PAULO HENRIQUE DA SILVA

ARANHA MARROM E LOXOSCELISMO: revisão bibliográfica e ação sobre os tecidos hematopoiético e renal

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Biologia Celular e Molecular, Programa de Pós-Graduação do Departamento de Biologia Celular, Setor de Ciências Biológicas da Universidade Federal do Paraná.

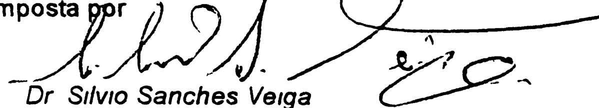
Orientador: Prof. Dr. Silvio Sanches Veiga
Co-orientador: Prof. Dr. Waldemiro Gremski

CURITIBA
2005

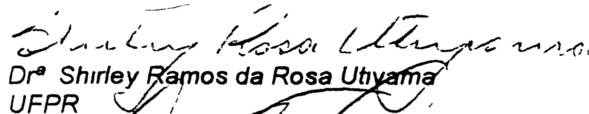
Departamento de Biologia Celular e Departamento de Fisiologia
Setor de Ciências Biológicas
Universidade Federal do Paraná
Instituto de Biologia Molecular do Paraná

PARECER

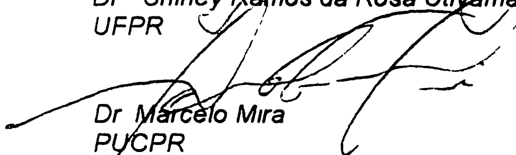
A banca examinadora, instituída pelo colegiado do Programa de Pós-Graduação em Biologia Celular e Molecular, do Setor de Ciências Biológicas, da Universidade Federal do Paraná, composta por



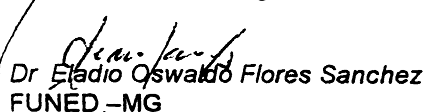
Dr Sílvio Sanches Veiga
Orientador da UFPR



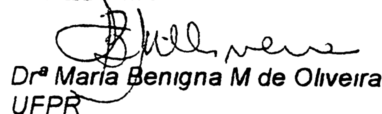
Drª Shirley Ramos da Rosa Utiyama
UFPR



Dr Marcelo Mira
PUCPR



Dr Eladio Oswaldo Flores Sanchez
FUNED –MG



Drª Maria Benigna M de Oliveira
UFPR

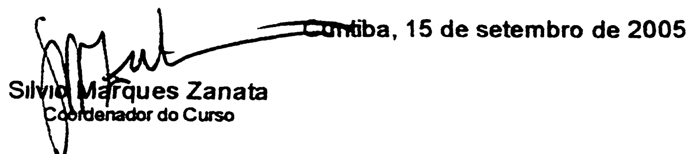
e tendo como suplentes,

Drª Célia Regina Cavichiolo Franco
UFPR

Dr Sílvio Marques Zanata
UFPR

após argüir o(a) doutorando(a) ***Paulo Henrique da Silva*** em relação ao seu trabalho de tese intitulado "***Aranha Marrom e Loxoscelismo***", é de parecer favorável à ***APROVAÇÃO*** do(a) acadêmico(a), habilitando-o(a) ao título de Doutor em Biologia Celular e Molecular, área de concentração em **Biologia Celular e Molecular.**
A obtenção do título de Doutor está condicionada à implementação das correções sugeridas pelos membros da banca examinadora e ao cumprimento integral das exigências estabelecidas nas Normas Internas deste curso de pós-graduação

Curitiba, 15 de setembro de 2005



Silvio Marques Zanata
Coordenador do Curso

CENTRO POLITECNICO – CEP 81.531-990-JARDIM DAS AMÉRICAS-Caixa Postal 19031-FONE: (41) 361-1676 FAX 266-2042 – E-Mail: ... – CURITIBA - PARANÁ

AGRADECIMENTOS

Inicialmente quero deixar registrado o meu agradecimento a todos os funcionários do biotério do Setor de Ciências Biológicas (Campus Centro Politécnico) da Universidade Federal do Paraná por terem tido uma participação fundamental na realização das coletas de medula óssea e sangue periférico dos animais utilizados durante o desenvolvimento da tese. Expresso meu agradecimento de forma especial ao funcionário Antonio Luiz de Andrade e ao bioterista biólogo Candido José Thomaz Pereira. Agradeço ao Dr. Carlos Roberto de Assis, médico veterinário, por nos ter ensinado a técnica de coleta de medula óssea e pela orientação nos anestésicos utilizados. Agradeço as bibliotecárias da biblioteca do Setor de Ciências Biológicas Campus Centro Politécnico e da biblioteca do Hospital de Clínicas por tornarem possível a realização do artigo de revisão.

Gostaria de corrigir uma injustiça que se faz quando a tese defendida leva somente o meu nome. O mérito da tese deve ser creditado a todos os componentes do Laboratório de Matriz Extracelular e Biotecnologia de Venenos, por terem participado ativamente na condução dos experimentos e pelo apoio que me foi dado. Quero agradecer especialmente a Márcia, Olga, Ana Isabel, Luiza, Kátia, Rafael e Juliana Dreyfuss.

Dedico esta tese a duas pessoas que foram muito importantes para a conclusão deste trabalho. A primeira pessoa é o Professor Yoshio Hashimoto, com quem tenho um relacionamento de amizade desde 1973 e participou de todos os momentos importantes da minha vida tanto pessoal como profissional e que acompanhou de modo especial a condução da tese, inclusive sendo co-autor de um dos trabalhos. A segunda pessoa é o Professor Doutor Silvio Sanches Veiga pelo modo como orientou a tese, mostrando-me o que é ser um orientador

preocupado com a qualidade científica do trabalho desenvolvido e por mostrar o compromisso que se deve ter com a verdade científica. O Prof. Sílvio é um pesquisador de grande quilate e responsável pela conclusão da tese com três artigos publicados até a data da defesa.

Por fim agradeço a minha família que acompanhou todo o desenrolar deste trabalho e por sempre acreditar em mim.

SUMÁRIO

RESUMO **iii**

ABSTRACT **v**

1. INTRODUÇÃO **1**

2. OBJETIVOS **11**

3. RESULTADOS **12**

I- Brown spiders and loxoscelism **13**

II- Hematological cell findings in bone marrow and peripheral blood of rabbits after acute exposure to *Loxosceles intermedia* (brown spider) venom **14**

III- Experimental evidence for a direct cytotoxicity of *Loxosceles Intermedia* (brown spider) venom in renal tissue **15**

4. DISCUSSÃO **16**

5. CONCLUSÃO **25**

6. REFERÊNCIAS BIBLIOGRÁFICAS **27**

RESUMO

Esta tese está baseada na publicação de três artigos científicos os quais versam sobre a ação do veneno da aranha marrom (*Loxosceles intermedia*) sobre células da medula óssea e sangue periférico, utilizando como modelo animal coelhos. Ação do veneno da aranha marrom sobre o tecido renal, utilizando camundongos como modelo animal, tendo como enfoque a participação de células da medula óssea e do sangue periférico na nefrotoxicidade e uma revisão sobre loxoscelismo em um período compreendido entre 1993 e 2003.

O objetivo do primeiro artigo publicado foi mostrar alterações celulares que ocorrem na medula óssea e sangue periférico de coelhos após a exposição aguda ao veneno da *L. intermedia*. Doses de 40 μg de proteínas do veneno (que corresponde a quantidade média de veneno inoculada pela aranha) foram injetadas na derme de 5 coelhos usando como controle outros 5 coelhos, tendo sido injetado nestes PBS, que também foi utilizado como solvente do veneno. Amostras de medula óssea e sangue periférico foram obtidas antes do envenenamento e 4, 8, 12, 24 e 48 horas e 5, 10, 15, 20 e 30 dias após o envenenamento. Na medula óssea observou-se a celularidade, células vermelhas nucleadas, megacariócitos e neutrófilos. No sangue periférico foi realizado o hemograma. O objetivo do trabalho foi verificar se o veneno tem um efeito direto sobre as células da medula óssea e sangue periférico, se as alterações são secundárias às necessidades teciduais e se há uma correlação entre a histopatologia e os achados hematológicos após a exposição ao veneno. A série vermelha não foi afetada pelo veneno, somente os eritroblastos apresentaram um decréscimo. A depleção medular de megacariócitos teve uma boa correlação com a trombocitopenia periférica, a qual correlacionou-se com os achados histopatológicos, obtidos a partir de biópsias da pele de coelhos. A queda da celularidade e dos neutrófilos medulares correlacionou-se significativamente com as alterações neutrofílicas periféricas. A trombocitopenia e a neutropenia em sangue periférico

são devidas a depleção medular pela migração plaquetária e neutrofílica ao sítio inflamatório. Se o veneno tem um efeito direto sobre a medula óssea este foi transitório.

No segundo trabalho publicado foi injetado intraperitonealmente 100 μl de veneno da *L. intermedia* diluído em PBS, equivalente a 1 mg de proteína/kg de peso, em 50 camundongos (grupo tratado). O grupo controle foi representado por 50 camundongos que receberam somente PBS. O camundongo foi utilizado como modelo animal pelo fato de não desenvolver lesão dermonecrótica, portanto qualquer lesão renal não pode ser atribuída a uma conseqüência secundária da lesão dermonecrótica. O objetivo do trabalho foi mostrar que o veneno induz diretamente o dano renal. Análises de biópsias renais ao microscópio óptico mostraram hialinização dos túbulos proximais e distais, eritrócitos nos espaços de Bowman's, colapso glomerular, bolhas e vacúolos em células epiteliais celulares, edema intersticial e deposição de material eosinofílico no lúmen dos túbulos. O veneno causou aumento da concentração plasmática de uréia e não houve decréscimo da fração C3 do complemento. Não causou hemólise evidente "in vivo" porque não houve alteração da contagem de eritrócitos, da concentração de hemoglobina e determinação do hematócrito entre o grupo controle e o tratado. Também não foi evidenciada hemoglobinúria ou hematúria. Os dados experimentais apresentados evidenciam uma ação nefrotóxica direta do veneno da *L. intermedia*.

O terceiro artigo publicado trouxe uma revisão sobre o loxoscelismo, nos últimos dez anos, enfocando a biologia e a epidemiologia da aranha marrom, os achados histopatológicos e alterações celulares após a exposição ao veneno. Mostra as toxinas identificadas e caracterizadas no veneno total, o mecanismo de desenvolvimento do quadro dermonecrótico e sistêmico causados pelo acidente com a aranha marrom, a imunogenicidade, as características clínicas, o diagnóstico e a terapia utilizada no tratamento do loxoscelismo. Por fim os produtos de biotecnologia já descritos e as proteínas clonadas até então.

ABSTRACT

This tesis is based in three papers. The purpose of the first work was to find out the cellular changes occuring in bone marrow and peripheral blood after acute exposure to the venom of *Loxosceles intermedia*. Doses of 40 μg of venom were injected intradermally into five rabbits, and five rabbits receiving only phosphate-buffered saline (PBS) were used as controls. Bone marrow and peripheral blood samples were obtained before the envenomation and 4, 8, 12, 24 and 48 h, and 5, 10, 15, 20 and 30 days after envenomation. In bone marrow samples we assessed cellularity, nucleated red cells, megakaryocytes and neutophils, and in peripheral blood we assessed red cells (red cell concentration, hemoglobin and hematocrit), leukocytes, neutrophils and platelets. Our objective was to find out if the venom has a direct effect on bone marrow and peripheral blood or if changes in both of them are secondary to the needs of tissues, and if there is a good correlation between histopathological and hematological findings. We found that the red cell parameters were not affected by the venom, except for nucleated red cells which decreased after venom exposure. The depression of megacaryocyte numbers and thrombocytopenia showed a strong correlation with the histopathological changes observed in skin biopsies obtained from the rabbits. The changes in cellularity and neutrophils of bone marrow were strongly correlated with those in peripheral blood and skin. The thrombocytopenia and neutropenia in peripheral blood are due to marrow depression, which may be a consequence of an extensive migration of platelets and neutrophils to the necrotic lesion or the marrow depression may be a transitory effect of evenoming by *L. intermedia*.

In the second work mice were exposed to *L. intermedia* venom with the aimed to show whether the venom directly induces renal damage. Mice were used as animal model because they did not develop dermonecrotic lesion. The experimental groups were composed of 50 mice as control and 50 mice that received the venom. Light microscopic analysis of renal biopsy specimens showed

alterations including hyalinization of proximal and distal tubules, erytrocytes in Bowman’s space, glomerular collapse, tubule epithelial cell blebs and vacuoles, interstitial edema, and deposition of eosinophilic material in the tubule lumen. We also found that the venom caused azotemia with elevation of blood urea levels but did not decrease C3 complement concentration or cause hemolysis in vivo, because neither red blood cell count, hemoglobin, or hematocrit values showed significant differences between the venom-treated and control groups. No hemoglobinuria or the presence of red blood cells in urine was observed. The data provide experimental evidence that *L. intermedia* venom is directly involved in nephrotoxicity.

The last work, a review, had the purpose to describe some insights into loxoscelism obtained over the last ten years. The biology and epidemiology of the brown spider, the histopathology of envenomation and the immunogenicity of *Loxosceles* venom are reviewed, as well as the clinical features, diagnosis and therapy of brown spider bites. The identification and characterization of some toxins and the mechanism of induction of local and systemic lesions caused by brown spider venom are also discussed. Finally, the biotechnological application of some venom toxins are covered.

1. INTRODUÇÃO

A aranha marrom pertence ao gênero Loxosceles, família Loxoscelidae, sub-ordem Labidognatha, ordem Araneida, classe Arachnida e filo Arthropoda (Ruppert and Barnes, 1996; Soerensen, 1996). O comprimento do corpo varia de 8 a 15 mm e as pernas podem medir entre 8 a 30 mm. A coloração varia de marrom claro (*L. laeta*) a marrom escuro (*L. gaucho*). A característica desse gênero é o formato do cefalotórax que lembra um violino (Futrell, 1992).

As aranhas do gênero *Loxosceles* foram descritas na América do Sul (Ministério da Saúde, Brasil, 1998; Sezerino et al., 1998; Schenone, 1998), América do Norte (Futrell, 1992; Escalante-Galindo et al., 1999), Europa (Nicholson and Graudins, 2003), África (Futrell, 1992; Nicholson and Graudins, 2003), parte da Ásia (Nicholson and Graudins, 2003), Israel (Borkkan et al., 1995; Cohen et al., 1999) e Austrália (Young and Pincus, 2001; Nicholson and Graudins, 2003). No Brasil são encontradas 7 espécies (Sezerino et al., 1998). A *L. gaucho* é encontrada em São Paulo e no Rio Grande do Sul. A *L. similis* é encontrada na Paraíba, São Paulo e Minas Gerais. A *L. adelaide* é encontrada no Rio de Janeiro. A *L. hirsuta* é encontrada no Rio Grande do Sul, Santa Catarina e no Paraná. A *L. intermedia* é encontrada desde o Rio Grande do Sul até o Rio de Janeiro. A *L. amazonia* é encontrada no Amazonas e no Ceará e a *L. laeta* é encontrada nas regiões sul e leste (Gertsch, 1983). Das sete espécies descritas três estão envolvidas com o envenenamento humano, a *L.intermedia*, *L. gaucho* e *L. laeta*.

São aranhas de hábito noturno que vivem sob pedras, troncos de árvores, restos de vegetais, telhas e pilhas de tijolos, e que adquiriram o hábito intra-domiciliar, sendo encontradas atrás de quadros, móveis, livros e no meio das roupas. O fato da aranha marrom ter adquirido o hábito intra-domiciliar tornou-se a principal causa de acidentes com o homem (Hite et al, 1960; Bücherl, 1960-62; Lucas et al., 1983-84; Rodriguez et al., 1986; Jorge et al., 1991). Elas não são agressivas, a picada ocorre quando elas são comprimidas contra a pele, o que pode ocorrer no ato de se vestir, de se enxugar ou durante o sono (Suarez, 1971; Lucas et al., 1983-84; Futrell, 1992). Alimentam-se de pequenos insetos e se

reproduzem com facilidade, mesmo em ambientes desfavoráveis. Podem sobreviver por vários dias ou até meses sem alimento e água, suportam temperaturas entre 8 à 43°C (Futrell, 1992). A incidência dos acidentes com o homem ocorre durante os meses mais quentes do ano, primavera e verão (74%) (Schenone and Letonja, 1975; Manfredini et al., 1993).

O quadro clínico provocado pela picada da aranha marrom é denominado de Loxoscelismo, o qual, no Brasil foi responsável por 36% dos 17.781 casos de acidentes com aranhas notificados ao Ministério da Saúde entre 1990 e 1993 (Sezerino et al., 1998). O loxoscelismo é especialmente predominante na região metropolitana de Curitiba, com cerca de 2.000 casos por ano (Málaque et al., 2002). A aranha marrom que predomina na região metropolitana de Curitiba e na região urbana do Paraná e Santa Catarina é a *L. intermedia* (Fischer, 1994; Mattosinho et al., 1997; Andrade et al., 2000).

O loxoscelismo pode apresentar dois quadros clínicos: quadro cutâneo ou dermonecrótico (aproximadamente 80% dos casos) e quadro cutâneo-visceral ou sistêmico. O desenvolvimento de um ou de outro tipo de quadro clínico está correlacionado com o estado nutricional, idade, local da picada, com a quantidade de veneno inoculado, susceptibilidade ao veneno e o tempo que o acidentado leva para iniciar o tratamento (Gajardo-Tobar, 1966; Barbaro et al., 1992). O veneno é essencialmente protéico com ação enzimática ou tóxica e pode ser comparado a alguns venenos de serpentes, onde os estudos demonstraram a presença abundante de enzimas em sua composição (Martinez-Vargas, 1987). Entre elas, fosfatase alcalina, 5’ ribonucleotideo fosfohidrolase e hialuronidase identificadas no veneno da *L. reclusa*, nenhuma delas produz lesão dermonecrótica em animais de laboratório (Futrell, 1992). A toxina melhor caracterizada bioquimicamente é a esfingomielinase D (32 kDa) envolvida na agregação plaquetária, hemólise intravascular e dermonecrose (Kurpiewski et al., 1981; Futrell, 1992), outras moléculas de 33, 34 e 35 kDa estão associadas com atividades nocivas do veneno (Barbaro et al., 1992; Geren et al., 1976; Tambourgi et al., 1995; Feitosa et al., 1998; Veiga et al., 1999). O veneno contém ainda outros fatores hemorrágicos, uma metaloprotease de 20-28 kDa (loxolisina A) com

efeitos sobre a fibronectina e fibrinogênio e uma metaloprotease gelatinolítica de 32-35 kDa (loxolisina B) provavelmente associada com a atividade dermonecrótica do veneno (Feitosa et al., 1998). O quadro dermonecrótico que é a manifestação mais característica do loxoscelismo, com morte celular e destruição dos constituintes intercelulares (matriz extracelular) na região lesionada, representa potencialmente a presença da atividade proteolítica das enzimas do veneno (Feitosa et al., 1998; Veiga et al., 1999 e Veiga et al., 2000). A trombocitopenia decorrente de uma agregação plaquetária induzida pelo veneno, bem como o quadro hemorrágico e a coagulação intravascular disseminada que surgem em alguns acidentados, representam fenômenos intimamente dependentes de moléculas da matriz extracelular tais como a fibronectina plasmática e o fibrinogênio (Ruoslahit, 1988; Veiga et al., 1996) e mais uma vez tais transtornos podem ser atribuídos à enzimas presentes no veneno com atividades proteolíticas sobre estes substratos (Feitosa et al., 1998; Zanetti et al., 2002).

A picada da aranha marrom é pouco dolorosa e geralmente passa desapercebida (Futrell, 1992). Após 2 a 8 horas da picada, inicia-se um quadro de dor local do tipo "queimação" ou ardência, podendo estar acompanhado de prurido, edema, eritema, sensação de mal estar geral e até febre. Posteriormente pode surgir uma lesão que varia de 1 a 30 cm, circundada por um halo vermelho e uma zona pálida, denominada de placa marmórea (Rodriguez et al., 1986; Futrell, 1992). Após 3 a 5 dias pode ocorrer acúmulo de leucócitos polimorfonucleares, necrose e formação de abcesso (Smith and Micks, 1970; Futrell, 1992). Pode ocorrer necrose com formação de úlcera necrótica ou mancha gangrenosa de difícil cicatrização e a cura não se completa em menos de um mês. No local da lesão permanece uma cicatriz que pode ser desfigurante (Jorge et al., 1991; Futrell, 1992). Em estudos realizados com coelhos, os exames histopatológicos mostraram após 3 horas, um acúmulo de leucócitos polimorfonucleares ao redor de vênulas e eritrócitos extravasculares sugerindo perda da integridade vascular. Nas arteríolas, foi verificado edema de células endoteliais. Após 6 horas, havia edema da derme e epiderme, infiltração de leucócitos polimorfonucleares nas paredes das vênulas, vasodilatação, coagulação intravascular, hemorragia

volumosa subcutânea e até mesmo no músculo, necrose, vacuolização das paredes das arteríolas e destruição da integridade das mesmas (Veiga et al., 2001; Ospedal et al., 2002). Após 48 horas, o infiltrado de leucócitos polimorfonucleares continua crescendo (Smith and Micks, 1970; Futrell, 1992). O quadro clínico descrito caracteriza o quadro cutâneo ou dermonecrótico.

O mecanismo pelo qual o veneno causa as lesões dermonecróticas não é totalmente conhecido. Os neutrófilos (polimorfonucleares) parecem ser a causa principal da necrose, porque uma depleção dos neutrófilos (usando nitrogênio mostarda, Smith and Micks, 1970) retarda os fenômenos de hemorragia, edema e necrose até que o "pool" de neutrófilos circulantes seja restabelecido (Smith and Micks, 1970). A participação do neutrófilo na dermonecrose está comprovada pelos achados histopatológicos em modelos animais (Elston et al., 2000; Ospedal et al., 2002) e em biópsias humanas (Yiannias and Winkelmann, 1992). A destruição tecidual está associada com a intensa reação inflamatória após o envenenamento. No entanto, o neutrófilo não é ativado diretamente pelo veneno, pelo contrário é inibido (Babcock et al., 1986; Majeski et al., 1977). O veneno ativa o endotélio vascular, a célula endotelial ativada seqüestra e ativa de maneira desregulada os neutrófilos circulantes (Berger et al.,1973; Geren et al.,1976; Patel et al., 1994). A presença da trombina e da histamina no local da picada induzem à expressão das P-selectinas, a partir dos corpos de Weibel –Palade, na superfície da célula endotelial. O neutrófilo possui receptores em sua membrana para as P-selectinas. Simultaneamente a célula endotelial sintetiza fator de ativação das plaquetas, o qual também é expresso na sua membrana citoplasmática e para o qual o neutrófilo também tem receptor. O neutrófilo torna-se ativado pela ação das P-selectinas e do fator de ativação plaquetário (Zimmerman et al., 1992; Bevilacqua and Nelson, 1993). Ocorre, também , uma expressão fraca de E-selectina pela célula endotelial, a qual está correlacionada com a ativação do neutrófilo (Zimmerman et al., 1992; Bevilacqua and Nelson, 1993). A célula endotelial ativada produz uma grande quantidade de interleucina-8 (IL- 8), a qual é um potente mediador da ativação e migração dos neutrófilos (Rot, 1992; Huber et al., 1991). O veneno da aranha

marrom também induz a uma produção bastante elevada, pela célula endotelial, de GM-CSF (fator estimulante de colônias de granulócitos e monócitos) (Borudy et al.,1986). A IL-8 e o GM-CSF estimulam a medula óssea a produzir uma quantidade maior de neutrófilos, a qual é proporcional a injúria tissular. A IL-8 e o GM-CSF são considerados fatores de crescimento para os neutrófilos porque regulam sua produção e maturação na medula óssea. Pacientes com mielossupressão são tratados com formas recombinantes (derivados de *E. coli*) de GM-CSF para estimular a produção e atividade de várias linhagens progenitoras na medula óssea (Vose and Armitage, 1995). O receptor para o GM-CSF (CD116) apresenta uma massa molecular de 75 a 85 kDa, está presente nos granulócitos, monócitos e seus precursores. Possui duas sub-unidades, α e β, ambos são membros da classe I da família de receptores de citocinas, possuem atividade de tirosinoquinase porque várias proteínas são fosforiladas quando ocorre a ligação do receptor ao GM-CSF (Lee et al., 1998). O receptor para interleucina-8 (IL-8) é o CD128, com massa molecular variando de 58 a 67 kDa e presente em neutrófilos, basófilos e linfócitos. A ligação da IL-8 ao seu receptor induz a quimiotaxia para neutrófilos, basófilos e linfócitos T. Ativa neutrófilos e basófilos aumentando sua adesão as células endoteliais (Lee et al., 1998). Os fatores de crescimento têm uma ação direta sobre a produção celular da medula óssea.

A medula óssea é um dos maiores orgãos do organismo humano e é o principal sítio da hematopoiese. Apresenta uma capacidade de produção celular impressionante. Um adulto normal produz cerca de 2,5 bilhões de eritrócitos, 2,5 bilhões de plaquetas e 1,0 bilhão de granulócitos por quilo de peso por dia (Testa and Molineux, 1993). A medula óssea que representa o tecido hematopoiético pode ser dividida em estroma e sinusóides, com uma circulação que permite que nutrientes cheguem a medula e que as células produzidas e maduras possam ganhar o sangue periférico. Uma artéria, denominada de nutriente, penetra a córtex óssea e ao longo desta córtex vai se ramificando, estas ramificações encontram-se com as ramificações de uma outra artéria, denominada artéria periόstea. A anastomose das ramificações formam capilares que dão origem aos

sinusóides da medula óssea, os quais, posteriormente vão desembocar num sinusóide central, que forma a veia emissária, que sai da medula óssea, levando células maduras e funcionais para a corrente circulatória. Entre os sinusóides formam-se os espaços hematopoiéticos, que é o estroma da medula óssea. As células que formam o estroma, as células endoteliais que revestem a camada íntima dos sinusóides, as células reticulares que revestem externamente os sinusóides, os osteoclastos, os osteoblastos e as células adiposas junto com as proteínas da matriz extracelular formam o microambiente da medula óssea. É neste microambiente que as células medulares encontram condições de proliferação e diferenciação. As células endoteliais representam a principal barreira no controle de substâncias químicas e partículas que entram ou deixam os sinusóides da medula óssea (Tavassoli and Shaklai, 1979). Elas expressam receptores para o fator de von Willebrand (Hasthorpe et al., 1992), colágeno tipo IV, laminina (Perkins and Fleischman, 1990) e duas moléculas de adesão; VCAM-1 (molécula de adesão celular vascular) e E-selectina (molécula de adesão leucocitária endotelial) entre outras (Schweitzer et al., 1996). As células reticulares formam a camada adventícia dos sinusóides, sintetizam fibras reticulares (argentofílicas) e seu citoplasma se estende pelos compartimentos hematopoiéticos formando uma malha na qual ficam as células hematopoiéticas (Beutler et al., 2001). Apresentam uma alta concentração de fosfatase alcalina, expressam CD10 e CD13 e antígenos HLA de classe I (Abboud et al., 1993). O CD10 é uma endopeptidase conhecida como metaloendopeptidase que modula a migração e adesão de neutrófilos. O CD13 é uma aminopeptidase que sinergiza a ação do CD10. Estas células regulam a migração de precursores hematopoiéticos para a circulação (Weiss and Geduldig, 1991). As células do estroma da medula óssea provavelmente são derivadas dos fibroblastos, elas tem um fenótipo único e características funcionais que as permitem participar do desenvolvimento das células hematopoiéticas em um microambiente altamente especializado (Torok-Storb, 1999).

A matriz extracelular que estruturalmente está dividida em membrana basal, matriz conectiva e matriz sangüínea caracteriza-se por ser uma estrutura

complexa formada por proteínas secretadas e glicoconjugados que interagindo tridimensionalmente originam uma rede molecular (Yurchenco and Schittny, 1990). A matriz extracelular interagindo funcionalmente com moléculas receptoras na superfície celular participa como suporte e na orientação dos processos biológicos onde existe adesão e locomoção celulares, coordenando importantes fenômenos fisiológicos no funcionamento homeostático dos tecidos (Albelda and Clayton, 1990; Meredith et al., 1993; Veiga et al., 1997). Na medula óssea a interação entre as células do estroma, as proteínas da matriz extracelular e as citocinas fazem a diferenciação das células tronco (células indiferenciadas que dão origem as células maduras e funcionais no sangue periférico) na linhagem linfóide (linfócitos T e B) e na linhagem mielóide (eritrócitos, plaquetas, monócitos/macrófagos e granulócitos). As principais moléculas da matriz extracelular encontradas na medula óssea são: proteoglicanos, fibronectinas, tenascinas, colágenos, lamininas, hemonectinas, trombospondinas e vitronectinas. O heparam sulfato proteoglicano, na medula óssea, em associação com a laminina e o colágeno do tipo IV tem um papel nas interações célula-célula, na apresentação de citocinas e na diferenciação celular (Ketaing and Gordon, 1988). Ele também media a ligação de células progenitoras ao estroma através da fibronectina (Minguell et al., 1992). A fibronectina está localizada nas interações entre as células do estroma da medula óssea e das células hematopoiéticas, exatamente nos sítios de desenvolvimento de granulócitos e monócitos (Sorrel, 1988). Ela está envolvida na adesão de células maduras como megacariócitos, mastócitos, linfócitos T, eosinófilos e neutrófilos. A tenascina media o sinal mitogênico para as células mononucleares da medula óssea (Seiffert et al., 1998). A laminina é o principal componente não colagênico da matriz extracelular e da membrana basal da medula óssea. Interage com o colágeno tipo IV, proteoglicanos e entactina e regula quimiotaxia leucocitária (Bryant et al., 1987). Progenitores de granulócitos CD34 +, monócitos maduros e neutrófilos se aderem à laminina. A hemonectina é uma glicoproteína que media a ligação dos granulócitos ao estroma da medula óssea (Campbell et al., 1987). A trombospondina foi inicialmente identificada nos grânulos α das plaquetas. Interage com o colágeno e fibronectina e participa da

ligação da célula tronco com o estroma da medula óssea (Long and Dixit, 1990). Ela é quimiotática para neutrófilos e monócitos (Mansfield and Suchard, 1994). A vitronectina é uma glicoproteína presente no plasma, nas plaquetas e no tecido conectivo. Sua principal contribuição na medula óssea é na diferenciação final dos megacariócitos e na formação de plaquetas (Beutler et al., 2001).

A medula óssea pode ser dividida em vários compartimentos celulares. O primeiro compartimento é o das células tronco, aderidas ao estroma da medula óssea, células com capacidade de autorenovação e que dão origem as células funcionais encontradas no sangue periférico. Estas células podem se comprometer com a diferenciação da linhagem linfóide ou mielóide. Quando comprometida com a linhagem linfóide dará origem aos linfócitos T e B. Quando está comprometida com a linhagem mielóide dá origem aos eritrócitos, as plaquetas, aos monócitos/macrófagos e aos granulócitos (eosinófilos, basófilos e neutrófilos) (Lee et al., 1998). O compartimento dos neutrófilos pode ser dividido em compartimento mitótico e pós-mitótico. O compartimento mitótico é formado pelos mieloblastos, promielócitos e mielócitos, células com capacidade de proliferação e autorenovação. O compartimento pós-mitótico é considerado um compartimento de reserva da medula óssea e é formado pelos metamielócitos, bastonetes e segmentados (a soma dos dois últimos representa os neutrófilos). Estas células não têm capacidade de divisão. De toda esta seqüência maturativa encontra-se no sangue periférico apenas os bastonetes (pequena quantidade) e os segmentados. Os neutrófilos do sangue periférico são divididos em dois sub-compartimentos: o sub-compartimento marginal e o circulante. A soma dos dois forma o compartimento periférico. Os neutrófilos marginados (sub-compartimento marginal) estão aderidos às células endoteliais e representam as células de reserva do compartimento periférico. Os neutrófilos são encontrados em grande quantidade circulando no sangue periférico (sub-compartimento circulante), uma outra quantidade de neutrófilos de igual tamanho da primeira encontra-se marginada e a medula óssea ainda dispõe de um compartimento de reserva. O neutrófilo representa a primeira linha de defesa do organismo humano contra processos inflamatórios e infecciosos e a medula óssea tem que estar preparada

para atender toda a demanda tecidual necessária (Lee et al., 1998). O veneno da aranha marrom representa uma injúria tissular caracterizada como um processo inflamatório agudo e grave. Este processo envolve as células do estroma, as células presentes nos sinusóides e as proteínas da matriz extracelular da medula óssea. As células do compartimento marginal migram para o local da lesão. Os neutrófilos ativados passam por entre as junções das células endoteliais e ganham o tecido injuriado, fazem a degranulação de seus grânulos primários e secundários caracterizando o quadro dermonecrótico que pode ou não evoluir para um quadro sistêmico. A migração ao tecido injuriado pode ser de tal magnitude que o neutrófilo destrói as células endoteliais e a membrana basal. Toda esta movimentação de células e consumo de plaquetas no sítio inflamatório deve alterar a celularidade da medula óssea de modo qualitativo e quantitativo. A literatura não descreve a ação do veneno da aranha marrom sobre as células da medula óssea.

No quadro clínico cutâneo-visceral ou sistêmico as primeiras manifestações surgem após 24 horas e os sintomas incluem astenia, febre, episódios eméticos, alterações sensoriais, cefaléia e insônia. Nos casos mais graves pode ocorrer alterações hematológicas incluíndo anemia hemolítica, trombocitopenia e coagulação intravascular disseminada, com conseqüente diminuição da concentração da hemoglobina e icterícia pelo aumento da bilirrubina indireta. Convulsões e coma também são descritas nos casos mais graves (Martinez-Vargas, 1987; Futrell, 1992; Bravo et al., 1993). A insuficiência renal aguda caracterizada por hemoglobinúria e hematúria e em casos extremos por obstrução da luz tubular, pode levar o paciente a óbito (Wasserman and Anderson, 1984; Jorge et al., 1991; Futrell, 1992). Alguns autores relatam que a insuficiência renal aguda é conseqüência das alterações hematológicas como a hemólise intravascular e a coagulação intravascular disseminada (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). Não se tem uma evidência experimental que confirme essa hipótese. O dano renal pode ser conseqüência de uma ação direta das toxinas do veneno sobre as estruturas renais, já demonstrado para vários tipos de veneno (Bjarnason and Fox, 1995; Veiga et al., 2000).

Josephine M. Futrell (Tulane University Medical School, Department of Dermatology, New Orleans, Louisiana, 1992) fez uma revisão sobre loxoscelismo mostrando a biologia da aranha marrom, a descrição do quadro clínico dermonecrótico e sistêmico, alterações histopatológicas do sítio inflamatório, o mecanismo da doença, o tratamento, a prevenção e os novos horizontes. Desde 1992 até os dias atuais muito conhecimento foi acumulado sobre o loxoscelismo, obteve-se toxinas de modo recombinante e produtos de biotecnologia e nenhum artigo juntando todo este conhecimento foi escrito.

2. OBJETIVOS

2.1. OBJETIVOS GERAIS

- Reunir os conhecimentos publicados sobre loxoscelismo nos últimos 10 anos e estudar as ações biológicas das toxinas do veneno da aranha marrom sobre os tecidos hematopoiético e renal.

2.2. OBJETIVOS ESPECÍFICOS

- Fazer um artigo de revisão mostrando o conhecimento acumulado sobre loxoscelismo de 1993 até 2003.

- Verificar a ação do veneno da aranha marrom (*Loxosceles intermedia*), em coelhos como modelo animal, sobre células da medula óssea e do sangue periférico,

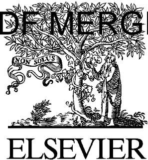
- Verificar o mecanismo de ação do veneno da aranha marrom (*Loxosceles intermedia*), em camundongos como modelo animal, na insuficiência renal aguda enfocando a participação hematológica na nefrotoxicidade.

3. RESULTADOS

Brown spiders and loxoscelism

Toxicon 44 (2004) 693-709

A-PDF MERGER DEMO



ELSEVIER

Toxicon 44 (2004) 693–709

TOXICON

www.elsevier.com/locate/toxicon

Review

Brown spiders and loxoscelism

Paulo Henrique da Silva[a], Rafael Bertoni da Silveira[a], Márcia Helena Appel[a], Oldemir Carlos Mangili[b], Waldemiro Gremski[a], Silvio Sanches Veiga[a,*]

[a]*Department of Cell Biology, Federal University of Paraná, Jardim das Américas 81531-990, Curitiba, Paraná, Brazil*
[b]*Department of Physiology, Federal University of Paraná, Paraná, Brazil*

Accepted 12 July 2004

Available online 1 October 2004

Abstract

Accidents caused by brown spiders (*Loxosceles* genus) are classically associated with dermonecrotic lesions and systemic manifestations including intravascular haemolysis, disseminated intravascular coagulation and acute renal failure. Systemic reactions occur in a minority of cases, but may be severe in some patients and occasionally fatal. The mechanisms by which *Loxosceles* venom exerts these noxious effects are currently under investigation. The venom contains several toxins, some of which have been well-characterised biochemically and biologically. The purpose of the present review is to describe some insights into loxoscelism obtained over the last ten years. The biology and epidemiology of the brown spider, the histopathology of envenomation and the immunogenicity of *Loxosceles* venom are reviewed, as are the clinical features, diagnosis and therapy of brown spider bites. The identification and characterisation of some toxins and the mechanism of induction of local and systemic lesions caused by brown spider venom are also discussed. Finally, the biotechnological application of some venom toxins are covered.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Loxoscelism; Brown spider; Venom

Contents

1. Introduction 694
2. Biology of the brown spider 694
3. Epidemiology of the brown spider 695
4. Histopathology and cellular changes after exposure to brown spider venom 696
5. Identification and characterisation of various toxins in brown spider venom 697
6. Mechanisms of local and systemic lesions caused by brown spider venom 700

* Corresponding author. Fax: +55-41-266-2042.
E-mail address: veigass@ufpr.br (S.S. Veiga).

0041-0101/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2004.07.012

7. Immunogenicity of Loxosceles spider venom . . . 700
8. Clinical features of brown spider bites . . . 701
9. Diagnosis of brown spider bites . . . 702
10. Therapy of brown spider bites . . . 703
11. Biotechnology of brown spider venom toxins. . . . 704
12. Future prospects . . . 705
Acknowledgements . . . 705
References . . . 705

1. Introduction

Bites from the *Loxosceles* genus (brown spiders) cause several clinical manifestations, especially necrotic skin degeneration and gravitational spread, renal failure and haematological disturbances (see Section 8). These spiders have a world-wide distribution and accidents have been described in America, Europe, Asia, Africa and Oceania (see Section 3). Brown spider venom has a highly complex composition, containing many different toxins. Some of these have been well-described biochemically and biologically, providing insights into the mechanism by which the venom causes its deleterious effects (for details, see Section 5). Numerous histopathological findings and host cellular changes triggered by the venom have been described in recent years and we now have good information about the cellular inflammatory response evoked after envenomation, the extent and time-dependency of reactions after envenomation and the effect of venom on endothelial cells, keratinocytes and tissue structures, such as basement membranes, as well as blood cellular alterations (discussed in Section 4). Clinical features, diagnosis and therapy have also been extensively studied in recent years. In the light of recent investigations into the effects of brown spider venom, some authors have reporting the design of immunologically based laboratory tests for the diagnosis of brown spider bite (see Section 9). Although little progress has been made in establishing an efficient protocol for therapy of loxoscelism in the past 10 years (see Section 10), biotechnology has been widely applied to the study and use of brown spider venom and the first products based on venoms or toxins have been described. Monoclonal antibodies and recombinant toxins are now available which may greatly enhance our knowledge of loxoscelism and perhaps provide some direct pharmaceutical applications (see Section 11). This review focuses on the last 10 years of literature data on loxoscelism.

2. Biology of the brown spider

Spiders of the genus *Loxosceles* (see Fig. 1) range from 8 to 15 mm in body length and their legs measure 8–30 mm. Their colour varies from a pale brown (*L. laeta*) to a dark chocolate (*L. gaucho*). A characteristic structure of this genus is the cephalothorax which resembles a violin (Futrell, 1992). These spiders can live for 3–7 years (Andrade et al., 2000). *Loxosceles* spiders have six eyes arranged in pairs with one anterior dyad and another lateral dyad on each side; this disposition of the eyes has been described as the best means of identifying the brown spider (Vetter and Visscher, 1998). These spiders are sedentary and nocturnal (Andrade et al., 1999). They build irregular webs that look like cotton thread (Futrell, 1992). The silk ribbons of *L. laeta* are organised in two sheets, a lower and an upper. The former is in contact with the substratum and the latter forms an exit hole for the spider and it is attached to the underside of objects. The sheets are composed of a meshwork of silk ribbons, which are anchored to surfaces by attached plaques derived from the pyriform glands. The silk ribbons are spun from glands which are homologous to the major ampullate gland of orb web spiders (Knight and Vollrath, 2002). All the ribbons in the web have the same width, suggesting that the web may consist entirely of this single type of silk ribbon structure. This ribbon morphology is unique among spiders (Coddington et al., 2001). The web glands are composed of a separate transverse zone, a duct and a spigot. The duct is short and the spigot is a highly elongated slit. The spinning mechanism in *Loxosceles* may be more ancestral than that in orb web spiders (Knight and Vollrath, 2002). The composition of the web silks of mature female *L. arizonica* and *L. laeta* is very similar and rich in glycine and alanine (Coddington et al., 2001). These spiders can withstand temperatures ranging from 8 to 43 °C and can survive for several days or even months without food and water (Futrell, 1992). Sandidge (2003) observed that *L. reclusa* prefers dead scavenged prey to live prey.

Fig. 1. Brown spider (*Loxosceles intermedia*) (magnification 2×).

According to the author, this may explain how immense populations of these spiders flourish even in adverse conditions. The brown spider is not aggressive, is retiring and prefers to live in dark areas (Futrell, 1992; Málaque et al., 2002). *L. intermedia* females produce more venom than the males and this may be related to their greater length and weight (see Fig. 1). Female venom causes a more severe dermonecrotic reaction in rabbits (Oliveira et al., 1999). *Loxosceles* venom is used to paralyse insect prey and also as a defence mechanism. The total venom volume is minute (about 4 μl) and contains 65 to 100 μg of proteins (Sams et al., 2001a). A 35 kDa dermonecrotic toxin named F35 protein (Tambourgi et al., 1995) is a toxic component of *L. intermedia* venom and starts to appear in its fully active form in third instar spiderlings, being absent in extracts of eggs or first and second instar spiderlings (Andrade et al., 1999).

The venom glands of *L. intermedia* are composed of two layers of striated muscle fibres, one external and the other internal in contact with an underlying structure that separates muscular cells from the epithelial cells of the venom glands, which lie internally on the secretory epithelium, a simple glandular epithelium. The epithelial cells are arranged side by side and send out projections to the lumen of the glands, which is rich in secretory vesicles containing venom. The morphology and cellular appearance of the venom glands suggest a holocrine mechanism controlling venom secretion. The basal lamina that separates the secretory epithelial cells of the glands from the muscular cells contains glycoproteins, glycosaminoglycan sulfated residues, laminin and entactin (Santos et al., 2000).

The genital female tract of *L. intermedia* contains a pair of elongated, sac-like ovaries, located in the ventral face of the abdomen among the digestive tract and silk glands. The mature oocyte reaches the uterus by travelling along the ovarian and oviduct lumen, rather than detaching from the pedicle, and is then liberated into the hemocoel. In *L. intermedia*, it is suggested that, after the oocyte enters the ovarian lumen, the pedicle cells are engulfed by, and appear to get lost among, the many folds of the retracting proteic band and the thinner basement membrane. Fertilisation may occur during the transit of the oocyte inside the uterus while it is covered by the viteline membrane and the granules of the future chorion. These coats permit sperm contact and probably act as a species-specific barrier. Oogenesis appears to be a continuous process in the mature *L. intermedia* female, and no regionalization of the ovary is seen (Morishita et al., 2003).

3. Epidemiology of the brown spider

Loxoscelism is a term used to represent accidents involving spiders of the genus *Loxosceles* and has been reported in South America (Ministry of Health, Brazil, 1998; Sezerino et al., 1998; Schenone, 1998), North America (Futrell, 1992; Escalante-Galindo et al., 1999), Europe (Nicholson and Graudins, 2003), Africa (Futrell, 1992; Nicholson and Graudins, 2003), the Middle East and some parts of Asia (Nicholson and Graudins, 2003), Israel (Borkkan et al., 1995; Cohen et al., 1999) and Australia (Young and Pincus, 2001; Nicholson and Graudins, 2003). In the USA, the range of *L. reclusa* extends from southeastern Nebraska to southernmost Ohio and south into Georgia and most of Texas; the spider is rarely found outside this range (Vetter and Bush, 2002a). *L. deserta* and *L. arizonica* inhabit Arizona, Nevada, New Mexico, Texas, Utah and Southern California. *L. rufescens* is found along the Gulf of Mexico coast (Sams et al., 2001a). Only *L. laeta* has been verified in Canada, being found in Vancouver, British Columbia and Ontario (Vetter and Bush, 2002a).

In Brazil, seven species have been reported, most of them in the South and South-east regions (Sezerino et al., 1998), but three (*L. intermedia*, *L. gaucho* and *L. laeta*) have

been mostly implicated in human envenomation (Málaque et al., 2002). *L. similes* has only been described in the State of Mato Grosso do Sul (Andrade et al., 2001). The brown spider is the only spider implicated in necrotizing-haemolytic syndrome (Sezerino et al., 1998). In the present decade, an extensive outbreak of *Loxosceles* envenomation and a high rate of intra-domiciliary infestation have been reported in Brazil (Ribeiro et al., 1993). Loxoscelism, in Brazil, was responsible for 36% of the 17,781 spider bites notified to the Ministry of Health between 1990 and 1993 (Sezerino et al., 1998). Loxoscelism is particularly prominent in the metropolitan area of Curitiba, Parana State, Brazil, with about 2000 cases per year (Málaque et al., 2002). *L. intermedia* is the predominant brown spider in the urban environment of Parana and Santa Catarina (southern states of Brazil) (Fischer, 1994; Mattosinho et al., 1997; Andrade et al., 2000). Accidents involving *Loxosceles* genus spiders represent around 4% of the total number caused by venomous animals in Argentina (Roodt et al., 2002a). Although the fertility of *L. laeta* is greater than that of *L. intermedia*, as the former has a greater total number of eggs and number per egg sac, it has been suggested that the expansion of *L. intermedia* (described above) may be due not to a great reproductive rate, but to environmental alterations in the South region of Brazil (Andrade et al., 2000).

In a retrospective study (Sezerino et al., 1998) carried out in Florianopolis, Santa Catarina State, Brazil between January 1985 and December 1995, 487 suspected cases were found, 267 of which fulfilled the criteria for inclusion in the study; 66.7% of these occurred during the warmest months of October–March and 80.5% occurred in the coastal region of the state, and 21.7% of the patients were getting dressed, 17.2% were sleeping and 15.4% were performing other activities inside the house. The average age of the patients was 25.1 years. Málaque et al. (2002) studied 359 cases of loxoscelism between January 1985 and December 1996 at the Butatan Institute, Sao Paulo, Brazil. The results are very similar to those described in the retrospective study of Sezerino et al. (1998), the difference being that 51 (14%) patients brought the spider with them and 28 were classified as *L. gaucho*, 5 as *L. laeta* and 18 as non-classified *Loxosceles*. In a restrospective study (1955–1995) of 1384 patients of probable spider bite or insect sting, Schenone (1996) reported that the main incidence of loxoscelism occurred in the warmest month. In 17.7% of cases, the spider was identified as *L. laeta*. In Curitiba, between 1993 and 2001, the incidence of brown spider bites was 1.4 cases per 1000 habitants and 23% of the bites were in the thigh, 16.7% in the trunk, 14% in the arm and 13% in the lower leg. Most of the cases were mild, only 1% being severe (data obtained from the Health Secretary, Curitiba, Parana, Brazil, 2002). Good housekeeping and care during dressing and before sleeping, especially when using bed clothes, can be effective in preventing accidents. Although spiders are arachnids and not insects, insecticides are effective in reducing the brown spider population, but re-infestation is a continuous problem and the toxic side effects for humans have to be considered (Forks, 2000).

4. Histopathology and cellular changes after exposure to brown spider venom

Clinical data for, and biopsies of, human patients after brown spider bites show an inflammatory infiltrate, thrombosis, haemorrhage, dermatitis, acute inflammation, induration of the lesioned region, erythema and liquefactive necrosis of the epidermis and dermis consistent with pyoderma gangrenosum (Futrell, 1992; Yiannias and Winkelmann, 1992). The histopathological findings described in experimental animal models using venom from different *Loxosceles* sp support the above mentioned pathological data. Histopathological findings of the effect of *L. intermedia* venom on rabbits were described by Ospedal et al. (2002). These authors described intravascular fibrin network deposition and thrombosis of the dermal blood vessels, degeneration of the blood vessel wall and infiltration and aggregation of inflammatory cells. The skeletal muscle was markedly infiltrated by neutrophil leukocytes and muscular oedema was present, with myonecrosis of some myofibrils. Destruction of the epidermis, a massive haemorrhage and necrosis of surrounding collagen near the epidermis were also described. Studying mice injected with *L. reclusa* venom, Sunderkötter et al. (2001) described local haemorrhage after 6 h accompanied by blistering of the ear skin. Histopathology showed a vasculitis reaction after 2 h of exposure. Histopathological findings 14 days after envenomation of rabbits with brown recluse spider venom were described by Elston et al. (2000). The major finding in the eschar of the inoculated rabbits was a mixed inflammatory cell infiltrate, coagulative tissue necrosis and vasculitis. All animals demonstrated a well-delineated zone of eosinophilic staining as 'mummified' coagulative necrosis of the epidermis and dermis, and a dense band of neutrophils bordered the zone of necrosis. The histological analysis of some organs from mice after envenomation with various doses of *L. intermedia* venom revealed remarkable alterations confined to the kidney. Acute tubular necrosis was seen in several nephrons, accompanied by deposition of eosinophilic material inside the proximal and distal renal tubules (Tambourgui et al., 1998a). Renal biopsies from *L. intermedia* venom-treated mice showed hyalinisation and erythrocytes in the Bowman's space, glomerular collapse, tubular epithelial cell cytotoxicity and deposition of eosinophilic material within the tubular lumen. Ultrastructural studies showed glomerular epithelial and endothelial cell cytotoxicity, alterations of the basement membrane and tubular epithelial cell degeneration (Luciano et al., 2004).

L. intermedia venom has a noxious effect on the endothelium of vessels, as shown by vessel instability, cytoplasmic endothelial cell vacuolisation and blebs in

biopsies of the skin of rabbits (Veiga et al., 2001a; Zanetti et al., 2002). This endothelial cell cytotoxicity was proved using rabbit aorta endothelial cells in culture, as *L. intermedia* venom treatment led to loss of cell adhesion to the culture substrate and the shedding of proteoglycans from the extracellular matrix and cell surface into the medium (Veiga et al., 2001a). Experiments using human umbilical vein endothelial cells (HUVEC) treated in culture with *L. reclusa* venom showed a potent endothelial cell agonist activity of the venom, which induced endothelial cell expression of E-selectin and the release of interleukin-8 and granulocyte macrophage colony-stimulating factor, resulting in a dysregulated inflammatory response (Patel et al., 1994). *L. deserta* venom induces the expression of vascular endothelial growth factor (VEGF) in human keratinocytes (Desai et al., 2000), suggesting that keratinocyte-derived VEGF may contribute to the vasodilation, oedema and erythema seen in brown spider envenomation. Exposure of HUVECs to *L. deserta* venom induces the production of interleukin-8, growth-related oncogene α and monocyte chemoattractant protein-1 via an NF-kB-dependent pathway (Desai et al., 1999; Gomez et al., 1999a). Primary cultures of human keratinocytes exposed to 100 ng/ml of *L. gaucho* venom release TNF-α into the medium after 6 h (Málaque et al., 1999).

Brown spider venom can disrupt basement membrane structures. This was demonstrated by the activity of *L. intermedia* venom on the murine tumour EHS (Engelbreth-Holm-Swarm) basement membrane, which was degraded and fragmented (Veiga et al., 2000a). The venom seems to have no activity on purified type IV collagen and laminin, but displays hydrolytic activity for entactin and heparan sulphate proteoglycan, two important constituents of basement membranes (Veiga et al., 2000a, 2001a,b). Double staining immunofluorescence using antibodies against type IV collagen or laminin and antibodies against venom toxins and confocal microscopy demonstrated the deposition and binding of venom toxins along the tubular and glomerular basement membrane of the kidney of mice exposed to *L. intermedia* venom (Luciano et al., 2004); ultrastructural analysis further demonstrated the collapse and destruction of glomerular basement membrane structures.

Cellular alterations in the bone marrow and peripheral blood caused by *L. intermedia* venom in rabbits have been studied (Silva et al., 2003). There were changes in the number of nucleated red cells, which initially showed a significant decrease, then recovered to normal values 10 days after venom administration. Marrow depression of megakaryocytes correlated with the thrombocytopenia in the peripheral blood observed at the beginning of envenomation, and the platelet count and number of megakaryocytes returned to normal after 10 days. Neutropenia in the peripheral blood, low neutrophil counts in the bone marrow and low leukocyte counts were consequences of marrow depletion, which may reflect an extensive neutrophil influx to the tissues. No changes were observed in eosinophil numbers. Fig. 2 depicts the histopathological findings evoked in rabbits by *L. intermedia* venom.

5. Identification and characterisation of various toxins in brown spider venom

Several enzymes, including alkaline phosphatase, 5′ ribonucleotide phosphohydrolase and hyaluronidase, have been identified in *L. reclusa* venom, but none of these produce necrotic lesions in experimental animals (Futrell, 1992). A biochemically well characterised component of *L. reclusa* venom is sphingomyelinase D (32 kDa), which, in laboratory animals, can produce necrotic lesions, haemolysis of red blood cells and platelet aggregation, resulting in death (Futrell,1992). Sphingomyelinase D exists in four active forms, each of which hydrolyses sphingomyelin and releases choline and *N*-acylsphingosine phosphate (Futrell, 1992).

L. gaucho, *L. laeta* and *L. intermedia* venoms have very similar SDS gel electrophoretic profiles. The major *L. laeta* bands are located between 32 and 30 kDa, while those in *L. gaucho* and *L. intermedia* are between 35 and 33 kDa (Barbaro et al., 1994a; Mota and Barbaro, 1995). Citrate is found in the venom of *L. reclusa* at a concentration of 1.5% based on dried venom solid (Fenton et al., 1995). The toxins of the venoms of *L. gaucho*, *L. laeta* and *L. intermedia* can be separated into three major fractions (A, B and C) and the dermonecrotic and lethal activities are found only in fraction A (higher molecular mass component) (Barbaro et., 1996b). The protein in fraction A has a molecular mass of about 35 kDa in *L. gaucho* and *L. intermedia* and 32 kDa in *L. laeta*. The high percentage identity of the N-terminal sequences of dermonecrotic toxins from *L. gaucho*, *L. intermedia* and *L. laeta* strongly suggests that they are similar proteins (Barbaro et al., 1996a, 1997). A 30 kDa fraction was purified from the venom of *L. gaucho* and two isoforms further characterised. These were called Loxnecrogin A (31.4 kDa) and Loxnecrogin B (31.6 kDa), both of which induce necrosis in rabbits, but the lesions are smaller than those produced by the total 30 kDa fraction (Cunha et al., 2003), showing that more than one protein is involved in the dermonecrotic mechanism. Loxnecrogin A shows a high similarity to recombinant LiD1 protein from *L. intermedia* (Kalapothakis et al., 2002; Cunha et al., 2003). Phospholipase D from *Loxosceles* venom resembles phospholipase D from *Corynebacterium*, both being basic proteins with similar isoelectric values and molecular masses (Bernheimer, 1996). Tambourgi et al. (1995) purified a fraction from *L. intermedia* venom named F35. This fraction has a molecular mass of 35 kDa on SDS-PAGE, but seems to be composed of three isoforms, which were named P1, P2 and P3. P1 and P2 have sphingomyelinase activity and can induce in vivo the effects seen with whole spider venom, including complement-dependent haemolysis and dermonecrosis, while P3 is completely inactive

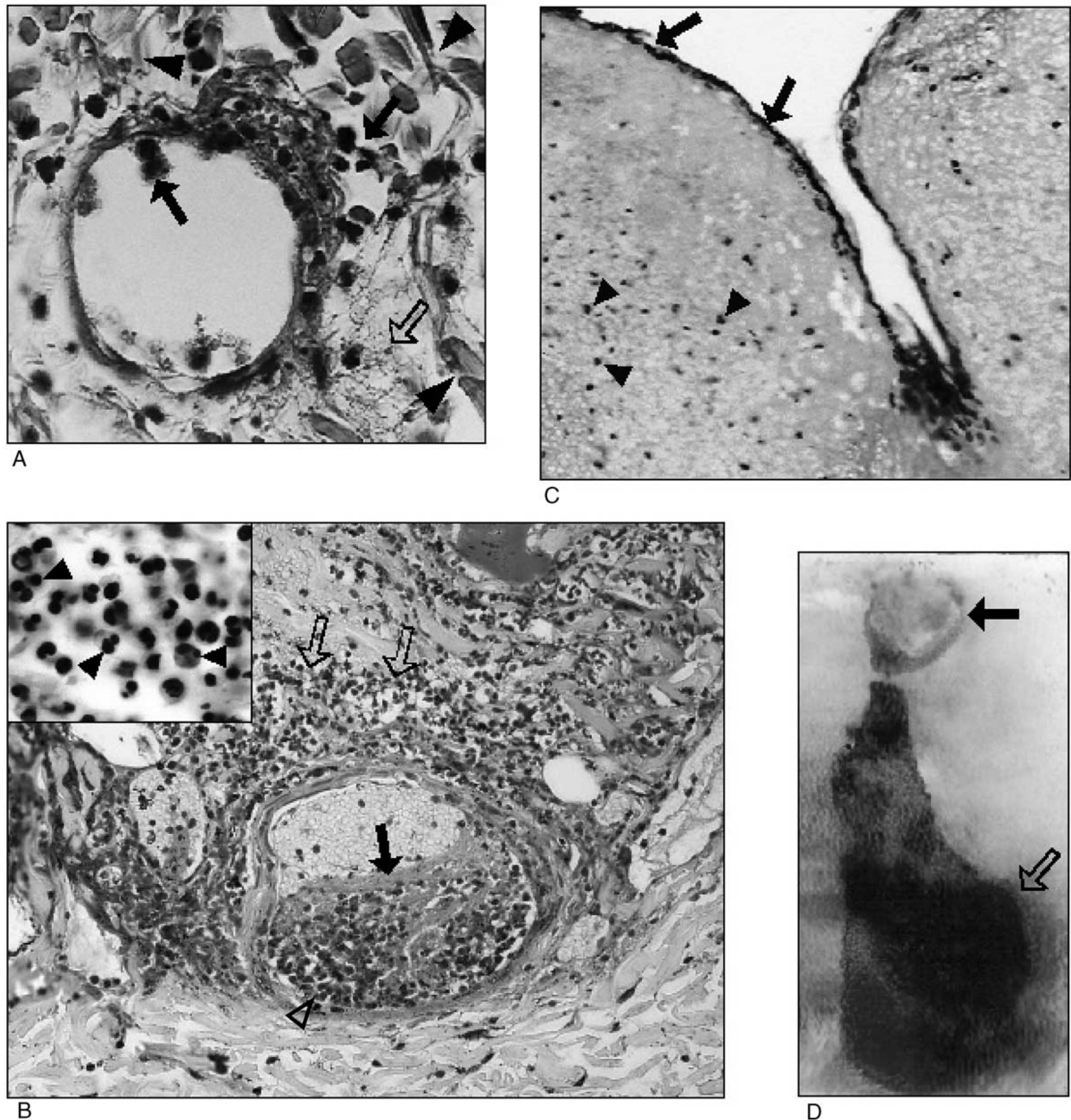


Fig. 2. Histopathological changes in the skin of rabbits after brown spider venom exposure. All panels show results from a rabbit injected with 40 μg of crude brown spider venom protein. (A) Blood vessel and connective tissue of the skin of a rabbit 30 min after exposure to brown spider venom. The black arrows indicate leukocytes interacting with endothelial cells of the blood vessel or infiltrating connective tissue. The white arrow indicates a proteinaceous network of fibrin in the dermis and the black arrowheads show profuse disorganisation of collagen fibres (magnification 1000×). (B) Details of a blood vessel of the skin of a rabbit 8 h after exposure to the venom showing inflammatory leukocytes accumulated in (white arrowhead) and around (white arrows) the vessel. The black arrow indicates intravascular clotting (magnification 200×). The inset shows details of the cellular inflammatory infiltrate represented by neutrophils (magnification 1000×). (C) Microscopic examination of the skin of a rabbit 2 days after brown spider envenomation. The black arrows indicate epidermal necrosis and the black arrowhead diffuse points of dermal necrosis (magnification 200×). (D) Macroscopic visualisation of dermonecrosis in the skin of a rabbit at 24 h after injection with venom. The black arrow indicates the site of venom injection and the white arrow gravitational spreading (hallmark of envenomation).

(Tambourgi et al., 1998b). A rapid assay for monitoring the lipolytic activity of brown recluse spider venom and toxin has been developed and the optimal reaction conditions determined (Merchant et al., 1998). These authors studied the venom- and toxin-mediated hydrolysis of yolk sphingomyelin in Triton X-100 or lysophosphotidylcholine micelles using phosphorous-31 nuclear magnetic resonance spectroscopy and found that an increased overall reaction rate

was observed not only with increased temperature, but also with decreased ionic strength. The presence of divalent calcium ions was found to be necessary for hydrolytic activity, but only in catalytic amounts (less than 1 mM).

Two metalloproteinases named Loxolysin A (20–28 kDa) and Loxolysin B (32–35 kDa) have been described in *L. intermedia* venom. The former has fibronectinolytic and fibrinogenolytic activity and the latter is gelatinolytic (Feitosa et al., 1998). The action of *L. intermedia* venom on fibrinogen shows a partial fibrinogenolytic activity on intact fibrinogen and a complete cleaving effect on the denatured molecule, supporting the idea of a conformation-dependent susceptibility of native fibrinogen. In the presence of 12.5 μg of venom, hydrolysis of the A α chains is first seen between 30 min and 1 h after venom exposure, with progressive proteolysis with time, whereas the B β chain begins to be cleaved after 4 h of venom treatment. This fibrinogenolytic activity is blocked by EDTA and 1,10-phenanthroline, suggesting that a divalent metal ion is critical for enzyme activity (Feitosa et al., 1998; Zanetti et al., 2002). This fibrinogenolytic effect and metalloprotease-dependent activity is seen in venom from *L. intermedia*, *L. laeta*, and *L. gaucho,* and a 30 kDa molecule with fibrinogenolytic activity has been purified from *L. intermedia* venom (Zanetti et al., 2002). The proteolytic activity of *L. intermedia* venom obtained directly from the venom glands was compared with that of venom obtained by electroshock (Silveira et al., 2002). A protein profile study of the two preparations showed that, in both cases, the venom is a mixture of proteins with a very similar electrophoretic profile, especially in the region of low molecular mass proteins (20–45 kDa), and that both showed the same fibronectinolytic and fibrinogenolytic activities. *L. intermedia* and *L. laeta* gland venom extracts have a similar level of metalloprotease-dependent proteolytic activity (gelatinolytic) (Silveira et al., 2002). *L. intermedia* venom shows basic aminopeptidase and prolyl-dipeptidyl aminopeptidase IV activities, analysed by fluorometric assay using naphthylamide substrate (Gasparello-Clemente and Silveira, 2002). Two 85 and 95 kDa serine proteases identified in *L. intermedia* venom have gelatinolytic activity in the pH range of 7.0–8.0, and act on casein to a lesser extent than on gelatin, but do not hydrolyse haemoglobin, immunoglobulin G, bovine serum albumin, laminin or fibrinogen (Veiga et al., 2000b). These proteases may be involved in the deleterious activities of the venom.

The oligosaccharide profile of *L. intermedia* venom shows asparagine-linked high mannose, complex type structures and N-linked fucosylated molecules. One serine/threonine-linked *N*-acetyl-galactosylated protein was detected. The venom contains no glycosaminoglycan, proteoglycan, galactose or sialic acid residues as complex structures (Veiga et al., 1999). Table 1 summarises the molecules described in the venom of *Loxosceles* sp. spiders.

Table 1
Molecules described in the venom of *Loxosceles sp.* spiders

Molecule	*Loxosceles* sp	Molecular mass	Activity	References
Alkaline phosphatase	*L. reclusa*	Not described	Not described	Futrell (1992)
Hyaluronidase	*L. rufescens*, *L. reclusa*	32.5 kDa	Digests hyaluronic acid	Futrell (1992) and Young and Pincus (2001)
5′ Ribonucleotide phosphohydrolase	*L. reclusa*	Not described	Not described	Futrell (1992)
Sphingomyelinase D	*L. reclusa*	30–32–35 kDa	Necrotic lesion	Futrell (1992) and Barbaro et al. (1994a, 1996a,b, 1997)
	L. rufescens		Platelet aggregation	
	L. gaucho, *L. laeta*		Haemolysis	Mota and Barbaro (1995)
	L. intermedia			Tambourgi et al. (1995)
Loxnecrogin A	*L. gaucho*	31.4 kDa	Necrosis in rabbits	Cunha et al. (2003)
Loxnecrogin B	*L. gaucho*	31.6 kDa	Necrosis in rabbits	Cunha et al. (2003)
LiD1 recombinant protein	*L. intermedia*	31.4 kDa	Sphingomyelinase D family without dermonecrotic activity, but with antigenic activity	Kalapothakis et al. (2002) and Cunha et al. (2003)
Loxolysin A	*L. intermedia*	20–28 kDa	Fibronectinolytic, fibrinogenolytic	Feitosa et al. (1998)
Loxolysin B	*L. intermedia*	32–35 kDa	Gelatinolytic	Feitosa et al. (1998)
Serine protease	*L. intermedia*	85 kDa	Gelatinolytic	Veiga et al. (2000b)
Proteases	*L. intermedia*	Not described	Hydrolysis of entactin, heparan sulphate proteoglycan and basement membrane	Veiga et al. (2000b, 2001a,b)
Metalloproteases	*L. rufescens*	Broad range	Caseinolytic, gelatinolytic, fibrinogenolytic	Young and Pincus (2001)

6. Mechanisms of local and systemic lesions caused by brown spider venom

The mechanisms by which brown spider venom causes local and systemic lesions are under investigation, but are currently unknown. In addition, there are no rational explanations why mice and rats do not develop dermonecrosis, while humans, guinea pigs and rabbits do. *L. deserta* venom causes a large necrotic lesion in swine and the size of the lesion is dose-dependent (Hobbs and Yealy, 1994). Some noxious activities of the venom can be attributed to proteolytic toxins, which degrade fibrinogen, fibronectin, entactin and heparan sulphate proteoglycan and disrupt basement membrane structures, resulting in local haemorrhage and gravitational spreading of the cutaneous lesions and systemic pathogenesis involving disseminated intravascular coagulation and renal failure (Feitosa et al., 1998; Veiga et al., 1999, 2000b, 2001a,b; Luciano et al., 2004). There is considerable evidence for the role of neutrophils in the dermonecrosis caused by the venom of *Loxosceles* spiders (for details, see Smith and Micks, 1970; Futrell, 1992) and this was proved by histopathological findings in animal models (Elston et al., 2000; Ospedal et al., 2002) and biopsies of human patients (Yiannias and Winkelmann, 1992). Neutrophil participation and the inflammatory response seem to be dependent on an endothelial cell agonist effect triggered by the venom which leads to an indirect and dysregulated neutrophil activation involved in dermonecrosis (Patel et al., 1994). The venoms from *L. gaucho*, *L. laeta* and *L. intermedia* have similar dermonecrotic activity (Barbaro et al., 1996a); in mice, the most lethal is that from *L. intermedia* and the least toxic that from *L. laeta*. The venoms of these three species have very low levels of caseinolytic and phospholipase A_2 activities even when large amounts are used, and have low myotoxicity (mouse model) and no direct haemolytic activity. *L. gaucho* venom causes increased degradation of red blood cell ghost protein 3 in patients with either the cutaneous or viscerocutaneous forms (Barretto et al., 2003); this is not inhibited by *N*-ethylmaleimide (a cysteine protease inhibitor) or phenylmethylsulphonyl fluoride (a serine protease inhibitor), but is inhibited by ethylenediaminetetraacetic acid (a metalloprotease inhibitor).

Bravo et al. (1993) concluded that *L. laeta* venom has a direct haemolytic action on red blood cells which is calcium- and complement-dependent, but antibody-independent. Tambourgi et al. (2000) studied the mechanism of induction of complement-dependent haemolysis by *L. intermedia* venom and proposed that the sphingomyelinase activity of the toxins induces the activation of an endogenous metalloproteinase, which then cleaves glycophorins, making the erythrocytes susceptible to lysis by human complement. They also compared the phospholipase activities of *Corynebacterium pseudotuberculosis* and *L. intermedia* venom and found that they have similar phospholipase D activity and that the purified phospholipase D induced similar clinical symptoms to those induced by envenomation (Tambourgui et al., 2002). Using the ECV304 cell line, van den Berg et al. (2002) showed that *L. intermedia* venom toxins induce cleavage of the complement regulator membrane co-factor protein (MCP/CD46) and the major histocompatibility complex I molecule from the cell surface by activation of a metalloproteinase of the adamalysin family and that this reduced MCP expression, resulting in an increased resistance to complement-mediated lysis.

Monteiro et al. (2002) isolated and identified many different bacteria, including *Clostridium perfringens*, from the fangs and venom of *L. intermedia* and compared the dermonecrotic lesions produced by venom alone and venom plus *C. perfringens*. The results showed that the combination resulted in a striking increase in the size of the dermonecrotic lesion, suggesting a role for *C. perfringens* in the severe dermonecrotic picture.

7. Immunogenicity of Loxosceles spider venom

Loxosceles venom is a mixture of different proteins. *L. intermedia* venom is enriched in proteins with low molecular masses in the range of 5–40 kDa and, to a lesser extent, proteins of high molecular mass (60–850 kDa), (Veiga et al., 2000b). The venom of *L. gaucho* is enriched in low molecular mass proteins (Barbaro et al., 1992). Because of its proteic nature, *Loxosceles* venom can induce antibody production. A characterisation of the antigenic cross-reactivity of *L. reclusa* and *L. deserta* venoms suggested that the venoms contain similar proteins, which show greater than 90% amino acid sequence identity and marked antigenic cross-reactivity (Gomez et al., 2001a). Antibodies in antisera raised against *L. gaucho* toxins in rabbits and horses also bound to *L. laeta* and *L. intermedia* toxins (Barbaro et al., 1996a), suggesting some antigenic conservation in these three venoms; the electrophoretic profile and immunoblotting of the proteins from these three venoms showed that almost all venom proteins, including the main 33–35 kDa component of *L. gaucho* venom responsible for the dermonecrotic and lethal activities, were recognised by the antisera. When the venom of each species of *Loxosceles* was incubated with these antisera, the dermonecrotic and lethal activities of each venom was completely neutralised (Barbaro et al., 1994a, 1996a).

Monoclonal antibodies produced against the 35 kDa dermonecrotic component of *L. gaucho* neutralised the dermonecrotic and lethal activities of the whole venom. The results of this study showed low cross-reactivity with heterologous toxins and that the antibodies failed to neutralise the toxic activities of *L. laeta* and *L. intermedia* venom, suggesting that different epitopes are present in the main proteins responsible for the toxic activity of *Loxosceles* venom (Guilherme et al., 2001). *L. gaucho* venom has an immunological adjuvant effect in rabbits which is

associated with high molecular mass components, which trigger inflammatory activity (Barbaro et al., 1994b; Mota and Barbaro, 1995).

The horse anti-arachnid antiserum from the Butantan Institute (Sao Paulo, Brazil), which is raised against a mixture of venoms from *Loxosceles gaucho*, *Phoneutria nigriventer*, *Tityus serrulatus* and *T. bahiensis*, did not inhibit human red blood cell haemolysis by *L. laeta* venom in vitro, and inhibition of the development of a skin necrotic lesion in rabbits was time-dependent (Bravo et al., 1994). On the other hand, Fab fragments of polyclonal antibodies raised in rabbits using *L. deserta* venom have been used therapeutically and injection within 4 h after envenomation inhibits dermal inflammation and necrosis in experimental animal models (Gomez et al., 1999b). A specific horse anti-*L. intermedia* antivenom was produced and compared with the horse polyvalent anti-arachnid antiserum from the Butantan Institute in order to evaluate their ability to block the development of the dermonecrotic lesion and the lethal activities of the venom; both kinds of antivenom were efficient in neutralising these actions, but the anti-*Loxosceles* antivenom was more efficient (Braz et al., 1999). In Argentina, a monovalent horse anti-*L. laeta* antiserum raised against spider venom gland homogenates was developed and its ability to neutralise both the toxic effect in mice and dermonecrotic activity in rabbits was at least as high as that of the anti-arachnid antivenom from the Butantan Institute (Brazil) or the anti-*Loxosceles* serum from the National Institute of Health (Peru) (Roodt et al., 2002b).

8. Clinical features of brown spider bites

Brown spider bites give rise to a classical well known picture of necrotic skin lesions with gravitational spreading that is the hallmark of envenomation. Futrell (1992) gave a clinical description of the bite of spiders of the genus *Loxosceles*, stating that the initial bite is relatively painless and the patient is often unaware that he has been bitten. Pain, varying from mild to severe, begins at 2–8 h. There may be small puncta and transient erythema with itching, swelling and mild to severe tenderness. This is followed by the appearance of a bleb or blister (12–24 h) surrounded by a halo of ischemic tissue and the bleb may become haemorrhagic. Over the following few days, the necrotised lesions become a fixed dull blue-violet colour, with a characteristic gravitational spread and, consequently, the blue area increases. An eschar may form at this time, roughly between 3 and 7 days. Over the following week, the area becomes indurated and the central area hardens. The eschar may drop off, leaving an ulcer that heals after varying lengths of time (6–8 weeks), but which sometimes requires skin grafting. Similar descriptions were made by Schenone (1996), Sezerino et al. (1998) and Málaque et al. (2002). Systemic involvement is much less common than cutaneous involvement and occurs in a minority of cases. It is generally mild and self-limited, although, in some cases, it may also be the cause of death, usually associated with renal failure, disseminated intravascular coagulation and intravascular haemolysis (Futrell, 1992; Sezerino et al., 1998). Brown spider venom manifestations vary from mild erythema to extensive necrosis and are influenced by the victim's health, degree of obesity, location of the bite and other factors (White et al., 1995; Barbaro et al., 1996a; Sams and King, 1999; Sams et al., 2001b; Vetter and Bush, 2002a). While erythema, pain and macule at the bite site are the most common local symptoms of a *L. reclusa* spider bite, fever is the most common systemic symptom. Bites on the extremities are more common and more severe (Clowers, 1996). The extension of the dermal inflammation seen in the necrotic arachnidism induced by *L. reclusa* venom is proportional to the amount of venom in the bite site. *Loxosceles* venom induces a inflammatory mechanism which is indirect, so venom diffusion defines the extent and magnitude of the dermonecrotic lesion (Gomez et al., 2001b). Cutaneous loxoscelism with an oedematous predominance occurs in about 4% of loxoscelism cases involving *L. laeta* (Schenone, 1998). Severe systemic loxoscelism occurs in far fewer than 1% of cases of focal necrosis of the skin induced by *L. reclusa* venom (Anderson, 1998). Brown spider bites can have nephrotoxic effects. The clinical and laboratory features observed in victims can include haemoglobinuria, haematuria and proteinuria (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). The pathological effect of the venom on the kidney may reflect haematological disturbances, such as intravascular haemolysis and disseminated intravascular coagulation, which are systemic manifestations of envenomation (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). Nevertheless, in mice exposed to *L. intermedia* venom, Luciano et al. (2004) were able to show direct binding of venom toxins to renal structures, suggesting that *Loxosceles* venom toxins act as direct and potentially nephrotoxic agents. Because of their biochemical and physicochemical properties, such as a positive charge and low molecular mass size, venom toxins can act as 'planted antigens' along the kidney structures, evoking nephrotoxicity.

A retrospective study was carried out on 111 patients in the Vanderbilt University Hospital (Nashville, Tennesee, USA) between May 1993 and October 1995 (Wright et al., 1997). The inclusion criteria for this study were the presence of a lesion consistent with brown spider bite and the identification of the spider or the patient reporting seeing a spider. Eighty-one percent of wounds involving the leg showed central discoloration at the bite site and 37% showed necrosis. Skin grafting in patients with necrotic lesion was rarely required. Sixteen of the patients (14%) complained of feeling ill and six (5%) were admitted to the hospital. Most patients did not have severe systemic involvement. Haemolytic anaemia was seen in one patient

and haemolysis and coagulopathy in another (Wright et al., 1997). In an observational retrospective study of 11 paediatric patients (Escalante-Galindo et al., 1999), there was a relationship between lesion severity and the time delay before the beginning of treatment. As reported by Cacy and Mold (1999), in the period between April 1996 and August 1998 in the Oklahoma Physicians Research Network (Oklahoma, USA), loxoscelism (*L. reclusa*) was more common in women (18–65 years), pain was a common sign and most of the bites were in the extremities. Erythema was always present, 40% of the patients developed necrosis, and some systemic signs and symptoms, such as nausea (7%), rash (5%), fever and fatigue (3%), were also described. A retrospective study was carried out in Israel between 1988 and 1997 on 11 patients hospitalised after *Loxosceles* spider bites (Cohen et al., 1999). All patients presented dermonecrotic lesions with haemorrhagic bulla and necrotic ulcers, six showed systemic involvement with an elevated body temperature, diarrhoea (only one patient), vomiting, hypotension, and a diffuse petechial rash. Three patients presented inguinal lymphadenitis. Sams et al. (2001b) carried out a retrospective study between 1987 and 1993 on 19 documented cases diagnosed as brown recluse spider envenomation. The extremities were most often affected and the main symptoms were pain and localised erythema at the bite site. Eleven patients (58%) had skin necrosis and six had areas of necrosis larger than 1 cm^2. The time to healing ranged from 5 days to more than 17 weeks.

In order to investigate intravascular haemolysis, Morena et al. (1994) performed a haptoglobin assay on 19 patients with the cutaneous form of loxoscelism and found no decrease in haptoglobin, demonstrating that these patients did not show haemolysis. Haemolytic anaemia was described in a 12-year-old female after a *L. reclusa* bite (Murray and Seger, 1994). The patient presented fever, headache, nausea and vomiting, and maculopapular rash and erythema were seen at the bite site. Haemoglobin levels were decreased and the haematocrit was 24%, indicating haemolysis as a systemic reaction to the venom. A 38-year-old man was bitten on his left upper extremity by a *L. reclusa* and presented a thrombocytopenia (17,000 platelets/μl that reverted in 4 days (110,000/μl) (Young, 1994). Two cases of *L. reclusa* loxoscelism were described by Williams et al. (1995); one was associated with extensive tissue necrosis, severe intravascular haemolysis and fulminant disseminated intravascular coagulation and the other with severe haemolysis and a positive direct antiglobulin test for IgG and complement. Leung and Davis (1995) described another case of systemic envenomation with haemolysis following a *L. reclusa* spider bite. Goto et al. (1996) described a 7-year-old boy with progressive cervical soft tissue swelling, necrosis and airway compromise after a spider bite in the neck; the spider was in his bed and was identified as *L. reclusa*. Bey et al. (1997) described a 13-year-old girl who developed shock following *L. arizonica* envenomation; the spider was identified by an entomologist as a female *L. arizonica*. The patient developed a cutaneous lesion and, after 15 h, the clinical signs were hypotension, tachycardia and delayed capillary refill. Yosef et al. (1998) described a 28-year-old man with a mutation in factor V (Factor V Leiden) and in the gene coding for methyl tetrahydrofolate reductase, both predisposing the patient to thrombogenic events, who was bitten by a brown spider and presented an extensive necrotic lesion, suggesting that these genetic mutations may be predisposing factors for severe necrosis induced by loxoscelism, but more research is required to confirm this. Lung and Mallory (2000) described a 7-year-old boy who presented systemic symptoms of viscerouscutaneous loxoscelism with no evidence of haemolysis, but with haematuria in the first 24 h, and who showed a high anti-streptolysin-O title and decreased C3 levels. Jarvis et al. (2000) described an 8-year-old girl who was bitten in the left eye by *L. reclusa*, resulting in periorbital erythema and oedema. The treatment included canthotomy and cantholysis and administration of methylprednisolone, dexamethasone, dapsone, erythromycin ointment, cefazolin and hyperbaric oxygen (HBO). A 17-year-old woman and a 4-year-old boy were admitted to the Hospital Vital Brazil and Hospital das Clinicas (Sao Paulo, Brazil) after *Loxosceles* bites (França et al., 2002). Both presented viscerocutaneous loxoscelism with severe oedema, erythema and dermonecrosis at the bite site, rhabdomyolisis and acute renal failure with elevated creatine kinase levels. A 3-week-old infant developed a sudden onset jaundice and unexplained haemolysis in the absence of the classically described necrotic cutaneous lesion probably caused by an *L. reclusa* bite (Hostetler et al., 2003), illustrating the importance of considering loxoscelism in the differential diagnosis of massive haemolysis, particularly in endemic areas.

9. Diagnosis of brown spider bites

The diagnosis of loxoscelism is rarely based on the identification of the spider, as the patients do not bring the spider, but on epidemiological and historical findings or clinical signs and symptoms (Wright et al., 1997; Vetter, 1999; Málaque et al., 2002) and the exclusion of other aetiologies (Sezerino et al., 1998). The diagnosis of recluse spider bites purely on the basis of clinical examination is always difficult and speculative. The diagnosis should be performed taking into account the natural geographic distribution and epidemiology of brown recluse spiders in North America. Brown recluse spider bites have been misdiagnosed because several of these medical reports have originated from regions of non-endemicity (Vetter, 1999; 2000; Nishioka, 2001; Vetter and Barger, 2002; Vetter and Bush, 2002a,b; Vetter et al., 2003). *Erythema migrans* is a lesion characteristic of Lyme disease (localised infection) and is very similar to the lesion of necrotic arachnidism,

and may mimic the systemic symptoms of loxoscelism. Osterhoudt et al. (2002) reported that a 9-year-old boy with Lyme disease was misdiagnosed as having been bitten by a brown recluse spider. Lyme disease may be considered as a differential diagnosis principally in regions in which *Ixodes* spp ticks are highly prevalent. Vetter and Bush (2002c) described a case of chemical burn misdiagnosed as a brown recluse spider bite. The patient was from Nevada City, California, where there are no populations of brown recluse spiders.

Nevertheless, based on basic research and histopathological findings from animal models and from clinical data, which present a typical profile of inflammatory cells (Futrell, 1992; Yiannias and Winkelmann, 1992; Ospedal et al., 2002), in case of doubt, physicians requiring a confirmatory diagnosis can use biopsies from lesioned skin. Barrett et al. (1993) developed a passive haemagglutination inhibition test specifically diagnosing *L. reclusa* envenomation using a collection of exudates taken from skin lesions of guinea pigs. According to the authors, the test sensitivity was 90% as late as 3 days after venom injection and the specificity was 100% compared to other spider species. An ELISA sandwich test for the detection of *Loxosceles* sp venom proteins has been developed, which, in addition to identifying mice injected with *L. intermedia*, *L. gaucho* or *L. laeta* venom, can also detect circulating antigens in the sera of patients envenomed by *L. intermedia* (Chávez-Olórtegui et al., 1998). Miller et al. (2000) reported a novel *Loxosceles*-specific competitive enzyme immunosorbent assay using a rabbit anti-*Loxosceles* venom polyclonal antiserum and used it to examine a dermal biopsy and hairs plucked from a skin lesion of a patient bitten by *L. deserta*. Using this technique, the authors reported that the venom was detected in the analysed samples for up 4 days after envenomation. A similar ELISA method has been used for the detection of *Loxosceles* species venom. The authors postulated that it may have clinical applications in the detection of *Loxosceles* venom in hair, aspirate and biopsy specimens (Gomez et al., 2002; Krywko and Gomez, 2002). Although these laboratory tests have potential for clinical application, they require more clinical correlation and statistical analysis and unfortunately are not commercially available.

10. Therapy of brown spider bites

A variety of treatments have been described for loxoscelism. Phentolamine, heparin and other substances, such as topical nitro-glycerine, cyproheptadine and HBO, have been used for therapy, but research on these therapies is inconclusive and their use is not recommended (Futrell, 1992; Wendell, 2003). The established therapy is dapsone, acetylsalicylic acid, antibiotics (erythromycin and cephalosporins), ice and elevation, avoidance of strenuous activity and heat and, when necessary, surgery. Early surgical excision has not been shown to be of benefit and, in most cases, delays healing (Futrell, 1992; Merigian and Blaho, 1996; Goddard, 1998). In Curitiba, Brazil, in which there were 3400 cases of brown spider bites between 1995 and 2001, prednisone, dapsone and antiserum therapy are used. Anti-*Loxosceles* antiserum is only administered in severe cases of viscerocutaneous loxoscelism. When the spider is identified as *L. intermedia* or *L. laeta*, prednisone treatment must be initiated (even in cases with no lesion), the patient must be monitored during the first 36 h and serum therapy may be used if the patient develops the severe form of envenomation. For severe cutaneous loxoscelism, dapsone in association with antiserum is indicated (data obtained from the Health Secretary, Curitiba, Parana, Brazil, 2002), but there is little evidence to support the effectiveness of anti-*Loxosceles* antivenom, especially against local effects. Systemic envenomation studies in animals and humans have demonstrated that the antivenom neutralises the deleterious effects of the venom. In countries in which the antivenom was introduced, paediatric mortality has been reduced (Isbister et al., 2003). The effectiveness of *Loxosceles* antivenom in the treatment of necrotic arachnidism resulting from the bite of recluse spiders is less clear, mainly due to the late presentation of victims (Nicholson and Graudins, 2003). The effectiveness seems to be time-dependent. Experimentally, anti-*Loxosceles* venom antibodies used within 4 h reduce *Loxosceles*-induced dermonecrotic lesions in rabbits (Gomez et al., 1999). Barrett et al. (1994) concluded that dapsone therapy is more effective than either electric shock or no therapy for *L. reclusa* envenomation in guinea pigs. An eyelid dermonecrotic lesion model was developed in rabbits using *L. reclusa* venom, the severity of the lesion being venom dose-dependent (Cole et al., 1995). When the rabbits were divided into three groups, which were treated with steroids, dapsone or antivenom, the best response was seen with dapsone or antivenom, with a decrease in erythema and necrosis. When animals were given combined treatment of steroids plus dapsone, steroids plus antivenom and dapsone plus antivenom, the dapsone plus antivenom group showed the best response, with a marked reduction in erythema and necrosis. Dapsone therapy is recommended by the Kentucky Regional Poison Center (KRPC, Louisville, Kentucky, USA) for all suspected cases of brown recluse spider envenomation. A retrospective study was conducted at the KRPC in 1994 and 38 patients with proven brown recluse spider envenomation were identified (Roos et al., 1995). In the 31 patients treated with dapsone, no haemolytic anaemia or methaemoglobinemia was reported and no complications of dapsone were identified. According to the authors, dapsone administration may decrease the need for surgical wound excision. Escalante-Galindo et al. (1999) reported that, in 11 paediatric patients, treatment with

dapsone and/or paracetamol and dicloxacilin was successful; one patient required surgical treatment, but no systemic loxoscelism occurred. Dapsone can cause a dose-dependent haemolytic anaemia. Rapid or severe haemolysis is not seen with dapsone unless the patient is deficient in glucose-6-phosphate dehydrogenase (G6PD), when a rapid and severe haemolysis is seen during the first week of therapy (Murray and Seger, 1994). Colchicine is another putative medicine currently being studied because of the potential for haemolysis with dapsone, especially in patients with G6PD deficiency (Elston, 2002), but there are no clinical data supporting its use in loxoscelism and more studies are required.

If the signs of the viscerouscutaneous form do not appear within 24 h, cutaneous loxoscelism may be the diagnosis and may have a good prognosis. Patients with symptoms of viscerocutaneous loxoscelism have a good prognosis if they survive for 48 h and are treated with antihistamines and corticosteroids for cutaneous loxoscelism and with corticosteroids for viscerocutaneous loxoscelism (Schenone, 2003). HBO therapy is controversial for loxoscelism, some studies reporting it to be effective, while others do not. Phillips et al. (1995) concluded that HBO did not decrease the severity of rabbit skin lesions resulting from *L. deserta* envenomation, and Hobbs et al. (1996) and Hobbs (1997) reached the same conclusion using swine and *L. reclusa* envenomation. Merchant et al. (1997) examined the effect of high pressure oxygen on the catalytic action of *L. reclusa* venom on sphingomyelin and concluded that the venom components or toxins responsible for sphingomyelinase D activity were not affected by HBO therapy under the test conditions. Beilman et al. (1994) used guinea pigs exposed to *L. deserta* venom and concluded that HBO resulted in decreased lesion sizes when compared to control and dapsone-pretreated animals. Similar results were described when the venom of *L. reclusa* was injected into rabbits and the skin lesions treated with HBO within 48 h; HBO therapy reduced skin necrosis and the wound was significantly smaller (Maynor et al., 1997). A 19-year-old man who was bitten on the glans penis by a brown spider brought the spider with him to the William Beaumont Army Medical Center, Houston (Broughton, 1996). The patient received intravenous diphenhydramine, methylprednisolone, calcium gluconate and famotidine, and oral dapsone treatment was begun immediately. After 24 h, HBO treatment was given twice daily for 5 days. Skin necrosis was avoided and no surgical intervention was required.

Topical nitro-glycerine is reported to prevent skin necrosis from brown recluse spider bites. Lowry et al. (2001) tested this hypothesis and concluded that it did not prevent skin necrosis and could increase inflammation and serum creatine phosphokinase levels. The results of their study did not support the use of topical nitro-glycerine in the treatment of *L. reclusa* envenomation and suggested that systemic toxicity could be increased.

11. Biotechnology of brown spider venom toxins.

McGlasson et al. (1993) have described a product named ARACHnase (Hemostasis Diagnostics International Co., Denver, CO, USA). This is normal plasma containing *L. reclusa* venom which mimics the presence of a lupus anticoagulant and may provide a positive control for lupus anticoagulant testing. The Butantan Institute, Sao Paulo, Brazil, produces anti-arachnid antiserum using a mixture of venoms from *L. gaucho*, *P. nigriventer*, *T. serrulatus*, and *T. bahiensis,* which also reacts with *L. intermedia*, and *L. laeta* toxins. The CPPI (Production Center of Immunobiologic Products, Parana, Brazil) has also produced an anti-*Loxosceles* antiserum using *L. intermedia* venom which is able to neutralise some noxious activities of *Loxosceles* venom. The National Institute of Health (Peru) produces anti-*Loxosceles* antiserum against *L. laeta* venom. All of these antisera have been used as bioproducts for serum therapy (Barbaro et al., 1994a, 1996a; Roodt et al., 2002; Health Secretary, Curitiba, Parana, Brazil, 2002). Guilherme et al. (2001) produced monoclonal antibodies against the 35 kDa dermonecrotic toxin of *L. gaucho* which neutralise the dermonecrotic effect and lethal activities of *L. gaucho*, but not those of a heterologous venom. M.H. Appel and O.M. Chaim (personnel communication), working at the Federal University of Parana, have prepared more than 30 hybridomas for the production of monoclonal antibodies to different *L. intermedia* toxins. Monoclonal antibodies against brown spider venom toxins are not only useful tools for clinical applications, but can also be used as reagents for the affinity purification or affinity screening of venom toxins, which may greatly enhance our knowledge of loxoscelism over the next few years. Molecular biological approaches have also been used to study and analyse brown spider venom toxins. Using recombinant venom proteins, scientists can generate enough material for research, diagnosis and treatment. The study of toxins is particularly difficult, as the amounts of venom collected are often limited. In this respect, the cloning and expression of recombinant toxins should be a very useful tool for better structural and functional studies on the mechanisms of loxoscelism. Recent studies reporting the cloning of venom toxins also offer potential biotechnological tools. Pedrosa et al. (2002) reported the cloning and expression of a functional dermonecrotic and complement-dependent haemolytic factor obtained from a cDNA library of *L. laeta* spider venom glands. By sequencing of aleatory clones obtained from the cDNA library, they identified at least three containing different inserts coding for proteins with a high similarity to known N-terminal sequences of *L. intermedia* sphingomyelinases.The entire sequences of the cDNAs for these three clones have been obtained and deposited at GenBank under Accession numbers AY 093599, AY093600 and AY093601. The longest cDNA cloned was used for expression of a mature *L. laeta* protein in a bacterial system. The recombinant toxin was expressed

as a fusion protein of approximately 32 kDa with a 6× His-tag at the N-terminus. The recombinant *L. laeta* protein exhibits similar biological properties to whole venom or purified sphingomyelinases, but has a decreased ability to bind to the erythrocyte surface. Kalapothakis et al. (2002) reported the cloning and expression of what they called LiD1, a protein present in the venom of *L. intermedia*. LiD1 was cloned from an expression cDNA library of *L. intermedia* spider venom glands and the partial cDNA sequence is available at GenBank under Accession no. AY340702. The deduced amino acid sequence reveals a mature protein belonging to a family of proteins with dermonecrotic activity. The aim of the study was to generate a fused nontoxic recombinant protein to be used for the production of an *L. intermedia*-specific antiserum. LiD1 was expressed as a β-galactosidase fusion protein, which was recognised by anti-dermonecrotic protein antibodies and was able to generate antibodies against native dermonecrotic proteins present in *L. intermedia* venom. This LiD1 recombinant protein was used in a study (Araujo et al., 2003) in which it was used to pre-immunise rabbits and mice and was found to neutralise the in vivo lethal and dermonecrotic effects of *L. intermedia* whole venom. The authors raised the possibility of using non-toxic recombinant proteins for the production of alternative neutralising antibodies and for the development of a vaccine. GenBank also contains three different sequences of exons of sphingomyelinase D obtained from genomic DNA of *L. arizonica* that are available under Accession Nos. AF512954, AF512955 and AF512956. The complete sequence of the cDNA for sphingomyelinase D from *L. arizonica* is also available under Accession No. AF512953.

12. Future prospects

Venom toxins can be used to investigate molecular and cellular mechanisms, as models for the design of new drugs and directly for therapeutic or diagnosis use. The development of recombinant *Loxosceles* toxins in the near future should provide insights into the understanding and putative applications of venom-derived molecules for use in pharmacology, medicine and other biological sciences.

Acknowledgements

This work was sponsored by grants from CNPq, CAPES and Paraná Tecnologia. We sincerely thank staff members of the libraries of Setor de Ciencias Biologicas and Hospital de Clinicas of the Federal University of Parana, Brazil, for their help with this review.

References

Anderson, P.C., 1998. Missouri brown recluse spider: a review an update. Miss. Med. 95, 318–322.

Andrade, R.M.G., Oliveira, K.C., Giusti, A.L., Silva, W.D., Tambourgi, D.V., 1999. Ontogenetic development of *Loxosceles intermedia* spider venom. Toxicon 37, 627–632.

Andrade, R.M.G., Lourenço, W.R., Tambourgi, D.V., 2000. Comparison of the fertility between *Loxosceles intermedia* and *Loxosceles laeta* spiders. J. Arachnol. 28, 245–247.

Andrade, R.M.G., Galati, E.A.B., Tambourgi, D.V., 2001. Presence of *Loxosceles similes* Moenkhaus, (Araneae, Sicariidae) in Bodoquena Range, State of Mato Grosso do Sul Brazil. Rev. Soc. Bras. Med. Trop. 34, 3.

Araújo, S.C., Castanheira, P., Alvarenga, L.M., Mangili, O.C., Kalapothakis, E., Olórtegui, C.C., 2003. Protection against dermonecrotic and lethal activities of *Loxosceles intermedia* spider venom by immunization with a fused recombinant protein. Toxicon 41, 261–267.

Barbaro, K.C., Cardoso, J.L., Eickstedt, V.R.D., Mota, I., 1992. Dermonecrotic and lethal components of *Loxosceles gaucho* spider venom. Toxicon 30, 331–338.

Barbaro, K.C., Eickstedt, V.R.D., Mota, I., 1994a. Antigenic cross-reactivity of venoms from medically important *Loxosceles* (*Araneae*) species in Brazil. Toxicon 32, 113–120.

Barbaro, K.C., Eickstedt, V.R.D., Mota, I., 1994b. Adjuvant effect of *Loxosceles gaucho* (South American brown spider) venom. Toxicon 32, 687–693.

Barbaro, K.C., Ferreira, M.L., Cardoso, D.F., Eickstedt, V.R.D., Mota, I., 1996a. Identification and neutralization of biological activities in the venoms of *Loxosceles* spiders. Braz. J. Med. Biol. Res. 29, 1491–1497.

Barbaro, K.C., Sousa, M.V., Morhy, L., Eickstedt, V.R., Mota, I., 1996b. Compared chemical properties of dermonecrotic and lethal toxins from spiders of the genus *Loxosceles* (Araneae). J. Protein Chem. 15, 337–343.

Barbaro, K.C., Ferreira, M.L., Cardoso, D.F., Souza, M.V., Morphy, L., Eickstedt, V.R.D., Mota, I., 1997. Study of *Loxosceles* spider venom. J. Ven. Anim. Toxins 3, 232.

Barrett, S.M., Romine-Jenkins, M., Blick, K.E., 1993. Passive hemagglutination inhibition test for diagnosis of brown recluse spider bite envenomation. Clin. Chem. 39, 2104–2107.

Barrett, S.M., Romine-Jenkins, M., Fisher, D.E., 1994. Dapsone or electric shock therapy of brown recluse spider envenomation?. Ann. Emerg. Med. 24, 21–25.

Barretto, O.C.O., Satake, M., Nonoyama, K., Cardoso, J.L.C., 2003. The calcium-dependent protease of *Loxosceles gaucho* venom acts preferentially upon red cell band 3 transmembrane protein. Braz. J. Med. Biol. Res. 36, 309–313.

Beilman, G.J., Winslow, C.L., Teslow, T.W., 1994. Experimental brown spider bite in the guinea pig: results of treatment with dapsone or hyperbaric oxygen. J. Wild. Med. 5, 287–294.

Bernheimer, A.W., 1996. Some aspects of the history of membrane-damaging toxins. Med. Microbiol. Immunol. 185, 59–63.

Bey, T.A., Walter, F.G., Lober, W., Schmidt, J., Spark, R., Schlievert, P.M., 1997. *Loxosceles arizonica* bite associated with shock. Ann. Emerg. Med. 30, 701–703.

Borkkan, J., Gross, E., Lubin, Y., Oryan, I., 1995. An outbreak of venomous spider bites in a citrus grove. Am. J. Trop. Med. Hyg. 52, 228–230.

Bravo, M.L., Puratic, O.S., Behn, C.T., Fardella, C.B., Contreras, A.F., 1993. Hemolysis induced by *Loxosceles laeta* venom. In vitro experience. Rev. Med. Chil. 121, 16–20.

Bravo, M.L., Oviedo, I.L., Schenone, H.F., 1994. Study of anti-loxosceles serum action on hemolytic and ulcero-necrotic cutaneous effects of *Loxosceles laeta* venom. Rev. Med. Chil. 122, 625–629.

Braz, A., Minozzo, J., Abreu, J.C., Gubert, I.C., Chávez-Olórtegui, C., 1999. Development and evaluation of the neutralizing capacity of horse antivenom against the Brazilian spider *Loxosceles intermedia*. Toxicon 37, 1323–1328.

Broughton, G., 1996. Management of the brown recluse spider bite to the glans penis. Mil. Med. 16, 1627–1629.

Cacy, J., Mold, J.W., 1999. The clinical characteristics of brown recluse spider bites treated by family physicians: an OKPRN study. Oklahoma Physicians Research Network. J. Fam. Pract. 48, 536–542.

Chávez-Olórtegui, C., Zanetti, V.C., Ferreira, A.P., Minozzo, J.C., Mangili, O.C., Gubert, I.C., 1998. Elisa for the detection of venom antigens in experimental and clinical envenomation by *Loxosceles intermedia* spiders. Toxicon 36, 563–569.

Clowers, T.D., 1996. Wound assessment of the *Loxosceles reclusa* spider bite. J. Emerg. Nurs. 22, 283–287.

Coddington, J.A., Chnzy, H.D., Jackson, C.L., Raty, G., Gardner, K.C.H., 2001. The unique ribbon morphology of the major ampullate silk of spiders from the genus *Loxosceles* (recluse spiders). Biomacromolecules 3, 5–8.

Cohen, N., Sarafian, D.A., Alon, I., Gorelik, O., Zaidenstein, R., Simantov, R., Blatt, A., Litinsky, I., Modai, D., Golik, A., 1999. Dermonecrotic loxoscelism in the Mediterranean region. J. Toxicol. Cut. Ocul. Toxicol. 18, 75–83.

Cole, H.P., Wesley, R.E., King, L.E., 1995. Brown recluse spider envenomation of the eyelid: an animal model. Ophtal. Plast. Reconstr. Surg. 11, 153–164.

Cunha, R.B., Barbaro, K.C., Muramatsu, D., Portaro, F.C.V., Fontes, W., Sousa, M.V., 2003. Purification and characterization of Loxnecrogin, a dermonecrotic toxin from *Loxosceles gaucho* brown spider venom. J. Prot. Chem. 22, 135–146.

Desai, A., Miller, M.J., Gomez, H.F., Warren, J.S., 1999. *Loxosceles deserta* spider venom induces NF-kB-dependent chemokine production by endothelial cells. Clin. Toxicol. 37, 447–456.

Desai, A., Lankford, H.A., Warren, J.S., 2000. *Loxosceles deserta* spider venom induces the expression of vascular endothelial growth factor (VEGF) in keratinocytes. Inflammation 24, 1–9.

Elston, D.M., Eggers, J.S., Schmidt, W.E., Storrow, A.B., Doe, R.H., McGlasson, D., Fischer, J.R., 2000. Histological findings after brown recluse spider envenomation. Am. J. Dermatopathol. 22, 242–246.

Elston, D.M., 2002. What's eating you? *Loxosceles reclusa* (brown recluse spider). Cutis 69, 91–95.

Escalante-Galindo, P., Montoya-Cabrera, M.A., Terroba-Larios, V.M., Nava-Juaréz, A.R., Escalante-Flores, I., 1999. Local dermonecrotic loxoscelism in children bitten by the spider *Loxosceles reclusa* (the violin spider). Gac. Med. Mex. 135, 423–426.

Feitosa, L., Gremski, W., Veiga, S.S., Elias, M.C.Q.B., Graner, E., Mangili, O.C., Brentani, R.R., 1998. Detection and characterization of metalloproteinases with gelatinolytic, fibronectynolitic and fibrinogenolytic activities in brown spider (*Loxosceles intermedia*) venom. Toxicon 36, 1039–1051.

Fenton, A.W., West, P.R., Odell, G.V., Hudiburg, S.M., Ownby, C.L., Mills, J.N., Scroggins, B.T., Shannon, S.B., 1995. Arthropod venom citrate inhibits phospholipase A_2. Toxicon 33, 763–770.

Fischer, M.L., 1994. Levantamento das espécies de *Loxosceles* Heinecken and Lowe, 1832 no município de Curitiba, Paraná, Brasil. Estudos de Biologia 3, 63–88.

Forks, T.P., 2000. Brown recluse spider bites. J. Am. Board Fam. Pract. 13, 415–423.

França, F.O.S., Barbaro, K.C., Abdulkader, C.R.M., 2002. Rhabdomyolisis in presumed viscero-cutaneous loxocelism: report of two cases. Trans. R. Soc. Med. Hyg. 96, 287–290.

Futrell, J., 1992. Loxoscelism. Am. J. Med. Sci. 304, 261–267.

Gasparello-Clemente, E., Silveira, P.F., 2002. Fluorometric assay using naphthylamide substrates for assessing novel venom peptidase activities. Toxicon 40, 1617–1626.

Goddard, J., 1998. Loxoscelism. N. Engl. J. Med. 339, 1944–1945.

Gomez, H.F., Miller, M.J., Desai, A., Warren, J.S., 1999a. *Loxosceles* spider venom induces the production of alpha and beta chemokines: implications for the pathogenesis of dermonecrotic arachnidism. Inflammation 23, 207–215.

Gomez, H.F., Miller, M.J., Trachy, J.W., Marks, R.M., Warren, J.S., 1999b. Intradermal anti-loxosceles Fab fragments attenuate dermonecrotic arachnidism. Acad. Emerg. Med. 6, 1195–1202.

Gomez, H.F., Miller, M.J., Waggener, M.W., Lankford, H.A., Warren, J.S., 2001a. Antigenic cross-reactivity of venoms from medically important North American *Loxosceles* spider species. Toxicon 39, 817–824.

Gomez, H.F., Greenfield, D.M., Miller, M.J., Warren, J.S., 2001b. Direct correlation between diffusion of *Loxosceles reclusa* and the extent of dermal inflammation. Acad. Emerg. Med. 8, 309–314.

Gomez, H.F., Krywko, D.M., Stoecker, W.V., 2002. A new assay for the detection of *Loxosceles* species (brown recluse) spider venom. Ann. Emerg. Med. 39, 469–474.

Goto, C.S., Abramo, T.J., Ginsburg, C.M., 1996. Upper airway obstruction caused by brown recluse spider envenomization of the neck. Am. J. Emerg. Med. 14, 660–662.

Guilherme, P., Fernades, I., Barbaro, K.C., 2001. Neutralization of dermonecrotic and lethal activities and differences among 32–35 kDa toxins of medically important *Loxosceles* spider venoms in Brazil revealed by monoclonal antibodies. Toxicon 39, 1333–1342.

Health Secretary, Curitiba, Parana, Brazil, 2002. Acidentes Loxoscélicos—Protocolo técnico e fluxo de atenção em Curitiba.

Hobbs, G.D., 1997. Brown recluse spider envenomation: is hyperbaric oxygen the answer?. Acad. Emerg. Med. 4, 165–166.

Hobbs, G.D., Yealy, D.M., 1994. Development of an acute swine model for Loxosceles envenomation. Vet. Hum. Toxicol. 36, 298–300.

Hobbs, G.D., Anderson, A.R., Greene, T.J., Yealy, D.M., 1996. Comparison of hyperbaric oxygen and dapsone therapy for *Loxosceles* envenomation. Acad. Emerg. Med. 3, 758–761.

Hostetler, M.A., Dribben, W., Wilson, D.B., Grossman, W.J., 2003. Sudden unexplained hemolysis occurring in an infant due to presumed loxosceles envenomation. J. Emerg. Med. 25, 277–282.

Isbister, G.K., Graudins, A., White, J., Warrel, D., 2003. Antivenom treatment in arachnidism. J. Toxicol. Clin. Toxicol. 41, 291–300.

Jarvis, R.M., Neufeld, M.V., Westfall, C.T., 2000. Brown recluse spider bite to the eyelid. Ophtalmology 107, 1492–1496.

Kalapothakis, E., Araujo, S.C., Castro, C.S., Mendes, T.M., Gomez, M.V., Mangili, O.C., Gubert, I.C., Chavez-Olórtegui, C., 2002. Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of *Loxosceles intermedia* spider venom. Toxicon 40, 1691–1699.

Krywko, D.M., Gomez, H.F., 2002. Detection of Loxosceles species venom in dermal lesions: a comparison of 4 venom recovery methods. Ann. Emerg. Med. 39, 475–480.

Knight, D.P., Vollrath, F., 2002. Spinning and elastic ribbon of spider silk. Phil. Trans. R. Soc. Lond. B Biol. Sci. 357, 219–227.

Leung, L.K., Davis, R., 1995. Life-threatening hemolysis following a brown recluse spider bite. J. Tenn. Med. Assoc. 88, 396–397.

Lowry, B.P., Bradfield, J.F., Carroll, R.G., Brewer, K., Meggs, W.J., 2001. A controlled trial of topical nitroglycerin in a New Zealand white rabbit model of brown recluse spider envenomation. Ann. Emerg. Med. 37, 161–165.

Luciano, M.N., Silva, P.H., Chaim, O.M., Santos, V.P., Franco, C.R.C., Soares, M.F.S., Zanata, S.M., Mangili, O., Gremski, O.C., Veiga, S.S., 2004. Experimental evidence for a direct cytotoxicity of *Loxosceles intermedia* (brown spider) venom on renal tissue. J. Histochem. Cytochem. 52, 455–467.

Lung, J.M., Mallory, S.B., 2000. A child with spider bite and glomerulonephritis: a diagnostic challenge. Int. J. Dermatol. 39, 287–289.

Málaque, C.M.S., Ori, M., Santos, S.A., Andrade, D.R., 1999. Production of TNF-α by primary cultures of human keratinocytes challenged with *Loxosceles gaucho* venom. Rev. Inst. Med. Trop. S. Paulo 1999;, 41.

Málaque, C.M.S., Castro-Valencia, J.E., Cardoso, J.L.C., França, F.O.S., Barbaro, K.C., Fan, H.W., 2002. Clinical and epidemiological features of definitive and presumed loxoscelism in São Paulo, Brazil. Rev. Inst. Med. Trop. S. Paulo 2002;, 44.

Mattosinho, S.G., Sezerino, U.M., Zannin, M., Grando, M., Cardoso, J.L.C., Eickstedt, V.R.D., França, F.O.S., 1997. Geographic distribution of loxoscelism in Santa Catarina (Brazil) and species of *Loxosceles* sp involved and existent in the state. J. Ven. Anim. Tox. 3, 99.

Maynor, M.L., Moon, R.E., Klitzman, B., Fracica, P.J., Canada, A., 1997. Brown recluse spider envenomation: a prospective trial of hyperbaric oxygen therapy. Acad. Emerg. Med. 4, 184–192.

McGlasson, D.L., Babcock, J.L., Berg, L., Triplett, D.A., 1993. ARACHnase. An evaluation of a positive control for platelet neutralization procedure testing with seven commercial activated partial thromboplastin time reagents. Am. J. Pathol. 100, 576–578.

Merchant, M.L., Hinton, J.F., Geren, C.R., 1997. Effect of hyperbaric oxygen on sphingomyelinase D activity of brown recluse spider (*Loxosceles reclusa*) venom as studied by ^{31}P nuclear magnetic resonance spectroscopy. Am. J. Trop. Med. Hyg. 56, 335–338.

Merchant, M.L., Hinton, J.F., Geren, C.R., 1998. Sphingomyelinase D activity of brown recluse spider (*Loxosceles reclusa*) venom as studied by ^{31} P-NMR: effects on the time-course of sphingomyelin hydrolysis. Toxicon 36, 537–545.

Merigian, K.S., Blaho, K., 1996. Envenomation from the brown recluse spider: review of mechanism and treatment options. Am. J. Ther. 10, 724–734.

Miller, M.J., Gomez, H.F., Snider, R.J., Stephens, E.L., Czop, R.M., Warren, J.S., 2000. Detection of *Loxosceles* venom in lesional hair shafts and skin: application of a specific immunoassay to identify dermonecrotic arachnidism. Am. J. Emerg. Med. 18, 626–628.

Ministry of Health-Brazil, 1998. Manual de diagnóstico e tratamento de acidentes por animais peçonhentos. Brasília, Fundação Nacional de Saúde.

Monteiro, C.L.B., Rubel, R., Cogo, L.L., Mangili, O.C., Gremski, W., Veiga, S.S., 2002. Isolation and identification of *Clostridium perfringens* in the venom and fangs of *Loxosceles intermedia* (brown spider): enhancement of the dermonecrotic lesion in loxoscelism. Toxicon 40, 409–418.

Morena, P., Nonoyama, K., Cardoso, J.L., Barreto, O.C.O., 1994. Search of intravascular hemolysis in patients with the cutaneous form of loxoscelism. Rev. Inst. Med. Trop. S. Paulo 36, 149–151.

Morishita, R., Ferreira, S.A., Filha, A.S., Faraco, C.D., 2003. Studies on oogenesis and oviposition in the brown spider *Loxosceles intermedia* (Araneae: Sicariidae). Anatom. Rec. 273, 575–582.

Mota, I., Barbaro, K.C., 1995. Biological and biochemical-properties of venoms from medically important *Loxosceles* (Araneae) species in Brazil. J. Toxicol. Tox. Rev. 14, 401–421.

Murray, L.M., Seger, D.L., 1994. Hemolytic anemia following a presumptive brown recluse spider bite. J. Toxicol. Clin. Toxicol. 32, 451–456.

Nicholson, G.M., Graudins, A., 2003. Antivenoms for the treatment of spider envenomation. J. Toxicol. Tox. Rev. 22, 35–59.

Nishioka, S.A., 2001. Misdiagnosis of brown recluse spider bite. West. J. Med. 174, 240.

Oliveira, K.C., Andrade, R.M.G., Giusti, A.L., Silva, W.D., Tambourgi, D.V., 1999. Sex-linked variation of *Loxosceles intermedia* spider venoms. Toxicon 37, 217–221.

Ospedal, K.Z., Appel, M.H., Neto, J.F., Mangili, O.C., Veiga, S.S., Gremski, W., 2002. Histopathological findings in rabbits after experimental acute exposure to the *Loxosceles* intermedia (brown spider) venom. Int. J. Exp. Pathol. 84, 287–294.

Osterhoudt, K.C., Zaoutis, T., Zorc, J.J., 2002. Lyme disease masquerading as brown recluse spider bite. Ann. Emerg. Med. 39, 558–561.

Patel, K.D., Modur, V., Zimmerman, G.A., 1994. The necrotic venom of the brown recluse spider induces dysregulated endothelial cell-dependent neutrophil activation: differential induction of GM-CSF, IL-8, and E-selectin expression. J. Clin. Invest. 94, 631–642.

Pedrosa, M.F.F., Azevedo, I.L.M.J., Andrade, R.M.G., Berg, C.W., Ramos, C.R.R., Ho, P.L., Tambourgui, D.V., 2002. Molecular cloning and expression of a functional dermonecrotic and haemolytic factor from *Loxosceles laeta* venom. Biochem. Bioph. Res. Commun. 298, 638–645.

Phillips, S., Kohn, M., Baker, D., Leest, R.V., Gomez, H., McKinney, P., McGoldrick, J., Brent, J., 1995. Therapy of brown spider envenomation: a controlled trial of hyperbaric oxygen, dapsone, and cyproheptadine. Ann. Emerg. Med. 25, 363–368.

Ribeiro, L.A., Eickstedt, V.R.D., Rubio, G.B., Konalsaissen, J.F., Handar, Z., Entres, M., Campos, V.A.F.P., Jorge, M.T., 1993. Epidemiologia do acidente por aranhas do gênero *Loxosceles* Heinecken. Lowe no Estado do Paraná (Brasil). Mem. Inst. But. 55, 19–26.

Roodt, A.R., Salomon, O.D., Lloveras, S.C., Orduna, T.A., 2002a. Poisoning by spiders of *Loxosceles* genus. Medicina 62, 83–94.

Roodt, A.R., Litwin, S., Dokmetjian, J.C., Vidal, J.C., 2002b. A reduced immunization scheme to obtain an experimental anti-*Loxosceles laeta* (violinist spider) venom. J. Nat. Toxins 11, 193–203.

Roos, M.P., Rodgers, G.C., Matyunas, N., 1995. Brown recluse spider envenomation: treatment with dapsone. Toxicol. Lett. 78, 71–72.

Sandidge, J.S., 2003. Arachnology: scavenging by brown recluse spiders. Nature 426, 6962.

Sams, H.H., King Jr., L.E., 1999. Brown recluse spider bites. Dermatol. Nurs. 11, 427–433.

Sams, H.H., Dunnick, C.A., Smith, M.L., King, L.E., 2001a. Necrotic arachnidism. J. Am. Acad. Dermatol. 44, 561–573.

Sams, H.H., Hearth, S.B., Long, L.L., Wilson, D.C., Sanders, D.H., King, L.E., 2001b. Nineteen documented cases of *Loxosceles reclusa* envenomation. J. Am. Acad. Dermatol. 44, 603–608.

Santos, V.L.P., Franco, C.R.C., Viggiano, R.L.L., Silveira, R.B., Cantão, M.P., Mangili, O.C., Veiga, S.S., Gremski, W., 2000. Structural and ultrastructural description of the venom gland of *Loxosceles intermedia* (brown spider). Toxicon 38, 265–285.

Schenone, H., 1996. Diagnosis in 1348 patients which consulted for a probable spider bite or insect sting. Bol. Chil. Parasitol. 51, 20–27.

Schenone, H., 1998. Cutaneous loxoscelism with an edematous predominance. Bol. Chil. Parasitol. 53, 78–83.

Schenone, H., 2003. Toxic pictures produced by spider bites in Chile: Latrodectism and Loxoscelism. Rev. Med. Chi. 131, 437–444.

Sezerino, U.M., Zannin, M., Coelho, L.K., Gonçalves, J., Grando, M., Mattosinho, S.G., Cardoso, J.L.C., Eickstedt, V.R., França, F.O.S., Barbaro, K.C., Fan, H.W., 1998. A clinical and epidemiological study of *Loxosceles* spider envenoming in Santa Catarina, Brazil. Trans. R. Soc. Trop. Med. Hyg. 92, 546–548.

Silva, P.H., Hashimoto, Y., Santos, F.A., Mangili, O., Gremski, W., Veiga, S.S., 2003. Hematological cell findings in bone marrow and peripheral blood of rabbits after experimental acute exposure to *Loxosceles intermedia* (brown spider) venom. Toxicon 42, 155–161.

Silveira, R.B., Filho, J.F.S., Mangili, O.C., Veiga, S.S., Gremski, W., Nader, H.B., Dietrich, C.P., 2002. Identification of proteases in the extract of venom glands from brown spiders. Toxicon 40, 815–822.

Smith, C.W., Micks, D.W., 1970. The role of polymorphonuclear leukocytes in the lesion caused by the venom of brown spider, *Loxosceles reclusa*. Lab. Invest. 22, 90–93.

Sunderkötter, C., Seeliger, S., Schönlau, F., Roth, J., Hallmann, R., Luger, T.A., Sorg, C., Kolde, G., 2001. Different pathway leading to cutaneous leukocytoclastic vasculitis in mice. Exp. Dermatol. 10, 391–403.

Tambourgi, D.V., Magnoli, F.C., Eickstedt, V.R.D., Benedetti, Z.C., Petricevich, V.L., Silva, W.D., 1995. Incorporation of a 35 kDa purified protein from *Loxosceles intermedia* spider venom transforms human erythrocytes into activators of autologous complement alternative pathway. J. Immunol. 155, 4459–4466.

Tambourgi, D.V., Petricevich, V.L., Magnoli, F.C., Assaf, S.L.M.R., Jancar, S., Silva, W.D., 1998a. Endotoxemic-like shock induced by *Loxosceles* spider venoms: pathological changes and putative cytokine mediators. Toxicon 36, 391–403.

Tambourgi, D.V., Magnoli, F.C., Berg, C.W., Morgan, B.P., Araujo, P.S., Alves, E.W., Silva, W.D., 1998b. Sphingomyelinases in the venom of the spider *Loxosceles intermedia* are responsible for both dermonecrosis and complement-dependent hemolysis. Biochem. Biophys. Res. Commun. 251, 366–373.

Tambourgi, D.V., Morgan, B.P., Andrade, R.M.G., Magnoli, F.C., Berg, C.W., 2000. *Loxosceles intermedia* spider envenomation induces activation of an endogenous metalloproteinase, resulting in cleavage of glycophorins from the erythrocyte surface and facilitating complement-mediated lysis. Blood 95, 683–691.

Tambourgi, D.V., Silva, M.S., Billington, S.J., Andrade, R.M.G., Magnoli, F.C., Songer, J.G., Berg, C.W., 2002. Mechanism of induction of complement susceptibility of erythrocytes by spider and bacterial sphingomyelinases. Immunology 107, 93.

van den Berg, C.W., de Andrade, R.M.G., Magnoli, F.C., Marchbank, K.J., Tambourgi, D.V., 2002. *Loxosceles* spider venom induces metalloproteinase mediated cleavage. Immunology 107, 102.

Veiga, S.S., Gremski, W., Santos, V.L.P., Feitosa, L., Mangili, O.C., Nader, H.B., Dietrich, C.P., Brentani, R.R., 1999. Oligosaccharide residues of *Loxosceles intermedia* (brown spider) venom proteins: dependence on glycosylation for dermonecrotic activity. Toxicon 37, 587–607.

Veiga, S.S., Feitosa, L., Santos, V.L.P., Souza, G.A., Ribeiro, A.S., Mangili, O.C., Porcionatto, M.A., Nader, H.B., Dietrich, C.P., Brentani, R.R., Gremski, W., 2000a. Effect of brown spider venom on basement membrane structures. Histochem. J. 32, 397–408.

Veiga, S.S., Silveira, R.B., Dreyfuss, J.L., Haoach, J., Pereira, A.M., Mangili, O.C., Gremski, W., 2000b. Identification of high molecular weight serine-proteases in *Loxosceles intermedia* (brown spider) venom. Toxicon 38, 825–839.

Veiga, S.S., Zanetti, V.C., Franco, C.R.C., Trindade, E.S., Porcionatto, M.A., Mangili, O.C., Gremski, W., Dietrich, C.P., Nader, H.B., 2001a. In vivo and in vitro cytotoxicity of brown spider venom for blood vessel endothelial cells. Throm. Res. 102, 229–237.

Veiga, S.S., Zanetti, V.C., Braz, A., Mangili, O.C., Gremski, W., 2001b. Extracellular matrix molecules as targets for brown spider venom toxins. Braz. J. Med. Biol. Res. 34, 843–850.

Vetter, R.S., 1999. Identifying and misidentifying the brown recluse spider. Dermatol. Online J. 5, 7.

Vetter, R.S., 2000. Myth: idiopathic wounds are often due to brown recluse or other spider bites throughout the United States. West. J. Med. 173, 357–358.

Vetter, R.S., Bush, S.P., 2002a. Reports of presumptive brown recluse spider bites reinforce improbable diagnosis in regions of North America where the spider is not endemic. Clin. Inf. Dis. 35, 442–445.

Vetter, R.S., Bush, S.P., 2002b. The diagnosis of brown recluse spider bite is overused for dermonecrotic wounds of uncertain etiology. Ann. Emerg. Med. 39, 544–546.

Vetter, R.S., Bush, S.P., 2002c. Chemical burn misdiagnosed as brown recluse spider bite. Am. J. Emerg. Med. 20, 68–69.

Vetter, R.S., Barger, D.K., 2002. An infestation of 2,055 brown recluse spiders (Araneae:Sicariidae) and no envenomation in a Kansas home: implications for bite diagnoses in nonendemic areas. J. Med. Entomol. 39, 948–951.

Vetter, R.S., Visscher, P.K., 1998. Bites and stings of medically important venomous arthropods. Int. J. Dermatol. 37, 481–496.

Vetter, R.S., Cushing, P.E., Crawford, R.L., Royce, L.A., 2003. Diagnoses of brown recluse spider bites (loxoscelism) greatly outnumber actual verifications of the spider in four western American states. Toxicon 42, 413–418.

Wendell, R.P., 2003. Brown recluse spiders: a review to help guide physicians in nonendemic areas. South. Med. J. 96, 486–490.

White, J., Cardoso, J.L.C., Fan, H.W., 1995. Clinical Toxicology of Spider Bites, in: Meier, J., White, J. (Eds.), Clinical Toxicology of Animal Venoms and Poisons. CRC Press, Boca Raton, pp. 259–330.

Williams, S.T., Khare, V.K., Johnston, G.A., Blackall, D.P., 1995. Severe intravascular hemolysis associated with brown recluse spider envenomation. Am. J. Clin. Pathol. 104, 463–467.

Wright, S.W., Wrenn, K.D., Murray, L., Seger, D., 1997. Clinical presentation and outcome of brown spider bite. Ann. Emerg. Med. 30, 28–32.

Yiannias, J.A., Winkelmann, R.K., 1992. Persistent painful plaque due to a brown recluse spider bite. Cutis 50, 273–275.

Yosef, M., Grotto, I., Gdalevich, M., Mimouni, D., Ashkenazi, I., Haviv, J., Shpilberg, O., 1998. Is the coexistence of mutations in the genes of factor V and MTHFR a predisposing factor for massive skin necrosis due to loxoscelism?. Public Health Rev. 26, 265–270.

Young, R.A., 1994. Letters to the editor. J. Emerg. Med. 12, 389.

Young, A.R., Pincus, S.J., 2001. Comparison of enzymatic activity from three species of necrotising arachnids in Australia: *Loxosceles rufescens*, *Badumna insignis* and *Lampona cylindrata*. Toxicon 39, 391–400.

Zanetti, V.C., Silveira, R.B., Dreyfuss, J.L., Haoach, J., Mangili, O.C., Veiga, S.S., Gremski, W., 2002. Morphological and biochemical evidence of blood vessel damage and fibrinogenolysis triggered by brown spider venom. Blood Coag. Fibrin. 13, 135–148.

Hematological cell findings in bone marrow and peripheral blood of rabbits after experimental acute exposure to *Loxosceles intermedia* (brown spider) venom

Toxicon 42 (2003) 155-161

ELSEVIER

Toxicon 42 (2003) 155–161

TOXICON

www.elsevier.com/locate/toxicon

Hematological cell findings in bone marrow and peripheral blood of rabbits after experimental acute exposure to *Loxosceles intermedia* (brown spider) venom

Paulo H. da Silva[a], Yoshio Hashimoto[b], Fábio A. dos Santos[c], Oldemir C. Mangili[d], Waldemiro Gremski[a], Silvio S. Veiga[a,*]

[a]*Department of Cell Biology, Federal University of Paraná, Jardim das Américas, 81531-990, Curitiba, Paraná, Brazil*
[b]*Department of Clinical Pathology, Federal University of Paraná, Paraná, Brazil*
[c]*Department of Periodontics; State University of Ponta Grossa, Ponta Grossa, Brazil*
[4]*Department of Physiology, Federal University of Paraná, Paraná, Brazil*

Received 5 December 2002; accepted 14 May 2003

Abstract

The purpose of this work was to find out the cellular changes occurring in bone marrow and peripheral blood after acute exposure to the venom of *Loxosceles intermedia*. Doses of 40 μg of venom were injected intradermally into five rabbits, and five rabbits receiving only phosphate-buffered saline (PBS) were used as controls. Bone marrow and peripheral blood samples were obtained before the envenomation and 4, 8, 12, 24 and 48 h, and 5, 10, 15, 20 and 30 days after envenomation. In bone marrow samples we assessed cellularity, nucleated red cells, megakaryocytes and neutrophils, and in peripheral blood we assessed red cells (red cell concentration, hemoglobin and hematocrit), leukocytes, neutrophils and platelets.

Our objective was to find out if the venom has a direct effect on bone marrow and peripheral blood or if changes in both of them are secondary to the needs of tissues, and if there is a good correlation between histopathological and hematological findings. We found that the red cell parameters were not affected by the venom, except for nucleated red cells which decreased after venom exposure. The depression of megakaryocyte numbers and thrombocytopenia showed a strong correlation with the histopathologic changes observed in skin biopsies obtained from the rabbits. The changes in cellularity and neutrophils of bone marrow were strongly correlated with those in peripheral blood and skin.

The thrombocytopenia and neutropenia in peripheral blood are due to marrow depression, which may be a consequence of an extensive migration of platelets and neutrophils to the necrotic lesion or the marrow depression may be a transitory effect of evenoming by *L. intermedia*.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: Loxosceles intermedia; Hematological cell findings; Peripheral and bone marrow blood

1. Introduction

The bite of the brown spider (*Loxosceles intermedia*) produces a local necrotic lesion which can result in a systemic involvement with renal failure, hemolytic anemia, thrombocytopenia and disseminated intravascular coagulation (Futrell, 1992; Veiga et al., 2000). Clinical and laboratory findings may include hemolytic anemia, hemoglobinemia, hemoglobinuria, elevated bilirubin, leukocytosis and proteinuria (Futrell, 1992). Histopathological changes in the necrotic lesions are characterized by polymorphonuclear (PMN) accumulation in and around venules and by extravascular red blood cells around the vessels, suggesting loss of vascular integrity and intravascular clotting (Smith and Micks, 1970; Futrell, 1992; Elston

* Corresponding author. Fax: +5541-266-2042.
E-mail address: veigass@ufpr.br (S.S. Veiga).

0041-0101/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0041-0101(03)00127-2

et al., 2000; Veiga et al., 2001; Ospedal et al., 2002). All of these observations were made at the clinical and histopathological level. The literature does not describe the effects of brown spider venom on the cellularity of bone marrow (BM) and peripheral blood after envenomation.

The red cell hemolysis is associated with brown spider venom in an in vitro model. Futrell et al. (1979) described a model for studying hemolysis associated with brown recluse venom. Hemolytic reaction has been observed in human and swine erythrocytes, but in rabbits and guinea pigs no hemolytic reaction occurs, indicating some variation in animal susceptibility.

Another in vitro model is the action of venom on platelets. The venom promotes platelet aggregation and thrombocytopenia evoked by sphingomyelinase D, a low molecular mass protein (Kurpiewski et al., 1981) and intravascular clotting is observed at the site of bite (Futrell, 1992; Ospedal et al., 2002).

In the leukocytes series, it has been well established that PMN have a critical role in necrotic lesions and migrate to the tissue, with consequent changes in the cellularity of BM and peripheral blood, but these hematological alterations have not been described after bites by brown spiders. It has also been established that PMN depletion protects against the pathologic process induced by the venom, but the venom does not activate PMN directly (Smith and Micks, 1970). Patel et al. (1994) confirmed that the venom activates endothelial cells which in turn activate PMN. The activated endothelial cells induce the migration of PMN which is responsible for the lesion at the tissue level.

Hematological cells are consumed at the site of bite, with the occurrence of a new balance between the production of cells by BM and their consumption by tissues, with changes in the cellularity of BM and peripheral blood (Lee et al., 1998). The objective of the present investigation was to the study cellular alterations at BM and peripheral blood level using rabbits as experimental animals, whose tissue changes are similar to those detected in humans and also to correlate such changes with the histopathological findings and venom cytotoxicity on hematological cells.

2. Materials and methods

2.1. Spider venom extraction

The venom was extracted from *L. intermedia* captured in nature and kept for 5 days without any kind of food. An electrostimulation of 15 V was applied to the cephalothorax of the spiders and the venom was collected directly from the fangs with a micropipette, dried under vacuum and frozen at −85 °C. A pool of venom collected from 1000 spiders was used in the experiments (Feitosa et al., 1998; Veiga et al., 2000).

2.2. Animals

Adult New Zealand rabbits weighing 3.0 and 4.0 kg from the Central Animal House of Federal University of Parana were used for marrow aspiration and blood sample collection.

2.3. Venom administration

Pooled venom (samples of 40 μg of protein from crude venom diluted in a final volume of 100 μl PBS, since 40 μg of protein represents the average venom concentration injected during natural bites) was injected intradermally into shaved skin areas of the rabbits. The animals were divided into two groups: control group (C) and test group (T). The control group consisted of five animals receiving only PBS and the test group consisted of five animals receiving 40 μg of *L. intermedia* venom. Peripheral blood and BM was collected before the envenomation (C0 and T0) and after 4 h (C4 and T4), 12 h (C12 and T12), 24 h (C24 and T24), 48 h (C48 and T48), 5 days (C5 and T5), 10 days (C10 and T10), 15 days (C15 and T15), 20 days (C20 and T20), and 30 days (C30 and T30). All animals were kept under the same experimental conditions.

2.4. Blood sample collection

Two ml of peripheral blood (PB) was obtained from ear vein and anticoagulated with EDTA-K3. Red cell, hemoglobin, hematocrit, leukocyte and platelet count were determined using an automated CELL-DYN 1.400 blood counter (Abbott Laboratories, IL, USA).

Two blood films were prepared for morphological examination of blood (red cell morphology, platelet morphology and leukocyte differentation) and stained by the May-Grünwald Giemsa technique (Lee et al., 1998). Morphology and differentation were observed by microscopic examination (Olympus BX-41) (Kyoto, Japan).

2.5. Bone marrow aspiration

The site of BM aspiration was the thighbone. Before marrow aspiration the animal was anesthetized with ketamine (Agribands, Paulínia-São Paulo) and acepromazin (Univet, São Paulo-São Paulo). The marrow needle was inserted through the skin, subcutaneous tissue and cortex of the bone with a slight twisting motion. Penetration of the cortex can be sensed by a slight, rapid forward movement accompanied by a sudden increase in the ease of the advancing the needle. The stylet of the needle is removed promptly, the hub is attached to a 20 ml syringe, and about 0.2–0.5 ml of fluid is aspirated and direct films were immediately prepared.

The direct marrow films were stained by the May-Grünwald Giemsa technique to determine the BM cellularity

and the differentation of the hematopoietic lines under microscopic examination, as described above.

2.6. Statistical analysis

The Student *t*-test for unpaired observations was used to detect statistically significant differences between control and test group as follow: 1-PB-red cell concentration (10^6 ml/blood); 2-PB-hemoglobin (g/dl blood); 3-PB-hematocrit (%); 4-BM-nucleated red cell; 5-PB-platelet (10^3 ml/blood); 6-BM-neutrophils (%); 7-PB-leukocytes (10^3 ml/blood); 8-PB-neutrophils (%). The threshold level for significance was 0.05. All statistical calculations were made using the GRAPHPAD INSTAT program version 3.00 for Windows 95. Frequency distributions (%) were calculated for megakaryocytes (BM) and cellularity (BM). The mean values obtained for five animals of each group (T and C) were plotted graphically.

3. Results

3.1. Effect of L. intermedia venom on red cell series

Fig. 1 shows the effect of venom on nucleated red cells in BM. The nucleated red cells were counted in relation of the total leukocyte count.

There were no consistent changes in erythrocytes count, hematocrit or hemoglobin when the two groups are compared at each time point (data not shown). Nucleated red cells were significantly decreased at times T12, T24 and T5 (significant differences, $p = 0.0066$, $p = 0.0001$, $p = 0.0498$, respectively-unpaired *t*-test). The venom has no effect on the morphology of red cell (data not shown).

3.2. Effect of L. intermedia venom on megakaryocytes and platelets

Fig. 2 shows the effect of brown spider venom on megakaryocytes and platelets: (A) the effect on platelet count in peripheral blood, (B) cellularity scores of megakaryocytes in BM (BM) of control group, and (C) the effect of spider venom on the cellularity scores of megakaryocytes in BM-test group. The megakaryocytes were scored as absent, rare, discrete (+), moderate (++) and intense (+++) under 10 × microscopic magnification.

There were consistent changes in platelet count when the two groups were compared (C and T). At times T4 and T12 a decreased count occurred (significant differences, $p = 0.0062$, $p = 0.0001$, respectively-unpaired *t*-test). A rebound began at T24 ($p = 0.2542$) and T48 ($p = 0.0041$) and high values were observed at T5 ($p = 0.0261$) and T10 ($p = 0.0398$).

Megakaryocytes changed in proportion to BM cellularity and the decrease of platelet count in peripheral blood. Changes were observed at times T4, T12, T24, T48, with a recovery of normal status at T5.

3.3. Effect of L. intermedia venom on cellularity and neutrophils in BM, and leukocytes and neutrophils in peripheral blood

Fig. 3 shows the effect of the venom on BM cellularity (A), the effect of venom on BM neutrophils (%) (B) and the effect of venom on leukocytes in peripheral blood (10^3/ml blood) (C) and the effect of venom on neutrophils in peripheral blood (10^3/ml blood) (D). BM cellularity was evaluated under microscopic magnification at 10 × and was classified as normocellular, decreased and hypocellular, using as reference of normocellular the control group.

At T4, two animals were normocellular, two had decreased cellularity, and one was hypocellular. At T12, three animals showed decreased cellularity and two showed hypocellularity. At T24, all animals showed hypocellularity. At T48, three animals showed decreased cellularity and two hypocellularity.

The number of neutrophils in BM was decreased at T24 ($p = 0.0037$) and T48 ($p = 0.0283$), with a recovery of their initial status at T5 ($p = 0.0055$).

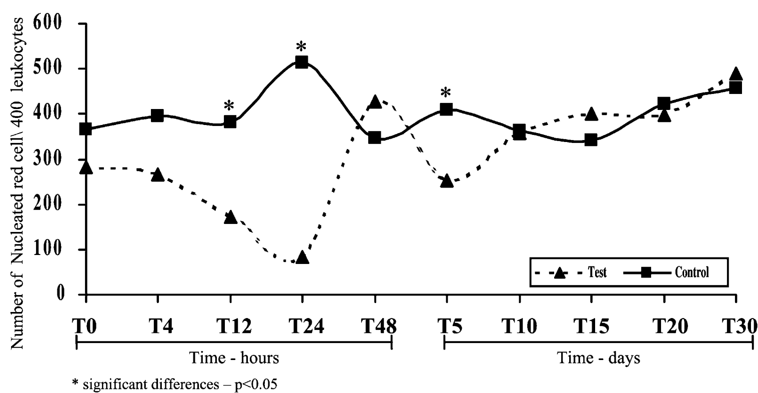


Fig. 1. The effect of *L. intermedia* venom on nucleated red cell in bone marrow.

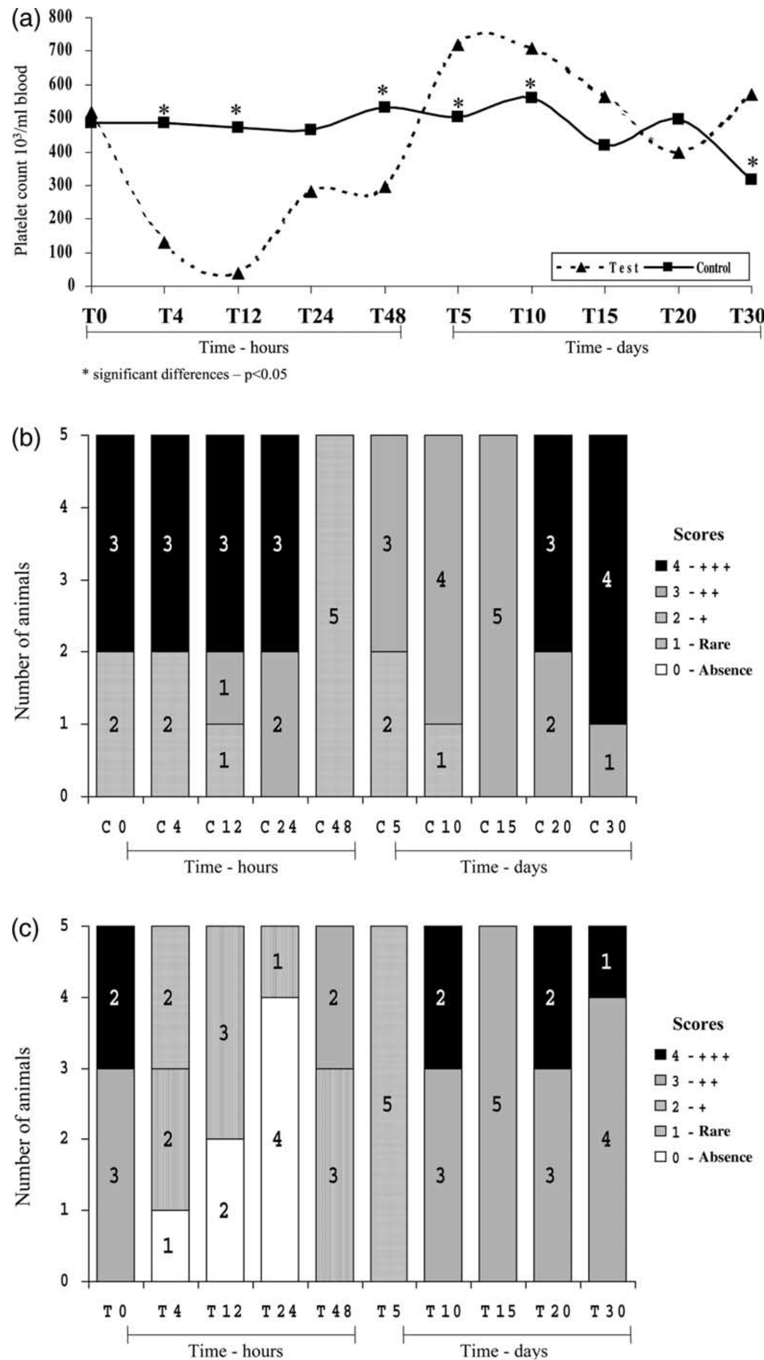


Fig. 2. *L. intermedia* venom activity upon megakaryocytes (BM) and platelets (PB). (a) The effect of *L. intermedia* venom on platelet count in peripheral blood (10^3/ml blood). (b) Cellularity scores of megakaryocytes in bone marrow (BM) of control group. Discrete (+), moderate (++) and intense (+++). (c) The effect of *L. intermedia* venom on megakaryocytes in bone marrow (BM) in test group. Discrete (+), moderate (++) and intense (+++).

Peripheral blood leukocytes were decreased at T24 ($p = 0.0008$), with high values at T5 ($p = 0.0386$) and T30 ($p = 0.0232$). Peripheral blood neutrophils were increased at T4 ($p = 0.0248$) and decreased at T24 ($p = 0.0014$), with recovery starting at T48 ($p = 0.0072$); at T30 ($p = 0.0257$) the values were increased. The venom had no effect on neutrophil morphology in BM or peripheral blood (data not shown).

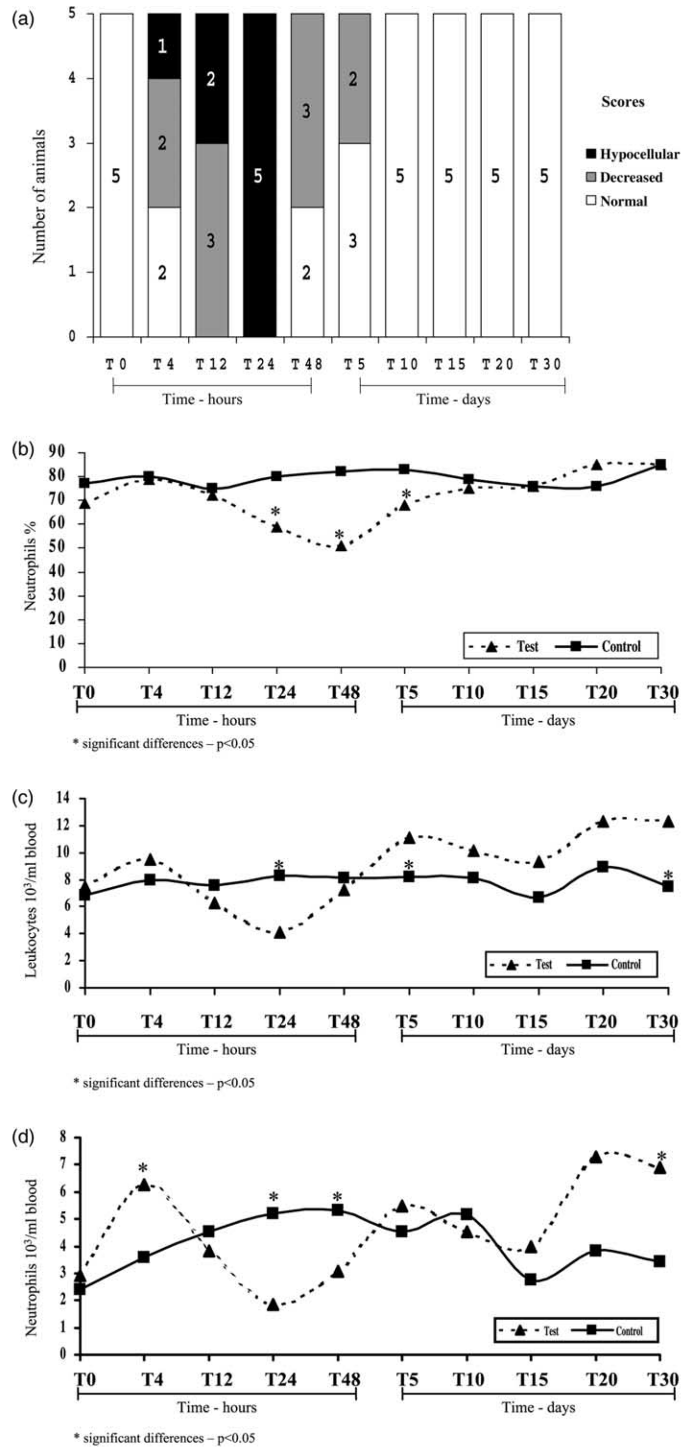


Fig. 3. *L. intermedia* venom activity upon bone marrow cellularity, leukocytes in peripheral blood and neutrophils in bone marrow and peripheral blood. (a) The effect of *L. intermedia* venom on bone marrow cellularity. (b) The effect of *L. intermedia* venom on bone marrow neutrophils (%). (c) The effect of *L. intermedia* venom on leukocytes in peripheral blood (10^3/ml blood). (d) The effect of *L. intermedia* venom on neutrophils in peripheral blood (10^3/ml blood).

4. Discussion

The whole mass of erythroid cells has been called the erythron (Lee et al., 1998). Under normal conditions the erythron represents the balance between the production by BM and the extravascular destruction by spleen. When this balance is disrupted, changes occur in the cellularity of BM and peripheral blood. Hemolytic anemia, extravascular red blood cells and massive hemorrhage are associated with brown spider envenomation (Smith and Micks, 1970; Futrell, 1992; Elston et al., 2000; Veiga et al., 2000; Zanetti et al., 2002).

Considering our results concerning the red cell series we can say that, in spite of the necrotic lesion and the hypocellularity of BM, there were no changes in red cell count, hemoglobin and hematocrit. These results are in agreement with Futrell (1992) who did not observe a hemolytic reaction in rabbits. On the other hand, nucleated red cells showed a significant decrease at T12, T24 and T5, recovering their normal values after T10. Nucleated red cells were not seen in peripheral blood. The putative explanation for this event could be a mitogen effect directed at the maintenance of adequate levels of red cells in peripheral blood or to a direct action of the venom on the nucleated red cells. The renal failure described during the Loxoscelism (Futrell, 1992) may have decreased the levels of erythropoietin, possibly explaining the decrease in nucleated red cells. The direct cytotoxic activity of venom toward blast cells, if confirmed, opens the possibility for the biotechnological use of toxins for investigations related to this type of cell.

One of the most serious effects of envenomation is the intravascular clotting that occurs early during the course of cutaneous lesion development (Futrell, 1992; Ospedal et al., 2002). The venoms of different species are able to promote platelet aggregation and thrombocytopenia, as evidenced for *L. reclusa*, *L. intermedia* and *L. laeta* (Kurpiewski et al., 1981; Bascur et al., 1982; Veiga et al., 1999). Elston et al. (2000) suggested that capillary thrombosis was an early event, followed by an influx of leucocytes. In the present study, the marrow depression of megakaryocyte was correlated with thrombocytopenia in peripheral blood, observed at T4 and T12. At T24 and T48 a rebound began, reaching a maximum at T5, with platelet count and megakaryocytes recovering their original status thereafter. The marrow depression of megakaryocyte with a consequent thrombocytopenia may be a consequence of an extensive consumption of platelets at the site of bite or a direct effect of the venom. If the latter is true, this effect is transitory since before T5 the original status was recovered.

After 4 h of envenomation, three animals showed a marrow depression. After 12 and 24 h the marrow depression was present in all the animals. The celullarity of BM started to recover after 48 h, reaching normal levels within 5 days. The neutropenia in peripheral blood (T24 and T48), the low neutrophils in BM (T24, T48 and T5) and the low leukocyte counts at T24 were consequences of marrow depletion. The marrow depression and the low white blood cell counts may be a consequence of an extensive neutrophil influx to the tissues. The hypothesis that Loxosceles venom is an endothelial agonist and that these activated endothelial cells in turn activate PMN was tested by Patel et al. (1994). If the venom has a direct effect of BM with changes in peripheral blood, this effect is transitory because the BM and peripheral blood recover the normal status before T5. The explanation of high values at T30 could be a secondary infection as consequence of necrotic lesion.

Elston et al. (2000) showed that the large numbers of eosinophils and neutrophils were common in dermal infiltrates and the degranulation of eosinophils adjacent to site of necrosis suggested that they play a role in tissue injury after experimental envenomation with brown recluse spider venom. In our study, there were no changes in eosinophil concentrations in BM or peripheral blood and no significant difference was observed between the test and control groups.

From our study, we can conclude that, the red cells series was not affected by the venom except the nucleated red cells. The thrombocytopenia shows a high correlation with the BM depression of megakaryocytes. The neutropenia (PB), the low count of neutrophils (BM) and the depression of BM were highly correlated with one another and with the data obtained at tissue level.

Acknowledgements

This research was supported by grants from Fundação Araucária-Paraná, CAPES, CNPq. We acknowledge LIPAPE (Laboratório Interdisciplinar de Pesquisa em Animais Peçonhentos, UFPR) for providing adult spiders for electrostimulated venom extractions. We are also grateful to Dr Carlos Roberto de Assis (Veterinary Clinic São Francisco de Assis, Curitiba-Paraná-Brazil) for teaching us how to obtain the bone marrow samples.

References

Bascur, L., Yevenes, I., Barja, P., 1982. Effects of *Loxosceles laeta* spider venom on blood coagulation. Toxicon 20, 795–796.

Elston, D.M., Eggers, J.S., Schmidt, W.E., Storrow, A.B., Doe, R.H., McGlasson, D., Fischer, J.R., 2000. Histological findings after brown recluse spider envenomation. Am. J. Dermatopathol. 22, 242–246.

Feitosa, L., Gremski, W., Veiga, S.S., Elias, M.C.Q.B., Graner, E., Mangili, O.C., Brentani, R.R., 1998. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in brown spider (*Loxosceles intermedia*) venom. Toxicon 36, 1039–1051.

Futrell, J., 1992. Loxoscelism. Am. J. Med. Sci. 304, 261–267.

Futrell, J.M., Morgan, B.B., Morgan, P.N., 1979. An in vitro model for studying hemolysis associated with the venom from brown recluse spider (*Loxosceles reclusa*). Toxicon 17, 355–362.

Kurpiewski, G., Forrester, L.J., Barrett, J.T., Campbell, B.J., 1981. Platelet aggregation and sphingomyelinase D activity of a purified toxin from the venom of *Loxosceles reclusa*. Biochem. Biophys. Acta 678, 467.

Lee, R.G., Foerster, J., Lukens, J., Paraskevas, F., Greer, J.P., Rodgers, G.M., 1998. Wintrobe's—Clinical Hematology, tenth ed., Lippincott Williams & Wilkins, New York, USA.

Ospedal, K.Z., Appel, M.H., Neto, J.N., Mangili, O.C., Veiga, S.S., Gremski, W., 2002. Histopathological findings in rabbits after experimental acute exposure to the *Loxosceles intermedia* (brown spider) venom. Int. J. Exp. Path. 84, 287–294.

Patel, K.D., Modur, V., Zimmerman, G.A., Prescott, S.M., McIntyre, T.M., 1994. The necrotic venom of brown recluse spider induces dysregulated endothelial cell-dependent neutrophil activation. J. Clin. Invest. 94, 631–642.

Smith, W.C., Micks, W.D., 1970. The role of polimorphonuclear leukocytes in the lesion caused by the venom of the brown spider *Loxosceles reclusa*. Lab. Invest. 22, 90–93.

Veiga, S.S., Gremski, W., Santos, V.L.P., Feitosa, L., Mangili, O.G., Nader, H.B., Dietrich, C.P., Brentani, R.R., 1999. Oligosaccharide residues of *Loxosceles intermedia* (brown spider) venom proteins: dependence on glycosylation for dermonecrotic activity. Toxicon 37, 587–607.

Veiga, S.S., Feitosa, L., Santos, V.L.P., Souza, G.A., Ribeiro, A.S., Manigili, O.C., Porcionatto, M.A., Nader, H.B., Dietrich, C.P., Brentani, R.R., Gremski, W., 2000. Effect of brown spider venom on basement membrane structures. Histochem. J. 32, 397–408.

Veiga, S.S., Zanetti, V.C., Franco, C.R.C., Trindade, E.S., Porcionatto, M.A., Mangili, O.C., Gremski, W., Dietrich, C.P., Nader, H.B., 2001. In vivo and in vitro cytotoxicity of brown spider venom for blood vessel endothelial cells. Thromb. Res. 102, 229–237.

Zanetti, V.C., Silveira, R.B.S., Dreyfuss, J.L., Haoach, J., Mangili, O.C., Veiga, S.S., Gremski, W., 2002. Hemorrhagic effects of brown spider venom. Blood Coag. Fibrinol. 13, 135–148.

Experimental evidence for a direct cytotoxicity of *Loxosceles intermedia* (brown spider) venom in renal tissue

Journal of Histochemistry & Cytochemistry 52(4): 455-467, 2004

Volume 52(4): 455–467, 2004
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Experimental Evidence for a Direct Cytotoxicity of *Loxosceles intermedia* (Brown Spider) Venom in Renal Tissue

Melissa N. Luciano, Paulo H. da Silva, Olga M. Chaim, Vera Lucia P. dos Santos, Célia Regina C. Franco, Maria Fernanda S. Soares, Silvio M. Zanata, Oldemir C. Mangili, Waldemiro Gremski, and Silvio S. Veiga

Department of Cell Biology, Federal University of Paraná (MNL,PHdS,OMC,CRCF,WG,SSV); Health Area, Campos de Andrade University (VLPdS); Department of Medical Pathology, Federal University of Paraná (MFSS); Department of Basic Pathology, Federal University of Paraná (SMZ); and Department of Physiology, Federal University of Paraná (OCM), Curitiba, Paraná, Brazil

SUMMARY Brown spider (*Loxosceles* genus) venom causes necrotic lesions often accompanied by fever, hemolysis, thrombocytopenia, and acute renal failure. Using mice exposed to *Loxosceles intermedia* venom, we aimed to show whether the venom directly induces renal damage. The experimental groups were composed of 50 mice as controls and 50 mice that received the venom. Light microscopic analysis of renal biopsy specimens showed alterations including hyalinization of proximal and distal tubules, erythrocytes in Bowman's space, glomerular collapse, tubule epithelial cell blebs and vacuoles, interstitial edema, and deposition of eosinophilic material in the tubule lumen. Electron microscopic findings indicated changes including glomerular epithelial and endothelial cell cytotoxicity as well as disorders of the basement membrane. Tubule alterations include epithelial cell cytotoxicity with cytoplasmic membrane blebs, mitochondrial changes, increase in smooth endoplasmic reticulum, presence of autophagosomes, and deposits of amorphous material in the tubules. We also found that the venom caused azotemia with elevation of blood urea levels but did not decrease C3 complement concentration or cause hemolysis in vivo. Confocal microscopy with antibodies against venom proteins showed direct binding of toxins to renal structures, confirmed by competition assays. Double-staining immunofluorescence reactions with antibodies against type IV collagen or laminin, antibodies to venom toxins, and fluorescent cytochemistry with DAPI revealed deposition of toxins in glomerular and tubule epithelial cells and in renal basement membranes. Two-dimensional electrophoresis showed venom rich in low molecular mass and cationic toxins. By immunoblotting with antibodies to venom toxins on renal extracts from venom-treated mice, we detected a renal binding toxin at 30 kD. The data provide experimental evidence that *L. intermedia* venom is directly involved in nephrotoxicity. **(J Histochem Cytochem 52:455–467, 2004)**

KEY WORDS
brown spider
venom
toxins
nephrotoxicity

Loxoscelism, the term used to describe lesions and reactions induced by bites from spiders of the genus *Loxosceles*, causes necrosis and local hemorrhage at the bite site, platelet aggregation, thrombocytopenia, disseminated intravascular coagulation, hemolysis, and renal failure at the systemic level (Futrell 1992; Elston et al. 2000; Lung and Mallory 2000; Veiga et al. 2001b; Zanetti et al. 2002). Symptoms such as weakness, fever, nausea, vomiting, and convulsions, which suggest some effect on the nervous system, have also been reported (Futrell 1992).

A number of enzymes and biologically active molecules that might contribute to the deleterious effects of the venom have been identified and biochemically characterized. A sphingomyelinase D of 32–35 kD isolated from brown spider venom can induce dermonecrosis, platelet aggregation, and experimental hemolysis (Futrell 1992). Metalloproteases of 32–35 kD and

Correspondence to: Silvio Sanches Veiga, Dept. of Cell Biology, Federal University of Parana, Jardim das Américas, 81531-990 Curitiba, Parana, Brazil. E-mail: veigass@ufpr.br
Received for publication August 4, 2003; accepted December 17, 2003 (3A6145).

© The Histochemical Society, Inc.

The Journal of Histochemistry & Cytochemistry

20–28 kD identified in the venom with gelatinolytic, fibronectinolytic, and fibrinogenolytic activities can also play a role in hemorrhage evoked by envenomation, such as hemorrhage into the dermis, injury of blood vessels, imperfect platelet adhesion, and the defective wound healing observed in some cases (Feitosa et al. 1998; Veiga et al. 2001a,b; da Silveira et al. 2002; Zanetti et al. 2002). A hyaluronidase with electrophoretic mobilities at regions of 33 kD and 63 kD is likely to contribute to the gravitational spread of dermonecrotic lesions, a hallmark of brown spider bites (Wright et al. 1973; Futrell 1992). A number of other molecules and activities have been identified in the venom, including a lipase, alkaline phosphatase, and proteolytic activities on entactin, basement membranes, and the protein core of a heparan sulfate proteoglycan from endothelial cells (Futrell 1992; Veiga et al. 2000,2001b). The mechanism underlying the involvement of these individual venom constituents or activities in the noxious effects of the venom on cell tissue has not been fully determined.

Some reports have indicated the participation of blood cells and molecular components in the noxious effects of the venom. The serum amyloid P component appears to be a target for platelet activation and ischemic effects and is likely to play a role in the necrosis caused by the venom (Gates and Rees 1990). Leukocytes, and especially polymorphonuclear cells (PMNs) such as neutrophils and eosinophils, appear to play a role in the dermonecrotic lesion evoked by the venom, because histopathological findings have revealed a massive infiltration of these cells into the dermis and related structures in the dermonecrotic regions induced by the venom (Elston et al. 2000; Ospedal et al. 2002). Depletion of leukocytes in the blood results in reduction of clinical signs in the skin injected with venom (Smith and Micks 1970). The complement system in the plasma also appears to participate in the deleterious activities of the venom, especially on erythrocytes, evoking a complement-dependent hemolysis (Futrell 1992).

Although some clinical signs of loxoscelism have been well described, and putative molecules in the venom and physiopathological events involved in cell destruction have been characterized as described above, data about renal disorders evoked by brown spiders have been limited to earlier reports describing clinical data from victims (Futrell 1992; Lung and Mallory 2000). We report here the effect of *L. intermedia* venom on kidney structures. Mice were used because these animals do not develop dermonecrotic lesions induced by *Loxosceles* venom, so that the occurrence of nephrotoxicity secondary to complications of dermonecrotic lesions can be ruled out. We hope to bring some insight into loxoscelism that could be useful to physicians who diagnose and treat the victims.

Materials and Methods

Reagents

Polyclonal antibodies to *L. intermedia* venom toxins were produced in a rabbit using crude venom as antigen and complete Freund's adjuvant (Sigma; St Louis, MO) for the first primary injection at one SC point and two IM points (20 μg of proteins as total antigens were used divided into three parts). As a booster for the second, third, and fourth injections, incomplete Freund's adjuvant (Sigma) was similarly injected at 2-week intervals. Twelve days after the last immunization, blood was collected and the production of specific antibodies analyzed by ELISA and Western blotting (Harlow and Lane 1988). Hyperimmune IgGs were purified from serum using protein–A Sepharose (Amersham Biosciences; Piscataway, NJ) as recommended by the manufacturer. Polyclonal antibodies that recognize type IV collagen were purchased from Chemicon International (Temecula, CA) and monoclonal antibodies against laminin were purchased from DAKO (Carpinteria, CA). Fluorescein- and rhodamine-conjugated anti-IgG antibodies were purchased from Chemicon. DAPI (4′,6-diamidino-2-phenylindole HCl) nucleic acid stain was purchased from Molecular Probes (Eugene, OR).

Spider Venom Extraction

The venom was extracted from spiders captured from the wild and kept for a week without any food but with water ad libitum. The venom was extracted from the fangs of spiders by electrostimulation (15 V) applied to the cephalothorax and was collected with a micropipette, dried under vacuum, and frozen at −85C until use. Pools of venom collected from 100 to 200 spiders in different batches were used during all the experiments, involving approximately 1000 spiders (Feitosa et al. 1998). Protein content was determined by the Coomassie Blue method (Bradford 1976).

Animals

Adult Swiss mice weighing approximately 25 g from the Central Animal House of the Federal University of Paraná were used for in vivo experiments with the venom. All experimental protocols using animals were performed according to the "Principles of Laboratory Animal Care" (NIH Publication 85-23, revised 1985) and Brazilian federal laws.

Venom Administration

Pooled crude venom and mouse samples of 1 mg of protein/kg were diluted in PBS (pH 7.3). These samples were injected IP in a volume of 100 μl in each mouse. The animals were divided into two groups, a control (C) group and a test (T) group. The control group consisted of five animals receiving only PBS and the test group consisted of five animals receiving *L. intermedia* venom. During the experimental procedures, the envenomation of animals was repeated at least 10 times, completing a number of 50 animals as controls and 50 animals that received the venom. All animals were kept under the same experimental conditions. All kidney and blood samples were collected from living animals.

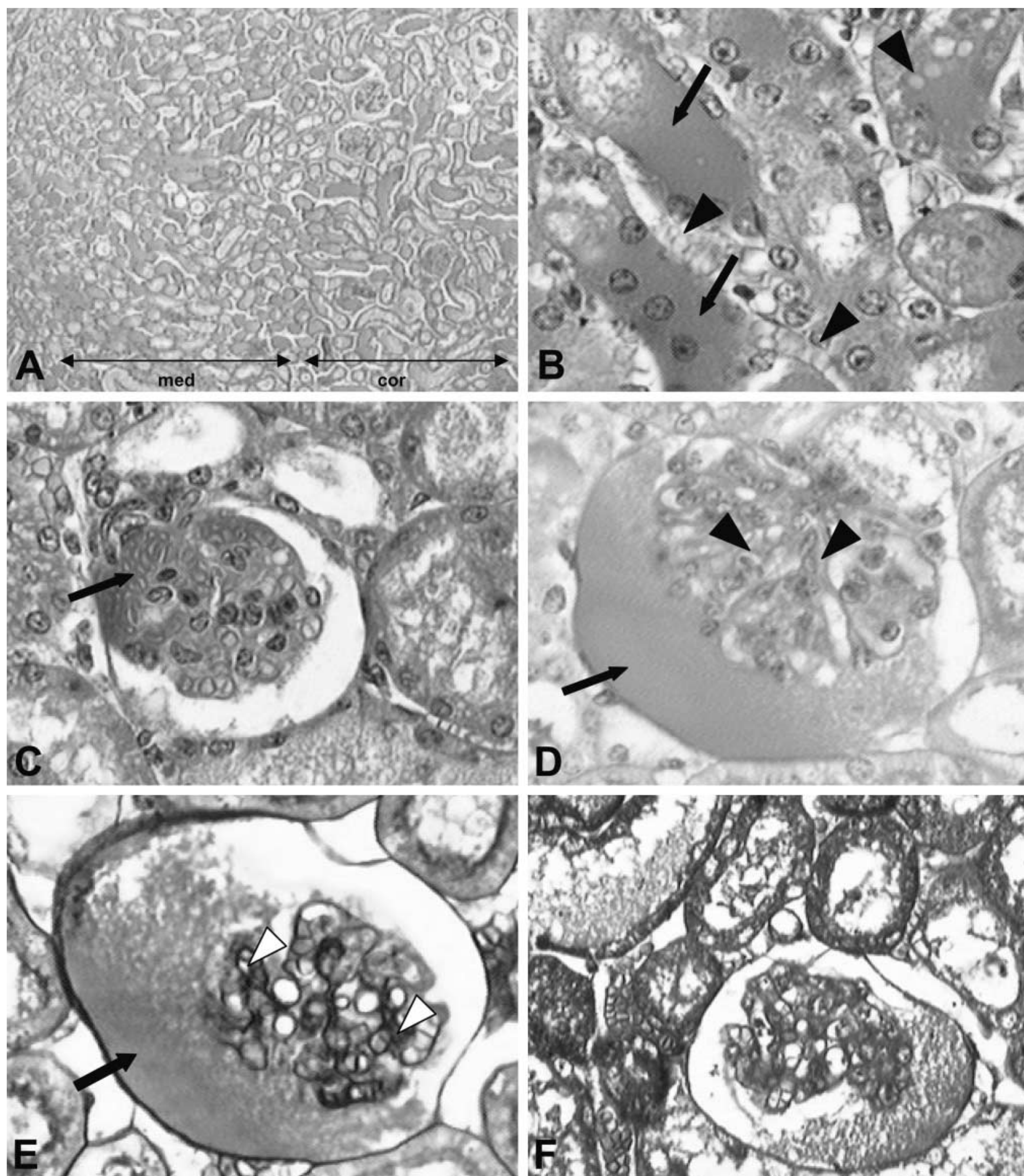
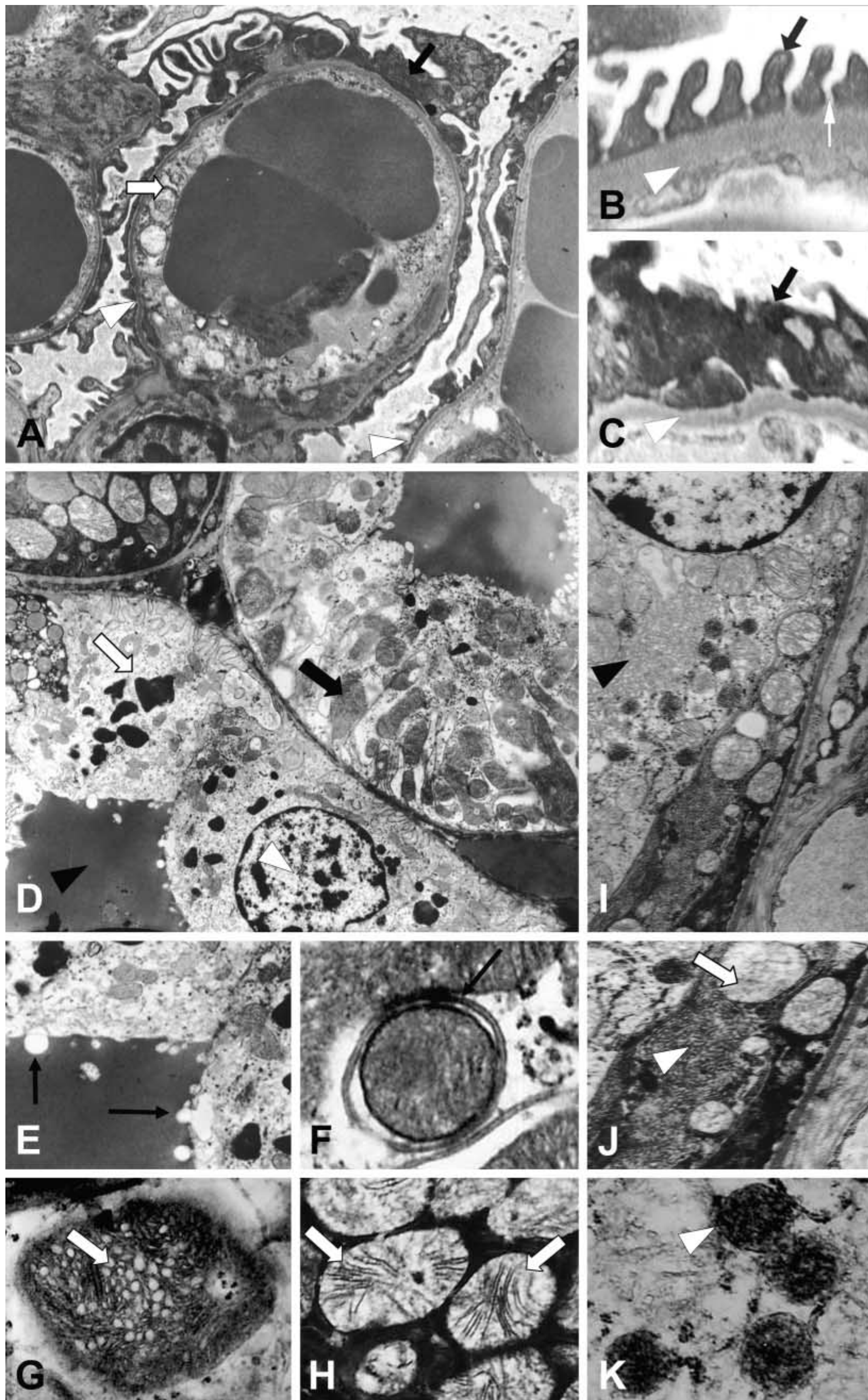


Figure 1 Light microscopic analysis of kidneys from venom-treated mice. Sections of kidneys from mice treated with *L. intermedia* venom were stained with a combination of dyes and analyzed by light microscopy. (**A**) Panoramic view of a kidney stained with hematoxylin and eosin, indicating diffuse alterations along medullary (med) and cortical (cor) regions. Magnification ×40. (**B**) Details of tubule structures stained with hematoxylin and eosin, showing accumulation of eosinophilic material within the tubule lumen (arrows) and vacuoles in the epithelial cells (arrowheads). (**C**) Inset of a cross-section of glomerular structures stained with hematoxylin and eosin, where focal intra-glomerular erythrocytes can be seen in Bowman's space (arrow). (**D,E**) Glomerular cross-sections stained with periodic acid Schiff reagent plus hematoxylin and silver impregnation, where collapse of basement membranes (arrowheads) and deposition of proteinaceous materials in Bowman's space (arrows) can be observed. (**F**) Cross-section of glomerular and tubule structures stained by the Rosenfeld method. No leukocyte infiltration can be observed. Magnifications ×400.


A
B
C
D
I
E
F
J
G
H
K

Kidney and Blood Sample Collections and Laboratory Analysis

Kidney and blood (directly from the heart) samples were obtained from mice anesthetized with ketamine (Agribands; Paulinia, SP, Brazil) and acepromazin (Univet; São Paulo, SP, Brazil). Blood was anticoagulated with EDTA-K3 and used for red cell, hemoglobin, hematocrit, leukocyte, and platelet counts that were determined with an automated CELL-DYN 1,400 blood counter (Abbott Laboratories; Chicago, IL). Urea and C3 complement were determined in serum. Assays were performed using standardized techniques and reagents as described by Kaplan and Pesce (1996) and Henry (2001).

Statistical Analysis

The Student's *t*-test for unpaired observations was used to detect statistically significant differences between control and test groups for red cell concentration (10^6 ml/blood), hemoglobin (g/dl blood), hematocrit (%), platelets (10^3 ml/blood), leukocytes (10^3 ml/blood), serum urea (mg/dl), and C3 complement (mg/dl). The threshold level for significance was $P=0.05$. All statistical calculations were done with the GraphPad InStat program version 3.00 for Windows 95. Morphometric analysis of sections stained with hematoxylin and eosin or from electronmicrographs were measured by use of software Jandel Sigma Scan Pro.

Gel Electrophoresis

Lysed renal cells were obtained by treatment of kidneys with lysis buffer (50 mM Tris-HCl, pH 7.3, 1% Triton X-100, 50 mM NaCl, 1 mM $CaCl_2$,1 mM phenylmethanesulfonyl fluoride, and 2 μg/ml aprotinin) for 15 min at 4C. The extract was clarified by centrifugation for 10 min at 13,000 × g. Renal extracts or crude venom (normalized for their protein contents) were submitted to electrophoresis under non-reducing conditions. Linear gradient 3–20% or 8–18% SDS-PAGE was performed as described by Laemmli (1970). For protein detection, gels were stained with Coomassie Blue. For immunoblotting, proteins were transferred to nitrocellulose filters overnight as described by Towbin et al. (1979). The molecular mass markers used were from Sigma. A two-dimensional gel was run with some modifications as previously described by the manufacturers using Immobiline Dry Strip Gel, pH range 3–10 (Amersham Biosciences). Crude venom 100 μg collected in water was diluted in rehydration solution (6 M urea, 2 M thiourea, 2% w/v CHAPS, 1% IPG buffer, and a trace of bromophenol blue) and applied to an IEF strip. The second dimension was carried out using 8–18% linear gradient SDS-PAGE under non-reducing conditions. Gel was stained with the silver method as described by Heukeshoven and Dernick (1986).

Histological Methods for Light Microscopy

Kidneys were fixed in modified Carnoy's fixative (5% acetic acid instead of 10% as originally proposed) for 3 hr. After fixation, tissues were processed for histology, embedded in paraffin, and cut into 4-μm sections. The sections were stained with hematoxylin and eosin, acid-Schiff (PAS), and silver and by the method of Rosenfeld (Culling et al. 1985; Beautler et al. 1995).

Transmission Electron Microscopy

Kidneys were fixed with modified Karnovsky's fixative (without calcium chloride and with glutaraldehyde 2.5%) (Karnovsky 1965) for 2 hr, washed in 0.1 M cacodylic acid buffer, pH 7.3, postfixed in 1% OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 hr, dehydrated with ethanol and propylene oxide, embedded in Epon 812, contrasted with uranyl acetate and lead citrate, and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV (Peabody, MA).

Immunofluorescence and Fluorescence Cytochemistry

For immunofluorescence microscopy, kidney tissues were fixed with 2% formaldehyde in PBS for 30 min at 4C, incubated with 0.1 M glycine for 3 min, and blocked with PBS containing 1% BSA for 1 hr at room temperature (RT). Histological sections were incubated for 1hr with specific antibodies raised against laminin (0.33 μg/ml), type IV collagen (1:40), and venom toxins (2.0 μg/ml) as described above. The sections were washed three times with PBS, blocked with PBS containing 1% BSA for 30 min at RT, and incubated with fluorescein- or rhodamine-conjugated anti-IgG secondary antibodies (Chemicon) at RT for 40 min. After washing with PBS, samples were observed under a confocal

◄

Figure 2 Ultrastructural findings of kidneys from venom-treated mice. Transmission electron micrographs of cross-sectioned kidneys exposed to the venom. **(A)** General view of glomerular structures showing alterations of foot processes (black arrow), basement membrane (white arrowhead), and capillary endothelial cells (white arrow). Magnification ×10,000. Comparison of glomerular filter structure between non-injected **(B)** and venom-treated mice **(C)** shows loss of foot processes (black arrows), alteration of filtration slits (white arrow), and fenestrated endothelium as well as reduced thickness of the basement membrane (white arrowheads). Magnification ×20,000. **(D)** Analysis of tubule epithelial cells shows signs of damage such as increased numbers of cytoplasmic electron-dense bodies (white arrow), mitochondrial injuries (black arrow), and deposition of amorphous substances in the tubule lumen (black arrowhead), although the nucleus is preserved (white arrowhead). Magnification ×10,000. **(E)** Details of tubule epithelial cells show blebs in the cytoplasmic membrane (arrows), providing further evidence of cell necrosis. Magnification ×12,000. **(F)** The enclosure of an organelle originating an autophagosome (arrow). Magnification ×15,000. **(G,H)** Details showing deranged and vacuolated mitochondria (arrows). Magnification ×20,000. **(I)** Details of the cytoplasm of a tubule epithelial cell where abundant smooth endoplasmic reticulum can be observed (arrowhead). Magnification ×12,000. **(J)** Again, another sign of cell damage shown by increased smooth endoplasmic reticulum (arrowhead) and an autophagosome of a damaged organelle (arrow). Magnification ×15,000. **(K)** Accumulation of electron-dense bodies in the cell cytoplasm (arrowhead). Magnification ×15,000.

fluorescence microscope (Confocal Radiance 2,100; BioRad, Hercules, CA) coupled to a Nikon–Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division; Melville, NY). For nuclear fluorescence cytochemistry, samples of renal tissue were incubated with DAPI (O.5 μg/mL diluted in PBS) for 5 min (Molecular Probes). The samples were washed and observed under a confocal microscope as above. For antigen competition assay, the immunofluorescence protocol was the same as described above except that the hyperimmune serum to venom toxins was incubated previously for 1 hr with 50 μg/ml of crude venom diluted in PBS. Then the mixture was incubated with renal biopsies identically as above.

Results

Histopathological Findings in Kidneys from Mice That Received *L. intermedia* Venom

To obtain information about the degree of renal damage caused by *Loxosceles* venom, mice were exposed IP to *L. intermedia* venom for 4 hr. As shown in Figure 1, the effects of the venom on the kidney revealed a complex pattern of nephrotoxicity. Light microscopy and histochemistry techniques revealed glomerular alterations consisting of collapse of basement membranes and deposition of hyaline and eosinophilic masses in the glomeruli (hyalinization), as well as focal intraglomerular erythrocytes. Tubule alterations were detected by accumulation of eosinophilic material in the lumen of cortical and medullary tubules, interstitial edema with extravascular red blood cells, and vacuolar degeneration of proximal and distal tubules. Morphometric analysis (Figure 1A) showed that 75–80% of the kidney area was affected by the venom. No glomerular or tubule leukocyte infiltration was detected.

Ultrastructural Evidence of Renal Injuries

To improve the evidence of the activity of brown spider venom toxin on kidney structures and to better understand these alterations, biopsies from venom-treated mice were studied by transmission electron microscopy. As shown in Figure 2, additional evidence of glomerular damage was provided by structural signs of podocyte cytotoxicity as shown by many disturbed foot processes (pedicels) and filtration slits. At the capillary level, fenestrated endothelial cells also showed signs of alterations, such as detachment from subendothelial basement membrane and structural signs of disorganization of the fenestra. The basement membrane was decreased compared to the control group. Morphometric analysis of glomerular basement membranes showed a general reduction of approximately 24%. Tubule injuries included deposition of amorphous electron-dense material in the distal tubule lumen. Necrotic destruction of tubule epithelial cells can be visualized by vacuolization and blebs on the cytoplasmic membrane, diffuse destruction of mitochondria, prominent smooth endoplasmic reticulum, and autophagosomes indicating necrotic cell destruction.

Table 1 Blood counts[a,b]

	WBC (10^3/μl)	RBC (10^6/μl)	HGB (g/dl)	HCT (%)	PLT (10^3/μl)[c]
Controls					
C1	5.0	9.6	15.7	47.4	682
C2	5.3	7.2	12.4	36.2	869
C3	3.0	7.7	12.9	35.7	818
C4	6.5	8.1	13.2	37.3	652
C5	4.1	8.0	14.2	40.2	900
Venom					
V1	5.3	8.5	13.3	36.7	265
V2	7.5	8.2	13.7	41.2	395
V3	3.8	8.1	13.0	39.8	261
V4	6.2	7.8	12.5	39.2	244
V5	6.6	8.0	12.5	38.6	418

[a]WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets.
[b]Significance is defined as $p<0.05$, Student's *t*-test.
[c]PLT significant differences ($p<0.0001$).

Laboratory Investigations After Administration of Brown Spider Venom

Our initial approach to study the involvement of venom-induced hemolysis in renal pathogenesis in the experimental model used was to determine blood cell counts, urinalysis, C3 complement, and serum urea and to compare venom-treated mice with a control group. As shown in Table 1, neither red blood cell count, hemoglobin, or hematocrit values showed significant differences between venom-treated and control groups, suggesting that the venom was unable to lyse mouse erythrocytes in vivo under the conditions assayed. In addition, no significant evidence of hemoglobinuria or of the presence of red blood cells in urine was observed in venom-treated mice (Table 2), indicating that hemolysis was not taking place. Table

Table 2 Urinalysis[a]

	Hemoglobinuria	Hematuria
Controls		
C1	−	−
C2	−	−
C3	−	−
C4	−	−
C5	−	−
Venom		
V1	−	+
V2	−	−
V3	−	−
V4	+	−
V5	−	−

[a]−, negative; +, trace.

Table 3 Complement (C_3)

	C_3 concentration (mg/dl)[a]
Controls	
C1	21.8
C2	16.7
C3	20.6
C4	20.5
C5	23.9
Venom	
V1	19.6
V2	16.6
V3	26.3
V4	19.0
V5	17.3

[a]Significance is defined as $p<0.05$, Student's *t*-test.

3 shows no significant differences in C3 complement component between venom-treated and control mice, demonstrating that the venom was unable to decrease this serum molecule in vivo under the conditions used. On the other hand, as shown in Table 4, serum urea was significantly increased in venom-treated mice compared to control group. Finally, renal biopsies from mice treated with the brown spider venom were negative for iron when submitted to histochemistry by the Prussian blue method (Figure 3), supporting the idea that venom-induced hemolysis was not involved in nephrotoxicity in the model used.

Evidence That *Loxosceles* Venom Toxins Bind Directly to Kidney Structures

To study the molecular pathways of venom pathogenesis in the kidney, we investigated the possibility of a direct interaction of venom toxins with kidney structures by submitting renal biopsies from venom-treated and control mice to immunofluorescence using affinity-purified hyperimmune IgG that reacts with venom toxins (Montero 2003). As shown in Figure 4, we

Table 4 Urea[a]

	Urea concentration[b] (mg/dl)
Controls	
C1	41.0
C2	37.0
C3	42.0
C4	36.0
C5	46.0
Venom	
V1	60.0
V2	59.0
V3	63.0
V4	70.0
V5	46.0

[a]Significance is defined as $p< 0.05$, Student's *t*-test.
[b]Significant differences ($p=0.0021$).

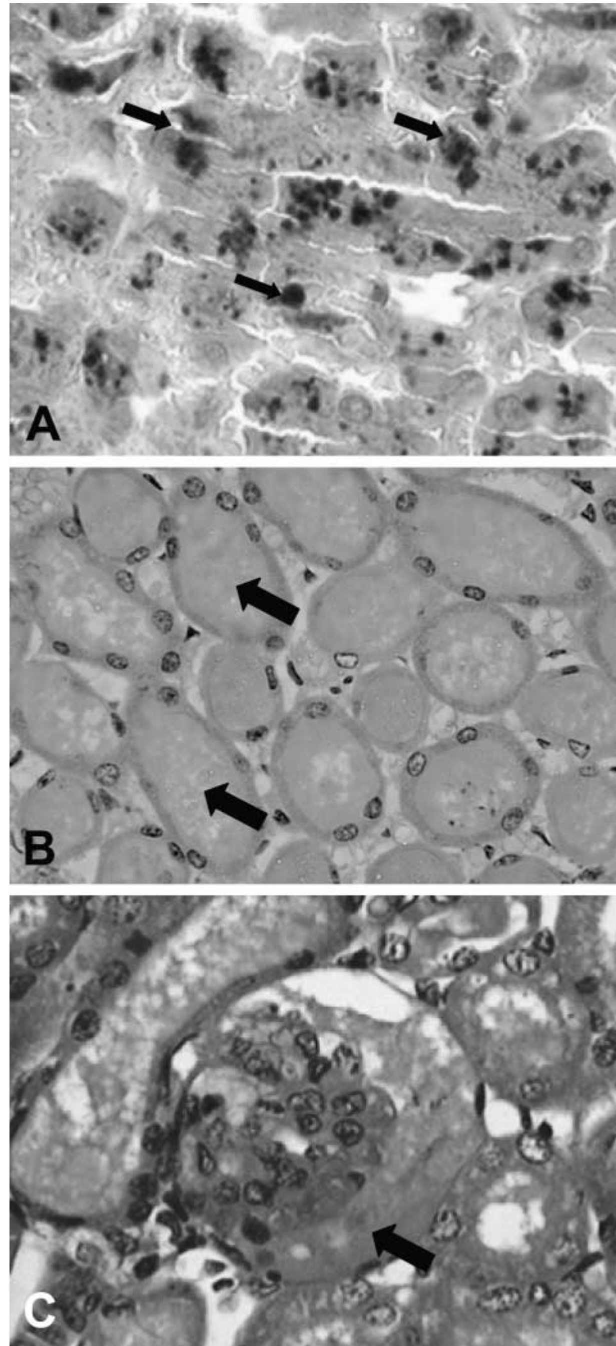


Figure 3 Sections from venom-treated kidneys stained with Prussian blue. *L. intermedia* venom-treated sections from mouse kidneys were stained with Prussian blue plus hematoxylin to detect iron (hemoglobin) deposition along the renal structures. Although deposition of proteinaceous materials in the tubule lumen (arrows) (**B**) and glomerular Bowman's space (arrow) (**C**) can be seen, the results were negative for iron (hemoglobin) because there was no precipitated blue material as found in the positive control group by using a liver section from a normal rabbit (arrows) (**A**). Magnification ×400.

The Journal of Histochemistry & Cytochemistry

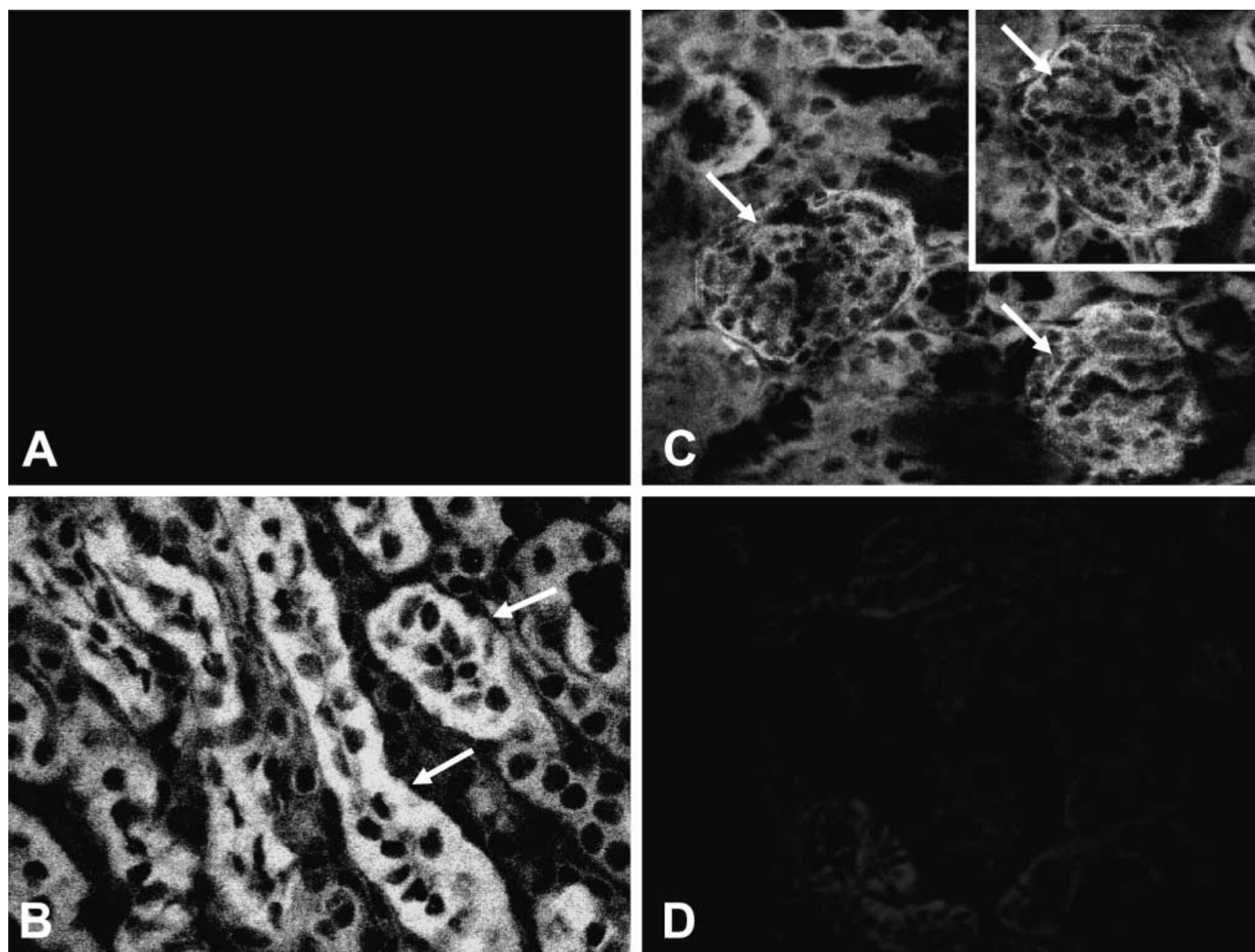


Figure 4 Confocal immunofluorescence microscopy analysis of kidney sections from venom-treated mice. Cross-sectioned kidneys immunolabeled with purified antibodies against *L. intermedia* venom toxins. (**A**) Section of a kidney from the control group that did not receive the venom. (**B,C**) Kidney sections from venom-treated mice, respectively showing regions rich in tubules and glomeruli (arrows) positive for binding of toxin antibodies. (**D**) Result of an antigen competition assay in which antibodies to venom toxins were previously incubated with crude venom in solution. A kidney section from a venom-treated mouse was exposed to the reaction mixture under conditions identical to those described above. The results indicated a highly representative decrease in immunolabeling and confirmed toxins as "planted antigens" in kidney structures from venom-treated mice. Magnifications ×200.

found that hyperimmune IgG produced a positive reaction in renal biopsies from venom-treated mice but did not react with biopsies from normal mice by immunofluorescence. In addition, confirm the antibody-specific interactions of venom toxins with the kidney, we repeated the same immunofluorescence approach, this time incubating hyperimmune IgG with a medium containing venom in solution and then exposing renal biopsies from venom-treated mice to this mixture (antigen competition assay). We found that soluble venom effectively inhibited binding of antibodies to the kidney, supporting the evidence of venom toxins as "planted antigens" bound to renal structures.

Renal Injuries by *Loxosceles* Venom Resulting from Binding of Toxins to Glomerular and Tubule Cells and Basement Membrane Structures

After confirming the direct binding of venom toxins to the kidney, we examined the possibility that intrinsic kidney components were targets of the toxins. We speculated that toxins can act as "planted antigens" and bind to renal structures as previously reported for several other antigens such as viral or bacterial products and drugs (Barnes 1989; Kerjaschki and Neale 1996; Cotran et al. 1999). We performed double staining and confocal microscopy immunofluorescence reactions on renal biopsies from venom-treated mice using antibodies that react with type IV collagen and

The Journal of Histochemistry & Cytochemistry

laminin (two molecular constituents of basement membranes; see Courtoy et al. 1982 and Rohrbach and Timpl 1993) and antibodies that react with venom toxins as well as fluorescent cytochemistry for chromosomes with DAPI. As shown in Figure 5, there was overlapping fluorescence of reactions using antibodies to laminin or type IV collagen and antibodies to venom toxins. Fluorescence staining cytochemistry of nuclei with the blue fluorescent dye DAPI revealed no overlapping with anti-venom fluorescence pattern. These data support diffuse binding and deposition of venom toxins along the glomerular and tubule cells, with prominent staining mainly in tubule cells and along renal basement membranes.

L. intermedia Venom Is Enriched in Cationic and Low Molecular Mass Proteins with Toxin(s) of Approximately 30 kD Binding to Intrinsic Renal Components

To corroborate the findings described above, we looked for the molecules in the venom involved in noxious influences on renal structures. For this purpose and to study venom toxins' physicochemical properties such as their charge and size, crude venom was submitted to a two-dimensional electrophoresis (isoelectric focusing and gradient SDS-PAGE) that was stained by the highly sensitive monochromatric silver method (Figure 6A). The venom was enriched in basic toxins at physiological pH, with low molecular mass in the range of 35–20 kD. Such cationic and low molecular mass properties might explain the localization of venom toxins in the kidney by interacting with components of the nephron. To strengthen such evidence, renal lysates from venom-treated mice were electrophoresed and immunoblotted with hyperimmune serum to venom toxins (see Materials and Methods). Antigen competition assays were also performed to substantiate the specificity of immune reactions. As shown in Figure 6B, we detected a band at 30 kD, in the venom-treated lysate, which specifically reacted with antibodies against venom toxins, confirming the above results and identifying toxin(s) from *L. intermedia* venom as a direct ligand on renal structures.

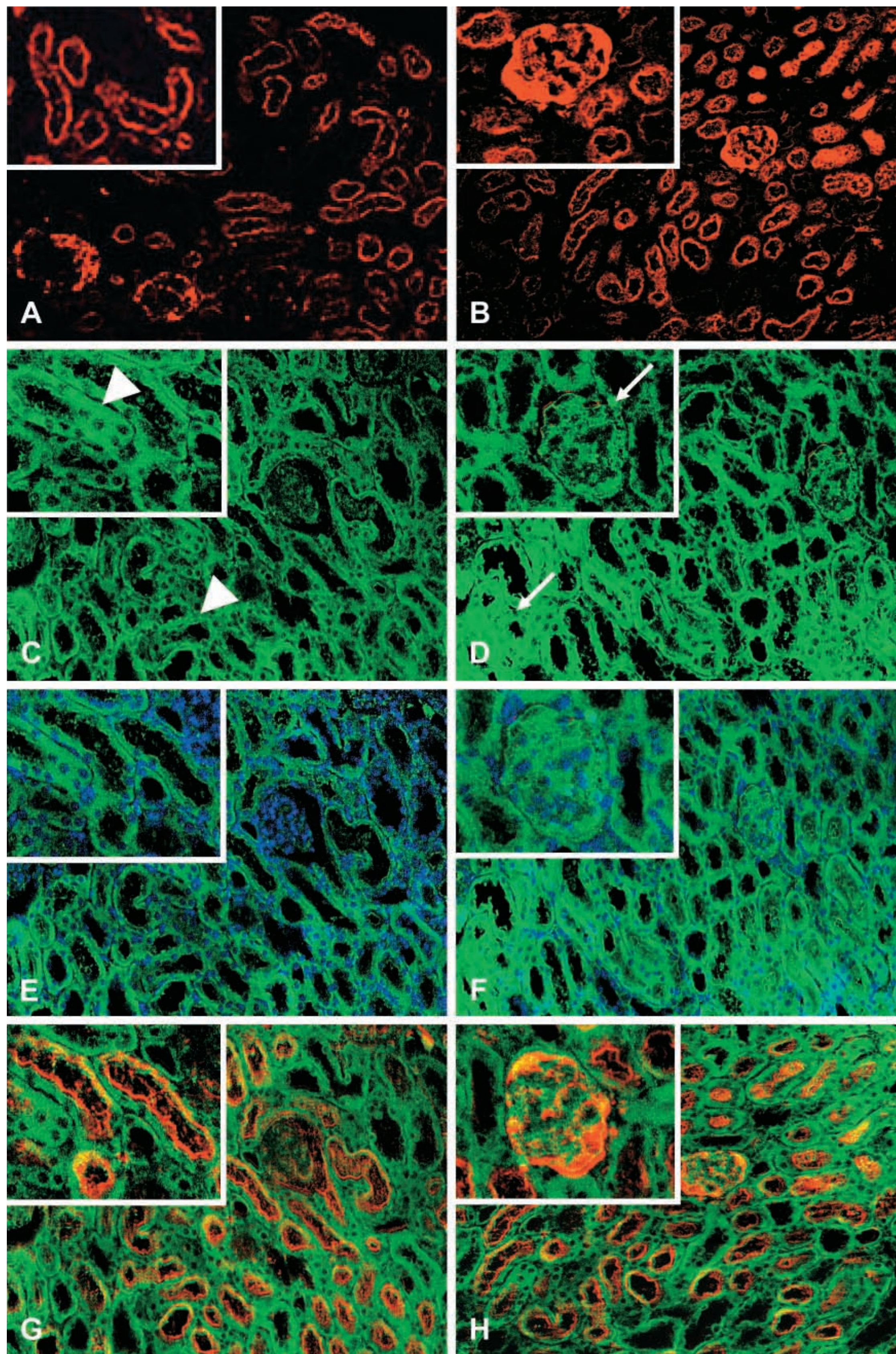
Discussion

Clinical data have shown that envenomation by brown spiders can have a severe nephrotoxic effect (Futrell 1992; Lung and Mallory 2000). This venom nephrotoxicity, although less common than necrotic lesions and gravitational spreading (which are the hallmark signals of poisoning), is generally involved in the complications that occur after spider bites (Futrell 1992; Lung and Mallory 2000). The clinical and laboratory features observed in victims may include hemoglobinuria and proteinuria (Futrell 1992; Williams et al. 1995; Lung and Mallory 2000). According to some specialists, the pathological processes in the kidney may reflect hematologic disturbances such as intravascular hemolysis and disseminated intravascular coagulation caused by envenomation, which may lead to renal failure (Futrell 1992; Williams et al. 1995; Lung and Mallory 2000). However, there is no direct experimental evidence confirming such a hypothesis. On the other hand, renal disorders may also be the consequence of toxic components of the venom that act directly on renal cells or structures such as glomerular and tubule basement membranes, as demonstrated for several venoms (Bjarnason and Fox 1995; Veiga et al. 2000). Experimentally, it was demonstrated that the venom is cytotoxic to rabbit blood vessel endothelial cells (CLPs) (Veiga et al. 2001b) and human umbilical vein endothelial cells (HUVECs) (Patel et al. 1994) in culture and also to endothelial cells from skin blood vessel walls of rabbits intradermally injected with the venom (Zanetti et al. 2002). The venom also acts on EHS (Engelbreth–Holm–Swarm tumor) basement membrane integrity and on purified basement membrane molecules such as entactin, fibronectin, and heparan sulfate proteoglycan (Feitosa et al. 1998; Veiga et al. 2000,2001b) but, again, there is no direct experimental evidence for venom cytotoxicity to renal cell lines or renal basement membrane structures in vivo.

To obtain additional information about the extent of renal damage and the mechanisms of the disorders induced by *L. intermedia* venom, we presented here laboratory and biochemical data and morphological data from examinations of kidney structures from mice exposed to the venom. Histological studies showed that envenomation induced a complex pattern of nephrotoxicity. A general view of renal tissue revealed alterations at the level of glomerular and tubule structures, supporting the nephrotoxic activity of venom toxins. The glomerular damage becomes apparent by the presence of extravascular red blood cells around the glomerular capillaries in Bowman's space. A protein-rich exudate inside Bowman's space confirms some damage at the glomerular level, suggesting loss of vascular integrity. In addition, tubule injuries are supported by the presence of proteinaceous material in their lumen in addition to tubule edema, causing the tubules to be susceptible to ischemic damage caused by occlusion. Ultrastructural features of nephrotoxicity were further supported by glomerular and tubule disorders. The azotemia detected by the increase in serum urea strengthened this evidence.

The mechanism by which *Loxosceles* venom induces nephrotoxic lesions has remained elusive and is currently unknown. In contrast to cutaneous lesions evoked by the venom, in which leukocytes (neutrophils) play an essential role in pathogenesis (Smith and

The Journal of Histochemistry & Cytochemistry



Micks 1970; Futrell 1992; Patel et al. 1994; Ospedal et al. 2002), in the model studied here there was no leukocyte infiltration in the presence of the renal injuries, which were not associated with inflammatory disease.

On the other hand, several case reports have described intravascular hemolysis associated with brown spider envenomation (Murray and Seger 1994; Williams et al. 1995), and many studies have indicated a direct hemolytic activity of *Loxosceles* venom on erythrocytes (Futrell 1992). Such hematological disturbances could be related to renal injuries as secondary agents (Futrell 1992; Williams et al. 1995; Lung and Mallory 2000). We found that, in the model and under the conditions used, despite the renal injuries caused by the venom there was neither a direct hemolytic effect in vivo nor hemoglobin deposition in the kidney structures. These results agree with those described by Futrell (1992), who pointed out the susceptibility of some animal species to venom-induced hemolysis. Human and swine erythrocytes are more susceptible to hemolysis than rabbit and guinea pig erythrocytes. We cannot rule out the involvement of venom hemolysis in the human renal failure evoked by the venom. Nevertheless, because the venom did not induce dermonecrosis (data not shown) or hemolysis in mice and was extremely active against renal integrity, we can speculate about a direct and primary activity of the venom on kidney structures.

We confirmed this possibility by confocal immunofluorescence microscopy using antibodies to venom toxins. We were able to detect toxins as "planted antigens" deposited along the kidney structures of animals exposed to the venom. A competition assay using crude venom toxins in solution blocked the immunofluorescence positivity in the kidney of venom-treated animals, further supporting this evidence and the idea of "planted toxins." The present results agree with several reports indicating the binding of exogenous molecules, such as bacterial products, viral antigens, and drugs, to intrinsic components of renal structures as etiological agents of renal injuries (Kerjaschki and Neale 1996; Cotran et al. 1999). In the model used here, we did not find a role for immune mechanisms in renal injuries. Because the biopsies were collected just 4 hr after exposure to the venom, there was no alteration in C3 complement levels in the serum of venom-treated animals compared to controls, and an immunofluorescence assay with an anti-mouse IgG was negative (data not shown), excluding the possibility of renal deposits of immunoglobulins. Taken together, the above results support the notion that venom toxins act as direct ligands in renal structures and as primary agents, playing a role in renal disorders.

In addition, our data also showed that venom toxins bind to glomerular and tubule cells and basement membranes but do not bind to kidney cell nuclei. This conclusion was based on double-staining immunofluorescence reactions that demonstrated co-localization of venom toxins with basement membrane constituents such as type IV collagen and laminin, but no co-localization along the chromosomes revealed by DAPI. The basement membranes are specialized extracellular matrices involved in several physiological events, especially those dependent on cell adhesion. In the kidney these structures play a role in glomerular filtration during urine formation in addition to organizing podocyte, endothelial, and epithelial cell adhesion (Courtoy et al. 1982; Rohrbach and Timpl 1993; Cotran et al. 1999). The deposition of venom toxins along the renal basement membranes can explain glomerular epithelial cell injury, fenestrated endothelial cell cytotoxicity, hyalinosis, and proteinuria, as well as tubule cell damage. Because some *L. intermedia* venom toxins are proteases, with the ability to degrade basement membrane constituents (as discussed above), such venom injuries can result from loss of renal basement membrane integrity with consequent cytotoxicity to epithelial and endothelial cells and detachment and loss of the glomerular basement membrane charge barrier.

Using two-dimensional electrophoresis, we observed that *L. intermedia* venom is enriched in basic proteins with molecular masses ranging from 35 to 20 kD. Immunoblotting analysis using antibodies to venom proteins identified venom toxin(s) at 30 kD as direct ligands of renal structures. Physicochemical properties

Figure 5 Venom toxin deposition along the glomerular and tubule cells and renal basement membranes detected by confocal double-staining immunofluorescence microscopy or fluorescence cytochemistry. Sections of kidneys from venom-treated mice were incubated with antibodies against basement membrane constituents such as laminin (**A**) or type IV collagen (**B**) and visualized with rhodamine-conjugated antibodies. Similar sections were incubated with antibodies against venom toxins and visualized with fluorescein-conjugated antibodies (**C,D**), respectively (arrows point to glomerular structures and arrowheads show tubule structures). These same sections were cytochemically labeled for nuclei with the blue fluorescent dye DAPI. (**E,F**) Confocal microscopic analysis and overlapping of immunostaining for venom toxins and cytochemistry using DAPI. There was no co-localization of images, supporting the idea that venom toxins do not bind to nuclei of renal cells. On the other hand, overlapping of immunostaining for basement membrane laminin and type IV collagen and immunostaining for venom toxins (**G,H**) respectively, indicated co-localization of toxins with laminin and type IV collagen along glomerular and tubule basement membranes (orange to yellow) as well as glomerular and tubule cells (green). Magnifications ×200; insets ×600).

The Journal of Histochemistry & Cytochemistry

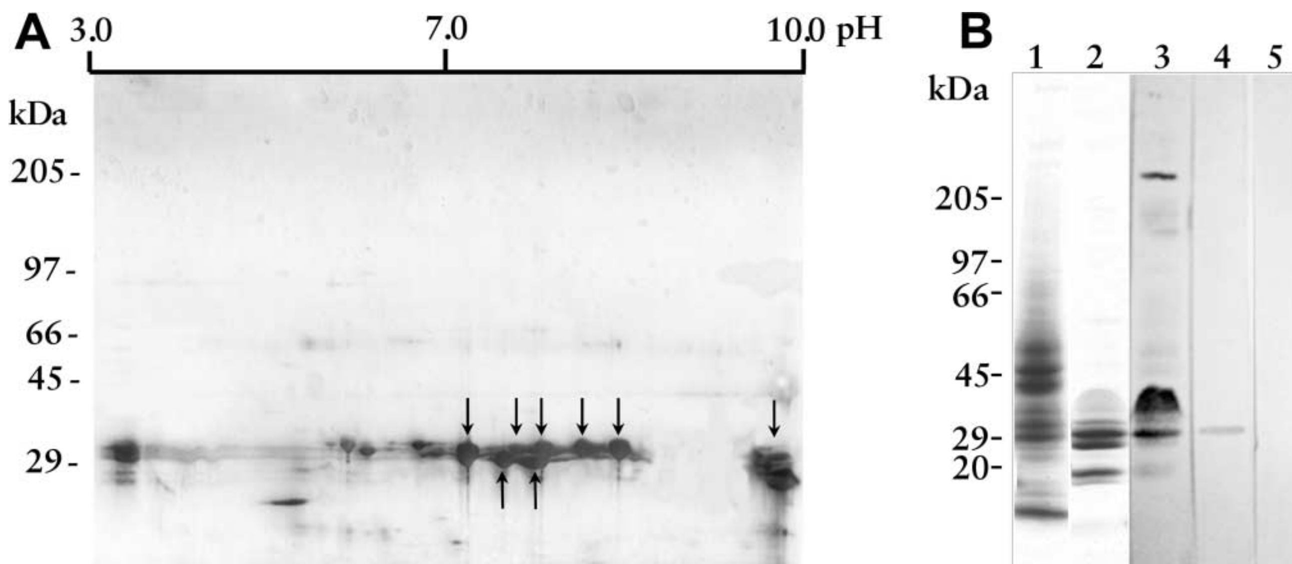


Figure 6 Identification of 30-kD venom toxin(s) as direct ligands of renal structures. Crude *L. intermedia* venom was separated by two-dimensional electrophoresis (isoelectric focusing and linear gradient 8–18% SDS-PAGE) under non-reducing conditions. The gel was stained by the silver method. Molecular mass protein standard positions are shown at left and pH gradient at the top. Arrows point to cationic proteins. (**B**) Renal extract from venom-treated mice (Lanes 1, 4, and 5) or *L. intermedia* Venom (Lanes 2 and 3) was separated by linear gradient 3–20% SDS-PAGE under non-reducing conditions. The gel was stained by the Coomassie Blue method (Lanes 1 and 2) or transferred to a nitrocellulose membrane that was exposed to purified antibodies against venom toxins (Lanes 3 and 4), or antibodies were previously incubated with crude venom in solution and the mixture was exposed to nitrocellulose membrane (Lane 5) in an antigen competition assay. Protein standard masses are shown at left.

such as the molecular charge and size of "planted antigens" along the kidney are very important factors that affect the interaction of these antigens along the renal basement membranes, especially with glomerular structures (Cotran et al. 1999). Highly cationic molecules (as is the case for *Loxosceles* venom toxins) tend to bind to glomerular basement membrane anionic sites (proteoglycans) (Cotran et al. 1999). In addition, the glomerular barrier function is dependent on the molecular mass of proteins. Molecules with mass larger than 70 kD are less permeable than low molecular mass proteins (as is the case for a large number of *Loxosceles* venom toxins) (Cotran et al. 1999). This charge and size properties of venom toxins can account for their binding to glomerular basement membranes in a first step and later to tubule structures accumulated in tubule epithelial cells.

On the basis of the above findings, we have identified a possible cellular and molecular mechanism for the nephrotoxicity that occurs after envenomation by *Loxosceles* spiders. Although the renal injuries occurring after envenomation can be increased by dermonecrotic products and hemolysis, we conclude that *Loxosceles* venom toxins are direct and potentially nephrotoxic agents.

We hope that this report will bring some insight into loxoscelism, opening the possibility for a rational basis for therapy after brown spider bites.

Acknowledgments

Supported by grants from CNPq, CAPES, Fundação Araucária-PR, FUNPAR-UFPR, and Parana Tecnologia.

We acknowledge LIPAPE for spider capture and venom extraction and Prof Marco A. F. Randi (Department of Cell Biology, Federal University of Paraná), who helped us during morphometic analysis.

Literature Cited

Barnes JL (1989) Glomerular localization of platelet secretory proteins in mesangial proliferative lesions induced by habu snake venom. J Histochem Cytochem 37:1075–1082

Beautler E, Litchman MA, Coller BS, Kipps TJ (1995) Williams Hematology. New York, McGraw–Hill

Bjarnason JB, Fox JW (1995) Snake venom metalloendopeptidases: reprolysins. Methods Enzymol 248:345–368

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cotran RS, Kumar V, Robbins SL (1999) Pathologic Basis of Disease. Boston, Elsevier

Courtoy PJ, Timpl R, Farquhar MG (1982) Comparative distribution of laminin, type IV collagen, and fibronectin in the rat glomerulus. J Histochem Cytochem 30:874–886

Culling CFA, Allison RT, Barr WT (1985) Cellular Pathology Technique. London, Butterworth

da Silveira RB, Filho JFS, Mangili OC, Veiga SS, Gremski W, Nader HB, Dietrich CP (2002) Identification of proteases in the extract of venom glands from brown spiders. Toxicon 40:815–822

Elston DM, Eggers JS, Schmidt WE, Storrow AB, Doe RH, McGlasson D, Fischer JR (2000) Histological findings after brown recluse spider envenomation. Am J Dermatopathol 22:242–246

Feitosa L, Gremski W, Veiga SS, Elias MCQB, Graner E, Mangili OC, Brentani RR (1998) Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in brown spider (Loxosceles intermedia) venom. Toxicon 36:1039–1051
Futrell J (1992) Loxoscelism. Am J Med Sci 304:261–267
Gates CA, Rees RS (1990) Serum amyloid P component: its role in platelet activation stimulated by sphingomyelinase D purified from the venom of brown recluse spider (Loxosceles reclusa). Toxicon 28:1303–1315
Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press
Henry JH (2001) Clinical Diagnosis and Management by Laboratory Methods. Philadelphia, WB Saunders
Heukeshoven J, Dernick R (1986) Silver staining of proteins. In Radola BJ, ed. Elektrophorese Forum '86. Technische Univerität München, Germany, 22–27
Kaplan LA, Pesce AJ (1996) Clinical Chemistry. Theory, Analysis, Correlation. St Louis, Mosby
Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A
Kerjaschki D, Neale TJ (1996) Molecular mechanisms of glomerular injury in rat experimental membranous nephropathy (Heymann nephritis). J Am Soc Nephrol 7:2518–2526
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685
Lung JM, Mallory SB (2000) A child with spider bite and glomerulonephritis: a diagnostic challenge. Int J Dermatol 39:287–289
Montero C (2003) The antigen-antibody reaction in immunohistochemistry. J Histochem Cytochem 51:1–4
Murray LM, Seger DL (1994) Hemolytic anemia following a presumptive brown recluse spider bite. Clin Toxicol 32:451–456
Ospedal KZ, Appel MH, Neto JF, Mangili OC, Veiga SS, Gremski W (2002) Histopathological findings in rabbits after experimental acute exposure to the Loxosceles intermedia (brown spider) venom. Int J Exp Pathol 83:287–294
Patel KD, Modur V, Zimmerman GA, Prescott SM, McIntyre TM (1994) The necrotic venom of brown recluse induces dysregulated endothelial cell-dependent neutrophil activation. J Clin Invest 94:631–642
Rohrbach DH, Timpl R (1993) Molecular and Cellular Aspects of Basement Membranes. San Diego, Academic Press
Smith WC, Micks WD (1970) The role of polymorphonuclear leukocytes in the lesion caused by the venom of the brown spider, Loxosceles reclusa. Lab Invest 22:90–93
Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76:4350–4354
Veiga SS, Feitosa L, Santos VLP, de Souza GA, Ribeiro AS, Mangili OC, Porcionatto MA, et al. (2000) Effect of Loxosceles intermedia (brown spider) venom on basement membrane structures. Histochem J 32:397–408
Veiga SS, Zanetti VC, Braz A, Mangili OC, Gremski W (2001a) Extracellular matrix molecules as targets for brown spider venom toxins. Braz J Med Biol Res 34:843–850
Veiga SS, Zanetti VC, Franco CRC, Trindade ES, Porcionatto MA, Mangili OC, Gremski W, et al. (2001b) In vivo and in vitro cytotoxicity of brown spider venom for blood vessel endothelial cells. Thromb Res 102:229–237
Williams ST, Khare VK, Johnston GA, Blackall DP (1995) Severe intravascular hemolysis associated with brown recluse spider envenomation. Am J Clin Pathol 104:463–467
Wright RP, Elgert K, Campbell BJ, Barret JT (1973) Hyaluronidase and esterase activities of the venom of the poisonous brown recluse spider. Arch Biochem Biophys 159:415–426
Zanetti VC, da Silveira RB, Dreyfuss JL, Haoach J, Mangili OC, Veiga SS, Gremski W (2002) Morphological and biochemical evidence of blood vessel damage and fibrinogenolysis triggered by brown spider venom. Blood Coag Fibrinol 13:135–148

The Journal of Histochemistry & Cytochemistry

4. DISCUSSÃO

O trabalho de revisão (da Silva et al., 2004) permitiu que todas as publicações sobre loxoscelismo entre 1993 e 2003 fossem reunidos em um único artigo, facilitando o acesso ao conhecimento acumulado ao longo destes 10 anos.

As aranhas do gênero *Loxosceles* tem como estrutura característica o cefalotórax (Futrell, 1992), vivem entre 3 a 7 anos (Andrade et al., 2000), têm como melhor característica de identificação a disposição dos pares de olhos (Vetter and Visscher, 1998). São aranhas sedentárias e noturnas (Andrade et al., 1999) as teias são irregulares lembrando um aspecto de algodão (Futrell, 1992), a morfologia da teia é única entre as aranhas (Coddington et al., 2001). A aranha marrom suporta temperaturas que variam entre 8 à 43ºC e sobrevivem até meses sem água e comida (Futrell, 1992) e preferem presas mortas (*L. reclusa*) às vivas (Sandidge, 2003), estas características explicam como a aranha marrom consegue proliferar em condições desfavoráveis. São aranhas não agressivas que preferem viver em lugares escuros (Futrell, 1992; Málaque et al., 2002). A fêmea produz uma quantidade maior de veneno que os machos, isto pode estar relacionado com a diferença de tamanho entre eles, o veneno da fêmea causa uma lesão dermonecrótica mais grave em coelhos (Oliveira et al., 1999). A quantidade de veneno injetada, por ocasião do acidente com a aranha, é muito pequena em torno de 4 μl e contém entre 65 a 100 μg de proteína (Sams et al., 2001a). O componente tóxico do veneno da *L. intermedia* é a proteína dermonecrótica (35kDa) chamada de proteína F35 (Tambourgi et al., 1995). A morfologia e a celularidade das glândulas do veneno sugerem um mecanismo holócrino de controle da secreção do veneno (Santos et al., 2000).

Na década de 90 houve um aumento significativo no número de acidentes com a aranha marrom e um grande aumento na infestação intra-domiciliar (Ribeiro et al., 1993). O loxoscelismo é particularmente proeminente em Curitiba com cerca de 2.000 casos notificados por ano (Málaque et al., 2002). A *L. intermedia* é a aranha marrom que predomina na região urbana do Paraná e

Santa Catarina (Fischer, 1994; Mattosinho et al., 1997). A fertilidade da *L. laeta* é maior que a da *L. intermedia* mas a expansão da *L. intermedia* não está correlacionada com alta fertilidade e sim com alterações ambientais na região sul (Andrade et al., 2000). Os acidentes com a aranha marrom ocorrem nos meses mais quentes do ano, entre outubro e março, e geralmente quando as pessoas estão se vestindo, dormindo ou em afazeres domésticos. A maioria das picadas ocorrem na coxa, tronco, braço e panturrilha com casos clínicos de gravidade leve, 1% são severos (Sezerino et al., 1998; Málaque et al., 2002; Schenone, 1996; Secretaria de Saúde de Curitiba, 2002).

Biópsias do sítio da picada pela aranha marrom, em humanos, mostram infiltrado inflamatório, trombose, hemorragia, dermatite, inflamação aguda, enduramento da região lesionada, eritema e necrose liquefativa da epiderme e derme (Futrell, 1992; Yiannias and Wilkelmann, 1992). A histopatologia da lesão de coelhos expostos ao veneno da *L. intermedia* mostra deposição de fibrina intravascular e trombose de vasos da derme, degeneração da parede dos vasos sangüíneos e infiltrado e agregação de células inflamatórias. A musculatura esquelética está infiltrada por polimorfonucleares com edema e mionecrose de algumas miofibrilas. Destruição da epiderme, hemorragia maciça e necrose de colágeno próximo a epiderme (Ospedal et al., 2002). O principal achado histopatológico após 14 dias de exposição ao veneno da aranha marrom, usando como modelo animal coelhos, foi um infiltrado de células inflamatórias, necrose tecidual coagulativa e vasculite (Elston et al., 2000). A histopatologia de camundongos injetados com o veneno da *L. reclusa* mostrou vasculite após 2 horas de exposição (Sunderkötter et al., 2001). A análise histológica de alguns orgãos de camundongos após o envenenamento com várias doses do veneno da *L. intermedia* mostrou alterações bastante evidentes restritas ao tecido renal (Tambourgui et al.,1998). Biópsias renais de camundongos expostos ao veneno da *L. intermedia* mostraram hialinização e eritrócitos nos espaços de Bowman's, colapso glomerular, citotoxicidade de células epiteliais tubulares e deposição de material eosinofílico no lúmen tubular (Luciano et al., 2004). O veneno da *L. intermedia* pode romper estruturas da membrana basal demonstrado pela

atividade do veneno sobre as células EHS (Engelbreth-Holm-Swarm) as quais são degradadas e fragmentadas (Veiga et al., 2000,2001).

Várias moléculas protéicas e enzimáticas foram descritas no veneno da aranha marrom , como: fosfatase alcalina, hialuronidase, 5'ribonucleotideo fosfohidrolase, esfingomielinase D, loxnecrogina A e B, proteína recombinante LiD1, Loxolisina A e B, serinoproteases e metaloproteases. O mecanismo pelo qual o veneno causa o quadro dermonecrótico e sistêmico não está esclarecido. Sabe-se que animais como camundongos e ratos não desenvolvem dermonecrose, enquanto humanos, cobaias, suínos e coelhos sim (Hobbs and Yealy, 1994). A atividade nociva do veneno pode ser atribuída as toxinas proteolíticas, as quais degradam a matriz extracelular e rompem estruturas da membrana basal causando hemorragia e espalhamento gravitacional, principais características da lesão dermonecrótica. A patogenia do quadro sistêmico envolve a coagulação intravascular disseminada e a insuficiência renal aguda (Feitosa et al., 1998; Veiga et al., 1999, 2000a, 2001; Luciano et al., 2004). Há evidências da participação dos polimorfonucleares na dermonecrose (Smith and Micks, 1970; Futrell, 1992). A participação dos polimorfonucleares parece ser dependente da ativação das células endoteliais que via citocinas levam a uma ativação neutrofílica desregulada (Patel et al., 1994). O veneno da *L. laeta* tem uma ação hemolítica direta a qual é cálcio e complemento dependente, mas anticorpo independente (Bravo et al., 1993). O mecanismo de indução da hemólise complemento dependente seria a ativação de uma metaloprotease endógena ativada pela esfingomielinase, que clivaria a glicoforina, tornando os eritrócitos susceptíveis a hemólise pelo complemento (Tambourgui et al., 2000).

O Instituto Butantan (São Paulo) desenvolveu um antisoro, em cavalos, a partir de uma mistura de venenos da *L. gaucho*, *Phoneutria nigriventer*, *Tityus serrulatus* e *T. bahiensis* que não inibe a hemólise de eritrócitos humanos pela *L. laeta*, mas inibe o desenvolvimento da lesão dermonecrótica sendo a inibição tempo dependente (Bravo et al., 1994). Fragmentos Fab de anticorpos policlonais obtidos a partir de coelhos, usando o veneno da *L. deserta*, foram usados de modo terapêutico injeções 4 horas antes do envenenamento inibiram a

inflamação dérmica e a necrose em animais de laboratório (Gomez et al., 1999). Um antiveneno anti-*L. intermedia* produzido em cavalos foi comparado com o antisoro do Instituto Butantan, com a finalidade de avaliar a eficiência em bloquear o desenvolvimento da lesão dermonecrótica e as atividades letais do veneno. O antiveneno anti-*Loxosceles intermedia* foi mais eficiente (Braz et al., 1999). Na Argentina foi desenvolvido um antisoro monovalente anti-*L. laeta*, em cavalos, a partir do homogenizado das glândulas do veneno e a inibição dos efeitos tóxicos em camundongos e da lesão dermonecrótica em coelhos foi semelhante ao antisoro do Instituto Butantan e do antisoro anti-*Loxosceles* do Instituto Nacional de Saúde do Peru (Roodt et al., 2002).

O diagnóstico do loxoscelismo dificilmente é baseado na identificação da aranha, porque os pacientes não trazem a aranha, baseia-se na epidemiologia, nos achados históricos e em sinais clínicos ou sintomas (Wright et al.,1997; Vetter, 1999; Málaque et al., 2002). Um teste de hemaglutinação passiva foi desenvolvido especificamente para diagnosticar o envenenamento por *L. reclusa* usando uma coleção do exsudato da lesão da pele de cobaias. A sensibilidade do teste foi de 90% até 3 dias após a injeção do veneno e a especificidade foi de 100%. Um teste de ELISA sanduiche foi desenvolvido para detectar o veneno da *L. intermedia*, *L. gaucho* e *L. laeta* em camundongos, mas foi capaz, também, de detectar antígenos circulantes no soro de pacientes envenenados pela *L. intermedia* (Chávez-Olórtegui et al., 1998). Foi desenvolvido um teste de imunoensaio de competição usando anticorpos policlonais anti-*Loxosceles*, a partir de coelhos, utilizado para análise de biópsias e de pelos retirados da lesão cutânea de pacientes picados pela *L. deserta*. O veneno pode ser detectado nas amostras analisadas até 4 dias após o envenenamento (Miller et al.,2000). Um teste similar ao ELISA foi usado para a detecção do veneno da *Loxosceles* no cabelo, aspirado e biópsias (Gomez et al., 2002; Krywko and Gomez, 2002). Todos estes testes são experimentais nenhum está disponível comercialmente.

O tratamento do loxoscelismo é muito variado tendo sido descrito a fentolamina, heparina, nitroglicerina tópica, ciproheptadina e oxigênio hiperbárico

como terapia, as quais apesar de descritas são inconclusivas e o seu uso não é recomendado (Futrell, 1992; Wendell, 2003). A terapia estabelecida é a dapsona, ácido acetisalicílico, eritromicina e cefalosporinas, gelo, evitar atividades estressantes e calor, quando necessário cirurgia (Futrell, 1992; Merigian and Blaho, 1996; Goddard, 1998). Em Curitiba o tratamento estabelecido é a prednisona, dapsona e o soro anti-*Loxosceles* feito na CPPI (Centro de Produção de Produtos Imunobiológicos, Secretaria de Estado da Saúde). O antisoro só é administrado em casos severos de loxoscelismo viscerocutâneo. Quando a aranha é identificada como *L. intermedia* ou *L. laeta* o tratamento com prednisona deve ser iniciado, mesmo na ausência da lesão dermonecrótica, o paciente deve ser monitorado durante as primeiras 36 horas e a soroterapia será utilizada se o paciente desenvolver a forma sistêmica. No quadro cutâneo severo há indicação de utilizar a dapsona com o antisoro (Secretaria Municipal da Saúde de Curitiba, 2002). Se os sinais do quadro sistêmico não iniciam dentro de 24 horas fica estabelecido o diagnóstico de quadro cutâneo e ele é de bom prognóstico. Pacientes com sintomas de loxoscelismo sistêmico tem bom prognóstico se sobrevivem as primeiras 48 horas (Schenone, 2003).

Produtos de biotecnologia têm sido obtidos a partir do veneno da aranha marrom. Um produto chamado ARACHnase (Hemostasis Diagnostics International Co.,Denver, CO, USA) que é um plasma normal com veneno da *L. reclusa*, é utilizado como controle positivo para a pesquisa do anticoagulante lúpico. Os antisoros produzidos são bioprodutos terapêuticos (Barbaro et al., 1994, 1996; Roodt et al., 2002; Secretaria de Saúde de Curitiba, 2002). Foram produzidos anticorpos monoclonais contra a toxina dermonecrótica da *L. gaucho*, o qual neutraliza o efeito dermonecrótico e a atividade letal do veneno da *L. gaucho* (Guilherme et al., 2001). Os anticorpos monoclonais desenvolvidos contra as toxinas do veneno da aranha marrom não são somente úteis para a aplicação terapêutica, podem ser utilizados na purificação e triagem das toxinas do veneno. É muito difícil obter quantidades significativas de veneno por eletrochoque ou por extração das glândulas de veneno, a clonagem e a expressão de toxinas recombinantes permitem a obtenção de grandes quantidades das toxinas do

veneno. O uso de proteínas recombinantes é uma ferramenta biotecnológica muito importante, tanto no esclarecimento dos mecanismos do loxoscelismo como na aplicação terapêutica. A clonagem e a expressão das proteínas dermonecróticas e do fator hemolítico dependente de complemento foi obtida a partir de biblioteca de cDNA do veneno extraído das glândulas da *L. laeta*. A proteína recombinante tem propriedades biológicas semelhantes ao veneno total e com as esfingomielinases purificadas (Pedrosa et al., 2002). A clonagem e a expressão de uma proteína recombinante foi obtida a partir de biblioteca de cDNA de glândulas do veneno da *L. intermedia*, a proteína recombinante foi chamada de LiD1 (Kalapothakis et al., 2002). A LiD1 foi utilizada para pré-imunizar coelhos e camundongos e neutralizou os efeitos letais e dermonecróticos do veneno total da *L. intermedia*. A LiD1 abre a possibilidade de utilizar proteínas recombinantes não tóxicas para a produção de anticorpos neutralizantes e o desenvolvimento de vacina (Araujo et al., 2003).

No artigo (da Silva et al., 2003) o objetivo foi estudar as alterações celulares ocorridas na medula óssea e no sangue periférico de coelhos, modelo animal, após exposição aguda ao veneno da *L. intermedia*. A finalidade do trabalho foi estabelecer um paralelo entre as alterações da medula óssea e sangue periférico com as histopatológicas no sítio inflamatório (lesão dermonecrótica). Outra finalidade foi a possibilidade de encontrar um produto biotecnológico, dependendo dos resultados obtidos, que pudesse atuar em alterações patológicas da medula óssea. O veneno da aranha marrom dissemina-se na corrente circulatória e chega a vários orgãos, entre eles, provavelmente, a medula óssea. O coelho foi escolhido como modelo animal porque as alterações ocorridas neste são semelhantes as ocorridas em humanos. O trabalho mostrou que a série vermelha, em sangue periférico, não foi afetada pelo veneno da aranha-marrom. A prova disto é que não houve mudança significativa na contagem de eritrócitos, determinação da hemoglobina e volume globular durante os trinta dias de acompanhamento. O veneno não causou alteração morfológica eritrocitária. O coelho mostra uma policromatofilia em sangue periférico moderada (++), o que corresponde a uma intensa atividade medular, confirmada pela presença de eritroblastos em medula óssea nos controles. Foi interessante notar

que após 12, 24 horas e 05 dias ocorreu uma diminuição significativa de eritroblastos na medula óssea sem que isto comprometesse o sangue periférico (figura 1). Um efeito mitogênico fazendo com que houvesse uma maior diferenciação dos eritroblastos para a manutenção dos níveis da série vermelha no sangue periférico, uma ação direta do veneno sobre os eritroblastos ou a falência renal descrita durante o loxoscelismo (Futrell, 1992) com queda do nível de eritropoietina podem ser hipóteses que expliquem a diminuição da série vermelha nucleada.

Um dos efeitos do veneno da aranha-marrom é a coagulação intravascular (Futrell, 1992; Ospedal et al., 2002) que ocorre na lesão dermonecrótica com consumo plaquetário. O veneno da aranha-marrom, de espécies distintas, causa agregação plaquetária (Kurpiewski et al., 1981; Bascur et al., 1982; Veiga et al., 1999). Foi inclusive sugerido que a agregação plaquetária é o evento inicial com posterior migração neutrofílica (Elston et al., 2000). A trombocitopenia em sangue periférico está de acordo com a depleção de megacariócitos na medula óssea (figura 2). Isto ocorreu com 4, 12, 24 e 48 horas após a exposição ao veneno, tanto na medula óssea (megacariócitos) como no sangue periférico (plaquetas). Após 5 e 10 dias ocorreu uma aumento na contagem das plaquetas isto é explicado pela ativação dos megacariócitos retornando aos valores basais após 15 dias. Pelo gráfico plaquetário e dos megacariócitos (figura 2) parece que as alterações são conseqüência do consumo de plaquetas no sítio inflamatório e não pela ação direta do veneno sobre os megacariócitos, talvez a ação direta possa ser verdadeira e em sendo é transitória.

O mielograma é o exame que avalia a celularidade (todas as células nucleadas), os tipos celulares e as quantidades de cada célula presente na medula óssea. Após 12 e 24 horas todos os animais expostos a ação do veneno mostraram depleção da celularidade medular (figura 3a), a recuperação da celularidade iniciou-se após 48 horas e retorna aos valores basais em 05 dias. A célula que determina a queda da celularidade é o neutrófilo. O gráfico do neutrófilo (figura 3b) na medula óssea está de acordo com a queda da celularidade (figura

3a) e com a queda de leucócitos totais (figura 3c) e neutrófilos em sangue periférico (figura 3d). A queda de celularidade, de neutrófilos medulares e de leucócitos e neutrófilos periféricos confirma os achados em tecidos (Elston et al., 2000) onde o número de neutrófilos é bastante elevado. Parece que a necessidade tecidual faz com que haja uma grande migração de neutrófilos ocasionando a perda do "pool" marginal e depleção medular. A perda do "pool" marginal é visualizada no gráfico (figura 3d) de neutrófilos em sangue periférico, onde nas primeiras quatro horas o número de neutrófilos aumenta sem que haja aumento do compartimento leucocitário periférico, ocorre uma neutrofilia sem leucocitose por consumo tecidual. No final dos trinta dias de acompanhamento ocorreu um aumento significativo de leucócitos e neutrófilos periféricos, a explicação é de aumento leucocitário as custas de neutrofilia por infecção secundária. Pelos resultados apresentados no trabalho parece que não há um efeito do veneno sobre as células da medula óssea, caso haja uma ação, ela é transitória. Este trabalho foi realizado simulando um acidente com a aranha-marrom porque o veneno foi injetado de modo intradérmico. Não foi feita uma exposição "in vitro" das células da medula óssea a diferentes concentrações do veneno.

O acidente com a aranha-marrom pode evoluir do quadro cutâneo para um quadro clínico sistêmico, com efeito nefrotóxico (Futrell, 1992; Lung and Mallory, 2000). Os acidentados que evoluem para o quadro sistêmico apresentam hemoglobinúria e proteinúria (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). Alguns autores descrevem que a patologia renal é conseqüência de alterações hematológicas como a hemólise intravascular e a coagulação intravascular disseminada (Futrell, 1992; Williams et al., 1995; Lung and Mallory, 2000). No entanto, não há uma evidência direta que possa confirmar esta hipótese. A insuficiência renal aguda pode ser causada pela ação direta de toxinas do veneno sobre células renais e membranas basais tubulares e glomerulares como já demonstrado com outros tipos de veneno (Bjarnason and Fox, 1995; Veiga et al., 2000). O artigo (Luciano et al., 2004) mostrou que há uma ação direta do veneno sobre as estruturas renais e que ela não é dependente de

alterações hematológicas. Camundongos foram utilizados como modelo animal porque não desenvolvem lesão dermonecrótica, a insuficiência renal não seria conseqüência do processo inflamatório cutâneo. Análises histológicas de camundongos expostos ao veneno mostraram um padrão de nefrotoxicidade, o dano glomerular foi notado pela presença de eritrócitos em torno dos capilares glomerulares nos espaços de Bowman's, um exsudato rico em proteínas nos espaços de Bowman's confirmou o dano glomerular sugerindo perda da integridade vascular (figura 1c,d,e). O dano tubular foi visualizado pela presença de material proteináceo no lúmen do túbulo, levando a oclusão tubular (figura 1b). O aumento da uréia plasmática denota o dano renal (tabela 4). No modelo animal estudado não houve infiltração leucocitária mostrando que o dano renal não está associado com o processo inflamatório, a contagem global de leucócitos se manteve a mesma no grupo controle e teste (tabela 1). No modelo animal estudado não houve efeito hemolítico e nem deposição de hemoglobina (hemólise intravascular) nas estruturas renais, isto afasta, neste modelo, a hemoglobinúria como causa da insuficiência renal. Estes dados foram confirmados pela manutenção do eritrograma (tabela 1) no grupo controle e teste e pela ausência de hemoglobinúria e hematúria (tabela 2) no último grupo. A hemólise intravascular pode estar envolvida na insuficiência renal aguda em humanos, mas o veneno pode ter uma ação direta sobre as estruturas renais. Utilizando anticorpos anti-veneno foi possível demonstrar toxinas do veneno depositadas ao longo das estruturas renais (figura 5). Também não não houve alteração da fração C_3 do complemento (tabela 3). A insuficiência renal pode ser exacerbada pelos produtos gerados na lesão dermonecrótica e pela hemólise, mas as toxinas do veneno da *Loxosceles* são agentes nefrotóxicos diretos.

5. CONCLUSÃO

5.1 artigo da Silva et al., 2004

1. O artigo de revisão trouxe os conhecimento acumulados sobre a aranha marrom de 1992 até 2004.
2. Foi revisada a biologia, epidemiologia, histopatologia e alterações celulares após a exposição ao veneno, identificação e caracterização de várias toxinas do veneno, o mecanismo local e sistêrmico causado pelo acidente com a aranha marrom, imunogenicidade, características clínicas, diagnóstico, terapia e biotecnologia.

5.2 artigo da Silva et al., 2003

1. A série vermelha em sangue periférico não foi afetada pelo veneno porque não houve alteração da contagem de eritrócitos, hemoglobina e volume globular e da morfologia eritrocitária durante os trinta dias de duração do experimento, no modelo ensaiado.
2. As células vermelhas nucleadas (eritroblastos da medula óssea) mostraram uma diminuição significativa após 12, 24 horas e 5 dias, no modelo ensaiado.
3. A trombocitopenia em sangue periférico correlacionou-se muito bem com a depleção de megacariócitos na medula óssea, observado nos tempos 4, 12, 24 e 48 horas após a exposição do veneno, no modelo ensaiado. O aumento ocorrido na contagem de plaquetas observado nos tempos 5 e 10 dias, é devido a recuperação dos megacariócitos na medula óssea. A ação do veneno sobre os megacariócitos foi transitória porque houve recuperação dos megacariócitos na medula óssea e plaquetas no sangue periférico.
4. A queda da celularidade na medula óssea ocorreu pela depleção de neutrófilos medulares, acompanhada de leucopenia e neutropenia periféricas e correlaciona-se com os achados em tecido onde o número de

neutrófilos é bastante elevado. A depleção medular e periférica é explicada pela necessidade tecidual.

5.3 artigo Luciano et al., 2004

1. O veneno foi nefrotóxico com dano glomerular e tubular mostrado a partir de análises histológicas de camundongos. O dano renal foi confirmado pelo aumento da uréia plasmática.
2. Não houve alteração da série vermelha, se houve hemólise esta foi discreta. Não houve alteração da contagem global de leucócitos, não houve infiltrado leucocitário em tecido, mostrando que o dano renal não está associado com o processo inflamatório. Não houve alteração da contagem global de leucócitos, não houve infiltrado leucocitário em tecido, mostrando que o dano renal não está associado com o processo inflamatório.
3. As plaquetas apresentam um decréscimo significativo entre o grupo controle e o tratado.
4. O dano renal não foi por conseqüência de hemólise porque não houve deposição de hemoglobina nas estruturas renais e pela ausência de hemoglobinúria e hematúria. As toxinas do veneno foram agentes nefrotóxicos diretos.

6. REFERÊNCIAS BIBLIOGRÁFICAS

Albelda, S.M., Clayton, A.B. Integrins and other cell adhesion molecules. **FASEB. J.** 4:2668-2680, 1990.

Abboud, C.N., Liesveld, J.L., Lichtman,M.A. The architecture of marrow and its role in hematopoietic cell lodgement, in **The Hematopoietic Microenvironment**, edited by MW Long, MS Vicha, 2-20. Johns Hopkins University Press, Baltimore and London, 1993.

Andrade, R.M.G., Oliveira, K.C., Giusti, A.L., Silva, W.D., Tambourgui, D.V. Ontogenetic development of *Loxosceles intermedia* spider venom. **Toxicon**, 37, 627-632, 1999.

Andrade, R.M.G., Lourenço, W.R., Tambourgi, D.V. Comparison of the fertility between *Loxosceles intermedia* and *Loxosceles laeta* spiders. **J. Arachnol.**, 28, 245-247, 2000.

Araujo, S.C., Castanheira, P., Alvarenga, L.M., Mangili, O.C., Kalapothakis, E., Olórtegui, C.C. Protection against dermonecrotic and lethal activities of *Loxosceles intermedia* spider venom by immunization with a fused recombinant protein. **Toxicon**, 41, 261-267, 2003.

Babcock, J.L., Marmer, D.J., Steele, R.W. Immunotoxicology of brown recluse spider (*Loxosceles reclusa*) venom. **Toxicon**,24:783-790, 1986.

Barbaro, K.C., Cardoso, J.L.C., Eickstedt, V.R.D., Mota, I. Dermonecrotic and lethal components of *Loxosceles gaucho* spider venom. **Toxicon**,30:331- 338, 1992.

Barbaro, , K.C., Eickstedt, V.R.D., Mota, I. Antigenic cross-reactivity of venoms from medically important *Loxosceles* (*Araneae*) species in Brazil. **Toxicon**, 32, 113-120, 1994.

Barbaro, K.C., Ferreira, M.L>, Cardoso, D.F., Eickstedt, V.R.D., Mota, I. Identification and neutralization of biological activities in the venoms of *Loxosceles* spiders. **Braz. J. Med. Biol. Res.**, 29, 1491-1497, 1996.

Bascur, L., Yevenes, I., Barja, P. Effects of *Loxosceles laeta* spider venom on blood coagulation. **Toxicon**, 20, 795-796, 1982.

Berger, R.S., Adelstein, E.H., Anderson, P.C. Intravascular coagulation: the cause of necrotic arachnidism. **J. Invest. Dermatol.**,61:142-150, 1973.

Bevilacqua, M.P., Nelson, R.M. Selectins. **J. Clin. Invest.**, 91: 379-387, 1993.

Beutler, E., Lichtman, M.A., Coller, B.S., Kipps, T.J., Seligsohn, U. **Williams – Hematology**, 6° ed., McGrawhill, 2001.

Bjarnason, J.B., Fox, J.W. Snake venom metalloendopeptidases: reprolysins. **Methods Enzymol.**, 248, 345-368, 1995.

Borkkan, J., Gross, E., Lubin, Y., Oryan, I. An outbreak of venomous spider bites in a citrus grove. **Am. J. Trop. Med. Hyg.,**52, 228-230, 1995.

Bravo, L.M., Puratic,S.O., Behn, T.C., Fardella, B.C., Contrepa, F.A. Estudio de la hemólisis inducida por veneno de *Loxosceles laeta.* Experiência *in vitro*. **Rev. Med. Chile**, Santiago, v.121, p.16-20,1993.

Braz, A., Minozzo, J., Abreu, J.C., Gubert, I.C., Chávez-Olórtegui, C. Development and evaluation of the neutralizing capacity of horse antivenom against the Brazilian spider *Loxosceles intermedia*. **Toxicon**, 37, 1323-1328, 1999.

Bryant, G., Rao, C.N., Brentani, M. A role for the laminin receptor in leucocyte chemotaxis. **J. Leucocyte Biol.**, 41;220, 1987.

Borudy, V.C., Kaushansky, K., Segal, G.M., Harlan, J.M., Adamson, J.W. Tumor necrosis factor type α stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. **Proc. Natl. Acad. Sci. USA** 83:7467-7471, 1986.

Bücherl, W. Aranhas do gênero *Loxosceles* e "loxoscelismo" na América do Sul. **Mem. Inst. Butantan**, São Paulo, v.30, p.167-186, 1960-62.

Campbell, A.D., Long, M.W., Wicha, M.S. Haemonectina: a bone marrow adhesion protein specific for cells of granulocytic lineage. **Nature**, 329; 445, 1987.

Chávez-Olórtegui, C., Zanetti, V.C., Ferreira, A.P., Minozzo, J.C., Mangili, O.C., Gubert, I.C. Elisa for the detection of venom antigens in experimental and clinical envenomation by *Loxosceles intermedia* spiders. **Toxicon**, 36, 563-569, 1998.

Coddington, J.A., Chnzy, H.D., Jackson, C.L., Raty, G., Gradner, K.C.H. The unique ribbon morphology of the major ampullate silk of spiders from the genus *Loxosceles* (recluse spiders). **Biomacromolecules**, 3, 5-8, 2001.

Cohen, N., Sarafian, D.A.,Alon, I., Gorelik, O., Zaidenstein, R., Simantov, R., Blatt, A., Litinsky, I., Modai, D., Golik, A. Dermonecrotic loxoscelism in the Mediterranean region. **J. Toxicol. Cut. Ocult. Toxicol.** 18, 75-83, 1999.

Elston, D.M., Eggers, J.S., Schmidt, W.E., Storrow, A.B., Doe, R.H., McGlasson, D., Fischer, J.R. Histological findings after brown recluse spider envenomation. **Am. J. Dermatopathol.**, 22, 242-246, 2000.

Escalante-Galindo, P., Montoya-Cabrera, M.A., Terroba-Larios, V.M>, Nava-Juaréz, A.R., Escalante-Flores, I. Local dermonecrotic loxoscelism in children bitten by the spider *Loxosceles reclusa* (the violin spider). **Gac. Med. Mex.,** 135, 423-426, 1999.

Feitosa, L., Gremski, W., Veiga, S.S., Elias, M.C.Q.B., Graner, E., Mangili, O.C., Brentani, R.R. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in brown spider (*Loxosceles intermedia*) venom. **Toxicon**, v. 36, p. 1039-1051, 1998.

Fischer, M.L. Levantamento das espécies de *Loxosceles* Heinecken and Lowe, 1832 no município de Curitiba, Paraná, Brasil. **Estudos de Biologia**, 3, 63-88, 1994.

Futrell, J. Loxoscelism. **Am. J. Med. Sci.**,304:261-267, 1992.

Gajardo-Tobar, R. R. Mi experiência sobre loxoscelismo. **Mem. Inst. Butantan**, v.33, n.3, p.689-698, 1966.

Geren, C.R., Chan, T.K., Howell, D.E., Odell, G.V. Isolation and characterization of toxins from brown recluse spider venom (*Loxosceles reclusa*). **Archs. Biochem. Biophys.,**174:90-99, 1976.

Gertsch, W.J., Ennik, F. The spider genus *Loxosceles* in North America, central America and West Indies (Aranae, Loxoscelidae). **Bull. Am. Mus. Nat. Hist.**, v.175, n.3, p.264-360, 1983.

Goddard, J. Loxoscelism. **N. Engl. J. Med.**, 339, 1944-1945, 1998.

Gomez, H.F., Miller, M.J., Trachy, J.W., Marks, R.M., Warren, J.S. Intradermal anti-loxosceles Fab fragments attenuate dermonecrotic arachnidism. **Acad. Emerg. Med.**, 6, 1195-1202, 1999.

Gomez, H.F., Krywko, D.M., Stoecker, W.V. A new assay for the detection of *Loxosceles* species (brown recluse) spider venom. **Ann. Emerg. Med.**, 39, 469-474, 2002.

Guilherme, P., Fernades, I., Barbaro, K.C. Neutralization of dermonecrotic and lethal activities and differences among 32-35 kDa toxins of medically important *Loxosceles* spider venoms in Brazil revealed by monoclonal antibodies. **Toxicon**, 39, 1333-1342, 2001.

Hasthorpe, S., Bogdanovski, M., Rogerson, J., Radley, J.M. Characterization of endothelial cells in murine long-term marrow culture:Implication for hemopoietic regulation. **Exp. Hematol.**, 20; 386, 1992.

Hite, J.M., Glany, W.J., Lancaster, J.C., Whitcomb, W.H. Biology of the brown recluse spider. **Arkansas Agri. Exp. St. Bull**., v.711, p.26, 1960.

Hobbs, G.D., Yealy, D.M. Development of an acute swine model for Loxosceles envenomation. **Vet. Hum. Toxicol.**, 36, 298-300,1994.

Huber, A. R., Kunkel, S.L., Todd, R.F., Weiss, S.J. Regulation of transendothelial neutrophil migration by endogenous inteleukin-8. **Science (Wash. DC),** 254: 99-102, 1991.

Jorge, T.M., Eickstedt, V.R.D., Knysak, I., Fisman, L.F.Z., Ribeiro, L. Curso sobre acidentes com animais peçonhentos. **Arq. Bras. Med.**, Philadelphia, v.65, n.5, 1991.

Kalapothakis, E., Araujo, S.C., Castro, C.S., Mendes, T.M>, Gomez, M.V., Mangili, O.C., Gubert, I.C., Chavez-Olórtegui, C. Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of *Loxosceles intermedia* spider venom. **Toxicon**, 40, 1691-1699, 2002.

Ketaing, A. & Gordon, M.Y. Hierarchical organization of hematopietic microenvironments: role of proteinoglicans. **Leukemia**, 2;766, 1988.

Krywko, D.M., Gomez, H.F. Detection of Loxosceles species venom in dermal lesions: a comparison of 4 venom recovery methods. **Ann. Emerg. Med.**, 39, 475-480, 2002.

Kurpiewski, G., Forrester, L.J., Barrett, J.T., Campbell, B.J. Platelet aggregation and sphingomyelinase D activity of a purified toxin from the venom of *Loxosceles reclusa*. **Biochem. Biophys. Acta** ,678:467-476, 1981.

Lee, R.G., Foerster, J., Lukens, J., Paraskevas, F., Greer, J.P., Rodgers, G.M. **Wintrobe's – Clinical Hematology**, 10° ed., Lippincott Williams & Wilkins, 1998.

Long, M.W. & Dixit, V.M. Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. **Blood**, 75; 2311, 1990.

Lucas, S., Cardoso, J.L., Moraes, A.C. Loxoscelismo: Relato de um caso atribuído a *Loxosceles amazonica* Gerstch, 1967. **Mem. Inst. Butantan**, São Paulo, v.7; n.48, p.127-131, 1983-84.

Luciano M.N., Silva, P.H., Chaim, O.M., Santos, V.P., Franco, C.R.C., Soares, M.F.S., Zanata, S.M., Mangili,O.C., Gremski, W., Veiga, S.S. Experimental evidence for a direct cytotoxicity of *Loxosceles intermedia* (brown spider) venom on renal tissue. **J. Histochem. Cytochem.**, 52, 455-467, 2004.

Lung, J.M., Mallory, S.B. A child with spider bite and glomerulonephritis: a diagnostic challenge. **Int. J. Dermatol.**, 39, 287-289, 2000.

Majeski, J.A., Stinnett, J.D., Alexander, J.W., Durst, G.G. Action of venom from the brown recluse spider (*Loxosceles reclusa*) on human neutrophils. **Toxicon,** 15: 423-427, 1977.

Málaque, C.M.S., Castro-Valencia, J.E., Cardoso, J.L.C., França, F.O.S., Barbaro, K.C., Fan, H.W. Clinical and epidemiological features of definitive and presumed loxoscelism in São Paulo, Brazil. **Rev. Inst. Med. Trop. S. Paulo**, 44, 2002.

Manfredini, L., Maluf, E.M.C.P., Carvalho, D.S., Opabe, I., Mangili, O.C. *Loxosceles*: A "aranha marrom". **Cadernos de Saúde da Prefeitura da Cidade de Curitiba**. v.1, n.2, 1993.

Mansfield, P.J., Suchard, S.J. Thrombospondin promotes both chemotaxis and haptotaxis in neutrophil of human peripheral blood monocytes. **J. Immunol.**, 153; 4219, 1994.

Martinez-Vargas, A.Z. Loxoscelismo, un problema de salud en el *Perú.* **Bol. San. Panam.**, Washington, v.103, n.4, p.378-386, 1987.

Mattosinho, S.G., Sezerino, U.M., Zannin, M., Grando, M., Cardoso, J.L.C., Eickstedt, V.R.D., França, F.O.S. Geographic distribution of loxoscelism in Santa Catarina (Brazil) and species of *Loxosceles* sp involved and existent in the state. **J. Ven. Anim. Tox.,** 3, 99, 1997.

Meredith, J.E. Jr., Fazeli, B., Schwartz, M.A. The extracellular matrix as a cell survival factor. **Mol. Biol. Cell.**, 4:953-961, 1993.

Merigian, K.S., Blaho, K. Envenomation from the brown recluse spider: review of mechanism and treatment options. **Am. J. Ther.**, 10, 724-734, 1996.

Miller, M.J., Gomez, H.F., Snider, R.J., Stephens, E.L., Czop, R.M., Warren, J.S. Detection of *Loxosceles* venom in lesional hair shafts and skin: application of a specific immunoassay to identify dermonecrotic arachnidism. **Am. J. Emerg. Med.**, 18, 626-628, 2000.

Minguell, J.J., Hardy, C., Tavassoli, M. Membrane associated chondroitin sulfate proteoglycan and fibronectin mediate the binding of hemopoietic progenitor cells to stroma cells. **Exp. Cell. Res.**, 201;200,1992.

Ministério da Saúde-Brasil. Manual de diagnóstico e tratamento de acidentes por animais peçonhentos. Brasília, **Fundação Nacional de Saúde**, 1998.

Nicholson, G.M., Graudins, A. Antivenoms for the treatment of spider envenomation. **J. Toxicol. Tox. Rev.,** 22, 35-59, 2003.

Oliveira, K.C., Andrade, R.M.G., Giusti, A.L., Silva, W.D., Tambourgui, D.V. Sex-linked variation of *Loxosceles intermedia* spider venoms. **Toxicon**, 37, 217-221, 1999.

Ospedal, K. Z., Appel, M.H., Neto, J.N., Mangili, O. C., Veiga, S.S., Gremski, W. Histopathological findings in rabbits after experimental acute exposure to the *Loxosceles intermedia* (brown spider) venom. **Int. J. Exp. Path.** 83: 287-294, 2002.

Patel, K.D., Modur, V., Zimmerman, G.A., Prescott, S.M., McIntyre, T.M. The necrotic venom of brown recluse spider induces dysregulated endothelial cell-dependent neutrophil activation. **J. Clin. Invest.** 94: 631-642, 1994.

Pedrosa, M.F.F., Azevedo, I.L.M.J., Andrade, R.M.G., Berg, C.W., Ramos, C.R.R., Ho, P.L., Tambourgui, D.V. Molecular cloning and expression of a functional dermonecrotic and haemolytic factor from *Loxosceles laeta* venom. **Biochem. Bioph. Res. Commun.**, 298, 638-645, 2002.

Perkins, S., Fleischman, R.A. Stromal cell progeny of murine bone marrow fibroblast colony-forming units are clonal endothelial-like cells that express collagen IV and laminin. **Blood**, 75; 620,1990.

Ribeiro, L.A., Eickstedt, V.R.D., Rubio, G.B., Konalsaissen, J.F., Handar, Z., Entres, M., Campos, V.A.F.P., Jorge, M.T. Epidemiologia do acidente por aranhas do gênero *Loxosceles* Heinecken. Lowe no Estado do Paraná (Brasil). **Mem. Inst. But.** 55, 19-26, 1993.

Rodrigues, C.J.H., Paras, Z.M.S., Nobrega, M.M., Torres, J.B. Acidentes com aracnídeos. **Revista HCPA**, v.6, n.2, p.91-96, 1986.

Roodt, A.R., Litwin, S., Dokmetjian, J.C., Vidal, J.C. A reduced immunization scheme to obtain an experimental anti-*Loxosceles laeta* (violinist spider) venom. **J. Nat. Toxins**, 11, 193-203, 2002.

Rot, A. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. **Immnunol. Today** , 13: 291-294, 1992.

Ruoslathi, E. Fibronectin and its receptors. **Ann. Rev. Biochem.**, 57:375-413, 1988.

Ruppert, E.E.; Barnes, R.D. **Zoologia dos invertebrados**. Ed. Roca, São **Paulo, 6ª ed., 1996.**

Sams, H.H., Dunnick, C.A., Smith, M.L., King, L.E. Necrotic arachnidism. **J. Am. Acad. Dermatol.** 44, 561-573, 2001.

Sandidge, J.S. Arachnology: scavenging by brown recluse bites. **Nature**, 426, 6962, 2003.

Santos, V.L.P., Franco, C.R.C., Viggiano, R.L.L., Silveira, R.B., Cantão, M.P., Mangili, O.C., Veiga, S.S., Gremski, W. Structural and ultrastructural description of the venom gland of *Loxosceles intermedia* (brown spider). **Toxicon**, 38, 265-285, 2000.

Schenone, H., Letonja, T. Notas sobre la biologia y distribuicion geográfica de las aranas del gênero *Loxosceles*. **Bol. Chil. Parasitol**., Chiba, n.30, p.27-29, 1975.

Schenone, H. Diagnosis in 1348 patients which consulted for a probable spider bite or insect sting. **Bol. Chil. Parasitol., 51, 20-27, 1996.**

Schenone, H. Cutaneous loxoscelism with an edematous predominance. **Bol. Chil. Parasitol.** 53, 78-83, 1998.

Schenone, H. Toxic pictures produced by spiders bites in Chile: Latrodectism and Loxoscelism. **Rev. Med. Chi.**, 131, 437-444, 2003.

Schweitzer, K.M., Drager, A. M., van der Valk, P. Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. **Am. J. Pathol.**, 148;165,1996.

Secretaria de Saúde, Curitiba, Paraná, Brasil. **Acidentes loxoscélicos-Protocolo técnico e fluxo de atenção em Curitiba.** 2002.

Seiffert, M., Beck, S.C., Schermutzki, F. Mitogenic and adhesive effects of tenascin-C on human hematopoietic cells are mediated by various functional domains. **Matrix Biol.**, 17; 47, 1998.

Sezerino, U.M., Zannin, M., Coelho, L.K., Gonçalves, J., Grando, M., Mattosinho, S.G., Cardoso, J.L.C., Eickstedt, V.R., França, F.O.S., Barbaro, K.C., Fan, H.W. A clinical and epidemiological study of *Loxosceles* spider envenoming in Santa Catarina, Brazil. **Trans. R. Soc. Trop. Med. Hyg.** 92; 546-548, 1998.

Smith, W.C., Micks, W.D. The role of polimorphonuclear leukocytes in the lesion caused bi the venom of the brown spider, *Loxosceles reclusa*. **Lab. Invest**., Baltimore, v.22, p.90-93, 1970.

Soerensen, B. **Acidentes por animais peçonhentos: reconhecimento, clínica e tratamento**. Editora Atheneu, 1996.

Sorrel, J.M. Ultrastructural localization of fibronectin in bone marrow of the embryonic chick and its relationship to granulopoiesis. **Cell. Tissue Res.**, 252;565, 1988.

Suarez, G., Biggermann, U., Schenone, H. Estudios bioquímicos del veneno de *Loxosceles laeta* y sus mecanismos de acción. **Bol. Chil. Parasitol**., Chiba, n.26, p.60-62, 1971.

Sunderkötter, C., Seeliger, S., Schönlau, F., Roth, J., Hallmann, R., Luger, T.A., Sorg, C., Kolde, G. Different pathway leading to cutaneous leukocytoclastic vasculitis in mice. **Exp. Dermatol.**, 10, 391-403, 2001.

Tambourgi, D.V., Magnoli, F.C., Eickstedt, V.R.D., Benedetti, Z.C., Petricevich, V.L., Silva, W.D. Incorporation of a 35-kilodalton purified protein from *Loxosceles intermedia* spider venom transforms human erythrocytes into activators of autologous complement alternative pathway. **J. Immunol.**,155:4459- 4466, 1995.

Tambourgui, D.V., Petricevich, V.L., Magnoli, F.C., Assaf, S.L.M.R., Jancar, S., Silva, W.D. Endotoxemic-like shock induced by *Loxosceles* spider venoms: pathological changes and putative cytokine mediators. **Toxicon**, 36, 391-403, 1998.

Tambourgui, D.V., Morgan, B.P., Andrade, R.M.G., Magnoli, F.C., Berg, C.W. *Loxosceles intermedia* spider envenomation induces activation of an endogenous metalloproteinase, resulting in cleavage of glycophorins from the eirthrocyte surface and facilitating complement-mediated lysis. **Blood**, 95, 683-691, 2000.

Tavassoli, M., Shaklai, M. Absence of tight junctions in endothelium of marrow sinuses: possible significance for marrow cell egress. **Br. J. Haematol.**, 41:303,1979.

Testa, N.G., Molineux, G. **Haemopoiesis: a practical approach**. IRL Press/ Oxford University Press, New York, 1993.

Torok-Storb, B., Iwata, M., Graf, L. Dissecting the marrow microenvironment. **Ann. N.Y. Acad. Sci.**, 872; 164,1999.

Veiga, S.S., Elias, M.C.Q.B., Gremski, W., Porcinatto, M.A., Nader, H.B., Brentani, R.R. Detection of post-translational sulfation of $\alpha_5\beta_1$ integrin and its role in integrin-fibronectin binding. **Braz. J. Med. Biol. Res.**,1235-1238, 1996.

Veiga, S.S., Elias, M.C.Q.B., Gremski, W., Procinatto, M.A., Silva, R., Nader, H.B., Brentani, R.R. Post translational modifications of $\alpha_5\beta_1$ integrin by glycosaminoglycan chains. The $\alpha_5\beta_1$ integrin is a facultative proteoglycan. **J. Biol. Chem.,**272:12529-12535, 1997.

Veiga, S.S., Gremski, W., Santos, V.L.P., Feitosa, L., Mangili, O.C., Nader, H.B., Dietrich, C.P., Brentani, R.R. Oligosaccharide residues of *Loxosceles intermedia* (brown spider) venom proteins: dependence of glycosylation for dermonecrotic activity. **Toxicon**, 37:587-607, 1999.

Veiga, S.S., Feitosa, L., Santos, V.L.P., Souza, G.A., Ribeiro, A. S., Manigili, O.C., Porcionatto, M. A., Nader, H.B., Dietrich, C.P., Brentani,R.R., Gremski,W. Effect of brown spider venom on basement membrane strutures. **The Histochemical Journal**. 32:397-408,2000.

Veiga, S.S., Da Silveira, R.B., Dreyfuss, J.L., Haoach, J., Pereira, A.M., Mangili, O.C., Gremski, W. Identification of high molecular weight serine- proteases in *Loxosceles intermedia* (brown spider) venom. **Toxicon**,38:825-839, 2000a.

Veiga, S.S., Zanetti, V.C., Franco, C.R.C., Trindade, E.S., Porcionatto, M.A., Mangili, O.C., Gremski, W., Dietrich, C.P., Nader, H.B. In vivo and in vitro cytotoxiicty of brown spider venom for blood vessel endothelial cells. **Thrombosis Research**, 102:229-237,2001.

Vetter, R. S., Visscher, P.K. Bites and stings of medically important venomous arthropods. **Int. J. Dermatol.** 37, 481-496, 1998.

Vetter, R.S. Identifying and misidentifying the brown recluse spider. **Dermatol. Online J.**, 5, 7, 1999.

Vose, J.M. & Armitage, J. O. Clinical applications of hematopoietic growth factors. **J. Clin. Oncol.**, 13: 1023-1035, 1995.

Yiannias, J.A., Winkelmann, R.K. Persistent painful plaque due to a brown recluse spider bite. **Cutis.** 50, 273-275, 1992.

Young, A.R., Pincus, S.J. Comparison of enzymatic activity from three species of necrotising arachnids in Australia: *Loxosceles rufescens*, *Badumma insignis* and *Lampona cylindrata*. **Toxicon,** 39, 391-400, 2001.

Yurchenco, P., Schittny, J. Molecular architecture of basement membranes. **FASEB J.**, 4:1577-1590, 1990.

Wasserman, G.S., Anderson, P.C. Loxoscelism and arachnidism. **J. Toxicol. Clin. Toxicol.,** 21:451-472, 1984.

Weiss, L., Geduldig, U. Barrier cells: stromal regulation of hematopoiesis and blood cell release in normal and stressed murine bone marrow. **Blood**, 78;975, 1991.

Wendell, R.P. Brown recluse spiders: a review to help guide physicians in nonenedemic areas. **South. Med. J.**, 96, 486-490, 2003.

Williams, S.T., Khare, V.K., Johnston, G.A., Blackall, D.P. Severe intravascular hemolysis associated with brown recluse spider envenomation. **Am. J. Clin. Pathol.**, 104, 463-467, 1995.

Wright, S.W., Wrenn, K.D., Murray, L., Seger, D. Clinical presentation and outcome of brown spider bite. **Ann. Emerg. Med.**, 30, 28-32, 1997.

Zanetti, V.C., Silveira, R.,B.,S., Dreyfuss, J.,L., Haoach, J., Mangili, O.C., Veiga, S.,S., Gremski, W. Hemorrhagic effects of brown spider venom. **Blood Coagulation and Fibrinolysis**. 13:135-148, 2002.

Zimmerman, G.A., Prescott, S.M., McIntyre, T.M. Endothelial interactions with granulocytes:tethering and signaling molecules. **Immunol. Today,** 13: 93-100, 1992.