caused the development of an inflammatory reaction at the site of injection. This effect has been attributed to the ability of this molecule to trap the venom for a long period preventing its diffusion systemically (Domingos et al., 2003). It is possible that the pool of molecules in the implant microenvironment was also able to keep the Loxosceles similis venom allowing for its prolonged action in the newly formed fibrovascular tissue. It may be argued that the highly permeable nature of the neovasculature would allow rapid diffusion of the venom. However, the lytic activity of the venom promoting blood
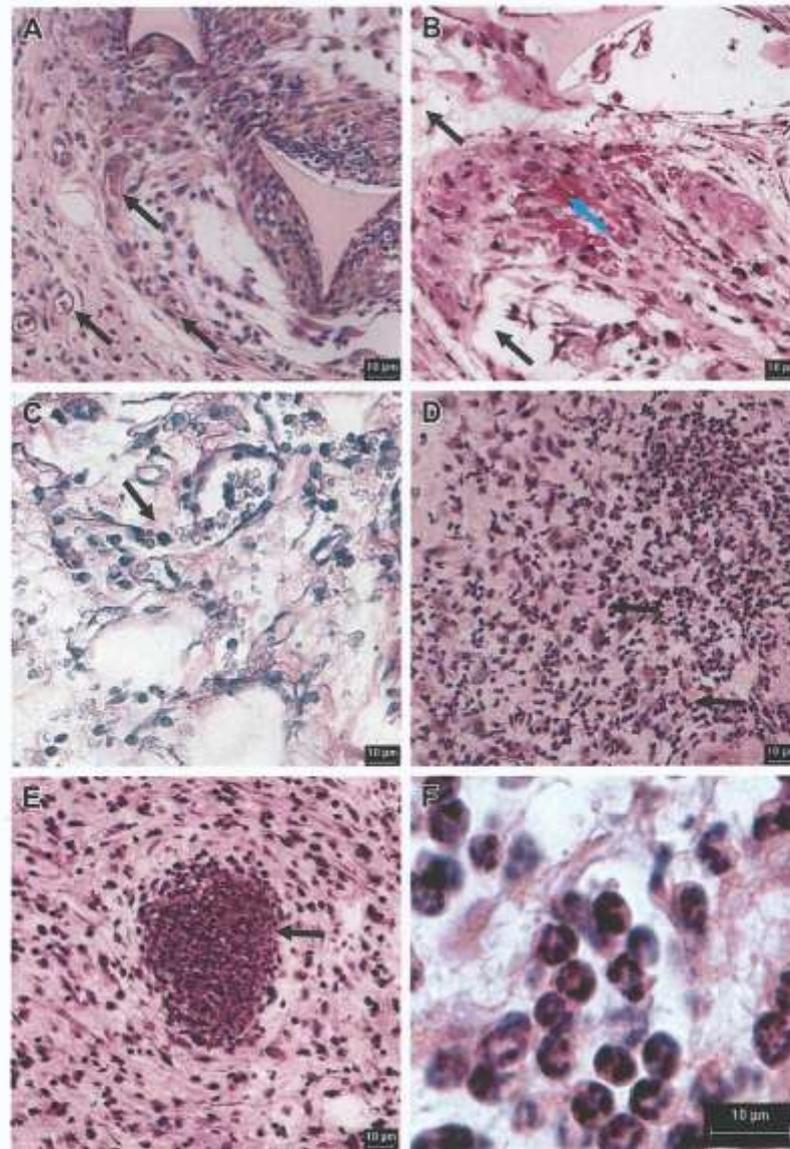
Fig. 3. Histological sections of implants: (A) Implant of control group at 4 h post injection – note the well delineated blood vessels (black arrows) (stain – HE); (B) implant of the venom-treated group at 1 h post injection of *L. similis* venom – observe the dissociation of fibers and cells (black arrows) (characterizing edema) and area of hemorrhage (blue arrow) (stain – HE); C–F – implant of the venom-treated group at 4 h post injection of *L. similis* venom; (C) note the rupture of the vessel wall (black arrow) (Stain – PAS); (D–F) – neutrophilic inflammatory infiltrate: (D) diffuse and (E) focal; (F) detail of the neutrophilic infiltrate (stain – HE) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

vessel wall rupture intraimplant prevented its release from the site of injection. Further investigation will be necessary to identify the nature of the molecules present in implant compartment responsible for keeping the venom and/or its active fraction.

The levels of cytokines VEGF (Vascular Endothelial Growth Factor) and TNF-α (Tumor necrosis factor-α) were also evaluated in the present study. There was a significant increase of these cytokines in the treated group 4 hours after injection compared with the control group. The VEGF is a multifunctional cytokine that exerts a variety of effects on endothelial cells that together promote the formation of new blood vessels, the protection of vascular cells, moreover can lead to increased vascular permeability and
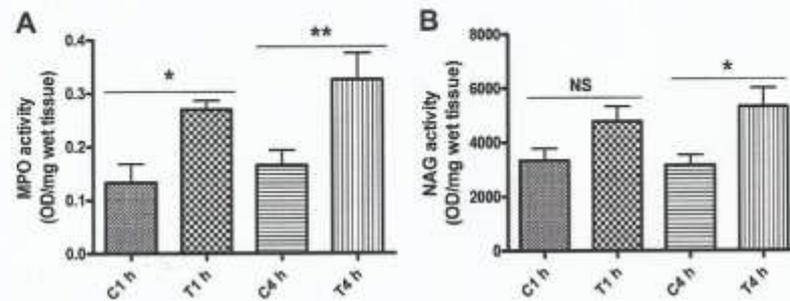
Fig. 4. Markers of inflammation in sponge implants. Neutrophils and macrophages recruited to the implant were determined through the activity of MPO (A) and NAG (B), respectively. Overall, the inflammatory enzyme activities were increased after venom inoculation. Results are expressed as mean ± standard error of mean. $^* P < 0.05$; $^{**} P < 0.01$; Newman–Keuls post test.

thrombogenicity (Robinson and Stringer, 2001). The high level of VEGF detected in the venom-treated implant supports the increase of permeability that induced the edema observed in the histological analysis. This result and is in agreement with Desai et al. (2000) that showed that *L. deserta* stimulated the expression of VEGF in cultured human keratinocytes. Various studies have shown that *Loxosceles* venom stimulates the production of various cytokines. The TNF-α (tumor necrosis factor-α) is a potent regulator of neutrophil chemotaxis, adhesion, priming, phagocytosis, inflammatory mediator release and superoxide generation (Ballou et al., 1996). Furthermore, Málaque et al. (1999) observed that *L. gaucho* venom causes

alterations in primary cultures of keratinocytes and stimulates TNF-α production. Recently, Souza et al. (2008) reported high levels of IL-6 and TNF-α in a patient bitten by *Loxosceles* spp. spider. Several cytokines have been involved in severe envenomation, TNF-α, IL-1b and IL-6 (Petricevich, 2004). In our study, the high level of this cytokine intra-implant induced by the venom may have contributed for the local neutrophil chemotaxis and the consequent neutrophilic infiltration observed in the histological analysis. The sensitivity of the method and its applicability to detect the effects of *Loxosceles* venom were strongly supported by histological and biochemical parameters. Thus, besides being less expensive and ease
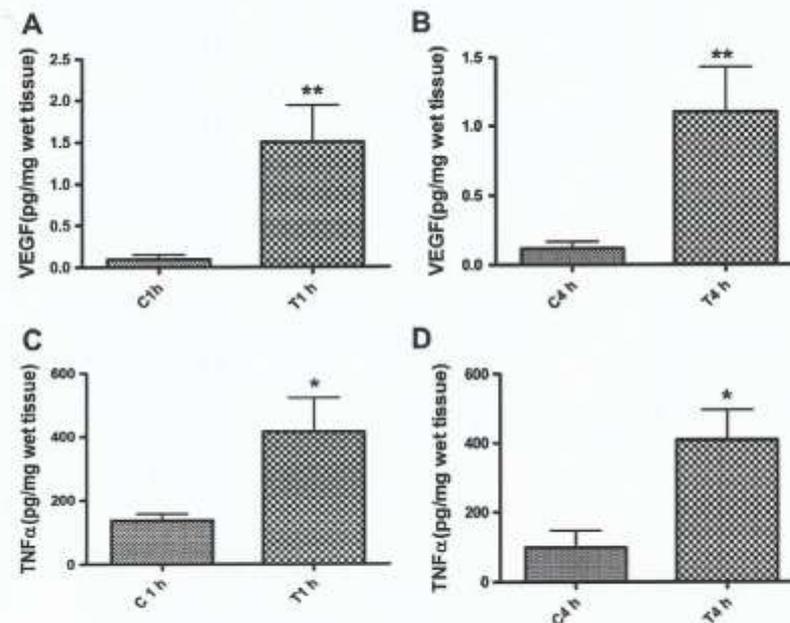

Fig. 5. Cytokines levels in sponge implants: the levels of both cytokines VEGF (A and B) and TNF-α increased at both time intervals studied after venom injection. The results are expressed as mean ± standard error of mean. VEGF p values $^{**} P < 0.006$ (1) and 0.009 (4 h); TNF-α p values $^* P < 0.04$ (1 h) and 0.02 (4 h); Unpaired t test.

handling the implantation technique induces a fibrovascular healing tissue that allows the characterization of molecular and cellular events associated with loxocelism in mice.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Capítulo 2

Apoptose, desgranulação de mastócito e degradação de colágeno na patogenia do loxoscelismo no modelo de implante subcutâneo de esponjas.

1. Justificativa

1 Justificativa

Estudos sobre o loxoscelismo têm sido facilitados através da utilização de modelos experimentais. A *L. similis*, espécie já encontrada em residências de Belo Horizonte (MACHADO *et al.,* 2005) e até pouco tempo pouco estudada, induz experimentalmente lesão cutânea semelhante àquela relatada para outras espécies de importância médica (PEREIRA *et al*., 2012b; CHATZAKI *et al*., 2012).

Estudos recentes do nosso grupo (Pereira *et al*., 2012b) mostraram que o modelo de implante subcutâneo de esponja em camundongo (Andrade *et al*., 1987, Andrade *et al.,* 1992) é útil para a avaliação dos efeitos do veneno de *L. similis*. Utilizando uma concentração de 0,5 μg de veneno de *L. similis* foi possível observar uma inflamação aguda caracterizada por intenso infiltrado neutrofílico, edema e hemorragia intensa, uma e quatro horas após a injeção do veneno intra-implante. Adicionalmente, a atividade de neutrófilos (MPO), macrófagos (NAG) e das citocinas (TNF e VEGF) foram maiores nos animais implantados e injetados com o veneno de *L. similis* se comparados com os animais controles.

Apesar de o modelo de implante subcutâneo de esponja fornecer um microambiente ideal para o estudo do loxoscelismo, faz-se necessário descrever os efeitos do veneno de *L. similis* em um tempo superior aos já estudados no modelo de implante, uma vez que que manifestações clínicas mais intensas tornam-se mais evidentes 12 horas após a picada. Ainda não há estudos que avaliem os efeitos da *L. similis* em um tempo superior a 8 horas no modelo de implante.

Além disso, os mecanismos pelos quais os venenos das aranhas *Loxosceles* provocam lesões dermonecróticas e/ou sistêmicas precisam ser mais investigados, pois os mesmos ainda não estão completamente elucidados.

2. Introdução

2 Introdução

2.1 Os mastócitos e o processo inflamatório

Os mastócitos são células derivadas da medula óssea, residentes no tecido conjuntivo que normalmente estão associados aos vasos sanguíneos e linfáticos, nervos e ductos glandulares, nas proximidades de superfície com interface para o meio externo, incluindo, a pele (METCALFE *et al*., 1997) que apresentam grânulos citoplasmáticos metacromáticos. Os mastócitos são as primeiras células a responder a um trauma e estimulam uma resposta imune através da liberação de mediadores biológicos pré-formados, que ficam estocados nos seus grânulos até que sejam ativados (METCALFE *et al*., 1997).

Os mastócitos quando ativados liberam vários mediadores, tais como, histamina, proteases neutras, fatores quimiotáticos, heparina (uma glicosaminoglicana sulfatada responsável pela metacromasia), serotonina, IL4, IL5, IL6, IL13, TNF-α, e mediadores inflamatórios, prostaglandina, leucotrienos e fator de ativação das plaquetas (SIRAGANIAN, 1988; LEVI-SCHAFFER, RUBINCHICK, 1995; *KATZ et al.*, 1995; OKAYAMA *et al*., 1995).

Em resposta a uma ferida os mastócitos desgranulam e a ativação do conteúdo dos seus grânulos estimula a proliferação de células endoteliais no tecido. As citocinas liberadas pelos mastócitos promovem a produção de citocinas pró-inflamatórias pelas células residentes, atraindo células inflamatórias. A liberação de aminas vasoativas estimula o aumento da permeabilidade dos vasos sanguíneos, promovendo um influxo de neutrófilos, macrófagos e mastócitos adicionais ao tecido. A ativação e a desgranulação de mastócitos residentes intensifica e prolonga a resposta inflamatória (CHEN *et al.,* 2014). Os mastócitos são evidentes em tecido cicatricial e geralmente permanecem na cicatriz até um ano após o ferimento. A ativação de mastócitos pode influenciar o processo de remodelamento da ferida com o excesso de inflamação e a produção de citocinas pode promover a formação de uma cicatriz. Além da histamina e serotonina os mastócitos liberam dos seus grânulos, triptases, que estimulam os fibroblastos a sintetizar colágeno (Chen *et al*., 2014).

Chen *et al.* (2014) mostram em seus estudos que camundongos deficientes de mastócitos apresentaram alterações no processo inflamatório, alterações na arquitetura das fibras colágenas (não apresentaram disposição e birrefringência normal). Alterações na cicatriz também foram observadas. Por outro lado, o aumento do número de mastócitos tem

sido descrito na diabetes melitus, fibrose hepática, artrites, queloides e tumores (NORRBY *et al.*, 1990; MALONE *et al.*, 1987).

2.2 Os mastócitos e o loxoscelismo

Estudos mostram que a injeção intradérmica do veneno de várias espécies de *Loxosceles* é capaz de causar hiperemia, aumento da permeabilidade vascular, edema, hemorragia, trombos, inflamação aguda intensa, rabdomiólise e destruição na parede dos vasos em modelos experimentais (OSPEDAL *et al.,* 2002; BUCARETCHI *et al.,* 2010; PEREIRA *et al.,* 2012 a,b; CHATZAKI *et al.*, 2012). No entanto, a patogenia do loxoscelismo não está completamente elucidada e mais estudos são necessários. O mecanismo de ação do veneno é bastante complexo, pois envolve a atividade de várias enzimas e o sinergismo de ação entre os vários componentes presentes no veneno.

Os mastócitos são participantes fundamentais no desenvolvimento de uma resposta inflamatória a danos teciduais e, portanto estão envolvidos na patogenia de um processo inflamatório (ZUO *et al.*, 2003; LIU *et al.*, 2007). A sua ativação promove a liberação de mediadores químicos responsáveis por importantes alterações teciduais, alterando a permeabilidade vascular, além de vasodilatação e quimiotaxia de neutrófilos. Algumas fosfolipases D, presentes na superfície de mastócitos, são responsáveis pela desgranulação de mastócitos. Estudos avaliando a participação e a importância dos mastócitos no loxoscelismo são escassos. Sabe-se que o veneno tanto bruto quanto a fração dermonecrótica de *L. intermedia* é capaz de causar em camundongos *Swiss* e em ratos a desgranulação de mastócitos e liberação de histamina e serotonina (RATTMANN *et al.*, 2001; PALUDO *et al.*, 2009). Tal abordagem ainda não foi avaliada para o veneno de *L. similis* e nem tampouco utilizando o modelo de implante subcutâneo de esponja para o estudo do loxoscelismo. Por isso, torna-se necessário uma análise mais detalhada da participação e importância dessas células no loxoscelismo e no presente estudo, utilizando como modelo, o implante subcutâneo de esponjas.

2.3 Apoptose

A morte celular por apoptose, também conhecida como "morte celular programada", envolve a eliminação de células por mecanismos genéticos. É um processo que depende de energia, síntese e degradação protéica para a sua execução (KERR & SEARLE, 1972; ELMORE, 2007).

Toda célula possui a maquinaria de apoptose e está pronta para ativar sua autodestruição, dessa forma, para que a célula se mantenha viva ela depende de estímulos específicos, tais como, adesão na matriz extracelular, adesão célula-célula, integridade do citoesqueleto, das mitocôndrias e do retículo endoplasmático. Além disso, é necessário também que a célula possua ATP e um fluxo intracelular de cálcio em concentrações equilibradas (MALLAT & TEDGUI, 2000; GROENENDYK & MICHALAK, 2005; CHEN *et al*., 2008; YAO & WOOD, 2009).

A apoptose é um mecanismo de eliminação controlada de células, que pode ser iniciado tanto por estímulos fisiológicos quanto por estímulos patológicos. Fisiologicamente a apoptose participa do desenvolvimento embrionário, organogênese, renovação de células epiteliais, involução dos órgãos reprodutivos na senilidade e hipotrofia induzida pela remoção de fatores de crescimento ou hormônios (KERR, 1993; KERR, 1999). Dentre os estímulos patológicos que podem induzir esse tipo de autodestruição celular estão: vários vírus (VASCONCELOS & LAM, 1994; VASCONCELOS & LAM, 1995; MORO *et al*., 2003a, NYKKY *et al.,* 2014), venenos de aranha (GAO *et al.*, 2005, PEREIRA *et al.*, 2012b) e serpentes (DEBNATH *et al.*, 2006). Adicionalmente, a apoptose têm sido observada também em algumas doenças neuro-degenerativas tais como: na doença de *Alzheimer* e de *Parkinson*, bem como em algumas doenças auto-imune (ELMORE, 2007; VENDEROVA & PARK, 2012).

À microscopia de luz de fase clara, as seguintes alterações características de apoptose são descritas: (a) retração celular e perda de adesão com outras células, ou com a membrana basal (anoiquia); (b) zeiose ou formação de projeções digitiformes na membrana citoplasmática; (c) condensação do citoplasma com diminuição do teor hídrico e do volume celular, (d) condensação nuclear com compactação da cromatina em massas densas uniformes, alinhadas na face interna da carioteca (crescentes) (PEITSCH *et al.,* 1993; VASCONCELOS, (2001); (e) convolução e posterior fragmentação da membrana nuclear e (f) fragmentação celular com a formação dos corpos apoptóticos (KERR *et al.*, 1972).

Os corpos apoptóticos que se formam são fagocitados pelas células circunjacentes (canibalismo celular) ou por macrófagos (SAVILL *et al.*, 1993) antes que ocorra a lise celular. Como não ocorre ruptura de células (SAVILL *et al.*, 1993), não há liberação de componentes celulares no espaço extracelular e, por conseguinte, não há indução de inflamação (FADOK & HENSON, 1998).

Alterações bioquímicas e moleculares também são observadas durante o processo de apoptose, tais como a clivagem do DNA genômico em fragmentos múltiplos de 180-200 pares de bases, que é bem típico do processo de apoptose (COHEN *et al.,* 1981; WYLLIE *et al*., 1980). Essa fragmentação nuclear é visualizada através da eletroforese do DNA em gel de agarose, produzindo o clássico "padrão em escada" (WYLLIE *et. al.,* 1980). Essa fragmentação pode ser observada *in situ* por meio da reação de TUNEL. Essa reação consiste na introdução de nucleotídeos marcados nas extremidades 3' OH do DNA fragmentado utilizando-se a enzima transferase terminal de desoxinucleotídeo – TDT (GAVRIELI *et al*., 1992).

Outras técnicas vêm sendo utilizadas como método de auxílio na identificação de células apoptóticas dentre elas destacam-se: a microscopia eletrônica (NOWATZKI, 2012) e a imunofluorescência (De RESENDE *et al*., 2006; MORAIS *et al*., 2012). Estudos de Blaschke *et al*. (2004) e De Resende et al., (2006) utilizaram a técnica de TUNEL associada com marcadores fluorescentes (DAPI). Adicionalmente, estudos conduzidos por De Resende *et al*. (2006) também utilizaram tripla marcação para TUNEL, DAPI em associação com anticorpo CD31, objetivando avaliar a apoptose de células endoteliais de músculo esquelético. Tais técnicas podem ser importantes ferramentas no estudo de células de difícil identificação e quantificação da apoptose.

O mecanismo de apoptose é altamente complexo e envolve uma cascata de eventos dependente de energia (ELMORE, 2007) que é ativada pela expressão de genes que controlam a síntese de várias enzimas, tais como: caspases, transglutaminases e endonucleases (FESUS *et al*., 1987; ARENDS & WYLLIE, 1991; TENNISWOOD *et al*., 1994).

A maioria das mudanças morfológicas observadas na apoptose (**Figura 1**) é mediada por uma cascata enzimática envolvendo proteases de cisteína denominadas caspases (THORNBERRY *et al*., 1997; HYMAN & YUAN, 2012). Essas enzimas possuem um resíduo de cisteína no sítio ativo e clivam substratos que apresentam resíduos de ácido aspártico em sequências específicas. Elas são sintetizadas como precursores inativos que são clivados proteoliticamente para gerar subunidades ativas. A ativação das caspases promove a

desmontagem da membrana nuclear e do arcabouço de laminas, a condensação da cromatina e a degradação proteolítica das estruturas nucleares e citoplasmáticas.
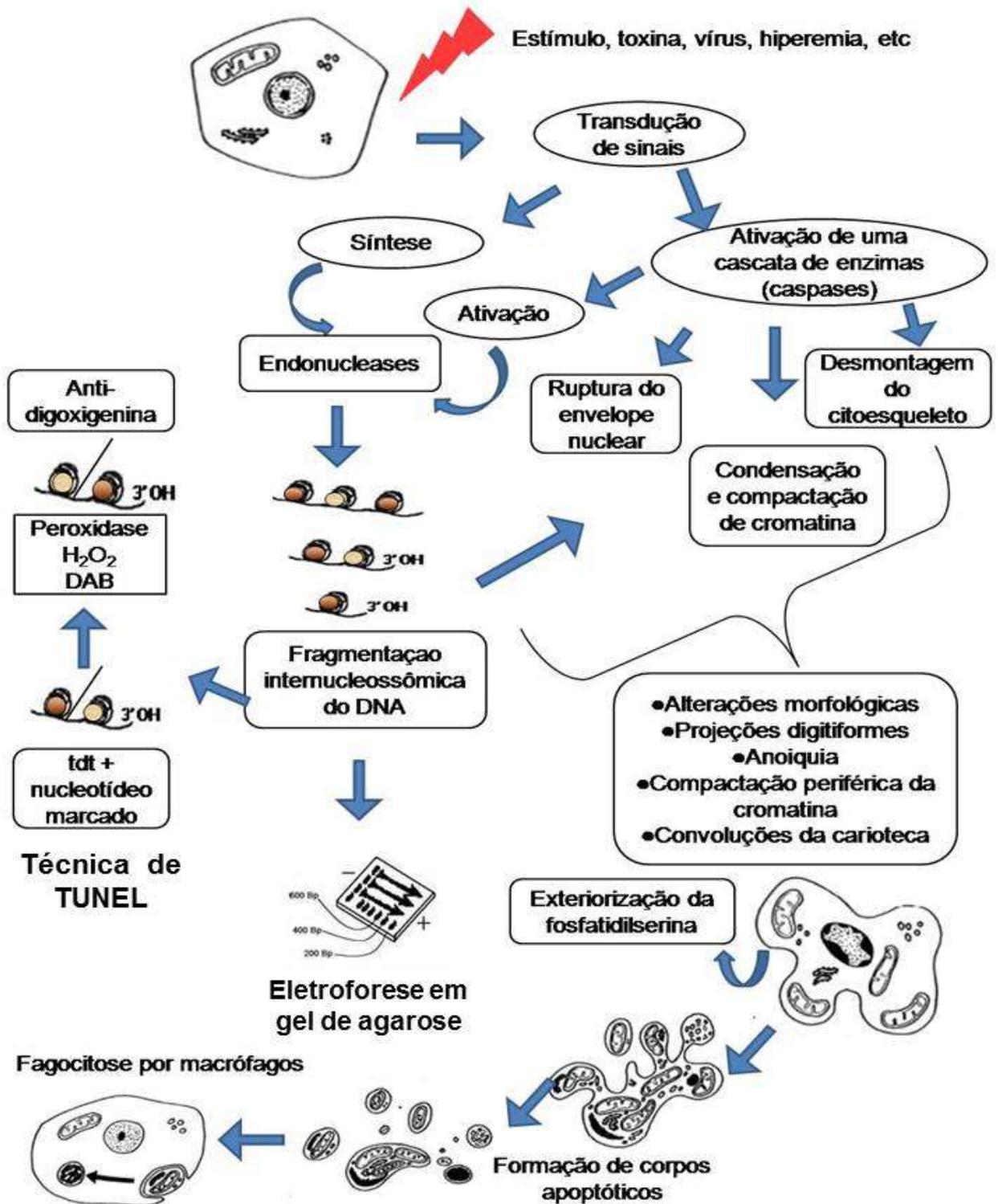

Figura 1: Sequência de eventos envolvidos no processo de morte celular por apoptose e métodos de detecção. Cedido pela profa. Luciana Moro com algumas modificações.

Essas alterações são comuns nas células em apoptose, independentemente do agente indutor do processo. Isto significa que a ação das caspases representa a via final comum que normalmente opera em células programadas para morrer (THORNBERRY *et al.*, 1997; HUANG *et al.*, 2011). As caspases que participam do processo de apoptose são divididas em duas classes: as iniciadoras (caspases 1, 2, 4, 5, 8, 9, 10, 11 e 12) e as executoras (caspases 3, 6, 7 e 14) (COHEN, 1992; ELMORE, 2007; HYMAN & YUAN, 2012).

Estudos mostram que a ativação de caspases pode ser controlada por membros da família Bcl-2. Uma característica importante da família Bcl-2 é a heterodimerização entre componentes anti-apoptóticos (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) e pró-apoptóticos (Bax, Bak, Bid, BIM, PUMA,), inibindo assim a atividade biológica dos outros membros (HYMAN & YUAN, 2012). Assim, as concentrações celulares proporcionais e a dimerização competitiva e seletiva entre as proteínas da família Bcl-2 determinam a susceptibilidade celular a apoptose (KORSMEYER, 1999; HYMAN & YUAN, 2012). A dimerização de Bax resulta em sua translocação (GROSS *et al.*, 1998) e inserção na membrana mitocondrial (SUZUKI *et al.,* 2000) com consequente disfunção mitocondrial e ativação de caspases (GROSS *et al.,* 1999).

Pesquisas indicam que existem duas vias apoptóticas: a via extrínseca ou a via do receptor de morte e a via intrínseca ou mitocondrial (**Figura 2**). Existe também uma via adicional que envolve células T como mediadoras de citotoxicicidade e a granzima perforina dependente da morte celular. Essas vias são iniciadas pela clivagem de caspase 3 e resultam em fragmentação do DNA, degradação do citoesqueleto e proteínas nucleares, formação de corpos apoptóticos e fagocitose de células (HYMAN & YUAN, 2012).

A via extrínseca, também conhecida como "via do receptor de morte", é induzida por sinais extracelulares. A ligação ao receptor de morte cliva a caspase 8 inativa, gerando a forma ativa da caspase 8, que é iniciadora do processo de apoptose. Essa ativação desencadeia a ativação de uma cascata de caspases, dentre elas a caspase 3 (executora), que ativa as endonucleases que iniciam o processo de clivagem do DNA.

A via intrínseca ou mitocondrial é ativada em resposta a vários tipos de estresse intracelular, tais como radiação, estresse no retículo endoplasmático, drogas, dentre outros. O estresse celular leva a alterações morfofuncionais na mitocôndria com consequente liberação do citocromo C. O citocromo C quando acoplado à molécula adaptadora (Apaf – fator de ativação da apoptose), ativa a pro-caspase 9 formando o apoptossomo, que é responsável pela ativação da caspase 9 que, ativará a caspase 3 (FIG.5).
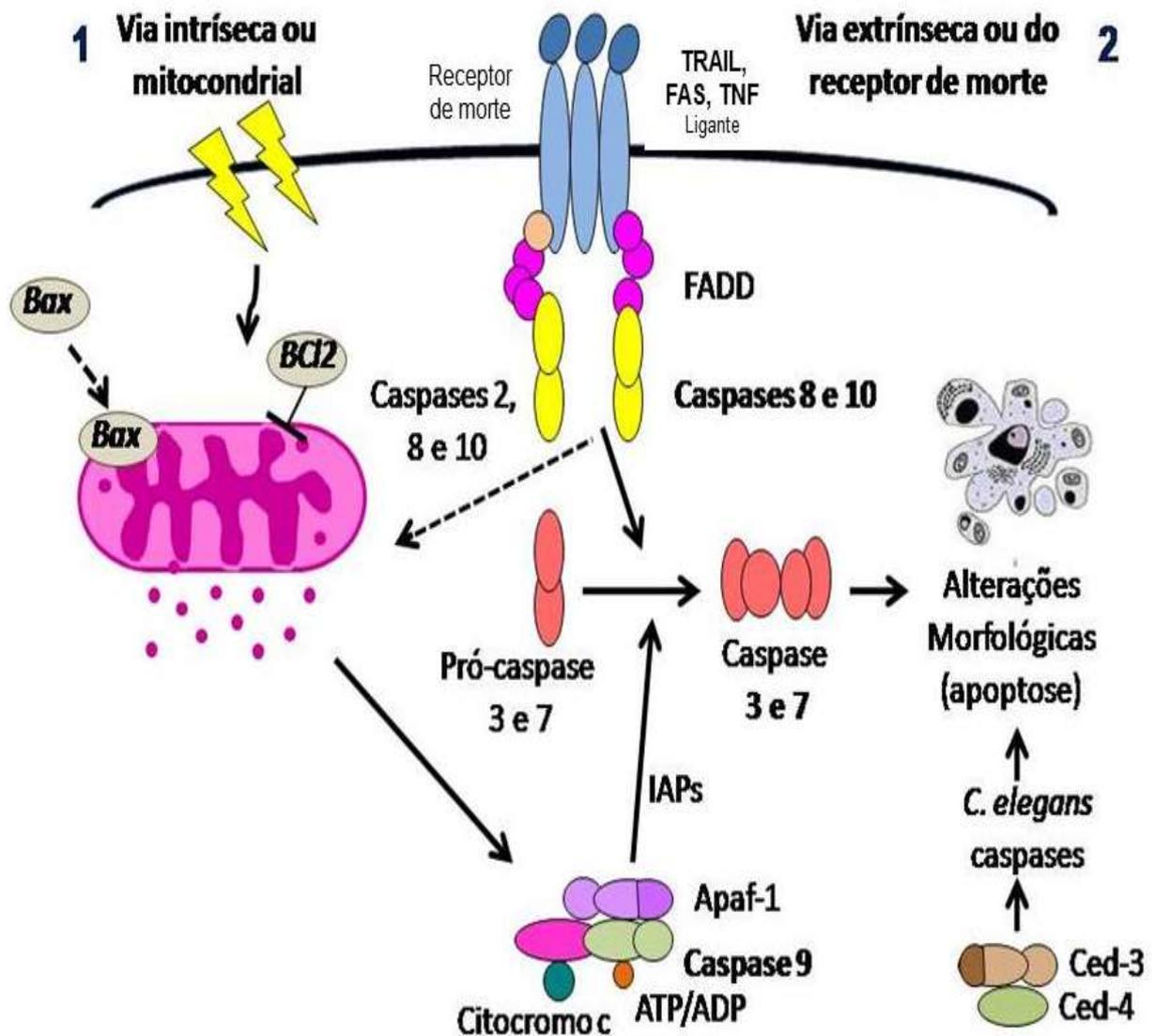
Figura 2 - Esquema representativo das vias apoptótica: (1) via intrínseca e (2) via extrínseca

2.4 Estudo da apoptose e da patogenia do loxoscelismo

Sabe-se também que a interação do veneno com o tecido ativa o sistema do complemento, induz a migração de leucócitos, particularmente polimorfonucleares, assim como a liberação de enzimas proteolíticas e de citocinas, a agregação plaquetária e alterações na hemostasia (PATEL, *et al.,* 1994; Da SILVA *et al.,* 2004; VAN DER BERG *et al.,* 2007; TAMBOURGI *et al.,* 2005).

Estudos de VEIGA *et al.* (2001a) observaram que o veneno tem ação direta sobre a integridade dos vasos, pois ele degrada o proteoglicano heparam sulfato e a entactina, envolvidos no processo de adesão celular. NOWATZKI (2009) demonstrou que o veneno de

L. intermedia é capaz de interagir com a integrina α5b1 que é um receptor específico para fibronectina. Sabe-se que a matriz extracelular (MEC) influencia diversos mecanismos celulares, tais como, adesão, migração, proliferação e diferenciação celular. Tais mecanismos são sinalizados por meio da interação de componentes da MEC com integrinas (NOWATZKI, 2009).

O veneno de *Loxosceles* induz lesão vascular no local da picada por agir tanto nas células endoteliais (VEIGA *et al.,* 2001a) quanto na membrana basal subendotelial, que é considerada uma estrutura de extrema importância para a integridade dos vasos (ZANETTI *et al.*, 2002). A injeção intradérmica de 40 µg do veneno de *L. intermedia* induz formação de digitações subendoteliais, vacúolos e degeneração da parede dos vasos após 4 horas pós-exposição. O estudo da ação do veneno de *L. intermedia* sobre células endoteliais *in vitro* tem mostrado que ela induz perda de adesão celular (VEIGA *et al.*, 2001a; NOWATZK *et al*., 2012) que se inicia 3 horas após a exposição (NOWATZK *et al*., 2012). Adicionalmente, Pereira *et al.* (2012b) avaliaram o efeito do veneno de *L. similis* (0,5 µg) injetando intradermicamente em coelhos e observaram a presença de células endoteliais retraídas, apresentando perda de adesão entre si e com a matriz extracelular (anoiquia), contendo núcleo retraído, carioteca irregular e cromatina condensada e compactada na periferia, caracterizando apoptose, 2 horas após a injeção do veneno. Adicionalmente, células endoteliais em apoptose estavam presentes na interface trombo-vaso a partir de 2 horas de injeção do veneno. A apoptose de células endoteliais pode induzir a perda de adesão celular e a descamação da parede do vaso, o que faz com que o colágeno subendotelial seja exposto, induzindo agregação plaquetária e ativação da cascata de coagulação. Uma vez que os trombos são formados, haverá uma isquemia e consequentemente ulceração, que comumente se observa nas lesões cutâneas do loxoscelismo.

Apesar de alguns estudos abordarem a patogenia do loxoscelismo, ainda há muito que se elucidar principalmente em tempos superiores a 12-24 horas, pós-injeção do veneno, tempos estes que mais comumente os pacientes levam para procurar atendimento médico. Além disso, como dito anteriormente, sabe-se o veneno atua na matriz extracelular degradando seus componentes (Veiga *et al*., 2000). Considerando que o processo de cicatrização do loxoscelismo ocorre por segunda intenção (HOGAN *et al*., 2004), sendo muito vagaroso, torna-se necessário avaliar também a apoptose de outras células presentes na matriz tais como macrófagos, células gigantes e fibroblastos, que poderá facilitar a compreensão dos mecanismos envolvidos no loxoscelismo. O loxoscelismo foi muito bem caracterizado no modelo subcutâneo de implante de esponjas, no entanto pesquisas relacionadas com a

patogenia do loxoscelismo ainda não foram conduzidas em tal modelo. Por se tratar de um modelo que mimetiza o processo de cicatrização de feridas e por similar a um cultivo celular *in vivo* o mesmo torna-se uma importante ferramenta e por essas razões torna-se pertinente essa abordagem.

2 Objetivos

3. Objetivos

3.2 Objetivo geral

Caracterizar a apoptose, a desgranulação de mastócitos e a degradação do colágeno na patogênese do loxoscelismo em esponjas implantadas subcutaneamente.

3.3 Objetivos específicos

(1) Descrever histologicamente os efeitos do veneno bruto de *L. similis* no tecido fibrovascular do implante subcutâneo de esponjas de 14 dias, com ênfase em 24 horas pós-injeção.

(2) Quantificar e mensurar sua atividade de desgranulação dos mastócitos no tecido fibrovascular do implante nos tempos de 1 e 4 horas após a injeção intra-implante de *L. similis*;

(3) Quantificar o colágeno total e os subtipos I e III no tecido fibrovascular do implante nos tempos de 1 e 4 horas após a injeção intra-implante de 0,5 µg (30µL) do veneno de *L. similis*;

(4) Verificar e quantificar a apoptose no tecido fibrovascular do implante nos tempos de 1, 4 e 24 horas após a injeção do veneno *L. similis*.

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5. Artigo

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Apoptosis, mast cell degranulation and collagen breakdown in the pathogenesis of loxoscelism in subcutaneously implanted sponges

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ARTICLE INFO

ABSTRACT

Envenomation by the Loxosceles spider causes loxoscelism, a pattern of signs and symptoms that primarily manifests in the dermonecrotic form. Our studies have shown that a mouse subcutaneous sponge implantation model may be useful in evaluating the effects of Loxosceles similis venom. This model provides an ideal microenvironment in which to study loxoscelism; however, it is still important to evaluate its pathogenesis and to observe the effects of L. similis venom for longer time periods than those in previous studies of this model. The aims of this study are: (1) to histologically characterize the effects of L. similis crude venom in a subcutaneous sponge implant; (2) to quantify the mast cells present in the implant and to measure their degranulation activity; (3) to quantify collagen subtypes I and III; and (4) to verify, quantify, and evaluate the effects of apoptosis in the implant on the pathogenesis of loxoscelism at 1 h, 4 h, and 24 h after injecting the venom. Thirty Swiss mice (6–8 weeks old, male) were subcutaneously implanted with polyester-polyurethane sponge discs. Fourteen days post-implantation, the animals were divided into six groups (5 animals per group): three control groups (C1h, C4h, and C24h), in which the mice received 30 μl injections of intra-implant saline, and three treated groups (T1h, T4h, and T24h), in which the mice received 30 μl (0.5 μg) injections of L. similis crude venom at 1 h, 4 h, and 24 h intervals. After each time interval, the animals were euthanized, and the implants were harvested and processed for light and electron microscopic analyses. The following results were observed in the implants harvested from the treated groups: acute inflammation with marked edema, thrombus, and vasculitis, as well as increased levels of mast cells and mast cell degranulation, and apoptosis in giant cells. Furthermore, degradation of collagen types I and III was observed. An analysis of the ultrastructure revealed apoptosis in various cell types. The present results suggest that apoptosis in some cell types

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associated with an increase in mast cell degranulation and the degradation of collagen fibers are important in the pathogenesis of loxoscelism therefore may explain the difficulty in repairing the ulcer is commonly observed in severe cases of loxoscelism cutaneous in humans.

1. Introduction

Loxoscelism describes the lesions and clinical manifestations that result from bites caused by *Loxosceles* spiders, commonly known as brown spiders. The clinical manifestations of loxoscelism are generally characterized by dermonecrosis at the bite site (67%–100% of cases) and may be followed by systemic effects that can cause acute renal failure and, occasionally, death (Hogan et al., 2004; Da Silva et al., 2004).

The bite is initially painless, but after 2–8 h, pain, moderate to severe edema, and erythema appear at the bite site. Within 12–24 h, the area becomes pallid because of ischemia and is surrounded by a white halo. This lesion, known as marble plate, may ulcerate, and in some cases, healing difficulties may necessitate surgical excision and skin grafting (Hogan et al., 2004; Da Silva et al., 2004).

The rabbit model is most frequently used to study dermonecrotic loxoscelism (Ospedal et al., 2002; Chatzaki et al., 2012; Pereira et al., 2012b). However, a recent study by our group (Pereira et al., 2012a) showed that a mouse subcutaneous sponge implantation model (Andrade et al., 1987, 1992) was useful in assessing the effects of *Loxosceles similis* (*L. similis*) venom because the resulting lesions were similar to those observed in experimental rabbits with loxoscelism. This model mimics *in vivo* cell growth; it consists of fibrovascular tissue that contains of loose connective tissue, newly formed vessels, inflammatory cells, mast cells, foreign-body giant cells, and *Langhans* giant cells.

Although the subcutaneous implantation of sponge provides an ideal setting in which to study the loxoscelism microenvironment, it is necessary to evaluate the pathogenesis of loxoscelism and to describe the effects of *L. similis* venom for longer periods than those used in the implant model. Previous studies (Pereira et al. 2012a) have shown the involvement of apoptosis in the pathogenesis of endothelial cells in skin loxoscelism in a rabbit model. We must now determine whether the same phenomenon occurs in a fibrovascular tissue implant. This study had four aims: (1) to histologically characterize the effects of *L. similis* venom on fibrovascular tissue after the subcutaneous implantation of sponges; (2) to quantify the mast cells present in the tissue implant and to measure their degranulation activities; (3) to quantify the total collagen and collagen subtypes I and III; and (4) to verify, quantify, and assess the effects of apoptosis in fibrovascular tissue implants on the pathogenesis of this model 1 h, 4 h, and 24 h after injecting *L. similis* venom.

2. Materials and methods

The present study was approved by the *Comitê de Ética em Experimentação Animal* (CETEA) of the Universidade Federal de Minas Gerais (UFMG) (process number 229/09) and by the *Comitê de Ética no Uso de Animais* (CEUA) at the Centro de Pesquisas René Rachou (CPqRR/Fiocruz Minas) (approval number 93/200-1).

2.1. Venom extraction

Maceration and centrifugation, according to Silvestre et al. (2005), was used to extract venom from the venom glands of adult animals.

2.2. Preparation and implantation of sponge discs to study loxoscelism

Polyether-polyurethane sponge discs (Vitafoam Ltd., Manchester, UK) measuring 6 mm thick and 11 mm in diameter (Fig. 1A) were soaked overnight in 70% v/v ethanol. Before implantation, the sponges were washed and boiled in distilled water for 20 min. The animals (thirty Swiss mice) were anesthetized with xylazine/ketamine (1 mg/kg, Syntec of Brazil). A trichotomy of the dorsal skin was performed, and skin antisepsis was performed with iodized alcohol. The sponge discs were aseptically implanted into a subcutaneous pouch through a 1 cm dorsal midline incision, which was sutured with n°5 silk thread. The animals were maintained for 14 days in the experimentation bioterium of the *Centro de pesquisas René Rachou* (CPqRR) and postoperatively monitored for signs of infection at the operative site, discomfort, and distress. The animals were given *ad libitum* access to water and food throughout the experiment.

2.3. Venom injection

Thirty Swiss mice (6–8 weeks old, male) received injections (saline or venom) fourteen days post-implantation. The animals were divided into six groups (5 animals per group): three control groups (C1h, C4h, and C24h), in which the mice received 30 μl injections of intra-implant saline, and three treated groups (T1h, T4h, and T24h), in which the mice received 30 μl (0.5 μg) injections of *L. similis* crude venom at 1 h, 4 h, and 24 h intervals.

2.4. Implant removal and macroscopical analysis

At 1 h, 4 h, and 24 h post-injection, the animals were euthanized, and the implants were removed and analyzed
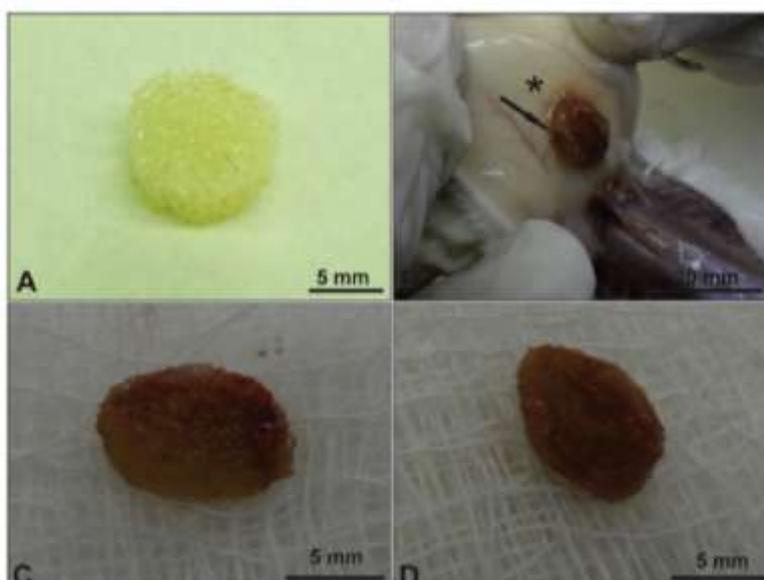
Fig. 1. A macroscopic analysis of the effects of the intra-implant injection of *L. similis* crude venom of the spongy matrix: (A) A polyester-polyurethane sponge before implantation and (B) 14 days post-implantation, surrounded by a fibrous reddish capsule (black arrow) and adhered to the skin (*); (C) implants in a control group (C24h) and (D) in a treated group (T24h), note that the implants were also surrounded by a reddish fibrous capsule. Barr = 5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

macroscopically. Subsequently, the implants were fixed in buffered formalin (pH 7.4), embedded in paraffin, and processed for histologic and morphometric analyses. Sponge fragments were also fixed in 2.5% glutaraldehyde and processed in phosphate buffer for 12 h at 4 °C for embedding in resin for ultrastructural analysis.

2.5. Light microscopy

At each time interval, implants from both groups (control and treated) were fixed in 10% buffered formalin (pH 7.4) and processed for paraffin embedding. Sections (5 mm thick) were stained with hematoxylin and eosin (HE), Dominici Blue, and Picrosirius Red for histological and morphometrical analyses.

2.6. Morphometric analysis

2.6.1. Assay for collagen quantification

Picrosirius staining, followed by polarized-light microscopy, was used to visualize and examine the collagen fibers. Picrosirius Red is an anionic composite that reveals the thickness and density of collagen fibers through coloration that is visible under polarized light (Puchtler et al., 1973; Junqueira et al., 1979; Campos et al., 2011). The thickest and strongest fibers, which are associated with type I collagen, emit colors of longer wavelengths, such as red and yellow. In contrast, the fine, dissociated fibers typical of type III collagen appear greenish. To perform the morphometric analysis, 5 cross-sectional images obtained

from whole slides were evaluated to determine the densitometric fibrovascular area (area = 443,592.6 μm^2). Collagen fibers stained with picrosirius and observed under polarized light microscopy (Olympus BX-640) were digitized using a JVC TK-1270/JCB micro-camera with a 10× objective, and the images were transferred to an Image-Pro Plus 4.5 image analyzer (Media Cybernects, Inc.).

2.6.2. Quantification of mast cells

To count the mast cells, we calculated the minimum number of representative microscopic fields in a single layer of each sample, and the number of mast cells per field was recorded and analyzed, according to Moro et al. (2003a). The Dominici Blue stained sections and images were captured using a 40× objective. The minimum sample size considered representative, which was defined as the sample size at which an increase in the number of fields did not result in a considerable reduction in the amount of the error or coefficient of variation, was 30 fields.

The index of mast cells (IMC) was calculated using the following formula:

IMC = number of mast cells per field/
total number of cells × 100.

2.6.3. Mast cell degranulation index (MCDI)

To determine the mast cell degranulation rate, we used 30 microscopic fields, as described in Section 2.6.2, to measure the MPI. The IDM was calculated using the following formula:

MCDI = number of degranulated mast cells per field/
total number of mast cells × 100.

2.6.4. Apoptosis in implants

2.6.4.1. Apoptosis of endothelial cells (AEC).
Apoptosis of endothelial cells was observed by ultrastructural analysis by transmission electron microscopy (ZEISS model AT-10). See details in item 2.8.

2.6.4.2. Index of apoptotic giant cells (IAGC).
The IAGC was determined by counting the total number of cells and the number of cells undergoing apoptosis. The following formula was used to calculate the apoptotic index:

IAGC = number of giant cells/total number of cells per field
× 100 cells per field.

To ensure that the giant cells could be easily visualized microscopically, we used 40× magnification. When establishing the apoptotic index of giant cells, we considered the following factors: (1) apoptosis in one or more giant cell nuclei and (2) apoptosis in all giant cells, with the consequent loss of giant cell adhesion with spongy and/or connective tissue.

2.7. In situ DNA fragmentation

To identify in situ DNA fragmentation, a terminal deoxynucleotidyl transferase uracil nick-end labeling
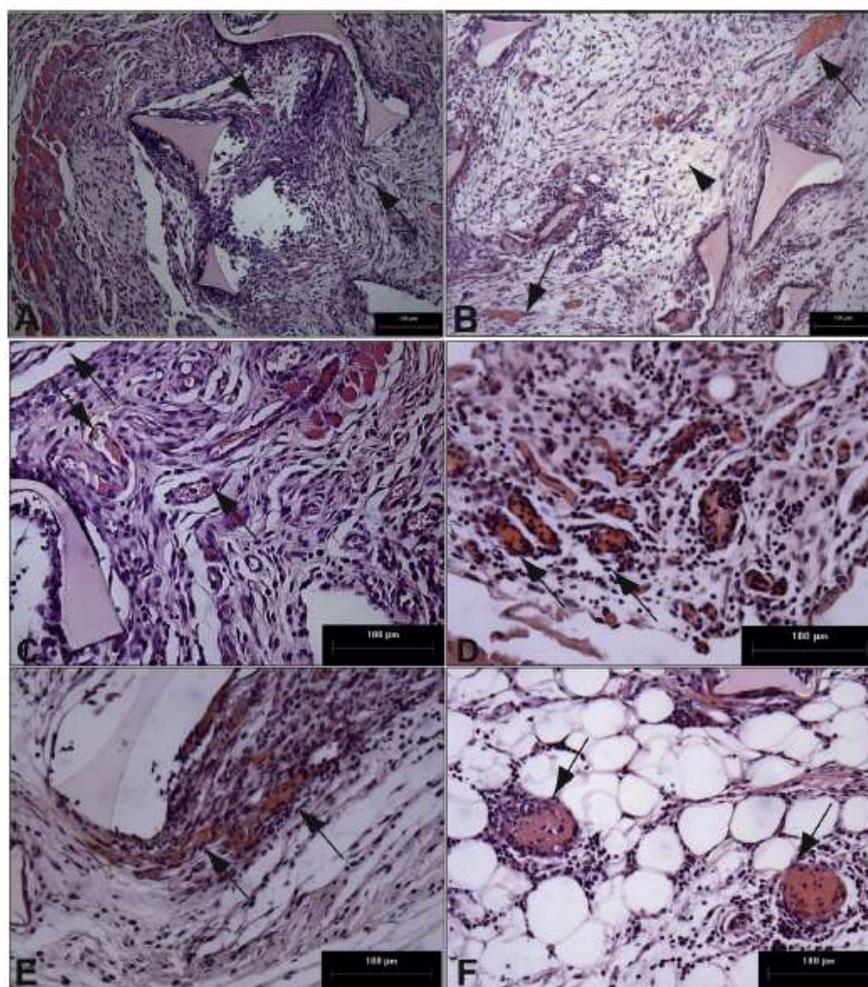

Fig. 2. Histological sections of subcutaneous sponge implants after 14 days. (A) Control group, 24 h after the saline injection; note that the sponge implant has been infiltrated by fibrovascular tissue (black arrows); (B) sponge implant group treated with L. similis venom (30 μL/0.5 mg of venom) 24 h after the injection, note the cell depletion, vasodilation, and hyperemia (black arrows), as well as the dissociation of fibers and cells (edema) (arrowhead); (C) control group, note the integrity of the blood vessels (D–F), presence of perivascular inflammatory infiltrates, and occlusive thrombus (white arrows) 1 h, 4 h, and 24 h after injecting L. similis crude venom, respectively. HE staining. Barr = 100 μm.

(TUNEL) (Calbiochem Kit catalog QIA33) analysis was used, according to Gavrieli et al. (1992) and the manufacturer's instructions.

2.8. Transmission electron microscopy (TEM)

For transmission electron microscopy, samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in osmium tetroxide, and embedded in EPON resin. The sponges were sectioned with a diamond knife, contrasted using uranyl acetate and lead citrate, and examined under a ZEISS model AT-10 transmission electron microscope (Chiarini-Garcia et al., 2014).

2.9. Statistical analysis

The results are presented as the means ± standard error (SE). The Kolmogorov–Smirnov test was performed to determine whether the data showed normal distribution. For normally distributed data, an analysis of variance (ANOVA) and the Newman–Keuls test were used to compare groups. For non-normally distributed data, the non-parametric Kruskal–Wallis test and Dunn's post test were applied. Values of $p < 0.05$ were considered to be significant. The statistical analysis was performed using the GraphPad Prism program, version 6.

3. Results

3.1. Macroscopic analysis

Macroscopically, on post-implantation day 14, the sponge discs were enveloped by a fibrous capsule (Fig. 1) and reddish in color, indicating implant vascularization activity in the control and treated groups 24 h post-injection. Hyperemia and mild hemorrhage were observed 24 h post-injection in the control and treated group. The implants were found closely attached to the animals' skin (Fig. 1).

3.2. Microscopic and morphometric analyses of the implants

3.2.1. Light microscopy

On post-implantation day 14, the sponges were involved with and infiltrated by fibrovascular tissue, as shown in Fig. 2. However, in the treated groups (24 h after the intra-implant injection of 30 μl (0.5 μg)) of L. similis venom, an acute inflammatory process was observed on the fibrovascular tissue implant. This process was characterized by predominantly neutrophilic inflammatory infiltration, hyperemia, intense edema, and mild hemorrhage; thrombus was observed on the occludentes (Fig. 2). The presence of infiltrate was common in and around vessel walls containing thrombus, which featured vasculitis.

Mast cells were also evaluated in the control (C1h, C4h, and C24h) and treated groups (T1h, T4h, and T24h). These cells were found alone or in groups, next to blood vessels, adipose tissue, fibroblasts, and muscle layers (Fig. 3).

3.2.2. Collagen analysis and quantification

Histological sections of sponge implants stained with Picrosirius Red were examined under polarized light to analyze the collagen fibers. Thinning of the collagen fibers was observed in all time intervals for the groups treated with L. similis venom (Fig. 4). The results of the collagen measurements (Fig. 5) showed that the amounts of both collagen types I and III were lower in the implants injected with L. similis venom compared with the control animals; the difference was significant at 24 h post-injection.

3.2.3. Mast cell index (MCI) and mast cell degranulation index (MCDI)

Mast cells were quantified morphometrically. The MCI and MCDI results (Fig. 6) showed that the groups treated with L. similis venom had significantly greater mast cell values than the control groups ($p < 0.0001$). The control groups had mast cell rates of 4.59% ± 0.53% (1 h), 4.66% ± 0.70% (4 h), and 4.087% ± 0.97% (24 h). The percentages were 26.52% ± 2.84% (1 h), 28.51% ± 2.29% (4 h), and 26.48% ± 1.72% (24 h) in the treated groups. The control mice showed mean mast cell degranulation values of 32.28% ± 4.06% (1 h), 41.17% ± 4.35% (4 h), and 32.28% ± 4.06% (24 h), and the treated animals showed mean values 76.24% ± 3.53% (1 h), 87.38% ± 2.72% (4 h), and 75.10% ± 3.08% (24 h).

3.3. Apoptosis in implants

3.3.1. Apoptosis of endothelial cells (AEC)

Retracted endothelial cells were observed in the ultrastructure, with a loss of adhesion (anoikis) and/or basement membrane and cytoplasmic fingering (zeiosis). These cells exhibited contracted nuclei with fingering of the nuclear membrane and condensed chromatin in the nuclear membrane, which are characteristic of apoptosis (Fig. 7).

3.3.2. Morphologic analysis and index of apoptotic giant cells (IAGC)

Under light microscopy, we observed that the apoptosis of multinucleated giant cells (AMGC) showed different patterns. IAGC was observed with apoptosis in one, several, or all implants in the treated and control groups (Fig. 8). However, in the treated animals, the presence of retracted IAGC was commonly observed, with strong eosin staining, cytoplasm shrinkage, loss of adhesion with the sponge matrix and fibrovascular tissue, and retracted nuclei containing basophilic chromatin characteristic of apoptosis (Fig. 8). Several of these cells were already fragmenting of apoptotic bodies (Fig. 8). These morphological differences were confirmed by the apoptotic index, the TUNEL assay, and TEM (Fig. 9).

The L. similis venom induced apoptosis in multinucleated giant cells at all analyzed time intervals: 1 h, 4 h, and 24 h after the intra-implant injection of venom (Fig. 10). The apoptotic indices of the giant cells in the control groups were as follows: after injecting the saline, 43.33% ± 4.99% (1 h), 31.1% ± 4.66% (4 h), and 42.19% ± 4.57% (24 h). In the treated groups, the values after injecting L. similis venom were as follows: 67.22% ± 5.75% (1 h), 82.28% ± 3.33% (4 h), and 84.25% ± 3.35% (24 h) (Fig. 8). Apoptosis in the giant
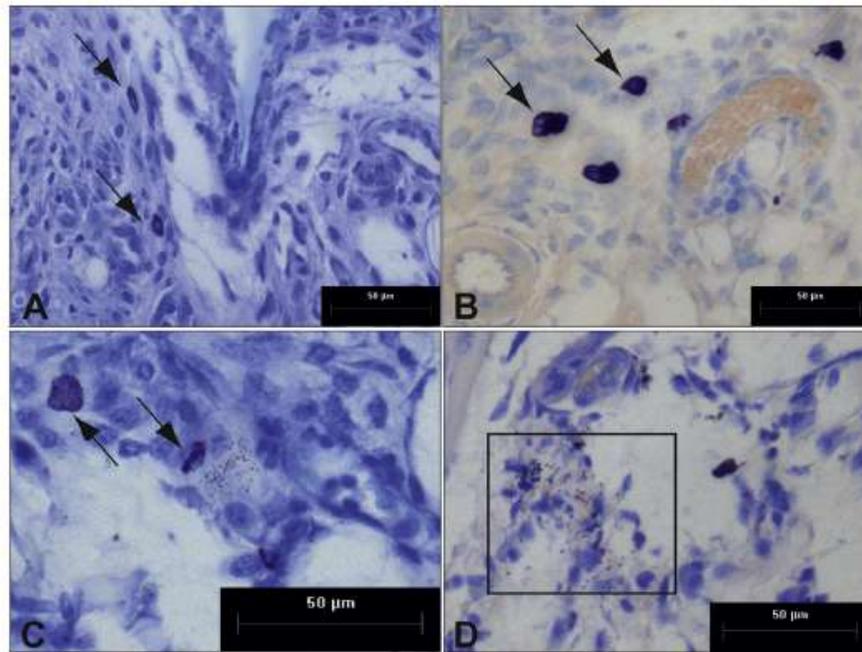
Fig. 3. Histological sections of subcutaneous sponge implants. (A) Control group 24 h after injecting 30 μl of saline, note the mast cells stained in purple (black arrows); (B–D) groups treated with *L. similis* venom (30 μL/0.5 μg); (B) note the increase in the number of mast cells (black arrows) from 1 h after the intra-implant injection of the venom; (C) note the mast cells with reduced numbers of intracytoplasmic granules; (D) note the degranulation of mast cells (marked by the rectangle) 24 h after injecting the venom. Dominici Blue staining. Barr = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
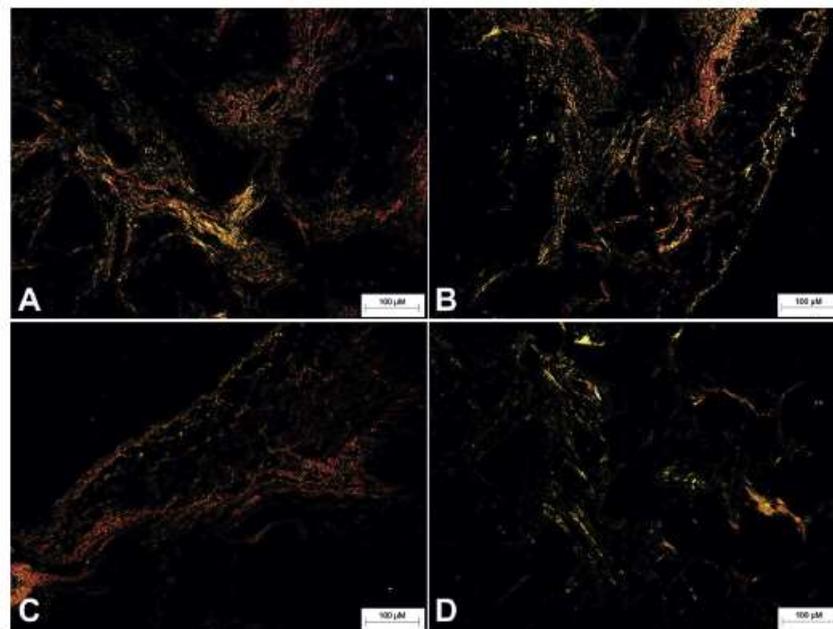

Fig. 4. Histological sections of fibrovascular tissue from the subcutaneous sponge implants. (A) Control group: note the pattern arrangement of the collagen fibers; (B–D) implants in the groups treated with *L. similis* venom (30 μL/0.5 μg of venom) 1, 4, and 24 h post-injection. There was a disruption and degradation of the collagen fibers in the treated groups. Staining: Picrosirius Red. Barr = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
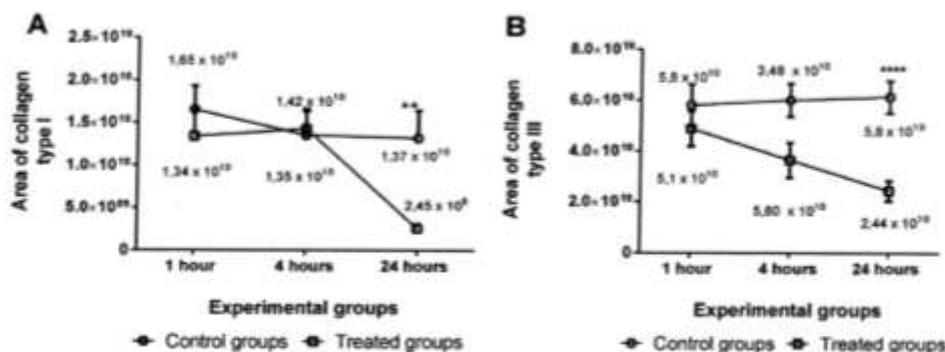
Fig. 5. Quantitative evaluations of collagen subtypes I and III in the sponge implants. (A) Collagen type I and (B) collagen type III in the control groups (C1h, C4h, and C24h) and the treated groups (T1h, T4h, and T24h). The plotted values represent the means ± SEM for groups of 3–4 animals at each time interval. * Represents a significant difference among the groups. ANOVA, $p < 0.05$.

cells was confirmed by TUNEL analysis and transmission electron microscopy (Fig. 9).

3.3.3. Apoptosis in other cells

Other cell populations, such as fibroblasts, macrophages, neutrophils, and retracted pericytes, also exhibited shrinkage of the cytoplasm, and some of these cells had fragmented into apoptotic bodies (Fig. 10). These observed features are consistent with the morphology of cells undergoing apoptosis.

4. Discussion

The subcutaneous implantation of sponges has proved to be a suitable model to study loxoscelism (Pereira et al., 2012a). The present study is the first to describe the histopathological changes that occur 24 h after the intra-implant injection of *L. similis* crude venom (0.5 μg/30 μL of venom). The microscopic alterations included acute inflammation characterized by intense edema and hyperemia. Additionally, we observed occlusive thrombosis, mild hemorrhage, and cell depletion as well as the disorganization and degradation of collagen fibers.

Another finding of our study was an increase in both the quantity and the degranulation (activation) of mast cells in implants injected with the venom of *L. similis* at all analyzed time intervals (1 h, 4 h, and 24 h). Mast cells play an important role in the pathophysiology of the inflammatory process because their activation promotes degranulation and the release of the mediators responsible for tissue changes, such as vasodilation, increased vascular permeability, and neutrophil chemotaxis (Chen et al., 2014). Our data are similar to those reported by Paludo et al. (2009) e Rattmann et al. (2008) and who studied histamine and serotonin and highlighted the importance of mast cells as a source of inflationary mediators in loxoscelism.

Our findings concerning the content and degranulation of mast cells at all time intervals may explain the maintenance of the inflammatory process in loxoscelism, which remains evident 24 h after the *L. similis* venom injection. The maintenance of acute inflammation for a prolonged period may explain some of the local and systemic effects of loxoscelism, which result in the release of chemical mediators, such as histamine and serotonin (Fig. 11).

This process also explains the reduction in skin lesions and the improvement in the acute inflammatory process,
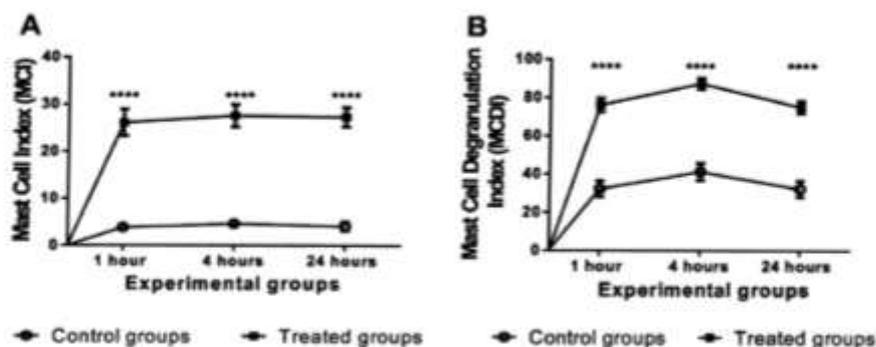

Fig. 6. The MCI and MCDI results. (A) The MCI for the control (C1h, C4h, and C24h) and treated groups (T1h, T4h, and T24h); the between-group difference was significant ($p < 0.001$) using the Kruskal Wallis test and Dunn's post hoc test. (B) The MCDI values of the control (C1h, C4h, and C24h) and treated groups (T1h, T4h, and T24h); the between-group difference was significant (****, $p < 0.001$) using the Kruskal–Wallis test and Dunn's post test.
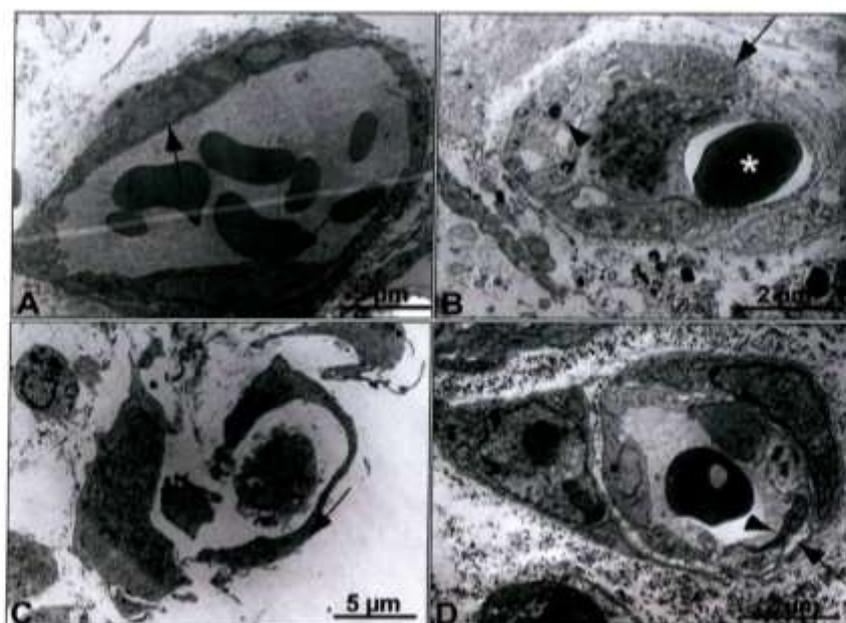
Fig. 7. Subcutaneous sponge implants under transmission electron microscopy. (A) Control group 4 h after the injection of saline. Note the presence of well-preserved endothelial cells (black arrows); (B) Treated group 1 h after the intra-implant injection of *L. similis* venom (0.5 mg/30 µL of poison). Note the endo-thelial cell apoptosis (black arrow indicating the fingering of the cytoplasmic membrane and the loss of adhesion with the adjacent cell) and the presence of occluding thrombus (asterisk); (C) ruptured blood vessel and endothelial cell with apoptosis (black arrow) 4 h after the intra-implant injection of *L. similis* venom (30 µL/0.5 mg of venom); (D) note the apoptosis in the endothelial cells (arrow), anoiquia (black arrow) and occluding thrombus (asterisk) 4 h after the intra-implant injection. Barr = 2 µm (B–D) or 5 µm (A–C).

which were observed for up to 8 h (Chatzaki et al., 2012) when the *L. similis* venom (3 µg) was pre-incubated with an anti-loxoscelic serum. Patients usually seek medical care more than 12 h after being bitten (Futrell, 1992; Pereira et al., 2011), which prevents treatment with the serum; only anti-histamines are effective because irreversible damage has already occurred. In this case, anti-histamines can only provide relief by reducing inflammation and swelling. Additionally, the present study found thrombus, perivascular cuffs, and a decrease in the total cell population (i.e., fibroblasts, macrophages, and giant cells) in groups T1h, T4h, and T24h. In the ultrastructure analysis, it was also observed that the intra-implant injection of *L. similis* venom disrupted the vessel wall and the apoptosis in endothelial cells at intervals of 1 h, 4 h and 24 h. Thrombus formation and the presence of perivascular cuffs and endothelial cell apoptosis are consistent with cutaneous loxoscelism and have been described in the rabbit model of cutaneous loxoscelism using *L. similis* venom (Pereira et al., 2012b).

According to Bombeli et al. (1997), apoptotic endothelial cells become pro-coagulant because of the increased expression of phosphatidylserine and the loss of anticoag-ulant membrane components. Thus, apoptotic endothelial cells contribute to the development of a pro-thrombogenic status (Bombeli et al. 1997). In addition, the shedding of apoptotic endothelial cells and the consequent exposure of

subendothelial collagen may contribute to platelet adhe-sion and aggregation, with subsequent thrombus formation.

These factors may contribute to thrombus formation in skin loxoscelism, which then leads to ischemia and results in necrosis. Additionally, other cell types, such as pericytes, macrophages, and fibroblasts, underwent apoptosis. The apoptosis in these cells may negatively influence the healing process. Perivascular pericytes are cells that stabi-lize blood vessels and are, thus, essential for the mainte-nance of metabolic, mechanical, and signaling functions in microvessels; in the case of loxoscelism, they can also explain the observed vascular lesions. Current evidence suggests that pericytes function as progenitors of resident tissue cells because of their ability to differentiate into various cell types, including osteoblasts, chondroblasts, adipocytes, fibroblasts, and macrophages (Hirschi and D'Amore, 1996). *In vitro* studies conducted by Horta et al. (2013) reported the occurrence of apoptosis in human fi-broblasts (HFF1) treated with crude *L. similis* venom or recLiD1 (pre-incubated with lysophosphatidylcholine) after 48 h of incubation.

This study also observed that the apoptosis in giant cells was significantly higher in the groups treated with *L. similis* venom. The giant cell is a cell type commonly observed in subcutaneous implants of biomaterials. These implants induce a chronic inflammatory process known as the
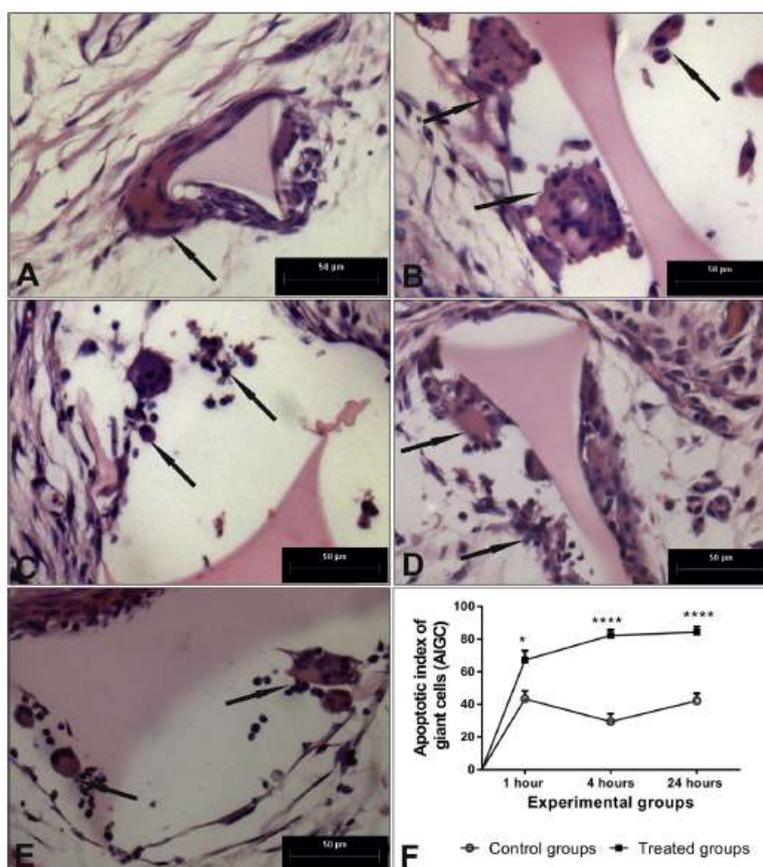
Fig. 8. Histological sections of subcutaneous sponge implants. Apoptosis in giant cells was examined in (A) the control groups (note the integrity of the giant cells) and the treated groups (note several giant cells with apoptotic features) (black arrow); (B) 1 h, (C) 4 h, and (D) 24 h after the intra-implant injection of *L. similis* venom; (E) the black arrows indicating the presence of apoptotic bodies; and (F) a graph showing the AGCI. Note the significant differences between the control groups (C1h, C4h, and C24h) and the treated groups (T1h, T4h, and T24h) using the Kruskal–Wallis test and Dunn's pos test. Barr = 50 μm.

foreign body reaction (Anderson et al., 2008). The formation of multinucleated giant cells (CGM) involves macrophages, which undergo homotypic fusion. These cells are relatively short-lived and are usually eliminated from the tissue by apoptosis. Studies have demonstrated apoptosis in these cells both in single nuclei and in numerous nuclei (Honma et al., 1996; Moro et al., 2003b). Although giant cells are not normally found in conventional models of loxoscelism, the apoptosis in this cell type supports the apoptosis in the macrophages described above. Macrophages have been described to have many functions in wounds, including host defense, the promotion and resolution of inflammation, the removal of apoptotic cells, and the support of cell proliferation and tissue restoration following injury (Koh and DiPietro, 2011).

Note that the role of macrophages is essential in resolving acute inflammation through programmed cell cleaning. Considering the source of the CGM, the induction of apoptosis in this type of cell may damage the cleaning of apoptotic programmed cell bodies in neutrophils and the resolution of the acute inflammatory process, thereby enabling the maintenance of acute inflammation. Savill and Fadok (2000) demonstrated that macrophages clean apoptotic neutrophils at inflammatory sites. Fadok et al. (2001) showed that the phagocytosis of apoptotic cells performed by macrophages promoted the release of anti-inflammatory cytokines, such as TGF-β and IL-10, which suppressed its pro-inflammatory activity. In addition, the authors proposed that defects in the 'scheduled cleaning' of apoptotic bodies could lead to chronic inflammatory diseases, such as cystic fibrosis, idiopathic fibrosis, pneumonia, bronchitis, and systemic lupus erythematosus. These authors showed that a deficiency in scheduled cleaning allowed apoptotic bodies to remain in place
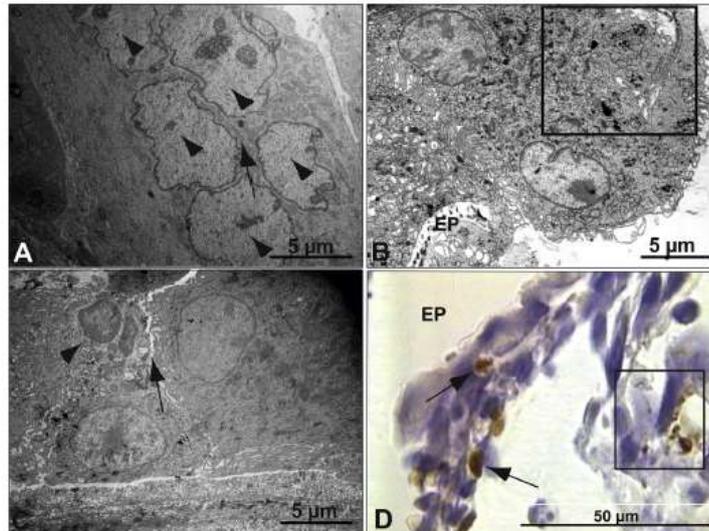
Fig. 9. Transmission electron microscopic and light microscopic examinations of subcutaneous sponge implants. (A) Control group: 4 h after the saline injection, note the giant cell nuclei in the quality standard (arrow head) and the complete fusion between cells (arrow); (B) T4h after the *L. similis* (30 µl/0.5 µg of venom) intra-implant injection, note the apoptotic giant cell and the formation of an apoptotic body (rectangle); (C) fragmentation of a giant cell with apoptotic body formation (black arrow), with a fragmented nucleus containing compressed and condensed chromatin typical of apoptosis (arrowhead) 24 h after the intra-implant injection of *L. similis* venom (0.5 µg venom/30 µL); (D) arrows indicate the nuclei of giant cells positive for TUNEL assay and the presence of apoptotic bodies (demarcated by the rectangle/positive; diaminobenzidine staining counterstained with HE. Barr=5µm (A-C) or 50µm (D).
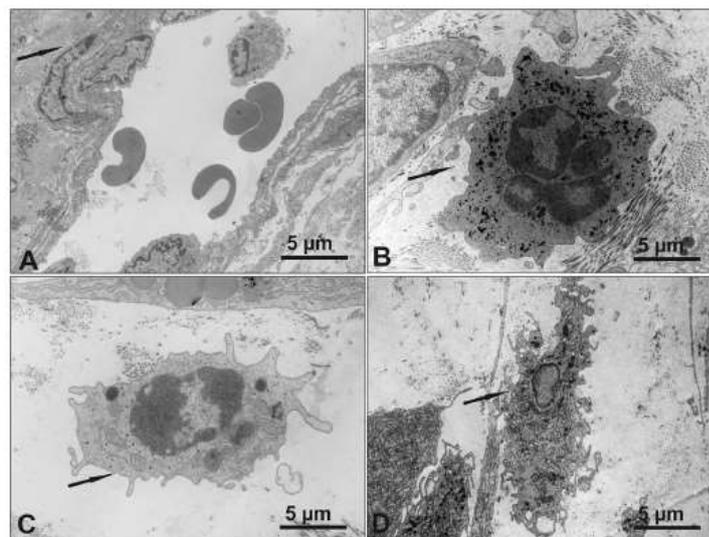

Fig. 10. Subcutaneous sponge implants under transmission electron microscopy. Note the chromatin condensation and shrinkage of the cytoplasm (apoptosis) in the groups injected with *L. similis* venom in various cells, such as (A) pericytes (black arrow), (B) neutrophils, (C) macrophages (black arrow), and (D) fibroblasts. Barr = 5 µm.
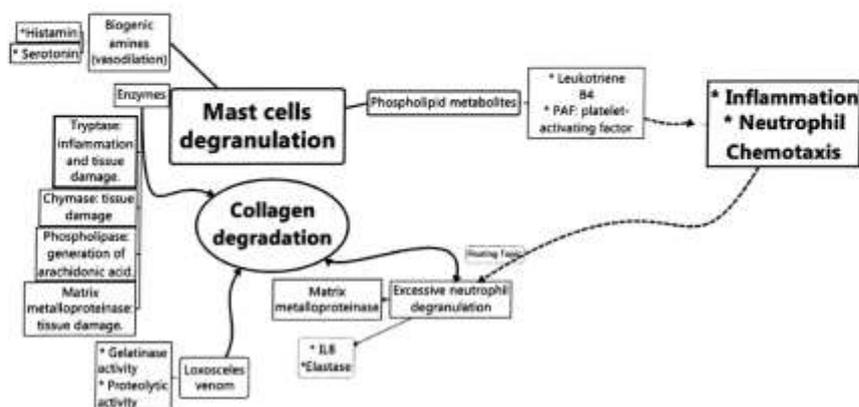
Fig. 11. A possible mechanism involved in loxoscelism: note the role of mast cells in the lesions caused by *L. similis* crude venom.
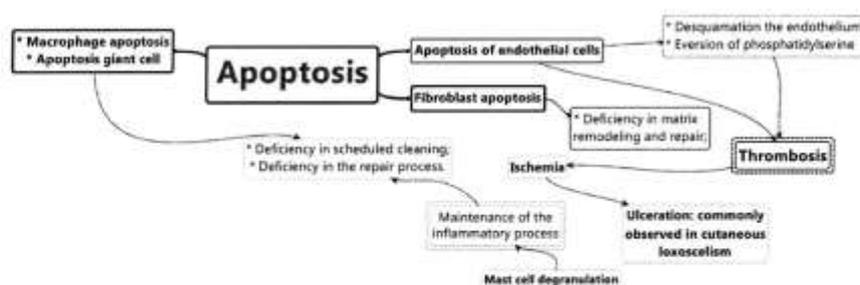

Fig. 12. A possible mechanism involved in loxoscelism: note the role of apoptosis in various cell types in the pathogenesis of loxoscelism.

longer and, consequently, to become disrupted, thereby releasing their contents and encouraging further inflammation.

In the case of loxoscelism, the intensity of the acute inflammatory process, which remains intense because of mast cell degranulation, is quite apparent. Whereas neutrophils physiologically die by apoptosis, a deficiency in the cleaning process because of a reduced macrophage population is a factor that may intensify the inflammatory reaction. It is known that the repair process occurs only after the acute inflammatory process is resolved. Thus, the results concerning apoptotic giant cells, macrophages, fibroblasts and pericytes, which are associated with the degradation of collagen types I and III, may help to clarify the difficult healing process that is commonly observed in loxoscelism (Fig. 12).

Ethical statement

The present study was approved by the *Comitê de Ética em Experimentação Animal* (CETEA) of the Universidade Federal de Minas Gerais (UFMG) (process number 229/09) and by the *Comitê de Ética no Uso de Animais* (CEUA) at the Centro de Pesquisas René Rachou (CPqRR/Fiocruz Minas) (approval number 93/200-1).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.03.003.

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Considerações finais

CONSIDERAÇÕES FINAIS

No presente estudo, foi possível observar alterações bioquímicas e histológicas induzidas pela injeção intra-implante de 0,5 µg of veneno bruto *L. similis*. Curiosamente, tais alterações são semelhantes ao observado por vários autores em humano, coelho e cobaia, mas não em camundongos. É possível que o conjunto de moléculas do microambiente do implante também foi capaz de manter o veneno da *L. similis* no implante, permitindo sua ação prolongada no tecido fibrovascular recém-formado. A sensibilidade do modelo e de sua aplicabilidade em detectar os efeitos de veneno de *L. similis* foram fortemente confirmados tanto por parâmetros histológicos quanto bioquímicos. Além de ser um modelo viável economicamente e de fácil manuseio, induz um tecido de cicatrização fibrovascular que permite a caracterização de eventos moleculares e celulares associados ao loxoscelismo em camundongos, poderá fornecer futuras contribuições na tanto patogenia e como até mesmo na soroterapia destas lesões.

Adicionalmente foi possível descrever pela primeira vez, no modelo de implante subcutâneo de esponjas, alterações hispatológicas 24 horas pós-injeção intraimplante de 0,5 µg de veneno bruto de *L. similis,* além de abordar outros enfoques nos tempos já analisados anteriormente (1 e 4 horas pós-injeção intraimplante) com ênfase na patogenia do loxoscelismo. Os dados deste estudo mostraram que a injeção intraimplante do veneno de *L. similis* (24 horas pós-injeção) causa inflamação aguda caracterizada por intenso edema e hiperemia. Adicionalmente, observou-se trombose oclusiva, hemorragia discreta, rarefação celular, além de desorganização e degradação das fibras colágenas.

A apoptose das células gigantes, macrófagos, fibroblastos e pericitos associados aos dados de degradação do colágeno dos tipos I e III podem contribuir para elucidar a dificuldade do processo de cicatrização comumente observadas no loxoscelismo.

Outros estudos qualitativos e quantitativos estão sendo conduzidos na população de neutrófilos, macrófagos e células endoteliais para uma avaliação mais aprofundada das vias apoptóticas envolvidas na patogenia do loxoscelismo, utilizando a tripla marcação para TUNEL, anticorpos anti-CD31, anti-macrófagos, anti-fibroblastos e DAPI. Além disso, as caspases também estão sendo avaliadas.

Capítulo 3

Este capítulo contém 2 artigos relacionados ao tema loxoscelismo, mas, que não estão diretamente relacionados com o projeto de tese, mas, que foram publicados durante o doutorado.

Outros artigos relacionados ao projeto de tese

ORIGINAL PAPER

Histopathological characterization of experimentally induced cutaneous loxoscelism in rabbits inoculated with *Loxosceles similis* venom

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Abstract: Envenomation by *Loxosceles* bites is characterized by dermonecrotic and/or systemic features that lead to several clinical signs and symptoms called loxoscelism. Dermonecrotic lesions are preceded by thrombosis of the dermal plexus. Recent studies show that atheromatous plaque is prone to thrombosis due to endothelial cell apoptosis. To the best of our knowledge, there are no reports of microscopic dermal lesion and endothelial cell apoptosis induced by *Loxosceles similis* venom in the literature. Thus, the aim of the present study is to describe histological lesions induced by *L. similis* venom in rabbit skin and to elucidate whether apoptosis of endothelial cells is involved in the pathogenesis of loxoscelism. Forty male rabbits were split into two groups: the control group (intradermally injected with 50 µL of PBS) and the experimental group (intradermally injected with 0.5 µg of *L. similis* crude venom diluted in 50 µL of PBS). After 2, 4, 6 and 8 hours of injection, skin fragments were collected and processed for paraffin or methacrylate embedding. Sections of 5 µm thick were stained by HE, PAS or submitted to TUNEL reaction. Microscopically, severe edema, diffuse heterophilic inflammatory infiltrate, perivascular heterophilic infiltrate, thrombosis, fibrinoid necrosis of arteriolar wall and cutaneous muscle necrosis were observed. Two hours after venom injection, endothelial cells with apoptosis morphology were evidenced in the dermal plexus. Apoptosis was confirmed by TUNEL reaction. It seems that endothelial cell apoptosis and its consequent desquamation is an important factor that induces thrombosis and culminates in dermonecrosis, which is characteristic of cutaneous loxoscelism.

Key words: *Loxosceles similis*, loxoscelism, dermonecrosis, cutaneous loxoscelism, apoptosis.

INTRODUCTION

Loxoscelism is a set of clinical signs and symptoms that result from the bite of spiders of the genus *Loxosceles* (1). Clinical manifestations are generally characterized by dermonecrosis at the bite site and sometimes followed by systemic effects, which may cause an acute renal failure and possible death (2-5). Accidents with spiders of the genus *Loxosceles* have been already described in all continents (2, 4, 6-10). In Brazil, loxoscelism is regarded as a serious public health problem with 51,865 accidents notified from 2001 to 2008 (11). In Minas Gerais, from 2001 to

2008 the number of *Loxosceles* accidents notably increased. Twenty-one accidents were reported in 2001 but the number rose to 269 and 229 cases in 2007 and 2008, respectively (data not published). From 2005, specimens of *L. similis* have been found inside residences in Belo Horizonte (Minas Gerais state) increasing the risk of domestic accidents (12).

Reports of *L. similis* lesion are scarce in the literature. Histopathological studies with three *Loxosceles* species of clinical importance, *L. intermedia*, *L. laeta* and *L. recluse*, showed that the venom induces vasodilation, edema, inflammatory infiltrate (mainly neutrophilic),

hemorrhage, cutaneous muscle necrosis, thrombosis and arteriolar walls degeneration (6, 13-15). It is necessary to elucidate whether the histological lesion induced by the *Loxosceles similis* venom is similar to that observed in other species of medical importance. Furthermore, it is important to determine the pathogenesis of the loxoscelic dermonecrotic lesion.

A previous study showed that *L. intermedia* venom has a cytotoxic effect on the endothelium and causes a basal membrane lesion (16). This structure is important to maintain the vessels integrity. According to Zanetti *et al.* (17) and Nowatzki *et al.* (18) who studied the action of the *L. intermedia* venom *in vitro* on endothelial cells, it was observed that 18 hours after the venom action, cells showed plasmatic membrane convolutions and chromatin condensation. These morphological aspects are characteristic of apoptosis, which is an active, energy-dependent kind of cell death that requires protein synthesis and degradation to take place. The necrosis of the cutaneous loxoscelism occurs due to thrombus formation in dermal plexus.

Recent studies have shown a strong relationship between endothelial apoptosis, denudation of the endothelium and the occurrence of thrombosis (19). Therefore, the present work, using an intradermal injection of 0.5 µg of *L. similis* crude venom in rabbits as an experimental model, had the following purposes:

- to report a histological lesion caused by the venom;
- to evaluate morphometrically the vasodilation, edema and dermal inflammatory infiltrate 2, 4, 6 and 8 hours after venom injection;
- to evaluate if the venom induces apoptosis in dermal endothelial cells.

MATERIALS AND METHODS

Venom

The venom used was extracted according to previous works and stored at –80°C (20-22). The protein content of the samples was determined by the Bradford method and confirmed by the Lowry method.

Animal Groups and Samples

Forty male New Zealand rabbits, *Oryctolagus cuniculus*, weighing between 3.0 and 3.5 kg were submitted to trichotomy on the interscapular dorsal region. Animals were divided in two groups: the control group (n = 20) – received an intradermal injection of 50 µL PBS (phosphate-buffered saline); and he experimental group (n = 20) – received intradermal injection of 0.5 µg of crude venom diluted in 50 µL PBS. Five animals per group were submitted to euthanasia in intervals of 2, 4, 6 and 8 hours after injection.

Histology and Morphometry

At necropsy, skin fragments were collected and fixed for 48 hours in 10% buffered formalin (pH = 7.4). Then, they were sectioned and processed routinely for paraffin inclusion. Sections of 5 µm were stained in HE and PAS (23). Skin fragments were also fixed in 4% paraformaldehyde and processed for methacrylate inclusion using embedding kit (Historesin Leica – specification 7022 31731) according to da Silva (24) and manufacturer's instructions. Semi-thin sections of 3 µm thick were stained with toluidine blue and HE (hematoxylin andeosin).

Edema measurement

Skin thickness was measured in order to quantify the edema. Skin slides were digitalized in stereo microscope, under a 4x objective and a 3.3 telemetric, which resulted in a final increase of 12x. The Pro-plus software calibration was performed by digitalization of a millimeter ruler. Fragment measurement was adapted (25). Skin thickness was calculated by measuring the distance between the epidermis and the muscle layer. Measurements were calculated in three random points of each skin fragment.

Vasodilation measurement

Vasodilation was quantified by measuring the mean diameter of the blood vessels of HE-stained skin sections from both experimental and control groups. Images were captured using an Olympus BX-640 microscope and digitalized with a JVC TK-1270/JGB camera with an increase of 10x. Images were transferred to an image analyzer software (Kontron Electronic, Carl Zeiss – KS300, version 2), where the mean diameter of vessels was measured in micrometers. Ten fields were used per animal both for control and for experimental groups, totalizing 50 fields per time interval of 2, 4, 6 and 8 hours (n = 5 animals per group).

Inflammatory infiltrate

In order to measure the inflammatory infiltrate, the minimal representative number of microscopic fields per sample was determined using one slide from which the number of inflammatory cells per field was analyzed and registered (26). Images were captured from sections stained with HE using a 40x objective. The mean and respective standard errors and coefficients of variation were calculated. Sample size was considered as the minimum representative number of 25 fields (27).

In situ DNA Fragmentation

In order to identify the *in situ* DNA fragmentation, TUNEL (terminal deoxynucleotidil transferase uracil nick end labeling) (Calbiochem Kit catalog QIA33) was used according to Gavrieli *et al.* (28) and the manufacturer's instructions.

Statistical Analysis

Results were presented as mean ± standard error, as they presented a normal distribution or not, respectively, according to the Kolmogorov-Smirnov test. When the distribution was normal, the analysis of variance (ANOVA) and the Newnam Keuls test were applied in order to compare groups. When data distribution was not considered normal, non-parametric Kruskal-Wallis test was applied and Dunn's post-test. Values of $p < 0.05$ were considered significant. Statistical analysis was carried out using GraphPad Prism program version 5.

RESULTS

Macroscopy

Macroscopically, the area injected with 0.5 μg of *Loxosceles similis* venom presented a purple, swollen, and touch-sensitive lesion which radius spread out over time (Figure 1).

Histology and Morphometry

Microscopically, a dissociation of fibers and cells in all dermal layers, which characterizes an edema, was observed in all animals of the experimental group and in all time intervals (Figure 2 – B). Angiectasis (Figure 2 – B), hyperemia, hemorrhage (Figure 2 – D), fibrin exudation (Figure 2 – D), occluding and semi-occluding thrombus (Figure 2 – F), on superficial, medium and deep plexus and

fibrinoid degeneration of vascular wall were also observed. In all time intervals, the presence of a multi-focal, inflammatory infiltrate was observed (Figure 2 – B and C), particularly in the medium and deep dermis, whose intensity increased over time. The inflammatory infiltrate spread through the muscular layer, followed by muscle necrosis (Figure 2 – H). From 6 hours after the venom injection, the infiltrate became more severe with the formation of perivascular cuffs, which infiltrated into the arteriolar walls characterizing vasculitis (Figure 2 – E).

Edema Measurement

Means of skin thickness from the control animals were 3.45 ± 0.12; 3.50 ± 0.20; 3.38 ± 0.17 and 2.85 ± 0.10 μm for intervals of 2, 4, 6 and 8 hours, respectively. Means of skin thickness of animals from the experimental group were: 4.54 ± 0.15 μm; 4.75 ± 0.21 μm; 5.08 ± 0.27 μm and 5.10 ± 0.28 μm for intervals of 2, 4, 6 and 8 hours, respectively (Figure 3). The difference between control and experimental groups was significant in all time intervals.

Vasodilation Measurement

In order to verify the presence of angiectasis, the mean vessel diameter was measured. Data have shown that mean vessel diameters were 50.97 ± 5.7 μm, 55.73 ± 6.4 μm, 57.61 ± 5.53 μm and 55.94 ± 5.45 μm for intervals of 2, 4, 6 and 8 hours for control groups (Figure 4). In the experimental groups, the mean vessel diameters were 91.91 ± 5.89 μm, 101.7 ± 8.90 μm, 109.80 ± 10.2 μm and 109.90 ± 9.42 μm for intervals of 2, 4, 6 and 8 hours, respectively. The difference between control and experimental groups was significant in all time intervals.

Inflammatory Infiltrate

Morphometric data of the inflammatory infiltrated from the skin of control animals were as follows: 0.77 cells ± 0.14; 2.50 cells ± 0.59; 1.67 ± 0.56 cells and 2.85 ± 0.89 cells for intervals of 2, 4, 6 and 8 hours, respectively. The data for experimental animals were: 28.61 cells ± 1.79 cells; 50.12 ± 1.63 cells; 64.75 ± 1.82 cells and 105.2 ± 1.53 cells for intervals of 2, 4, 6 and 8 hours, respectively (Figure 5). The difference between control and experimental groups was significant in all time intervals.

In situ DNA Fragmentation

From two hours of venom injection, the presence of shrunken endothelial cells was observed in some vessels, with loss of cell adhesion (anoikis), retracted nucleus, irregular karyotheca and condensed chromatin (compacted against the karyotheca) characterizing apoptosis (Figure 6 – A and B). Furthermore, apoptotic endothelial cells were present in the thrombus-vessel interface from two hours of venom injection. Besides,
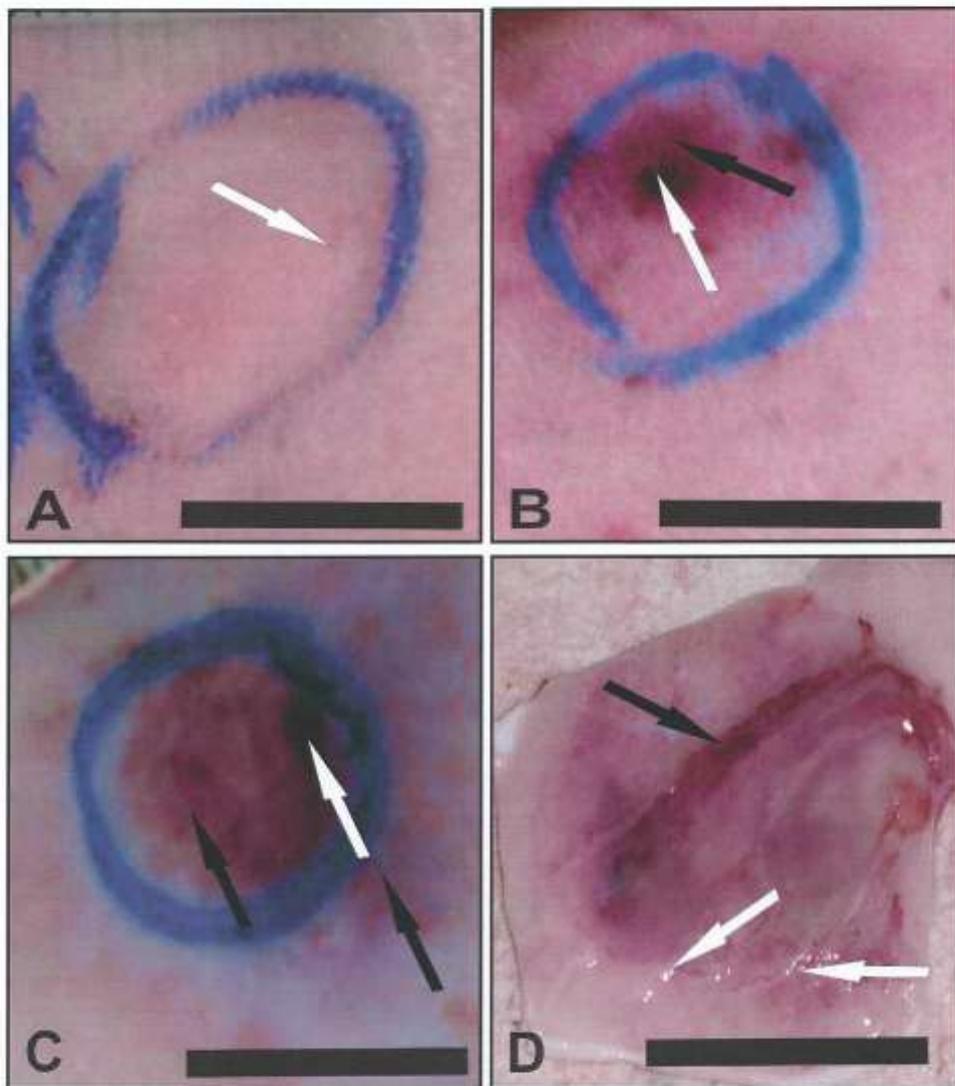

Figure 1. Macroscopic picture of rabbit skin: (A) control group – note the site of injection of 50 μL de PBS (white arrow); (B) experimental group – note the site of venom injection (white arrow) and the purple area (black arrow) two hours after injection of 50 μL (0.5 μg) of crude venom of *L. similis*; (C) experimental group - 8 hours after the injection of 50 μL (0.5 μg) of venom, note how the lesion spreads radially (black arrows); (D) experimental group – 8 hours after the injection of 0.5 μg of venom, note the edema (white arrow) and hemorrhage (black arrow) on the hypodermis. Bar = 1 cm.

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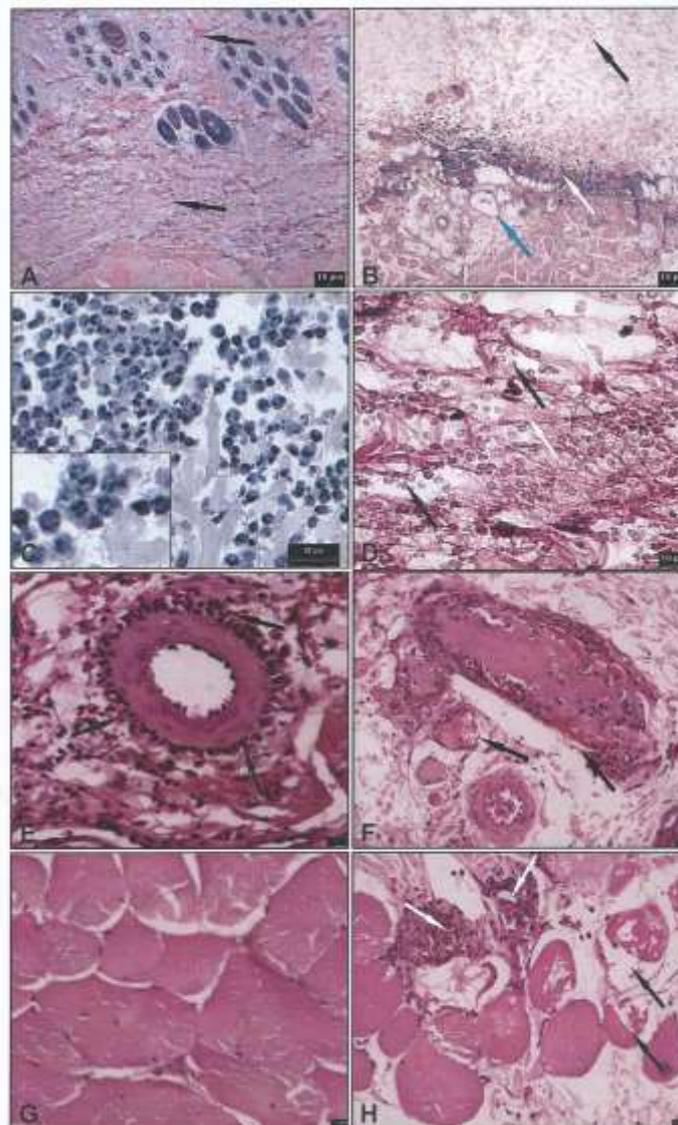
Figure 2. Microscopic picture of rabbit skin: (A) control group – the black arrow indicates the integrity of the collagen fibers; (B) experimental group – note the dissociation of fibers and cells characterizing edema (black arrows), angiectasis (blue arrow), intense inflammatory infiltrate, predominantly heterophilic (white arrow); (C) details of the inflammatory infiltrate (methacrylate, semi-thin section); (D) experimental group – fibrin deposition (white arrows) and hemorrhage (black arrow); (E) perivascular cuff observed 6 hours after venom injection (black arrows), (F) occluding thrombus (black arrows), (G) control group – cutaneous muscle; (H) experimental group – necrosis of the cutaneous muscle (white arrow) and heterophilic infiltration (black arrow). Staining: A, B, C, E and F = HE; D = PAS counter-stained with hematoxylin. Bar = 10 μm.
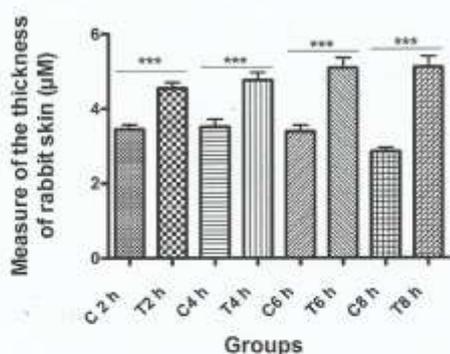
Figure 3. Skin thickness measurement for the edema quantification of the control and experimental groups 2, 4, 6 and 8 hours after injection of PBS or venom. The difference is statistically significant (***p < 0.001/ANOVA and Newman-Keuls tests). C2h, C4h, C6h and C8h = control groups 2, 4, 6 and 8 hours after PBS injection, respectively. T2h, T4h, T6h and T8h = experimental groups 2, 4, 6 and 8 hours after the venom, respectively.
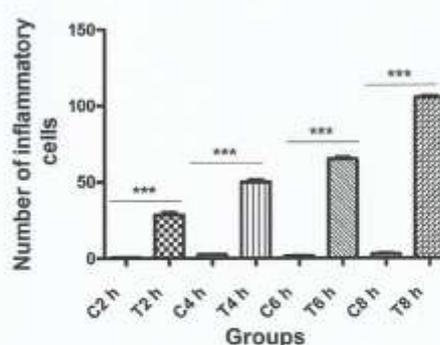
Figure 5. Quantitative analysis of the number of inflammatory cells 2, 4, 6 and 8 hours after PBS (control group) or venom (experimental group) injection. The difference is statistically significant (***p < 0.001/Kruskal–Wallis/Dunn's post-test). C2h, C4h, C6h and C8h = control groups 2, 4, 6 and 8 hours after PBS injection, respectively. T2h, T4h, T6h and T8h = experimental groups 2, 4, 6 and 8 hours after venom injection, respectively.
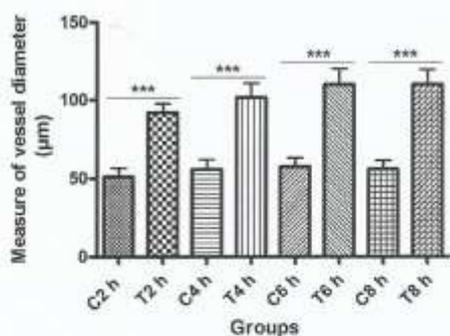
Figure 4. Diameter vessel measurement of control and experimental groups at 2, 4, 6 and 8 hours after PBS or venom injection. The difference is statistically significant (***p < 0.001/Kruscal-Wallis/Dunn's post-test). C2h, C4h, C6h and C8h = control groups 2, 4, 6 and 8 hours after PBS injection, respectively. T2h, T4h, T6h and T8h = experimental groups 2, 4, 6 and 8 hours after venom injection, respectively.

dermal endothelial cells, positively labeled by TUNEL reaction, were observed and confirmed the morphological evaluation (Figure 6 – C and D). The same did not occur in the skin of control animals.

DISCUSSION

In the present study, the intradermal injection of 0.5 μg of *Loxosceles similis* venom in rabbits induced a warm, swollen, purplish-red and touch-sensitive lesion (acute inflammation), also described by other authors for cutaneous loxoscelism (6, 29). Macroscopically, the radius of the injured area increased over time as previously related in loxoscelism induced by other *Loxosceles* species (1, 6, 9). Notably, the venom concentration used in this work was very low (0.5 μg) compared with our previous study of 3 μg of venom, but it was enough to induce a very similar and severe lesion (22). Microscopically, a severe fibrinous-hemorrhagic multi-focal acute dermatitis and a necrotic acute myositis were observed. The edema was intense and thrombosis was observed as early as two hours after injection. These lesions
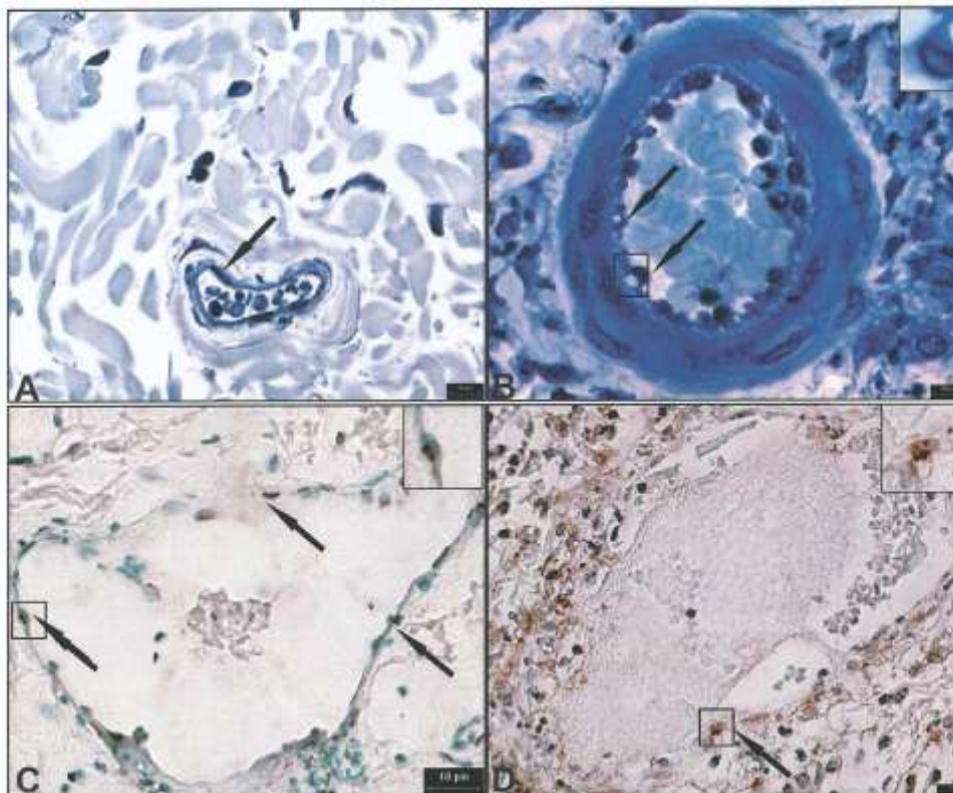
Figure 6. Microscopic picture of rabbit skin: (A) control group – blood vessel – endothelial cell (black arrow); (B) apoptotic endothelial cells (black arrows), (details – insertion on the top right corner); (C) experimental group, two hours after venom injection – presence of apoptotic endothelial cells, labeled positive with TUNEL reaction (black arrow) (details – insertion on the top right corner); (D) experimental group – two hours after venom injection – positive cells for TUNEL reaction in the thrombus-vessel interface (details – insertion on the top right corner). Stainings: toluidine blue (A and B) and TUNEL reaction (C and D) DAB chromogen counter-stained by methyl green. Bar = 10 μm.

are similar to those observed for other medically important species of *Loxosceles* (13-15).

In this study, morphometric analyses were carried out in order to follow the progression of the histological lesion through the measurement of edema, vasodilation, and inflammatory infiltrate present in the lesion, considering since there are few reports in the literature regarding these phenomena in loxoscelism. The average diameter of blood vessels was significantly higher ($p < 0.05$) in experimental animals compared with the control ones, in all time intervals. These data show that *L. similis* venom has a vasodilator action, already observed for other *Loxosceles*, and which is mediated by histamine (30, 31). The edema was significantly greater at 2 hours after venom injection. The present results are similar to those of Rattmann *et al.* (30) using *L. intermedia* venom in rat experimental model.

According to our data, the inflammation increases progressively from two hours and then becomes severe eight hours post *L. similis* crude venom injection corroborating the already existing studies with *L. laeta* and *L. intermedia* venom (15, 17, 32). Morphometrical parameters showed here can be used to monitor the process

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dynamics, and also any treatment and sera effectiveness studies (22, 31, 33, 34). In the present study, the heterophils/neutrophils were the predominant cells observed as mentioned by others for loxoscelism. The term heterophil was used instead of neutrophil in the results of this study, as in rabbits and in other species such as guinea pigs, chickens, reptiles and birds; the heterophil is functionally analogous to neutrophils (35-37).

Apoptotic endothelial cells, surrounded by a halo, with shrunken and fragmented nuclei, containing condensed chromatin were observed as early as two hours after the *L. similis* crude venom injection. The morphology was corroborated by means of a positive labeling of the endothelial cells through the TUNEL reaction. In addition, endothelial cells in apoptosis were present in the thrombus-vessel. Under transmission electron microscopy, Veiga *et al.* (38) observed the presence of endothelial cell degeneration, characterized by vacuoles on the vessel walls of rabbits skin, four hours after the injection of 30 μg of *L. intermedia* crude venom. Despite of the morphological evidences of apoptosis, the authors did not consider the occurrence of this kind of death cell in that study (38). According to them, loxoscelic venom has a deleterious effect due to destruction of components that are responsible for endothelial cells adhesion. As stated by Bombeli *et al.* (39), apoptotic endothelial cells become procoagulant due to increased expression of phosphatidylserine and loss of anticoagulant components of the cytoplasmic membrane. Thus, apoptotic endothelial cells contribute to the development of a pro-thrombogenic state (38). Consequently, the presence of apoptotic endothelial cells can contribute to the thrombosis pathogenesis on the cutaneous loxoscelism. According to the Virchow's triad, thrombus formation results from the pathological activation of the blood coagulation process due to rheological blood flow alterations, hypercoagulability and/or endothelial cell lesions.

In the cutaneous loxoscelism, thrombus formation is related to endothelial lesion by the venom direct effect and subendothelial basal membrane alteration (16). Some authors observed that there is a strong relationship among endothelial cell apoptosis, endothelium denudation and thrombosis (19). Besides, they showed that the atheroma plaque desquamation

via apoptosis predisposes to thrombosis which is a common consequence of atherosclerosis. So, we suggest that the apoptotic process may also induce loss of cell adhesion and desquamation from the vessel wall, which causes denudation of the vascular surface, exposure of the subendothelial collagen that induces platelet adhesion and aggregation and coagulation cascade activation (40). Once thrombi are formed, dermal necrosis with ulceration takes place and characterizes the loxoscelism cutaneous lesion.

CONCLUSION

The present data show that the experimental injection of the crude venom of the *Loxosceles similis*, intradermally in rabbits, induce a histological lesion pattern very similar to that related in the literature for other species of *Loxosceles*.

Additionally, the results suggest that the induction of endothelial cells apoptosis by the *Loxosceles similis* crude venom is involved in the pathogenesis of the thrombosis, necrosis and ulceration all characteristics of the cutaneous loxoscelism. Moreover, the dermal inflammation that intensifies over time seems to be an aggravating factor that may contribute to the development of a hard-to-heal ulcer observed in humans.

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CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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Cutaneous loxoscelism caused by *Loxosceles similis* venom and neutralization capacity of its specific antivenom

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ARTICLE INFO

ABSTRACT

Members of the spider genus *Loxosceles* pose a marked health risk to humans because of the seriousness of the necrotic and systemic effects of their bite, known as loxoscelism. The recent confirmation of *Loxosceles similis* in residences of Belo Horizonte in Minas Gerais Province, Brazil increases the local potential risk of loxoscelism at higher levels. The first characterization of the venom from this species showed that its main biological effects had a similar intensity as other species (e.g. *Loxosceles intermedia*, *Loxosceles laeta*, and *Loxosceles gaucho*). Therefore, we wished to further analyse the biological activity of the *L. similis* venom as well as the capacity of anti-*L. similis*-venom serum to reduce dermonecrotic effects to rabbit skin. Histological analysis of rabbit skin 2, 4 and 8 h after intradermal injection of *L. similis* venom demonstrated a dense inflammatory infiltrate, edema, degeneration and necrosis of the skin muscle, dissociation of collagen fibers, and disruption of reticular fibers. Importantly, pre-incubation of the venom with anti-*L. similis*-venom serum significantly decreased all of these effects. Anti-*L. similis* antivenom generated antibodies that were strongly reactive to *L. similis* venom and capable of neutralizing the dermonecrotic effects in rabbits caused by this venom. Moreover, the antivenom significantly reduced the sphingomyelinase activity of *L. similis* crude venom. Venoms produced by male and female spiders were equally reactive towards anti-*L. similis* and anti-*L. intermedia* antivenoms, but female venom induced larger lesions on rabbits. In contrast, female venom acted as an immunization enhancer and protected animals from *L. similis* envenomation to a greater degree than male venom. In conclusion, the results shown in this study for *L. similis* antivenom merits a more in depth study of its properties, which may become a valuable tool against loxoscelism.

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1. Introduction

The spider genus *Loxosceles* (Araneae, Sicariidae) is comprised of 101 species worldwide located in the temperate and tropical zones of North, Central, and South America as well as Europe, Asia, Africa, and Australia (Platnick, 2011). Several members of the genus have attracted the scientific interest of researchers, including *Loxosceles reclusa* (Gertsch and Mulaik, 1940), *Loxosceles gaucho* (Gertsch, 1967), *Loxosceles laeta* (Nicolet, 1849), and *Loxosceles intermedia* (Mello-Leitão, 1934), mainly due to the health risk to humans from the necrotic and systemic effects of their bite (loxoscelism). The three latter species are prominent in most of the southern

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provinces/states of Brazil, and *L. laeta* is also found in the state of Bahia. In addition, *L. similis* (Moenkhaus, 1898) has been found in the state of Minas Gerais, Brazil (Machado et al., 2005).

Extensive studies have been conducted on this genus in recent years and have revealed the biological effects of the venom (Barbaro et al., 2005; De Oliveira et al., 2005; Gomez et al., 2001; Silvestre et al., 2005) or of specific, isolated fractions of the protein components (Chaim et al., 2006; Guilherme et al., 2001; Tambourgi et al., 1995, 1998), the mechanism of action (Dias-Lopes et al., 2010b; Gomes et al., 2011), and the particular involvement of these proteins towards the production of broadly used and effective antivenoms (De Oliveira et al., 2005; Dias-Lopes et al., 2010a; Pauli et al., 2006; Olvera et al., 2006; Tambourgi et al., 2004).

Molecular cloning of the genes that code for these proteins and their particular biological effects on mammals has also been the focus of several studies in this scientific area (Castro et al., 2004; Kalapothakis et al., 2002; Silvestre et al., 2005; Tambourgi et al., 2004). Kalapothakis et al. (2007) described several new proteins from the most lethal family of toxins expressed in the venom gland of *Loxosceles* spiders, known as Loxtox, and also described important characteristics of this group. The highly conserved antigenic profile from the *Loxosceles* species has been shown by both amino acid sequence similarities and by high cross-reactivity between antivenoms and crude or purified fractions of individual species (Barbaro et al., 1994, 1996, 2005; Olvera et al., 2006; Silvestre et al., 2005; Tambourgi et al., 2004; Toro et al., 2006).

Several *Loxosceles* species are synanthropic and presumably have a strong ability to colonize urban areas due to their high dispersal competence and ecological features, such as tolerance to starvation and lack of water, great longevity, avoidance of extreme temperatures, and preference for relative aridity (Fischer and Vasconcellos-Neto, 2005a,b,c). This ecological profile, in combination with the increasingly high numbers of envenomations reported annually by the Brazilian Ministry of Health (Ministério da Saúde, Governo Federal), calls for more detailed research not only on known species, but also on other species that may prove to be a threat to human health in the future.

In line with this approach, *L. similis* (Moenkhaus, 1898) has been the focus of some recent biological studies (Machado et al., 2005; Silvestre et al., 2005). This species is one of the three reported in the state of Minas Gerais, Brazil, together with *L. laeta* and *L. anomala* (Mello-Leitão, 1917). Based on morphology, this species belongs to the *gaucho* group, together with *L. gaucho*, *L. adelaida*, and *L. variegata* (Gertsch, 1967). Until recently, it was thought to be mainly a cave-dwelling spider that frequented the areas of Pará, Bahia, Minas Gerais, Mato Grosso do Sul, and São Paulo (Andrade et al., 2001; Ferreira et al., 2000, 2005; Trajano and Gnaspini, 1990). However, Machado et al. (2005) reported its presence inside residences of Belo Horizonte in Minas Gerais Province, which added another species to the list of synanthropic members of this genus and increased the potential risk of loxoscelism at higher levels. Because of this, and because of an ongoing interest in speleology and touristic activities around the caves of Minas Gerais, Silvestre et al. (2005) conducted the first

characterization of the *L. similis* venom and identified its main biological effects. *L. similis* venom is capable of inducing haemolysis of human erythrocytes, dermonecrotic lesions in rabbits, and lethality in mice at a relatively low LD_{50} (0.32 mg/kg). Importantly, these biological effects are of similar intensity to those of other species, such as *L. intermedia*, *L. laeta*, and *L. gaucho*.

Recently, the number of incidents of loxoscelism caused by *L. similis* has markedly increased in one of the biggest cities of Brazil, Belo Horizonte. This increase in occurrence has justified additional investigation of the *L. similis* venom, sex-linked variation of its potency, and the neutralization effect of anti-*L. similis*-venom on rabbit skin.

2. Materials and methods

2.1. Spiders, venoms, and animals

L. similis spiders (350 individuals) were collected in a country house in the area of Sabará (Minas Gerais, Brazil) and identified using the method described by Gertsch (1967). Venom glands were removed, macerated, and centrifuged, and the cleaned supernatant was stored at −80 °C before use. Protein quantification of venom was performed using the Bradford technique (Bradford, 1976). Bovine serum albumin (BSA) was used as a protein standard. Absorbance was measured at 600 nm with a Spectra MAX 340 microplate spectrophotometer system (Molecular Devices, CA, USA). Adult female New Zealand white rabbits (2.0–2.5 kg) were used in this study and received water and food under controlled environmental conditions throughout the experimental study. Ethical approval regarding the management of the animals used in this study was obtained by the Internal Ethics Committee for Animal Experimentation (CETEA) from the Federal University of Minas Gerais.

2.2. Antivenom production and cross reactivity

After collection of pre-immune serum, rabbits were initially injected subcutaneously with 30 μg of *L. similis* crude venom emulsified in complete Freund's adjuvant at four different points. Four consecutive boosters, each containing 50 μg venom, were then emulsified in incomplete Freund's adjuvant and administered at fifteen day intervals to each rabbit. Immunizations were performed using samples of venom that originated from male or female spiders separately or pooled. One week after each booster, blood samples were taken from the ears of the rabbits, and serum was extracted and stored at −20 °C before use. One week after the last injection, blood was withdrawn and a serum titration was performed by ELISA as described in Chavez-Olortegui et al. (1997). In parallel, the titration of anti-*L. intermedia*-venom serum was evaluated as a comparative control.

2.3. Neutralization assays

2.3.1. In vivo neutralization assay
Rabbits immunized with *L. similis* venom extracted from male or female spiders were challenged 7 days after the last immunization by injecting 10 μg of *L. similis* venom diluted into 100 μl of phosphate-buffered saline (PBS) intradermally

on a shaved area of their dorsum. For the challenge, venom extracted from male or female spiders was used separately to investigate any sex-linked potency. The same protocol was performed in non-immunized rabbits as control. All rabbits received an injection of 100 μl of phosphate-buffered saline (PBS), which was used as a negative control. Dermonecrosis caused by venom was macroscopically observed in rabbit skin 24 and 72 h after administration.

2.3.2. In vitro neutralization assay

The *L. similis* sphingomyelinase activity was determined using different concentrations of *L. similis* venom (0.125, 0.25, 0.5, and 1 μg). Neutralization of sphingomyelinase activity was evaluated by pre-incubating 1 μg of the venom with 100 μl of anti-*L. similis*-venom serum at different dilutions (1:100, 1:500, and 1:2500) for 1 h at 37 °C. Incubation of the venom with pre-immune serum (1:100 dilution) in the same conditions was performed as a control. Purified sphingomyelinase from *Bacillus cereus* was used as a positive control and the kit reaction buffer without sphingomyelinase was used as negative control. Samples were assayed in triplicate. Hydrolysis of sphingomyelin was detected indirectly using the Amplex® Red Sphingomyelinase Assay Kit (Molecular Probes, Invitrogen, OR, USA). Fluorescence was measured with the Cary Eclipse fluorescence microplate reader (Varian, Agilent Technologies, CA, USA) using excitation at 530 nm and detection at 590 nm.

2.4. Histological analysis

Rabbits received an intradermal injection of 3 μg *L. similis* venom (pooled male and female venom) that was pre-incubated for 1 h at 37 °C with 100 μl anti-*L. similis*-venom serum or pre-immune serum in a shaved region of the back. PBS was used as a negative control. Animals were then euthanized at 2, 4, and 8 h post-injection, and skin samples were taken for histological studies. Rabbit skin samples were fixed in 10% buffered formalin solution, pH 7.4, and then embedded in paraffin. Sections of 4 μm thickness were stained with hematoxylin-eosin (H&E), Masson Trichrome (with aniline blue, Easypath Special Stain Kit, Brazil) and Reticulin (Easypath Special Stain Kit, Brazil) according to the manufacturer's instructions.

Fixed tissue samples were evaluated by light microscopy. Qualitative microscopic analysis evaluated the presence of necrosis, edema, hyperemia, hemorrhage, and thrombosis. Moreover, inflammatory cell infiltrate was quantitatively assessed using the following approach: a cell count was performed in 50 random fields and the standard deviation was calculated using 10 samples according to Moro et al. (2003). We determined that the coefficient of variation was stabilized after counting cells in 30 fields (data not shown). Therefore, the total number of inflammatory cells was determined in 30 fields per slide using a digital image analyser. Morphometry was performed using the specific software Image-Pro Plus 5.0 (Media Cybernetics, MD, USA).

2.5. Statistical analysis

The two-way analysis of variance (two-way ANOVA) with Bonferroni post hoc test was used to compare the titration curves of rabbit sera. For the *in vitro* neutralization assay, the one-way ANOVA with Dunnett's Multiple Comparison post hoc test was used to compare the sphingomyelinase activity of the venom with antivenom group versus the venom only group. For morphometry, the two-way ANOVA with Bonferroni post hoc test was used to test how the inflammatory cell infiltrate count was affected by the groups (venom or serum) or time (2, 4, or 8 h). The level of significance was set at $p < 0.05$ and statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, CA, USA).

3. Results

3.1. Antivenom production and cross reactivity

The immunization protocol was performed in rabbits by injecting several boosters of *L. similis* venom. We observed that after the third booster, no statistically significant difference was found between the boosters given to the
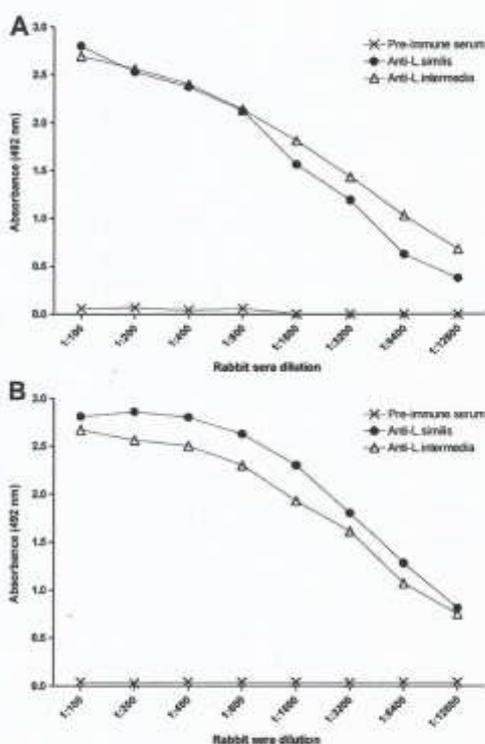

Fig. 1. Reactivity of *Loxosceles similis* venom (from male and female spiders) against anti-*L. similis*-venom (●) or anti-*L. intermedia*-venom (Δ) sera. Pre-immune serum (×) was used as a negative control. ELISA plates were pre-coated with *L. similis* venom (5 μg/ml) extracted from male (A) or female (B) spiders. The graphs show the titration of rabbit sera with a range of dilutions from 1:100 to 1:12,800. The values given are the means of duplicates.
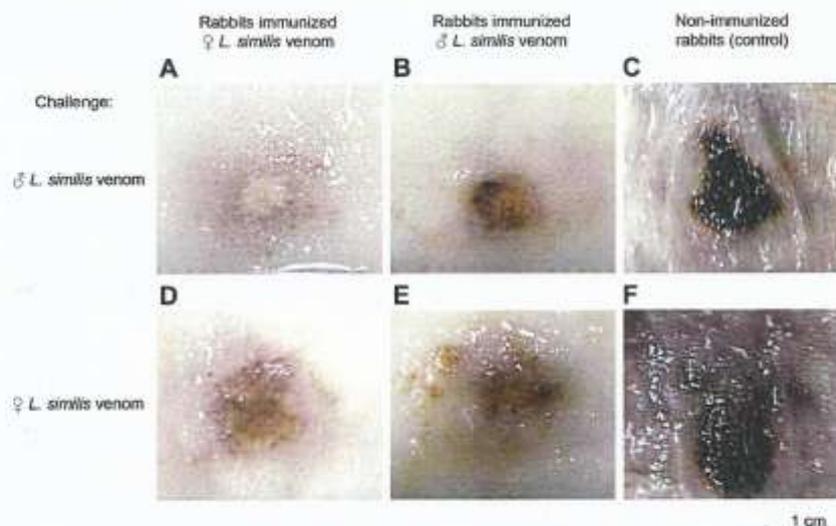
Fig. 2. In vivo neutralization assay. Dermonecrotic lesions were induced in immunized and control rabbits by Loxosceles similis venom. Rabbits were immunized with venom from female ♀ (A, D) or male ♂ (B, E) L. similis. C and F represent the control group of non-immunized rabbits. The rabbits were then challenged with 10 μg of L. similis venom extracted from male (A, B, and C) or female (D, E, and F) spiders. Macroscopic visualization of dermonecrosis was evaluated 24 h after the challenge.

same animal (data not shown). The titration of rabbit sera generated against L. similis venom is shown in Fig. 1. Anti-venom antibodies strongly reacted against L. similis venom extracted from male and female spiders (Fig. 1A and B, respectively). No significant differences were observed between reactions of anti-L. similis and anti-L. intermedia venom sera. The absorbance values of anti-L. similis-venom serum against L. similis male venom (Fig. 1A) were not statistically different from those obtained for anti-L. similis-venom serum against L. similis female venom (Fig. 1B).

3.2. Neutralization assays

3.2.1. In vivo neutralization assay

The neutralization assay was evaluated in rabbits immunized with L. similis venom extracted from female spiders (Fig. 2A, D) and rabbits immunized by venom obtained from male spiders (Fig. 2B, E). Small lesions were observed 24 h after injection of 10 μg of spider venom on the dorsum of rabbits. On rabbits immunized with male spider venom, lesions covering an average area of 15.7 mm^2 when male spider venom was injected (Fig. 2B) and 38.46 mm^2 when female spider venom was injected (Fig. 2E) were observed. In general, the lesions on rabbits immunized with female spider venom were slightly smaller and covered an average area of 12.56 mm^2 and 28.26 mm^2 using male (Fig. 2A) and female (Fig. 2D) venom, respectively. The lesions produced by female venom were larger and surrounded by substantial erythema compared to those produced by male venom on rabbits immunized by venom of either sex. All lesions markedly diminished after 72 h and almost disappeared after 5 days.

3.2.2. In vitro neutralization assay

The sphingomyelinase activity was measured using different doses of L. similis venom (0.125, 0.25, 0.5, and 1 μg; data not shown), and substantial sphingomyelinase activity was observed with 1 μg of L. similis venom (Fig. 3). To
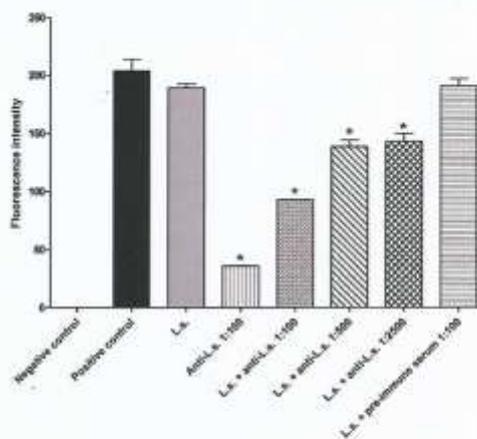

Fig. 3. In vitro neutralization assay. Sphingomyelin was incubated with Loxosceles similis venom (1 μg) and fluorescence was then measured (excitation at 530 nm; detection at 590 nm). For neutralization of sphingomyelinase activity, L. similis venom (1 μg) was pre-incubated with different dilutions of anti-L. similis-venom serum (1:100, 1:500, and 1:2500) or pre-immune serum (1:100). Purified sphingomyelinase from Bacillus cereus was used as a positive control and the kit reaction buffer without sphingomyelinase was used as a negative control. The results are mean ± S.D. of triplicates. *p < 0.05 compared to L.s.

investigate the neutralization capacity of the anti-*L. similis*-venom antibodies, 1 μg of *L. similis* venom was incubated for 1 h at 37 °C with 100 μl of antivenom diluted over a range of 1:100 to 1:2500. All dilutions of venom incubated with antivenom showed a significant reduction of sphingomyelinase activity compared to *L. similis* not incubated with antivenom. In contrast, the control pre-immune serum did not alter the sphingomyelinase activity of the venom (Fig. 3).

3.3. Histological analysis

Histological analysis of rabbit skin after intradermal injection of *L. similis* venom pre-incubated with pre-immune serum showed dense inflammatory infiltrate with the presence of numerous neutrophils and occasional eosinophils deep in the dermis. Edema, hyperemia, hemorrhage, and thrombosis were also observed. The
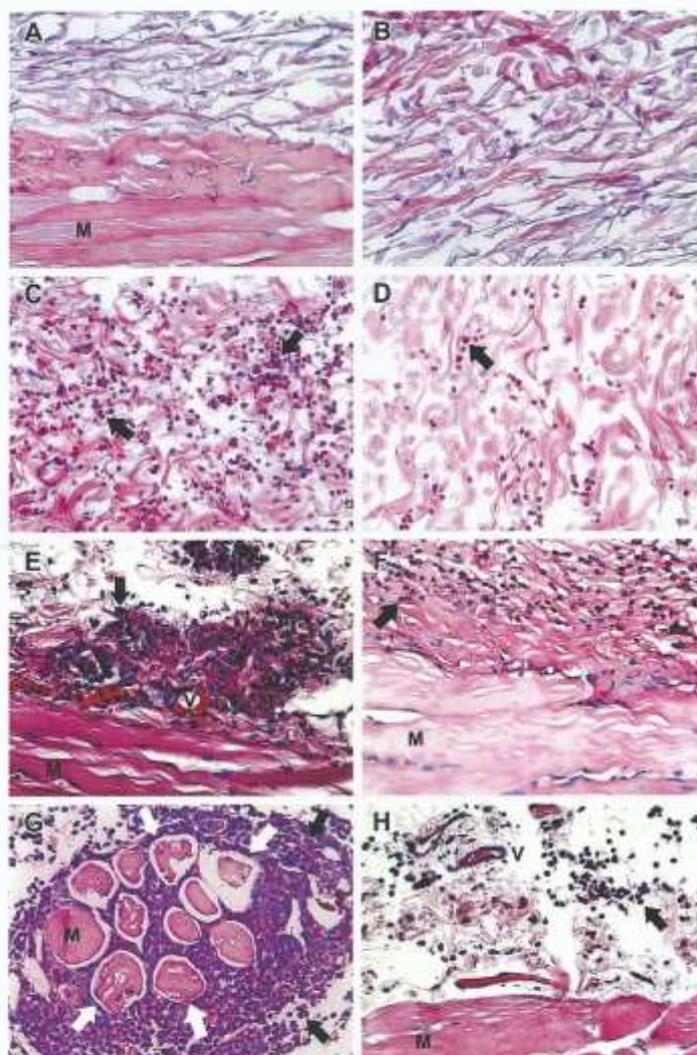

Fig. 4. Light microscopic analysis of skin sections from rabbits stained with hematoxylin-eosin (magnification ×400). Control sites were injected with PBS (A, B). Dense inflammatory infiltrate (black arrows) was detected in the dermis at 2, 4, and 8 h after 3 μg *Loxosceles similis* venom was injected (C, E, and G, respectively). The venom caused degeneration and necrosis of the skin muscle (white arrows) after 8 h (G). Pre-incubation of the venom with anti-*L. similis*-venom serum significantly decreased inflammatory cell infiltrate after 2, 4, and 8 h (D, F, and H, respectively) compared to the same periods of action of the venom. Degeneration and necrosis of the skin muscle were also prevented when venom was pre-incubated with antivenom (H). Notes: M = muscle; V = vessel.

inflammatory cell infiltrate was initially detected 2 h after venom injection and continued after 4 and 8 h (Fig. 4C, E, and G). The inflammatory cell infiltrate count was significantly higher after 8 h than 2 and 4 h post-injection, but no significant difference was observed in the cell count between 2 and 4 h (Fig. 5).

Masson Trichrome stained specimens showed dissociation of collagen fibers in the dermis due to marked edema predominantly at 8 h after the *L. similis* venom injection (Fig. 6C, E, and G).

The venom caused degeneration and necrosis of the skin muscle after 8 h (Fig. 4G). In addition, the reticular fibers of skin muscle, which act as mesh work and give support to muscle cells, were clearly disrupted (Fig. 7B).

Pre-incubation of the venom with anti-*L. similis*-venom serum significantly decreased inflammatory cell infiltrate after 2, 4, and 8 h post-injection compared to the same periods of action for the venom pre-incubated with preimmune serum (Figs. 4 and 5). Treatment with antivenom also prevented the degeneration and necrosis of the skin muscle (Fig. 4H) and reduced the severity of edema (Fig. 6H). Finally, the reticular fibers were mostly preserved after injection of *L. similis* venom pre-incubated with the antivenom (Fig. 7C).

4. Discussion

Loxoscelism is the term used to describe accidents, lesions, and symptoms induced by bites from spiders of the *Loxosceles* genus. The major focus of scientific studies of this genus have been focused on a family of proteins called Loxtox which comprise the most lethal toxins in the venom of *Loxosceles* spiders. The majority of isolated (native or recombinant) Loxtox proteins can reproduce several of the

effects observed when whole venom is used in biological assays (Kalapothakis et al., 2007). However, it is expected that other groups of proteins in the venom can also affect biological tissues and contribute to the overall functions of the venom, which aids spider nutrition by dissolving biological tissues and killing prey.

The production of high quality and effective antivenoms is difficult. Some venom components are more immunogenic than others (Calvete et al., 2009; Maria et al., 2005) and may not be relevant in the production of neutralizing antibodies. To optimize antivenom potency, the most lethal and immunogenic components of whole venom have to be characterized, identified, and selected against other peptides. Venomic/antivenomic technologies (Calvete et al., 2009) may help produce polyvalent antivenoms with multiple targets and higher potency by allowing for the selection of the most important elements of heterologous venoms and the creation of antivenom cocktails for multiple purposes. This task, however, is often complicated. For example, using a systematic effort to correlate the most toxic antigenic components of *Tityus serrulatus* (Ts) scorpion venom with the neutralizing capacity of various horse anti-Ts-venom sera, Maria et al. (2005) demonstrated the complexity of associating the reactivity between specific toxin antigens and sera antibodies with the neutralization potency of the corresponding antivenoms. The authors overruled the expected result that higher reactivity between antigens and sera antibodies corresponds to more potent antivenoms and suggested that current techniques, such as ELISA, are not sufficient for making accurate estimations in this regard.

Antivenom is the only venom-specific treatment used for loxoscelism, and all other treatment options mainly act by limiting secondary infections and side effects or directly treating the bite on the skin. Although serotherapy is extensively used, there are controversial opinions as to the efficacy of antivenoms to treat necrosis, especially in relation to the long time interval that usually exists between the time that the bite occurs and access to this treatment. For antivenoms to be used over a wider context, it is important to show their ability to neutralize local effects and improve both local and systemic symptoms of patients in realistic terms of time lapse and other conditions. Pauli et al. (2009) showed that treatment with anti-*L. intermedia* antivenom may effectively reduce cutaneous and systemic effects when applied up to 12 h after the bite occurs, but only reduces cutaneous symptoms if applied up to 48 h after the bite occurs.

The extent of the biological effects of spider venoms on their victims depends on factors relating to the victims (species, age, bite location, and genetic variations; see extensive literature in Pauli et al., 2006) and the characteristics of spiders that exhibit inter- and/or intra-specific variation. The interspecific variation of systemic and dermonecrotic effects of *Loxosceles* bites has been broadly analysed by several groups (Barbaro et al., 2005; De Oliveira et al., 2005; Gomez et al., 2001; Olvera et al., 2006; Silvestre et al., 2005; Toro et al., 2006). Intraspecific variation of venom toxicity is mainly due to differences in the sex and age of the spider (De Oliveira et al., 1999; Gonçalves de Andrade et al., 1999) and is rather neglected in the
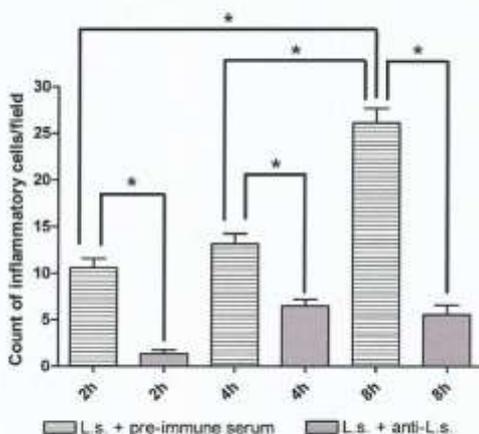

Fig. 5. Inflammatory cell infiltrate count in skin sections from rabbits at 2, 4, and 8 h after 3 μg *Loxosceles similis* venom was injected with or without anti-*L. similis*-venom serum. Pre-incubation of the venom with anti-*L. similis*-venom serum significantly reduced the inflammatory cell infiltrate count. The data are represented as mean ± S.E.M. of cell count in 30 fields. * $p < 0.05$.

literature, although it has been demonstrated in other venomous animals, such as snakes (Daltry et al., 1996; Furtado et al., 2006; Pahari et al., 2007) and scorpions (Badhe et al., 2006). Sex-linked differences in the toxins quantity, concentration of toxic elements, cross-reactivity, and biological effects have been reported for *L. intermedia* (De Oliveira et al., 1999; Gonçalves de Andrade et al., 1999) and *L. laeta* (De Oliveira et al., 2005), but not for the

medically important *Loxosceles* in South Africa, namely *L. spinulosa* and *L. parrami* (Newlands et al., 1982). In our study, sex-linked variation of *L. similis* venom potency was evident for dermonecrotic and neutralization effect on rabbits.

Our neutralization assay demonstrated that female spider venoms of *L. similis* induced larger lesions, but also protected animals to a greater degree as immunization
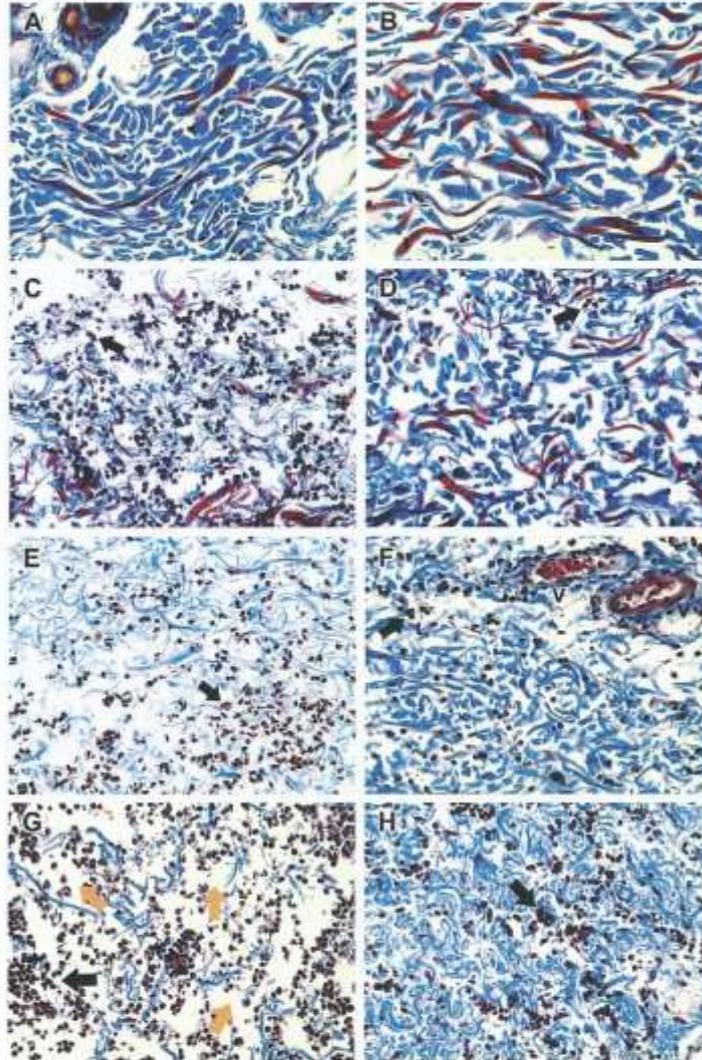

Fig. 6. Light microscopic analysis of skin sections from rabbits stained with Masson Trichrome (magnification ×400). Control sites were injected with PBS (A, B). In addition to dense inflammatory infiltrate (black arrows), dissociation of collagen fibers in the dermis due to edema was observed at 2, 4, and 8 h after 3 μg *Loxosceles* similis venom was injected (C, E, and G, respectively). Orange arrows point to marked edema observed at 8 h. Pre-incubation of the venom with anti-*L. similis*-venom serum significantly decreased edema and inflammatory cell infiltrate after 2, 4, and 8 h (D, F, and H, respectively) compared to the same periods of action of the venom. Note: collagen fibers are stained blue; V – vessels.

enhancers when compared to male venoms of the same species. In addition, female spider venom also provided greater protection against L. intermedia envenomation (data not shown). These results are in concordance with those by De Oliveira et al. (2005) showing the intraspecific variation of biological effects of L. intermedia and L. laeta. De Oliveira et al. (1999) also showed that in female individuals of L. intermedia, there was a higher concentration of the F35 toxin, which is one of the key elements that enhance the toxicity of this venom. This correlated with the larger size and higher quantities of venom produced by female spiders of this species. Although the venom quantity produced by

Fig. 7. Light microscopic analysis of skin sections from rabbits stained with Reticulin (magnification ×400). The control site was injected with PBS (A). Red arrows point to reticular fibers of skin muscle whose disruption can be observed 8 h after injection with 3 µg Loxosceles similis venom (B). Preserved reticular fibers of skin muscle were detected 8 h after injection of venom with anti-L. similis-venom serum (C). Note: reticular fibers are stained black; M — muscle.

female spiders in our study was also slightly higher than that produced by male spiders (12.49 and 13.93 mg/ml of venom in male and female spiders, respectively), we hypothesize that the difference between male and female venom potency is mainly qualitative and relies on differences in the presence of the most lethal toxins and other important elements for the dermonecrotic effects. Further investigation must be performed in order to clarify this point. In addition, it is not yet clear why female spiders have evolved with higher levels of venom toxicity to animals, but could be related to motherhood and greater longevity.

In the present investigation, we showed that anti-L. similis-venom was capable of reducing the disruption of the connective tissue and edema in the rabbit skin as well as partially preserving collagen and reticulin fibers. The action of L. similis venom on two important fibers of the connective tissue, collagenous and reticular fibers, was evaluated in vivo. To better characterize the initial action of this venom, rabbit skin was inoculated with a low dose of venom (3 µg) and analysed after 2, 4, and 8 h post-injection. Histopathological changes included diffuse edema of the dermis, proteinaceous exudation and massive and diffuse collection of inflammatory cells, and muscular necrosis. Importantly, we eliminated enzymes representing contamination of venom with egested stomach contents by using venom obtained from glands extracted from the spider's cephalothorax.

The disruption of connective tissue by components of the venom alters the extracellular matrix homeostasis, which causes the classical symptoms of loxoscelism. Metalloproteinases and serine proteases with gelatinolytic, fibronectinolytic, and fibrinogenolytic activity have been described as important components of L. intermedia venom. Degradation of entactin and the heparin sulfate protein core as well as the release of laminin from basement membranes were also observed; however, effects on laminin and type I and type IV collagen were not detected. An interesting characteristic of the L. intermedia venom is the presence of pro-enzymes (metallo and serine proteases) that are activated by APMA and trypsin (Veiga et al., 2000). The complexity of basement membranes and the extracellular matrix with different types and/or isoforms of collagens, laminins, proteoglycans, nidogen/entactin, and several other types of molecules (Kim et al., 2011; Kruegel and Miosge, 2010; Yurchenco, 2011) suggests that several other venom targets could be identified in the future.

In summary, the present study has provided data that advances our understanding of L. similis venom. These results reinforce the positive neutralization capacity of antivenom on many actions of the venom, such as connective tissue alterations, inflammatory cell infiltrate, and sphingomyelinase activity. Our results suggest that any study that provides improvements in the quality of antivenoms must be considered a higher priority for further analyses.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Capítulo 4

Este capítulo contém os certificados emitidos pelos Comitês de
ética

Anexos

1. Certificado de exame de qualificação

Ata do exame de qualificação a que se submeteu a doutoranda **NÚBIA BRAGA PEREIRA** nos termos do art. 2°, item VI da Resolução n° 05, de 10/03/83, do Conselho Federal de Educação e das Normas Gerais da Pós-Graduação da UFMG.

Ao primeiro dia do mês de outubro de dois mil e doze, convocada pelo Colegiado do Programa de Pós-Graduação em Patologia – Área de Concentração em Patologia Geral – compareceu a doutoranda **NÚBIA BRAGA PEREIRA** para submeter-se ao exame de qualificação com a tese intitulada: **"IMPLANTE SUBCUTÂNEO DE ESPONJAS EM CAMUDONGOS SWISS COMO MODELO DE ESTUDO PARA O LOXOSCELISMO"**, perante a Comissão Examinadora composta pelas professoras: Roselene Ecco – UFMG e Mary Suzan Varaschin – UFLA. Participou da sessão, como ouvinte, a professora Luciana Moro, orientadora da tese. A sessão constou da exposição oral e projeção de vídeo com a presença das professoras acima citadas. Após a exposição da candidata, as professoras participantes da Comissão Examinadora fizeram comentários sobre a apresentação, o material didático utilizado e o conteúdo do trabalho. Após a arguição, a Comissão Examinadora do exame de qualificação considerou a estudante APTA a se submeter à defesa de tese. Para constar, lavrou-se a presente ATA, que segue assinada pela Comissão Examinadora. Belo Horizonte, 1° de outubro de 2012.

Profa. Roselene Ecco _____

Profa. Mary Suzan Varaschin _____

Profa. Rosa Maria Esteves Arantes/Coordenadora _____

2. Certificado aprovação CETEA/UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 229/2009**, relativo ao projeto intitulado *"Avaliação da apoptose de células endoteliais induzida pelo veneno da L. Similis no modelo de implante subcutâneo de esponjas em camundongos Balb/c"*, que tem como responsável(is) **Luciana Moro** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo *Comitê de Ética em Experimentação Animal* (CETEA/UFMG), tendo sido aprovado na reunião de **9/06/2010**.

Este certificado expira-se em **9/ 06/ 2015**.

CERTIFICATE

We hereby certify that the Protocol nº 229/2009, related to the project entitled *"Apoptosis evaluation of the endothelial cells induced by Loxosceles similis poison in sponge models subcutaneously implanted in Balb/c mice"*, under the supervisors of Luciana Moro, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the *Ethics Committee in Animal Experimentation* (CETEA/UFMG), and was approved in **June 9, 2010**.

This certificate expires in June 9, 2015.

Belo Horizonte, 19 de Junho de 2010.
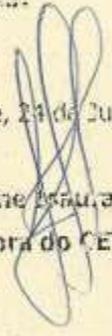
Profª. Jacqueline Isaura Alvarez-Leite
Coordenadora do CETEA/UFMG

Universidade Federal de Minas Gerais
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(Mod.Cert. v1.0)

3. Protocolo da Comissão de ética no uso de animais/ Fiocruz
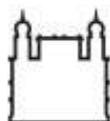
Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

PROTOCOLO No. 93/10-2

CEUA
Fiocruz

**Comissão de Ética
no Uso de Animais**

Vice-presidência de Pesquisa e
Laboratórios de Referência

Título do projeto :

Avaliação da apoptose de células endoteliais induzida pelo veneno da Loxosceles similis no modelo de implante subcutâneo
de esponjas em camundongos Swiss webster

Palavras Chave :

Implante de esponjas, células endoteliais, apoptose, loxoscelismo

Tempo de execução : 1 Ano(s)

Solicitante :

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    Formação / Titulação : DOUTORADO EM CIÊNCIA ANIMAL

Responsável pelo PROTOCOLO :

      Nome : PATRICIA MARTINS PARREIRAS

      Matricula Siape :

      Unidade : CENTRO DE PESQUISAS RENE RACHOU

      Email :

      Cargo :

      Titulação :