UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

RENATA MARIA SOARES TERRA

VENÔMICA: IDENTIFICAÇÃO DE PROTEÍNAS ENVOLVIDAS NA FISIOPATOLOGIA DE ENVENENAMENTOS ANIMAIS

2010

RENATA MARIA SOARES TERRA

VENÔMICA: IDENTIFICAÇÃO DE PROTEÍNAS ENVOLVIDAS NA FISIOPATOLOGIA DE ENVENENAMENTOS ANIMAIS

Tese submetida ao Programa de Pós-graduação em Biologia Celular e Molecular do Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do grau de Doutor em Ciências.

Orientador Prof. Dr. Jorge Almeida Guimarães

Co-Orientador Prof. Dr. Jay William Fox

Porto Alegre

2010

RENATA MARIA SOARES TERRA

VENÔMICA: IDENTIFICAÇÃO DE PROTEÍNAS ENVOLVIDAS NA FISIOPATOLOGIA DE ENVENENAMENTOS ANIMAIS

BANCA EXAMINADORA

Dr. Jorge Almeida Guimarães

UFRGS - Orientador

Dr. Jay William Fox

University of Virginia - Co-orientador

Dr. Richard Hemmi Valente

FIOCRUZ - Membro da Banca

Dra. Ana Maria Moura da Silva

Instituto Butantan - Membro da Banca

Dr. Henrique Bunselmeyer Ferreira

UFRGS - Membro da Banca

Dra. Célia Regina Ribeiro da Silva Carlini

UFRGS - Suplente

Dr. Antônio Frederico Michel Pinto

UFRGS - Revisor

A presente tese foi desenvolvida no Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul e no *Microbiology Department* da *University of Virginia* com colaboração de pesquisadores do Instituto Clodomiro Picado da Universidad de Costa Rica e suporte financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Ao Antônio, meu grande companheiro nesta jornada.

AGRADECIMENTOS

Agradeço ao meu orientador, Dr. Jorge Almeida Guimarães, pelos incontáveis ensinamentos nesses anos e, principalmente, pelo incentivo constante e o zelo com a minha carreira.

Ao meu co-orientador, Dr. Jay William Fox, pela oportunidade e os meios de realizar este trabalho.

Ao Antônio Pinto pela cumplicidade, companheirismo, amor e pelas longas discussões a respeito destes e dos diversos outros trabalhos em colaboração, mas acima de tudo pelo suporte emocional constante e indispensável.

À minha família por proporcionar os meios e acreditar incondicionalmente nas minhas escolhas, permitindo a realização dos meus sonhos.

Aos meus grandes e insubstituíveis amigos Markus Berger, José Reck Jr., Walter Beys e Lucélia Santi pelas infinitas horas fazendo ciência e falando bobagem.

Ao Dr. Nicholas E. Sherman, Dra. Nena Fox, Dr. Bojan Dragulev, Dra. Kristi Nelson e aos demais integrantes do Biomolecular Research Facilities da University of Virginia pelo suporte e colaboração.

Aos professores do Programa de Pós-graduação em Biologia Celular e Molecular e membros da minha comissão de acompanhamento Dra. Célia Carlini e Dr. Guido Lenz.

Aos secretários da pós-graduação Luciano Saucedo e Silvia Centeno e demais colegas.

Aos professores Dra. Ana Moura da Silva, Dr. Richard Valente, Dr. Henrique Ferreira e Dra. Célia Carlini por gentilmente aceitarem o convite para comporem esta banca.

"*Pode-se viver no mundo uma vida magnífica, quando se sabe trabalhar e amar: trabalhar pelo que se ama e amar aquilo em que se trabalha*."

Liev Tólstoi

ÍNDICE

LISTA DE ABREVIATURAS

ADAM - *Adamalysin and Metalloprotease*

ADP - Adenosina difosfato

CID - Coagulação Intravascular Disseminada

SINAN - Sistema de Informação de Agravos de Notificação

SVMP - *Snake Venom Metalloprotease* (metaloprotease de veneno de serpente)

uPAR - Ativador de plasminogênio tipo uroquinase

WHO - *World Health Organization* (Organização Mundial da Saúde)

APRESENTAÇÃO

Brevemente, meu treinamento científico iniciou-se no final da minha graduação em Farmácia na Pontifícia Universidade Católica do Rio Grande do Sul. Após conclusão do curso de graduação, ingressei no grupo de pesquisa do Dr. Jorge Almeida Guimarães onde realizei o mestrado. Neste período, tive a oportunidade de desenvolver projetos com modelagem molecular e ensaios *in vitro* e *in vivo* de agregação plaquetária e coagulação com diferentes toxinas e venenos. No doutorado, obtive o treinamento em proteômica na University of Virginia, durante estágio de doutorado sanduíche no grupo de pesquisa do Dr. Jay William Fox.

A presente tese é o resultado do trabalho desenvolvido no período de quatro anos no Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM) da Universidade Federal do Rio Grande do Sul. Os trabalhos aqui apresentados foram realizados sob a orientação do Prof. Dr. Jorge Guimarães e co-orientação do Dr. Jay Fox.

Esta tese encontra-se estruturada em uma introdução geral seguida dos objetivos e da seção de resultados. Os resultados estão divididos em três capítulos que se referem a cada um dos trabalhos apresentados: (I) um artigo publicado no periódico Toxicon, (II) um manuscrito em fase final de redação e (III) uma comunicação de resultados que compõem um manuscrito em fase de redação.

Dentro de cada capítulo há uma breve introdução específica do assunto tratado acompanhada do manuscrito. As referências ao final da tese referem-se às introduções, geral e específicas, tendo cada artigo sua seção própria de referências. Por fim, considerações finais são feitas à respeito dos resultados obtidos.

RESUMO

Acidentes com animais venenosos constituem um problema de saúde pública negligenciado em todo o mundo. Estimativas indicam que, considerando-se apenas acidentes com serpentes venenosas, ocorram mundialmente cerca de 2,5 milhões de casos, causando 85.000 óbitos anuais. O desenvolvimento do quadro patológico dos envenenamentos é o resultado conjunto de potentes atividades biológicas exercidas, principalmente, por proteínas e peptídeos que compõem os venenos animais.

A proteômica aplicada à caracterização de componentes de venenos animais, denominada venômica, é uma metodologia essencial na identificação não apenas dos componentes tóxicos, mas também valiosa na investigação molecular dos mecanismos patológicos dos envenenamentos. Através de metodologias proteômicas, descrevemos a caracterização protéica dos venenos animais das serpentes *Bothrops jararaca* e *Bothrops lanceolatus* e da lagarta *Lonomia obliqua*. Ainda, avaliamos através da proteômica de tecido experimentalmente envenenado as ações tóxicas de um componente isolado do veneno de *Bothrops jararaca*.

Através de análise comparativa e semi-quantitativa da composição protéica dos venenos de *B. jararaca* e *B. lanceolatus*, descrevemos uma diferença qualitativa na distribuição dos componentes tóxicos. Enfatizando o grupo protéico majoritário, metaloproteases (SVMP), descrevemos diferentes abundâncias relativas entre os

subtipos destas enzimas. Esta diferença explicaria as repercussões clínicas opostas durante o envenenamento humano, sendo um veneno hemorrágico e o outro pró-trombótico.

Componentes tóxicos capazes de gerar um quadro hemorrágico também foram avaliados através da análise proteômica das secreções tóxicas de *L. obliqua*. O estudo comparativo entre hemolinfa e extrato de espículas demonstrou que, diferentemente dos venenos botrópicos, as secreções tóxicas são compostas majoritariamente de inibidores de proteases, predominantemente serpinas. Além disso, descrevemos pela primeira vez a presença de novos componentes potencialmente tóxicos, como metaloproteases.

Por fim, a proteômica de tecidos foi aplicada à investigação dos efeitos locais da injeção da metaloprotease do veneno de *B. jararaca*, jararagina. O efeito direto da ação da metaloprotease foi observado através da identificação de proteínas presentes em maior ou menor abundância, denotando infiltração ou degradação, respectivamente. Hemorragia, edema e estresse oxidativo foram evidenciados por pronunciado aumento em proteínas envolvidas nesses processos, mas, acima de tudo, identificamos degradação de proteínas relacionadas à manutenção da integridade da matriz extracelular e estabilização de coágulos, sugerindo novos mecanismos relacionados à atividade tóxica a serem investigados.

De uma maneira geral, o presente trabalho descreve componentes tóxicos de venenos animais causadores de síndromes hemorrágicas e gera novas hipóteses em relação a mecanismos moleculares envolvidos no desenvolvimento da patofisiologia dos envenenamentos.

ABSTRACT

Accidents with venomous animals are a neglected health issue worldwide. Global estimates points to the occurrence of 2,500,000 envenomation cases, causing 85,000 deaths per year. The pathological envenomation condition is a result of strong biological activities caused mainly by the action of venom's proteins and peptides components.

Proteomics applied to the characterization of animal venom active principles, so called venomics, is an essential tool to the identification of toxic molecules as well as to help understanding the molecular mechanisms underlying pathological envenomation conditions. Through a proteomic methodology, here we describe the characterization of venoms from the snakes *Bothrops jararaca* and *Bothrops lanceolatus* and the caterpillar *Lonomia obliqua*. Moreover, from a tissue proteomic perspective we were able to evaluate the toxic effects of a *B. jararaca* venom component upon experimentally envenomed skin.

Using a comparative semi-quantitative proteomic analysis, we described a qualitative difference in toxic components distribution between *B. jararaca* and *B. lanceolatus* venoms. Focusing on snake venom metaloproteases (SVMPs) distribution, we observed different relative abundance of these enzymes subgroups. Those differences could explain the opposite clinical envenomation characteristics, since one venom is hemorrhagic and the other induces a prothrombotic profile.

Pro-hemorrhagic venom toxins were also characterized through the proteome of *L. obliqua* venomous secretions. Hemolymph and bristle extract were analyzed showing that, different from bothropic venoms, the toxic secretions composition are mainly protease inhibitors, especially serpins. Moreover, we were able to demonstrate for the first time the presence of new putative toxins, such as metalloproteases.

Finally, we applied tissue proteomics to the investigation of local snakebite pathology by jararhagin, a metalloprotease from *B. jararaca* venom. The metalloprotease direct effect was observed through the increase or decrease in protein identification, indicating infiltration or degradation respectively. Hemorrhage, edema and oxidative stress were characterized by enhance in correlated proteins but, most of all, we identified degradation in proteins important to extracellular matrix integrity and clot stabilization, indicating novel mechanism of toxicity to be further evaluated.

In a general perspective, the present work describes toxic components from venomous animals that cause hemorrhagic syndromes and generates new testable hypothesis of the mechanisms of action involved in the development of envenomation pathophysiology.

1. INTRODUÇÃO

1.1 Acidentes envolvendo animais venenosos ou peçonhentos

Animais venenosos é o termo que denomina animais capazes de produzir peçonha ou secreções venenosas que estão distribuídos em diversas classes incluindo milhares de espécies de serpentes, aranhas, sapos, lagartas e escorpiões, entre outros. Distribuídos amplamente no mundo, são causadores de acidentes em seres humanos principalmente em regiões tropicais e subtropicais, onde a abundância de espécies é maior. Em abril de 2009, através de uma mobilização de cientistas e profissionais da saúde que há muitos anos relatam casos sérios e frequentes de envenenamentos por serpentes, a Organização Mundial da Saúde (*World Health Organization* - WHO) incluiu o envenenamento por picada de serpente (no inglês, *snakebite*) como parte da lista de doenças tropicais negligenciadas (http://www.who.int/neglected_diseases/diseases/en/) e permitiu a criação de uma iniciativa global - *Global snakebite initiative* (Williams et al., 2010).

Assim como as demais doenças tropicais - *e.g.* Chagas, dengue, lepra e leishmaniose - as picadas por serpentes atingem mais amplamente a população pobre de áreas rurais de países da África, Ásia, Oceania e América Latina. Estima-se que sejam pelo menos 421.000 casos de envenenamento ocasionando 20.000 mortes anualmente. Diferentemente das doenças infecciosas, os envenenamentos

por serpentes e outros animais venenosos não são obrigatoriamente reportados às instituições de saúde, acreditando-se que ocorram, de fato, quase 2,5 milhões de envenenamentos por serpentes, deixando 250.000 pessoas com sequelas e matando cerca de 85.000 vítimas anualmente. A distribuição estimada de casos por continente é mostrada na Figura 1 (Gutierrez et al., 2009c). Comparando-se estas estimativas com os relatórios da WHO de incidência e morte por outras doenças tropicais negligenciadas, constatam-se números alarmantes especialmente quando consideramos que estes acidentes não possuem o caráter epidêmico de doenças infecciosas e parasitárias (Tabela I) (Williams et al., 2010).

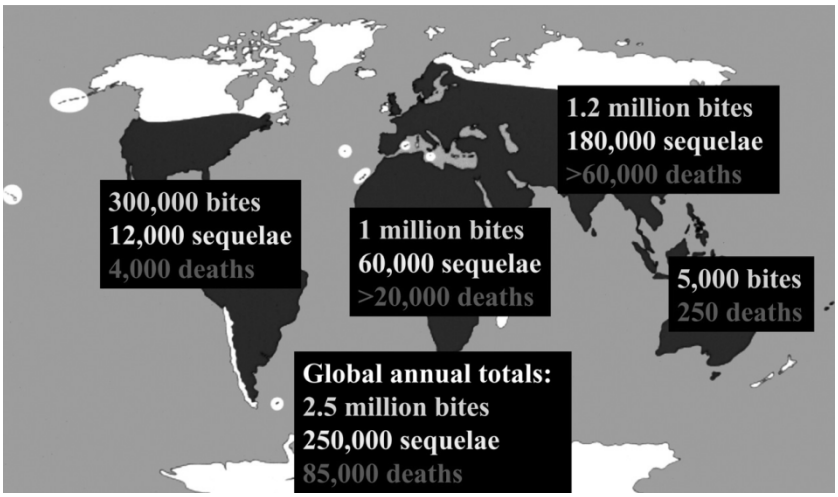


Figura 1 - Estimativa da morbidade e mortalidade causadas por envenenamentos com serpentes e sua distribuição regional e mundial (Gutierrez et al., 2009c).

Tabela I - Comparação das taxas mundiais de incidência e mortalidade de doenças tropicais negligenciadas reconhecidas pela WHO (adaptado de Williams *et al.* 2010).

	Incidência	**Mortes**
Doença de Chagas	217.000	14.000
Cólera	178.000	4.000
Dengue hemorrágica	73.000	19.000
Leishmaniose	1.691.000	51.000
Encefalite japonesa	44.000	14.000
Schistosomose	5.733.000	15.000
Picada de serpente	420.000 - 2.682.000	20.000 - 125.000
Febre amarela	100 - 2100	60 - 100

Não diferente do panorama mundial, os acidentes com animais venenosos no Brasil também representam um problema de saúde pública. A Secretaria de Vigilância em Saúde, órgão do Ministério da Saúde, vem monitorando os casos de envenenamentos por serpentes, aranhas, escorpiões, lagartas e abelhas através do Sistema de Informação de Agravos de Notificação (SINAN). Assim como nas demais regiões do mundo, ocorre a sub-notificação de casos e, em um esforço crescente, busca-se o treinamento dos profissionais responsáveis pela assistência e vigilância no intuito de mapear os casos em todo o território nacional. A Tabela II mostra as notificações de acidentes com animais venenosos em 2008 (http://portal.saude.gov.br/portal/arquivos/pdf/boletim_epidemiologico_zoonoses_06 2009.pdf).

Baseada na alta incidência de casos, nos baixos níveis de notificação e na alta taxa de morbidade e mortalidade causadas por envenenamentos, a iniciativa global propôs uma série de medidas a serem adotadas por organizações de saúde do mundo todo. Dentre estas medidas está, primeiramente, tornar os acidentes uma doença notificável. Além disso, é necessário promover a educação comunitária a respeito de prevenção e tratamento de acidentes, treinar profissionais de saúde

para o manejo das manifestações clínicas e minimização das sequelas. Mas, fundamentalmente, é preciso criar programas de financiamento da pesquisa básica e clínica para permitir melhor entendimento do desenvolvimento da patologia do envenenamento e promover a melhoria de anti-venenos e tratamentos alternativos ou de suporte, favorecendo assim todas as demais medidas paliativas citadas.

Tabela II - Notificações de acidentes causados por animais venenosos no Brasil no ano de 2008.

ACIDENTES CAUSADOS POR ANIMAIS PEÇONHENTOS - DADOS de 2008*										
BRASIL		SERPENTES		ARANHAS		ESCORPIÕES		LAGARTAS		ABELHAS
	Número de Acidentes	26.156		20.993		37.862		3.968		5.605
	Casos por Milhão de Habitantes	130		110		220		20		30
	Óbitos	119		19		87		5		13
	Letalidade %	0,5		0,10		0,23		0,10 - 0,30		0,23
ACIDENTES POR REGIÃO	CASOS POR MILHÃO DE HABITANTES									
REGIÃO NORTE	520		27		136		7		14	
REGIÃO NORDESTE	130		10		326		2		20	
REGIÃO CENTRO-OESTE	150		20		100		4		20	
REGIÃO SUDESTE	80		55		201		15		31	
REGIÃO SUL	110		557		36		80		58	
* Fonte: SINAN/SVS/MS - Boletim Epidemiológico Eletrônico, Junho 2009.										

A pesquisa básica em toxinologia vem sendo desenvolvida desde a primeira observação dos efeitos biológicos dos venenos animais. Tratados sobre animais venenosos e peçonhentos vêm sendo publicados desde o século XVIII e até hoje a pesquisa em toxinologia tenta esclarecer os mecanismos pelos quais ocorre o dano

aos mais diferentes tecidos durante o envenenamento, como impedi-los e tratá-los e, ainda, buscar responder como e porque algumas espécies não são afetadas pelos venenos (Fox & Serrano, 2009). Considerando a pesquisa com venenos de serpentes como modelo ao estudo de outros venenos animais, no final do século XIX a natureza protéica dos princípios ativos dos venenos começou a ser reconhecida e passou a ser estudada através do desenvolvimento das técnicas bioquímicas apropriadas (Fox & Serrano, 2009).

Dentre as metodologias e estratégias de estudo hoje disponíveis, a proteômica (que no caso específico de venenos animais recebeu o nome de venômica), é hoje a técnica de identificação mais utilizada no estudo dos componentes protéicos de venenos.

1.2 Vênomica: estratégias proteômicas para o estudo de venenos animais

Venenos animais são misturas únicas e complexas de proteínas e peptídeos bioativos cujas características físico-químicas e propriedades biológicas resultaram do processo evolutivo das espécies dotando tais indivíduos de eficientes mecanismos de proteção para defesa e alimentação em presas e predadores. As toxinas animais são capazes de interagir com enzimas, receptores e canais iônicos provocando a desestabilização de sistemas fisiológicos essenciais à sobrevivência das vítimas ou presas (Calvete, 2009). Essa característica vem incentivando pesquisadores há muitos anos em busca de moléculas com aplicações farmacológicas para o tratamento de diversas patologias, assim como para o desenvolvimento de terapias capazes de reverter os efeitos dos envenenamentos acidentais.

O estudo de venenos animais como fonte de moléculas bioativas ou para o entendimento básico da patofisiologia dos envenenamentos por muito tempo dependeu unicamente do isolamento pontual de cada toxina e avaliação da sua atividade ou possível papel no envenenamento. Até a década de 1990, os protocolos baseavam-se em métodos clássicos de caracterização bioquímica utilizando métodos cromatográficos seguidos de eletroforese unidimensional. O desenvolvimento de técnicas de *high-throughput* - *e.g.* proteômica - proporcionou um novo panorama no estudo destas complexas misturas. Com a revitalização das técnicas de eletroforese bidimensional e o avanço da espectrometria de massas para análise de proteínas em grande escala, foi possível observar a complexidade destes venenos e as nuances no que diz respeito às variações ontogenéticas e até mesmo individuais (Fox & Serrano, 2008).

Diferentes protocolos foram criados de maneira a permitir a identificação do maior de componentes protéicos (revisados em Fox & Serrano, 2008). Basicamente, tais protocolos dependem de pelo menos uma etapa de decomplexação da amostra, que pode ser realizada por cromatografia, eletroforese uni- ou bidimensional aliada ou não a técnicas de marcação com anticorpos ou colorações específicas. Paralelamente, técnicas de clonagem e expressão podem auxiliar na identificação dos princípios ativos componentes do veneno. De fato, o avanço da transcriptômica foi essencial na geração de bancos de dados de toxinas animais em larga escala. Porém, devido às características intrínsecas da técnica de transcriptômica (como a geração de pequenas sequências), a identificação exata de uma proteína de veneno animal ainda é problemática. Sendo assim, o sequenciamento da porção N-terminal por química de Edman e o sequenciamento *de novo* são técnicas complementares à

análise proteômica em larga escala baseada na busca de homólogos em bancos de dados (Calvete et al., 2007, Fox & Serrano, 2008).

Recentemente, adicionou-se ao termo venômica a expressão antivenômica. A antivenômica é a análise proteômica de componentes de venenos animais que formam ou não complexos antígeno-anticorpo com os soros clinicamente utilizados. Esta metodologia de imuno-reconhecimento dos princípios ativos dos venenos possibilita classificar os componentes totalmente reconhecidos, os parcialmente reconhecidos e os não-reconhecidos pelo soro. Admitindo-se, então, que exista uma relação direta entre o reconhecimento e a inativação das toxinas, é possível produzir anticorpos altamente específicos, melhorando a qualidade e eficiência dos anti-venenos (Calvete et al., 2009).

2. OBJETIVO

No desenvolvimento deste trabalho de doutorado objetivou-se aplicar técnicas proteômicas na identificação de proteínas envolvidas no desenvolvimento fisiopatológico dos envenenamentos produzidos por toxinas de origem animal. A presente tese dividiu-se em duas linhas para a abordagem do problema. Num primeiro momento, usamos três venenos animais (dois venenos de serpentes e um de lagarta) como objeto de estudo quanto à composição protéica. Em seguida, focando diretamente no problema do envenenamento buscou-se identificar como componentes de venenos interferem e afetam as estruturas-alvo no tecido do animal como consequência do envenenamento experimental.

Objetivos específicos:

1. Analisar comparativamente os componentes protéicos dos venenos das serpentes *Bothrops jararaca e Bothrops lanceolatus* buscando entender, com bases moleculares e bioquímicas, o desenvolvimento dos quadros de hemorragia e trombose característicos desses envenenamentos;

2. Analisar as secreções venenosas da lagarta *Lonomia obliqua*, hemolinfa e extrato de espículas, com o objetivo de caracterizar seus componentes protéicos

envolvidos no desenvolvimento da síndrome hemorrágica resultante do envenenamento;

3. Caracterizar molecularmente o efeito biológico direto da injeção intradérmica de Jararagina, uma metaloprotease de *Bothrops jararaca*, através da proteômica de tecidos visando identificar e caracterizar proteínas envolvidas no desenvolvimento da sintomatologia local do envenenamento.

3. RESULTADOS

3.1 CAPÍTULO I

ENVENENAMENTOS POR SERPENTES: ANÁLISE PROTEÔMICA DOS VENENOS DE SERPENTES DO GÊNERO *Bothrops* COM CONTRASTANTES ATIVIDADES BIOLÓGICAS

3.1.1 Acidentes com serpentes

Serpentes são o grupo de animais venenosos que causam os mais graves acidentes, resultando em irreversíveis sequelas, mutilações e outros agravos à saúde das vítimas, frequentemente resultando em óbitos. Tais acidentes ocorrem praticamente em todo o mundo, considerando-se como de maior importância médica os acidentes envolvendo as famílias Viperidae (Cascavéis, Jararacas, Surucucu, etc.) e Elapidae (Najas, Mambas, Coral, etc.). De uma maneira geral, acidentes com viperídeos são caracterizados por hemorragia, edema e necrose tecidual, resultantes da ação dos componentes ativos dos venenos sobre a pele, músculo e endotélio. Observam-se distúrbios de coagulação, agregação plaquetária e falência renal. Apesar de algumas serpentes da família Elapidae também causarem necrose tecidual e hemorragia, os componentes destes venenos apresentam, majoritariamente, ação neurotóxica, com parálise descendente que pode levar à morte por falência respiratória. A falência renal também é observada nestes casos.

No Brasil, os acidentes com serpentes da família Viperidae (*Bothrops*, *Crotalus* e *Lachesis*) são mais graves e mais frequentes que acidentes com serpentes da família Elapidae. Segundo o SINAN, em 2009, foram 24.733 notificações de acidentes com viperídeos e 205 com elapídeos, sendo as serpentes do gênero *Bothrops* responsáveis por 21.603 (87%) acidentes. Sabidamente, tais índices são subestimados devido à larga sub-notificação dos acidentes junto aos serviços especializados.

3.1.2 Acidentes com serpentes do gênero *Bothrops*

As serpentes do gênero *Bothrops* encontram-se amplamente distribuídas nas Américas. São um grupo de cerca de 30 espécies responsáveis por cerca de 90% dos envenenamentos por serpentes. O acidente botrópico, assim como a maioria dos acidentes com viperídeos, se caracteriza pela desestabilização do finamente auto-regulado controle do sistema hemostático das vítimas causando, principalmente, hemorragias local e sistêmica e necrose tecidual (Figura 2). Ocorre o que se denomina coagulopatia de consumo, ou seja, a ativação rápida do sistema pró-coagulante em sinergia com uma pronunciada fibrinólise, do que resulta rápida depleção do fibrinogênio. Os venenos botrópicos são também capazes de interferir na reatividade plaquetária, produzir vasodilatação e degradação da matriz extracelular desestabilizando o endotélio vascular. No geral, todos os processos contribuem para estabelece um profundo quadro hemorrágico. Além disso, ocorre lise das células sanguíneas, em especial hemácias, o que colabora com o desenvolvimento subsequente de falência renal aguda.

Porém, nem todas as espécies de *Bothrops* são capazes de causar acidentes hemorrágicos. De fato, existem duas serpentes do gênero *Bothrops* - *Bothrops lanceolatus* e *Bothrops caribbaeus* – cujos venenos, em humanos, desencadeiam predominantemente as reações pró-trombóticas.

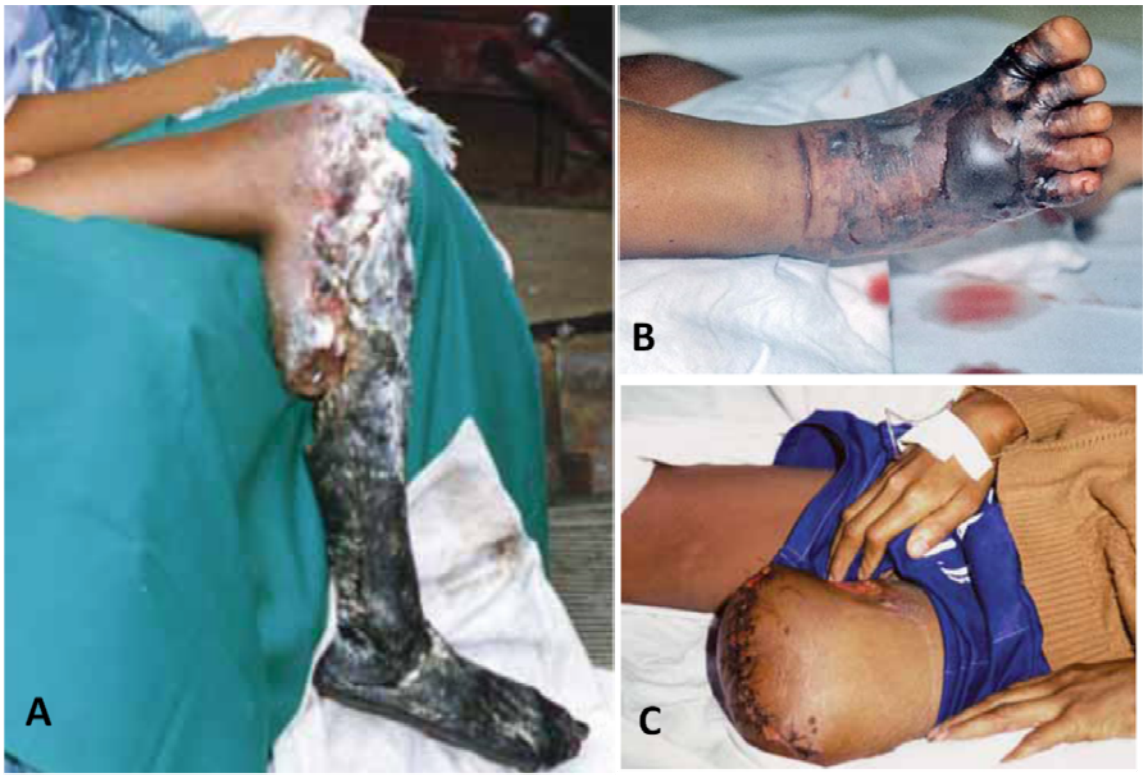


Figura 2 - Acidente botrópico com necrose (**A**), formação de bolhas (**B**) e amputação do membro afetado (**C**). (Fonte: WHO, 2010)

3.1.2.1 *Bothrops jararaca*

A serpente *Bothrops jararaca*, popularmente conhecida como Jararaca, está distribuída na região sudeste da América Latina, do sul do estado da Bahia, Brasil, até o norte da Argentina (Figura 3). É a serpente responsável por cerca de 90% dos registros de acidentes devido a sua alta adaptabilidade a diferentes habitats (floresta, campos abertos, propriedades rurais com intensa exploração agrícola e até cidades) (Antunes et al., 2010, da Silva et al., 2003). A população mais atingida é composta por indivíduos jovens, do sexo masculino, trabalhadores de áreas rurais, caracterizando os envenenamentos como acidentes ocupacionais.

Clinicamente, o envenenamento pode ser dividido em sintomas locais e sistêmicos. Os sintomas locais são dor e edema no local da picada, formação

ocasional de bolhas, necrose tecidual e hemorragia. Sistemicamente ocorrem hemorragia, sangramentos espontâneos de mucosas e falência renal. Nos exames laboratoriais do sangue de acidentados, observa-se trombocitopenia nas primeiras 48 horas pós-acidente, prolongados tempos de coagulação e a diminuição da contagem de células vermelhas (Santoro et al., 2008). Além da trombocitopenia, a reatividade plaquetária também é alterada com hipoagregabilidade a ADP e ristocetina (Sano-Martins et al., 1997).

Figura 3 - Distribuição geográfica da serpente *Bothrops jararaca*.

Do ponto de vista experimental, as atividades tóxicas do veneno de *B. jararaca* são amplamente estudadas. Em modelos animais, é possível reproduzir os distúrbios hemostáticos com prolongamento dos tempos de coagulação e de sangramento, as alterações plaquetárias e os danos teciduais, além de ser possível também reproduzir o edema, inflamação e danos renais (de Roodt et al., 2003, Francischetti et al., 1998, Picolo et al., 2002, Sano-Martins et al., 1995, Santoro & Sano-Martins, 2004, Zingali et al., 1990).

Os componentes protéicos responsáveis pelas atividades biológicas do veneno da jararaca já foram descritos por diversas técnicas, incluindo a proteômica

e a transcriptômica (Cidade et al., 2006, Fox et al., 2006, Serrano et al., 2005, Terra et al., 2009). O veneno é composto por diversas famílias de proteínas, principalmente metaloproteases, serinoproteases, fosfolipases, desintegrinas, lectinas, e L-amino oxidases (Terra et al., 2009). Dentro de cada família existe uma série de isoformas dessas proteínas capazes de atuar sinergicamente e com alta especificidade, compondo, no conjunto, as bases bioquímicas dos processos que desencadeiam o desenvolvimento do quadro patológico.

Dentre as famílias que compõem os venenos botrópicos, as metaloproteases são as enzimas mais estudadas. A literatura específica atribui a este grupo de enzimas grande parte dos efeitos patológicos locais e sistêmicos, porque tanto a hemorragia como a necrose são causadas por degradação proteolítica de componentes da membrana basal envolvidos na manutenção da integridade estrutural do tecido (Fox & Serrano, 2005). As metaloproteases de venenos de serpente, denominadas SVMP (*Snake Venom Metalloproteases*) compreendem um subgrupo de reprolisinas da classe M12 de metaloproteases e são estruturalmente relacionadas às ADAMs (*adamalysin and metalloprotease*) de mamíferos (Bjarnason & Fox, 1995). As SVMPs são classificadas de acordo com a presença de domínios além do domínio catalítico (Figura 4). SVMPs da classe PI possuem apenas o domínio metaloprotease, PII possuem em sua estrutura domínios metaloprotease e desintegrina, PIII (a,b e c) são compostas pelo domínio metaloprotease, um domínio tipo-desintegrina e um domínio rico em cisteína e, finalmente, PIIId são sintetizadas com estrutura similar às demais PIII com a adição de dois domínios tipo lectina ligados por pontes dissulfeto (Bjarnason & Fox, 1995, Fox and Serrano, 2009). Apesar da hemorragia ser o efeito biológico mais observado, algumas metaloproteases não são hemorrágicas e afetam a hemostasia de diferentes

maneiras, como inibindo a agregação plaquetária ou ativando diretamente fatores da coagulação (Fox & Serrano, 2005).

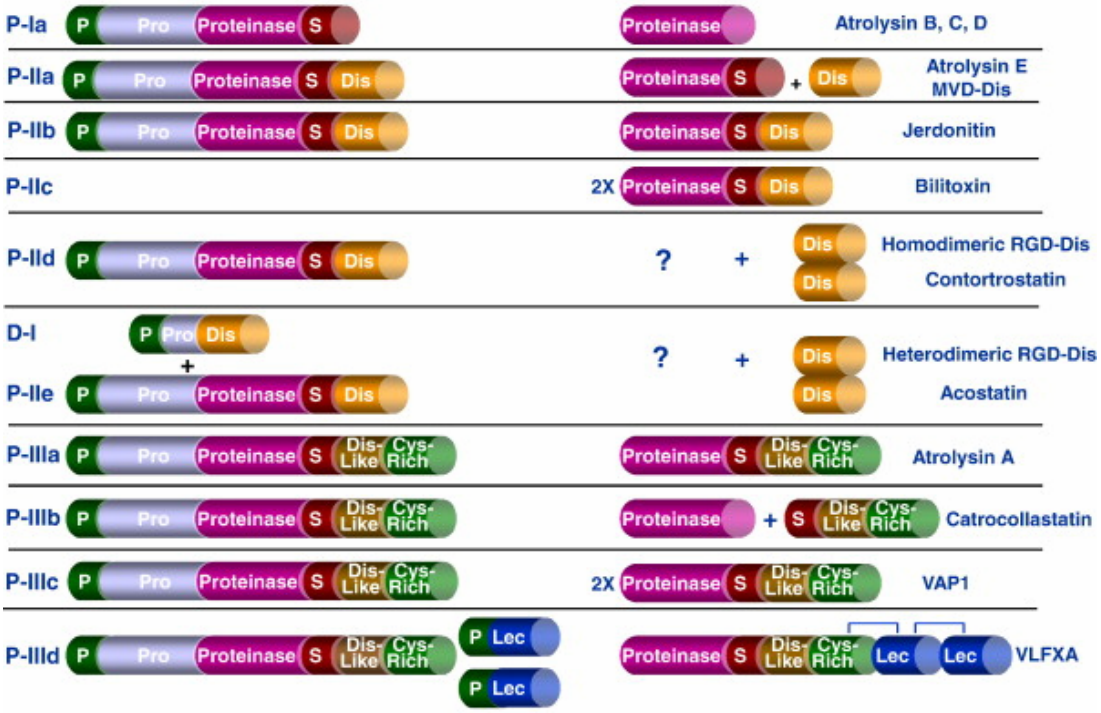


Figura 4 - Esquema das classes de SVMP de acordo com a presença de domínios não-catalíticos (Fox & Serrano, 2009). P (pré) e Pro (pró-peptídeo); S (espaçador); Dis (desintegrina); Dis-like (domínio tipo desintegrina); Cys-rich (domínio rico em cisteínas); Lec (domínio tipo lectina).

3.1.2.2 *Bothrops lanceolatus*

A serpente *Bothrops lanceolatus*, popularmente chamada de *Fer-de-lance* (Ponta- ou Cabeça-de-lança), é uma espécie endêmica da ilha Caribenha de Martinica (Figura 5). Na ilha, esta serpente é a única espécie peçonhenta e, portanto, é responsável pela totalidade dos envenenamentos. Anualmente, ocorrem de 10-20 casos de envenenamentos e cerca de 30% das vítimas evoluem para complicações pró-trombóticas em até uma semana pós-acidente (Thomas et al., 2006).

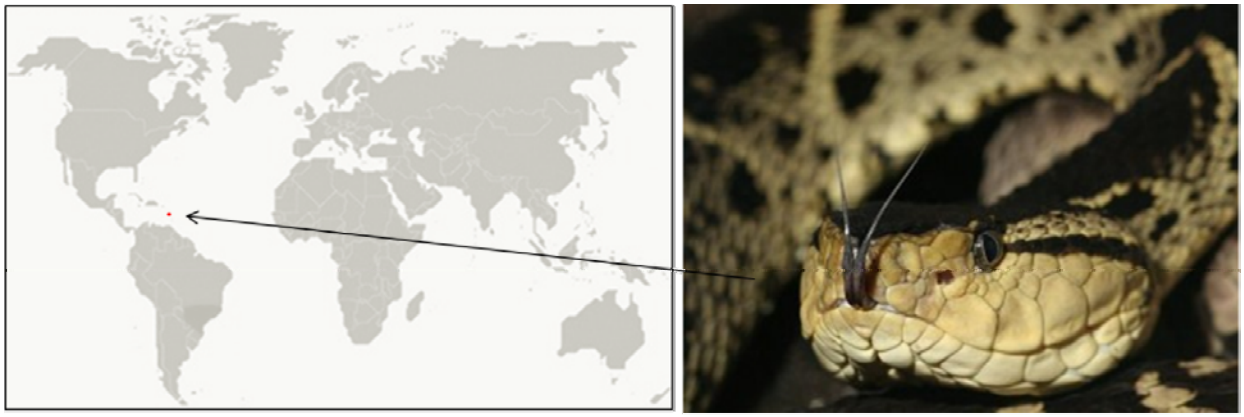


Figura 5 - Distribuição geográfica da serpente *Bothrops lanceolatus*.

Diferentemente dos demais envenenamentos botrópicos observados nas Américas Central e do Sul, acidentes com *B. lanceolatus* evoluem para uma trombose sistêmica, ao contrário de uma síndrome hemorrágica. As vítimas apresentam discreto edema no local da picada. Hemorragia e necrose tecidual são eventos raros, acometendo apenas 5% e 3% dos casos, respectivamente (Thomas et al., 1995, Terra et al., 2009). As manifestações pró-trombóticas ocorrem tardiamente, entre 12 - 96 horas pós-acidente, e acometem tanto pacientes com

envenenamentos graves como os casos moderados. As complicações podem evoluir para múltiplos infartos cerebrais, pulmonares e do miocárdio, levando o paciente a morte (Thomas et al., 1995).

Os pacientes que desenvolvem o quadro clínico pró-trombótico não apresentam alteração no tempo de coagulação, porém cerca de 50% dos pacientes apresentam trombocitopenia e/ou coagulação intravascular disseminada (CID) (Merle et al., 2005). Embora hajam indicações de que a atividade pró-trombótica se deva a uma ativação endotelial seletiva que induziria a adesão e agregação das plaquetas nos microvasos, não estão esclarecidos os mecanismos envolvidos no desenvolvimento da síndrome pró-trombótica. Como também não há um modelo animal capaz de reproduzir apropriadamente esta sintomatologia (Gutierrez et al., 2008), os estudos que poderiam confirmar tal hipótese constituem um desafio a ser vencido. Contrariamente, experimentos *in vivo* com ratos e camundongos demonstraram atividade edematogênica e pró-inflamatória do veneno com aumento da permeabilidade vascular e surgimento do quadro hemorrágico (de Araujo et al., 2000, de Faria et al., 2001, Guimaraes et al., 2004, Gutierrez et al., 2008).

Dentre os componentes tóxicos isolados do veneno da *Fer-de-lance* já foram identificados uma metaloprotease hemorrágica (da classe PI), uma fibrinogenase e uma fosfolipase A2 (Lobo de Araujo et al., 1998, Stroka et al., 2005). Não obstante, isoladamente, nenhuma destas proteínas é capaz de explicar a atividade pró-coagulante deste veneno. Neste sentido, em trabalho recente, Gutierrez e colaboradores, usando técnica proteômica identificaram componentes do veneno de *B. lanceolatus* e relacionaram as atividades tóxicas em camundongos (hemorragia e edema) à elevada presença de metaloproteases, serinoproteases e fosfolipases (Gutierrez et al., 2008). Ressalta-se que estes efeitos observados no

envenenamento experimental divergem do quadro patológico observado em seres humanos.

3.1.3 Perfil Proteômico de Metaloproteases de Veneno de Serpente (SVMPs): *Insights* na Patologia Induzida pelo Veneno

Proteomic Profiling of Snake Venom Metalloproteinases (SVMPs): Insights into Venom Induced Pathology

Renata M. S. Terra, Antônio F. M. Pinto, Jorge A. Guimarães e Jay W. Fox

Periódico *Toxicon (2009), volume 54(6), páginas 836-44*

Resumo - Acidentes envolvendo serpentes do gênero *Bothrops* representam 90% dos casos de enevenenamentos nas Américas do Sul e Central. Os acidentes com a serpente *Bothrops jararaca* são caracterizados por edema, hemorragia e necrose primariamente causados pela ação proteolítica de metaloproteases (SVMPs). Surpreendentemente, acidentes com a serpente *Bothrops lanceolatus* possuem um perfil pró-trombótico com raros casos de necrose e hemorragia. Neste trabalho, descrevemos abordagens bioquímicas e proteômicas aplicadas à comparação destes venenos, focando na presença e atividade de SVMPs.

Os resultados demonstraram que a abundância relativa de SVMPs é muito similar, com a diferença entre os venenos restando na distribuição dos subtipos de SVMPs. O veneno de *B. lanceolatus* possui uma abundância relativa maior de metaloproteases do tipo PI (que apresentam apenas o domínio catalítico) enquanto o veneno de *B. jararaca* possui uma abundância maior de PIII SVMPs (que apresentam além do domínio catalítico, um domínio tipo desintegrina e um domínio rico em cisteína). A atividade gelatinolítica na região de massa de PIII SVMPs também é mais pronunciada em *B. jararaca*, indicando uma degradação mais

efetiva de colágeno. De acordo com os dados obtidos, é possível especular que a diferente distribuição de subgrupos de SVMPs e suas características bioquímico-farmacológicas são os dois fatores colaborando com a discrepância na evolução clínica dos envenenamentos destas serpentes.

Este trabalho foi desenvolvido na Biomolecular Research Facility (University of Virginia) em colaboração com o Laboratório de Bioquímica Farmacológica (UFGRS). Este trabalho foi submetido à revista *Toxicon* em Abril de 2009 e aceito para publicação em Junho de 2009.

Toxicon 54 (2009) 836–844

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Proteomic profiling of snake venom metalloproteinases (SVMPs): Insights into venom induced pathology

Renata M.S. Terra [a,b], Antônio F.M. Pinto [a], Jorge A. Guimarães [b], Jay W. Fox [a,*]

[a] Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, VA, USA
[b] Laboratório de Bioquímica Farmacológica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 21 April 2009
Received in revised form 8 June 2009
Accepted 9 June 2009
Available online 17 June 2009

Keywords:
Bothrops jararaca
Bothrops lanceolatus
Snake venom
SVMP
Metalloproteases
Proteome

ABSTRACT

Bothrops sp. snakebites account for the majority of envenomations in South and Central America. *Bothrops jararaca* accidents are characterized by edema, hemorrhage and necrosis, mainly attributed to the action of hemorrhagic snake venom metalloproteinases (SVMPs). Interestingly, accidents involving *Bothrops lanceolatus* (Fer-de-Lance) have a prothrombotic profile with necrosis and hemorrhage rarely reported. Here we describe biochemical and proteomic approaches to compare the venom composition of these snakes, focusing on the presence and activity of SVMPs. The total relative amount of SVMPs was found to be approximately the same in the venom of both species, the difference being in the distribution of SVMPs subgroups. Fer-de-Lance venom has relatively more PI SVMPs peptides identified (23–16%) while Jararaca venom has a higher amount of PIII SVMPs (54–43%). Gelatinolytic activity in the PIII mass range is also higher in Jararaca venom. Interestingly, the homologous band region in the Fer-de-Lance zymogram was only very weakly gelatinolytic. According to these findings it is feasible that the different distribution of SVMPs subgroups and their particular biochemical and pharmacological characteristics are two of the main factors contributing to these two radically different venom induced pathologies.

1. Introduction

Snakebite envenomation is considered a relevant and neglected issue worldwide (Gutierrez et al., 2006). In South and Central America the *Bothrops* genus is responsible for the majority of the venomous accidents (Fernandez and Gutierrez, 2008; Pinho et al., 2004). In Brazil, *Bothrops* sp. accidents account for 85% of the total accidents and *Bothrops jararaca* is the most common snake involved in snakebite envenomation (Cidade et al., 2006).

B. jararaca (Jararaca) accidents are clinically characterized by edema, systemic bleeding and necrosis. Biochemically, this is the result of sequential consumption of coagulation factors, especially fibrinogen, with a drastic increase in fibrinolysis, thrombin–antithrombin complex, presence of fibrinogen degradation products (FfDP) and severe thrombocytopenia (Santoro et al., 2008; Santoro and Sano-Martins, 2004). In Jararaca envenomation these coagulation disturbances seem to be initiated by the enzymatic activity of venom components acting as Factor X and prothrombin activators (Berger et al., 2008; Santoro and Sano-Martins, 2004), while local hemorrhage and tissue necrosis are mainly related to the action of hemorrhagic snake venom metalloproteinases (SVMPs) (Bjarnason and Fox, 1994).

Interestingly, in the Caribbean island of Martinique, accidents involving *Bothrops* snakes show a prothrombotic profile. *Bothrops lanceolatus* (Fer-de-Lance) is the only snake involved in Martinique envenomation cases. The clinical profile is characterized by systemic thrombosis that can lead to cerebral, myocardial or pulmonary infarctions (Malbranque et al., 2008; Thomas et al., 2006). These

* Corresponding author.
E-mail address: jwf8x@virginia.edu (J.W. Fox).

0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.06.010

manifestations can occur in patients that have no coagulation disturbances and thrombocytopenia seems to be the only significant event in all cases (Thomas et al., 1995). Although a low molecular weight hemorrhagic SVMP has been isolated from Fer-de-Lance venom (Stroka et al., 2005), necrosis and hemorrhagic complications are rare events (5% and 3% of the cases, respectively) (Thomas et al., 1995).

Snake venom metalloproteases are a subgroup of the reprolysins and have been shown to participate in the hemorrhagic process by proteolytic degradation of endothelial cell surface proteins and extracellular matrix components involved in the maintenance of capillary structure and integrity, leading to disruption of capillary networks, edema and hemorrhage (Escalante et al., 2006; Fox and Serrano, 2005). SVMPs classification is based on their different domain structures, presented as follows: P-I (SVMPs with only a metalloprotease domain), P-IIa to P-IIe (containing metalloprotease and disintegrin domains; might be present as dimers or only dimeric disintegrins), PIIIa to PIIIc (containing metalloprotease, disintegrin-like and cysteine-rich domains, can also be present as dimers), and finally PIIId, formally known as PIV (containing the PIII structure and two C-Type lectin domains connected by disulfide bonds) (Bjarnason and Fox, 1995; Fox and Serrano, 2008b).

Proteomic analysis of snake venoms has been proven to be an important tool to enhance the understanding of their molecular composition; synthesis and post-translational modification and processing; their interaction with each other; and the mechanisms underlying their physiological/pharmacological actions (Fox and Serrano, 2008a). Currently many groups are utilizing this approach to understand the taxonomic, ontogenetic, geographical and individual variations of snake venoms (Angulo et al., 2008; Bazaa et al., 2005; Calvete et al., 2007; Georgieva et al., 2008; Serrano et al., 2005). Furthermore, continued development of mass spectrometry as applied to proteomics has given new insights into the molecular basis of distinct envenomation-induced clinical patterns.

Recently, the proteome of *B. lanceolatus* was analyzed and compared to the closely related snake *Bothrops caribbaeus* (Gutierrez et al., 2008). Toxicological and enzymatic profiles were also studied. Of significance was the fact that the procoagulant activity observed in human as thrombosis could not be reproduced in mice (Gutierrez et al., 2008). Proteomic analyses have indicated that the toxicological effect of Fer-de-Lance venom may be associated with the relative abundance of toxins such as serine proteinases, phospholipases and metalloproteinases.

In this work we combine biochemical and proteomic approaches to compare the differences between *B. jararaca* and *B. lanceolatus* venoms, focusing on SVMPs, in order to better understand the clinical significance of the venom's protein components and their role in these radically different envenomation pathophysiologies.

2. Material and methods

2.1. Snake venoms

B. jararaca and *B. lanceolatus* venoms were purchased from Latoxan (Valence, France). All other venoms were generously donated by Centro de Informação Toxicológica (CIT-RS, Brazil). Venoms were kept lyophilized at −20 °C until use. All protein determinations were done with NanoOrange Protein Quantitation Kit (Invitrogen – Carlsbad, CA).

2.2. Proteolytic activity

Proteolytic activity of *B. jararaca* and *B. lanceolatus* venoms was assayed with azocasein as substrate (Sigma–Aldrich – St. Louis, MO) according to Wang et al. (Wang et al., 2004) with modifications. Briefly, different concentrations of snake venoms were diluted in 50 μL of 20 mM Tris HCL, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 buffer and added to 50 μL of a 4 mg/mL azocasein solution. The mixture was incubated for 2 h at 37 °C. The reaction was then stopped with 100 μL of 10% trichloroacetic acid solution. Samples were centrifuged for 15 min at 4000 rpm, 4 °C. Supernant (100 μL) was mixed with 0.5 M NaOH (1:1) and absorbance measured at 440 nm.

For inhibitory assays 2 mM PMSF (phenylmethanesulphonyl fluoride) or OPA (o-phenanthroline) was added to venoms solutions 15 min prior to addition of the substrate.

Data are expressed as mean ± SEM three independent samples. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test correction. A p value of less than 0.05 was considered significant.

2.3. SDS-PAGE and gelatin zymography

SDS-PAGE (12%) was performed according to Laemmli (Laemmli, 1970) under non-reducing conditions. Protein bands were detected after staining with Coomassie brilliant blue.

Gelatin zymography was performed in 12% SDS-PAGE gels containing 1 mg/mL gelatin (Sigma–Aldrich – St. Louis, MO). The gel was equilibrated in 2.5% Triton X-100 for 1 h at room temperature and then for 30 min in 20 mM Tris HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4. The buffer was replaced by a fresh buffer solution and the gel incubated overnight at 37 °C. Gel was Coomassie brilliant blue stained.

2.4 LC/MS/MS analysis

SDS-PAGE gels sections were excised according to the size distribution of the major subgroups of snake venom metalloproteinases (SVMPs): Section 1 (>75 kDa), representing high molecular weight SVMPS, mainly PIIId and dimeric PII; Section 2 (75–37 kDa), representing PIIIa and Section 3 (<37 kDa), representing PI and free disintegrins/disintegrin-like cys-rich domains.

Gel sections and selected bands from 1D SDS-PAGE were destained for 2 h. The proteins in the sections were reduced for 30 min (30 μl 10 mM DTT) and alkylated for another 30 min in the dark (30 μl 50 mM iodoacetamide) at room temperature. Gel sections were washed with 100 mM ammonium bicarbonate and dehydrated with 100% acetonitrile before being dried in speed vac. Gel sections were then hydrated in a solution of Promega modified trypsin

(20 ng/mL) in 50 mM ammonium bicarbonate for 30 min on ice. Excess trypsin was removed and the digestion was carried on for 18 h at 37 °C. The tryptic peptides were extracted from gel sections twice with 30 μL of 50% acetonitrile/5% formic acid solution. The combined extracts were dried to a volume of 15 μL for mass spectrometric analysis.

LC/MS/MS was performed using a Thermo Electron LTQ ion-trap mass spectrometer. Depending on the intensity of the bands, 20–50% of the in-gel digest was injected into the mass spectrometer at 300 nL/min. Peptides were eluted from the C18 column using an acetonitrile/0.1 M acetic acid gradient (2–80% acetonitrile). The instrument was programmed to acquire a cycle of one mass spectrum followed by MS/MS on the ten most abundant ions in a data-dependent mode with exclusion of 120 s.

The mass spectra were extracted and analyzed utilizing Bioworks Sequest 3.11 software. Searches were performed against a snake venom protein database containing sequences deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/). Spectra generated on the LTQ were searched using 1.5 Da parent tolerance and 1 Da fragment tolerance. All hits were required to be from complete tryptic peptides.

Scaffold (version 2.01.01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they contained at least one identified peptide. Quantifications were based on number of unique peptides or number of unique spectra. Protein families' abundance was considered "high" if they represent more than 5% of the total number of unique peptides count in the whole venom, while low abundance was considered families representing less than 5% of the total unique peptides content.

3. Results

3.1. Venoms enzymatic characterization

In order to evaluate the proteolytic action of *Bothrops* venoms, enzymatic activity was measure by two different assays: azocasein degradation and gelatin zymography. Proteolytic activity of *B. lanceolatus* venom was shown to be higher than *B. jararaca* on azocasein assay (Fig. 1A). The activity of both venoms was inhibited by 2 mM o-phenanthroline to varying extents (94% and 81% inhibition for *B. jararaca* and *B. lanceolatus*, respectively), suggesting a major role of metalloproteinases in substrate degradation for both venoms (Fig. 1B). Serine proteinase activity (measure by inhibition with 2 mM PMSF) accounted for only 12% of *B. jararaca* and 7% of *B. lanceolatus* total venom proteinase activity (Fig. 1B). Gelatin zymography was chosen as a method to partially represent collagen proteolysis which may be related to *in vivo* matrix degradation. Jararaca venom presented overall greater gelatin degradation, especially in the 37–75 kDa range (Fig. 2A). Gelatinolytic activity could also be observed in the mass ranges above 75 kDa and below 37 kDa. In contrast, Fer-de-Lance venom gelatinolytic activity was concentrated on the region above and around 75 kDa and particularly on the 25 kDa band. When compared to other hemorrhagic venoms from the *Bothrops* family, *B. lanceolatus* has a lower gelatinolytic activity in the 37–75 kDa range, corresponding to the PIII SVMPs mass range (Fig. 2B). Interestingly there is a non-gelatinolytic, major band around 40 kDa, similar in intensity to the jararhagin band in *B. jararaca* venom.

To further elucidate the presence or absence of SVMPs, an antibody raised against Atrolysin A (AtroA), a hemorrhagic PIIIa from *Crotalus atrox* venom was used in Western blot analysis of the two venoms. As seen in Fig. 3, the amount of PIII SVMPs is much greater in Jararaca then in Fer-de-Lance. More interestingly, the non-gelatinolytic (~40 kDa) band in *B. lanceolatus* venom is recognized by the PIII antibody.

3.2. Comparative proteomic analysis

Based on the enzymatic characterization and in order to elucidate the protein distribution profiles of the two *Bothrops* snakes venoms we combined the non-reducing 1D-gel electrophoresis and LC/MS/MS analysis. The protocol utilized was designed to give a comparative, semi-quantitative methodology highlighting the SVMPs presence in these venoms. *B. jararaca* and *B. lanceolatus* crude venoms were separated by SDS-PAGE and the proteomic analysis followed by two approaches: (1) analysis of gel sections chosen to represent SVMPs subgroups based on their molecular size distribution and (2) analysis of single bands of interest in *B. lanceolatus* venom.

LC/MS/MS analysis of the first gel section (>75 kDa, Fig. 4) showed a difference of 9% in the distribution of unique peptides content of SVMPs between the two venoms, high molecular weight metalloproteinases content appeared to be higher in *B. jararaca* venom (Fig. 5). On the other hand, snake venom serine proteinase (SVSP) content is more abundant in *B. lanceolatus* (12%) than in Jararaca (5%). L-amino acid oxidase (LAAO) and C-type lectin-like proteins (CLP) abundance seems to be the equivalent in both venoms, but the presence of phospholipase A2s (PLA2) was observed in this mass range only in *B. lanceolatus* venom. A single unique peptide of a cystatin in *B. jararaca* and a three-finger toxin (3FTx) in *B. lanceolatus* venom were also identified (Supplementary Tables SI and SII).

Proteomic analysis of the second section (between 37 and 75 kDa) followed the same comparative distribution as Section 1, with higher amounts of SVMPs (54%) and lowers amounts of SVSP peptides (12%) in Jararaca venom, when compared to Fer-de-Lance venom (43% of SVMP and 26% of SVSP). The major difference in this mass range is the relatively greater abundance of CLP peptides (10% of the gel section) in *B. jararaca* venom. Among the low abundance protein families, two unique peptides from the cysteine-rich secretory protein family (CRISP) were identified only in *B. jararaca*; the presence of peptides from 3FTx, vascular endothelial growth factor (VEGF) and coagulation factor V (Cu-oxidases) was observed in both venoms.

The third gel section (<37 kDa) showed an opposite distribution of metalloproteinases in relation to the previous sections. Although the distribution of SVMPs seems to be the same when grouping together the metalloproteinase and disintegrin/disintegrin-like/cysteine-rich

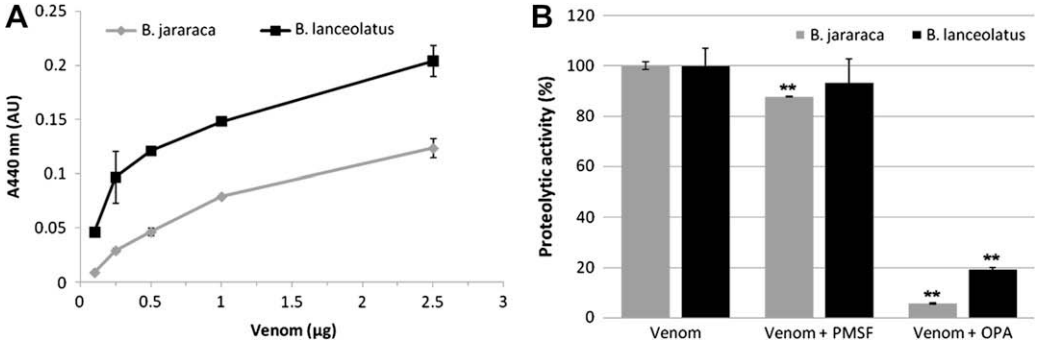


Fig. 1. Proteolytic activity of *B. jararaca* and *B. lanceolatus* venoms. A. Azocasein degradation. Different concentrations of venoms were incubated with azocasein for 2 h at 37 °C. Reactions were stopped and absorbance monitored at 440 nm. Activity is shown as arbitrary units. Results are presented as mean ± SD for three independent samples. B. Effect of inhibitors on venoms proteolytic activity. Venoms (2.5 µg) were incubated with 2 mM PMSF or 2 mM OPA for 15 min prior to addition of substrate. Activity was measured by monitoring absorbance at 440 nm. Inhibition is shown as residual activity of the control (no inhibitors). Each bar represents the mean of 3 independent experiments and vertical lines show SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test correction.

domains (around 23–28% of the proteins in the section) the amount of metalloproteinases domains is more abundant in *B. lanceolatus* (23%) than in *B. jararaca* venom (16%). In this mass range it is necessary to consider that most, if not all, SVMPs belong to the PI subgroup of metalloproteinases. In addition, we see a difference in SVSP distribution (about 5%), following the tendency of the other sections, in which this class of proteins was also more abundant in *B. lanceolatus* venom. The relative abundance of CLPs and PLA2 is higher in *B. jararaca* while LAAO is lower. At this mass range, we found a relatively large amount of CRISP peptides in *B. jararaca*. In addition, several unique peptides of venom nerve growth factors (vNGF) were found in both venoms. Among the very low abundance proteins, it was identified the presence of peptides from 5′-nucleotidases, cystatins and VEGF in Jararaca venom and bovine pancreatic trypsin inhibitor (BPTI), CNF-like inhibitor, bradykinin-potentiating peptides, and a myotoxin in Fer-de-Lance venom.

The combined unique peptides from all gel sections provide a view of the overall distribution of protein families in the venoms (Table 1 and Fig. 5). SVMPs are present in the same relative amount in both venoms, and show a higher abundance of PIIIa and PIIId SVMPs in *B. jararaca* and PI SVMPs in *B. lanceolatus*. SVSPs and LAAOs are present in slightly greater abundance in Fer-de-Lance venom (5% and 4% difference, respectively). On the other hand, CLP and PLA2 are more abundant in Jararaca venom. CLP represent 13% of *B. jararaca* and 4% of *B. lanceolatus* venom, while PLA2 represent 8% of Jararaca and 4% of Fer-de-Lance venom.

Comparison of unique spectra count was also performed in order to evaluate the best relative quantitation

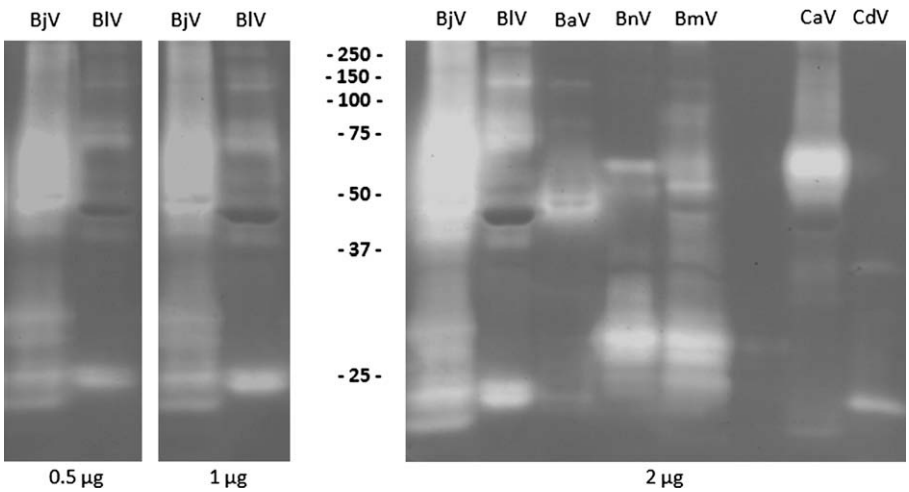


Fig. 2. Gelatinolytic activity of SVMPs in *B. jararaca*, *B. lanceolatus* and other bothropic and crotalid venoms. Left panel – Gelatin zymography. Gelatinolytic activity was observed in gel. BjV (*B. jararaca* venom) and BlV (*B. lanceolatus* venom) in two different amounts were separated by gelatin SDS-PAGE and activity was determined by incubation (18 h at 37 °C) in 20 mM Tris HCl, 150 mM NaCl, 4 mM $CaCl_2$, pH 7.4. Gels were Coomassie blue stained. Right panel – Comparison among other bothropic and crotalid venoms gelatinolytic activity. BjV (*B. jararaca* venom), BlV (*B. lanceolatus* venom), BaV (*B. alternatus* venom), BnV (*B. neuwiedi* venom), BmV (*B. moojeni* venom), CaV (*C. atrox* venom) and CdV (*C. durissus* sp. venom).

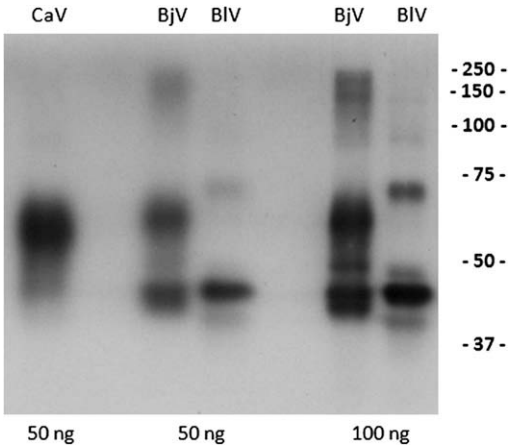


Fig. 3. Antibody recognition of SVMPs. Presence of PIII SVMPs on BjV and BlV was evaluated by western blotting with a polyclonal antibody raised against Atrolysin A, a PIIIa SVMP from *C. atrox* venom (CaV). Molecular markers are represented on the right.

parameter and no significant difference was observed between the two methods (supplementary material – Fig. S1). Detailed information with all peptides identified is show in supplementary material (Tables SI and SII).

The electrophoretic profiles of both venoms generally appear somewhat similar with the presence of a significant protein staining in the region between 37 and 50 kDa, corresponding to the PIII SVMP jararhagin, in Jararaca venom and a SVMP similar to jararhagin and acutolysin-E with 10 and 3 peptides identified, respectively, in Fer-de-Lance venom (Fig. 4). Other three single bands (around 200, 125 and 65 kDa, Fig. 4) were chosen to be sequenced based on their gelatinolytic activity. The top two database hits for each band are shown and all these bands were also identified as SVMPs.

4. Discussion and conclusion

In this work we report a comparative, semi-quantitative proteomic analysis of two *Bothrops* species (*B. jararaca* and *B. lanceolatus*) with different intriguing biological venom activities. We describe here a novel proteomic approach based on one dimension gel electrophoresis (1D-gel) followed by LC/MS/MS analysis designed to evaluate the presence and importance of a major protein family of interest: snake venom metalloproteases (SVMPs). The proteomic analysis were designed, compared and correlated to classical biochemical approaches in order to generate a better understanding between presence of snake venom metalloproteinases and the catalytic activity of the crude venoms.

Bothrops snakes envenomations are well known by their dramatic symptomology of generalized bleeding

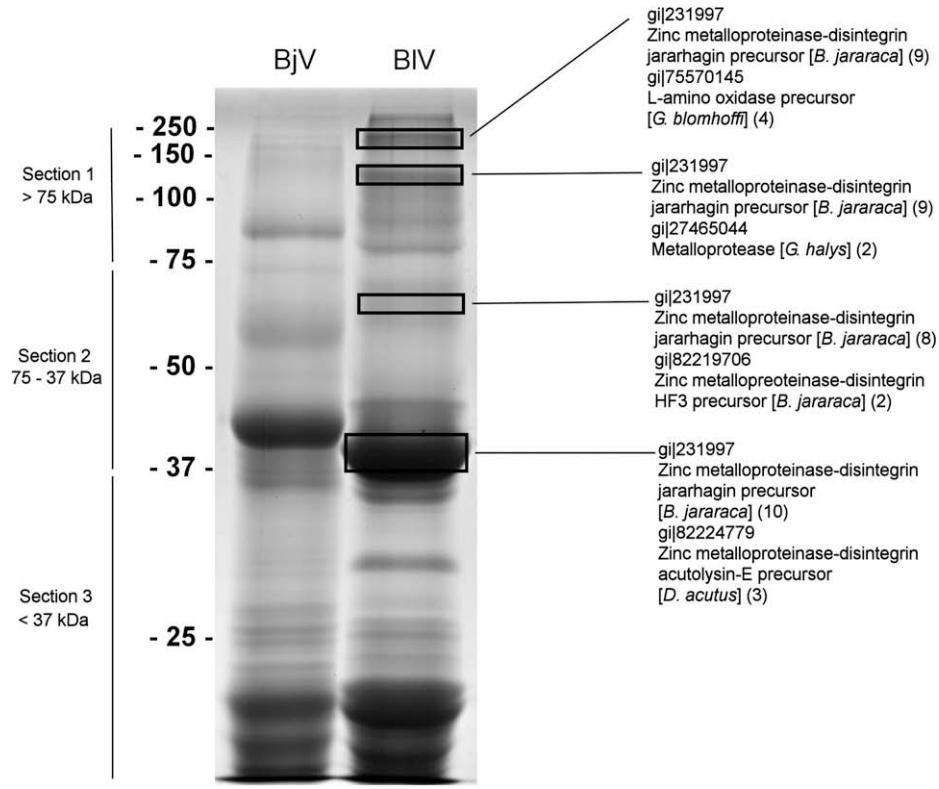


Fig. 4. SDS-PAGE of *B. jararaca* and *B. lanceolatus* venoms. Thirty micrograms of the venoms were separated by SDS-PAGE. Three sections (Sections 1–3, left) covering the full molecular weight range were cut and submitted to trypsin digestion prior to analysis by LC/MS/MS. Also, individual bands from *B. lanceolatus* venom were selected, digested and analyzed by LC–MS/MS. The two top hit identification of each band are represented on the right.

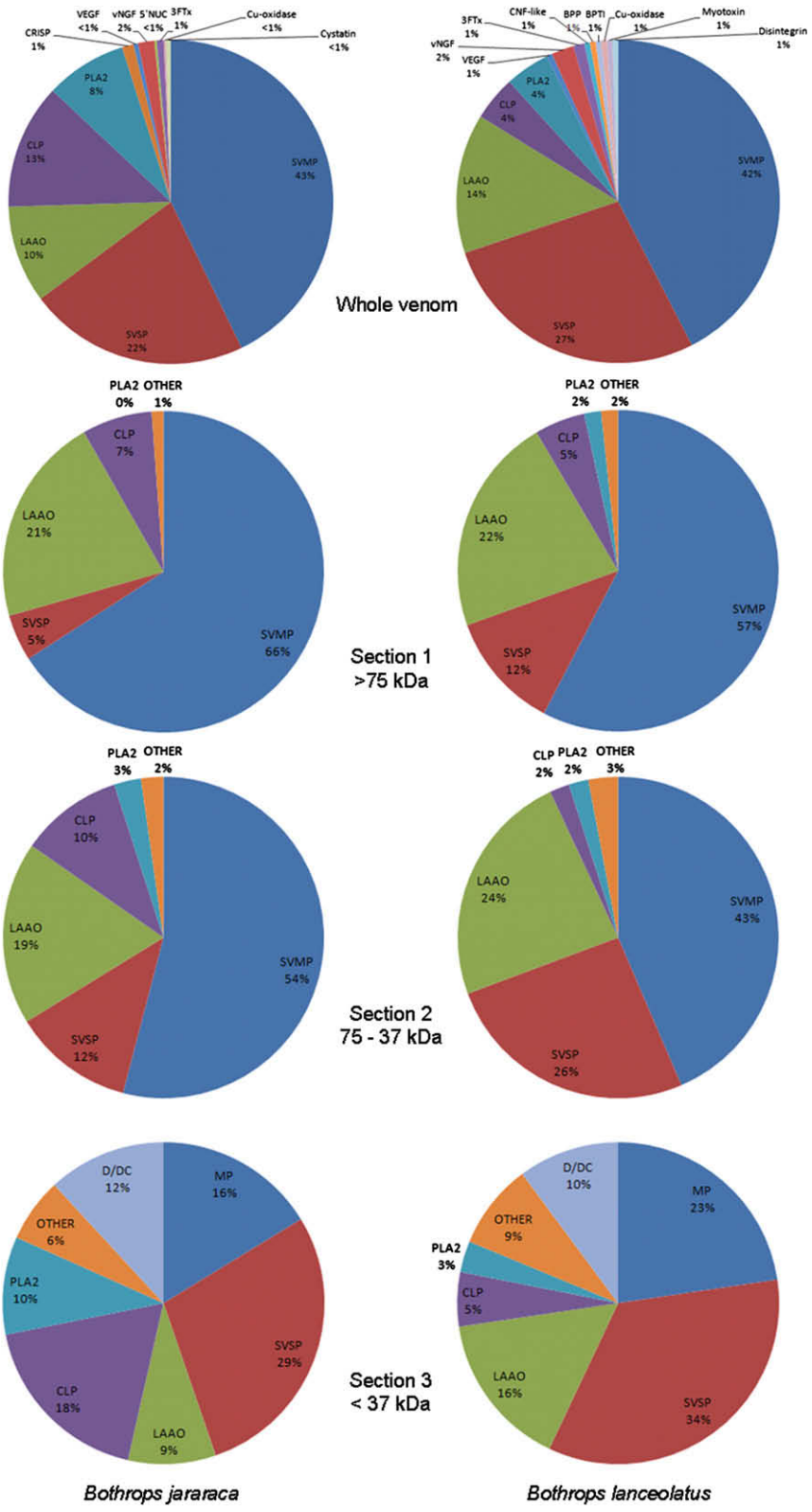


Fig. 5. LC/MS/MS identification of venom proteins. Sections cut from SDS-PAGE (Fig. 4) were submitted to LC/MS/MS analysis. Pie charts representing internal distribution of venom proteins by class are shown. Abundance of protein families was based on the number of unique peptides. SVMPs (Snake venom metalloproteinases), SVSP (Snake venom serine proteases), LAAO (L-amino acid oxidases), CLP (C-type lectin-like proteins), PLA2 (Phospholipases A2), CRISP (Cysteine-rich secretory proteins), VEGF (Vascular endothelial growth factors), vNGF (Venom nerve growth factors), 5′NUC (5′-nucleotidases), 3FTx (Three-finger toxins), Cu-oxidase (Coagulation factors V – copper oxidases), BPP (Bradykinin-potentiating peptides), BPTI (Kunitz/Bovine pancreatic trypsin inhibitors), CNF-like (*Crotalus* PLA2-neutralizing factor-like), MP (metalloprotease domain), D/DC (Disintegrin, Disintegrin-like, Cysteine-rich domains).

Table 1
Overview of protein families' distribution in the venoms of *B. jararaca* and *B. lanceolatus*. Percentage of the total combined unique peptide count of all three gel sections (Fig. 4).

Protein family	*Bothrops jararaca* abundance (%)/unique peptides (n)	*Bothrops lanceolatus* abundance (%)/unique peptides (n)
SVMP	42.8%/185	42.4%/76
SVSP	22%/95	27.4%/49
LAAO	9.7%/42	14%/25
CLP	12.5%/54	4.5%/8
PLA2	8.1%/35	4.5%/8
CRISP	1.1%/5	n.i.
VEGF	>1%/2	>1%/1
vNGF	1.6%/7	2.2%/4
5′NUC	>1%/1	n.i.
3FTx	>1%/3	>1%/2
Other	>1%/3	3.3%/6

accompanied by local tissue edema and necrosis (de Roodt et al., 2003). Central and South American *Bothrops* species (including *B. jararaca*) share the similar enzymatic activity profiles that leads to a consumption coagulopathy with a decrease in fibrinogen and other plasmatic proteins levels as well as severe thrombocytopenia. Distinguishing themselves from this "classical" clinical envenomation profile are the venoms of two geographically isolated Central American *Bothrops* species: *B. lanceolatus* and *B. caribbaeus*. In these cases hemorrhage is rarely observed and, more interestingly, 25% of the patients have thrombotic complications that can evolve to death.

The comparison of envenomation characteristics between *B. jararaca* and *B. lanceolatus* provides an interest perspective for differentiating the actions of snake venom metalloproteinases. These enzymes have been identified and isolated from all viperid snake venoms and likely represent the most important protein class contributing to hemorrhage and necrosis.

The caseinolytic activity of venom is one of the simplest ways to describe its proteolytic potential. When compared with *B. jararaca*, the venom of *B. lanceolatus* presented a slightly greater caseinolytic activity. This data could suggest that this venom would display a more effective action when exposed to biologically relevant substrates. However, when the crude venom was tested on the more appropriate substrate, gelatin, a form of denature collagen, this was not the case. Fer-de-Lance venom, though possessing the potential ability to degrade generic protein substrates, such as casein, showed low activity for gelatinolysis which may be associated with a lack of activity towards critical extracellular matrix (ECM) proteins whose degradation are associated with hemorrhage (Baramova et al., 1989; de Roodt et al., 2003). In addition, the general proteolytic activity in both venoms was shown to be mainly due to metalloproteinase activity, as highlighted by OPA inhibition assay. Moreover, when proteolytic activity using gelatin zymography was compared, there was a clear difference in the contribution of SVMPs with different molecular weights. Fer-de-Lance venom gelatinolytic activity is concentrated mostly in a low molecular range (above 25 kDa) while Jararaca activity is distributed mainly in the mid-range molecular weight (between 37 and 75 kDa). This was the first indication of different distribution of SVMPs subgroup in the two venoms.

Snake venom metalloproteases classification is primarily based on domain components and, therefore associated with their molecular weight. It is well known that, in general, PIIIa SVMPs are more hemorrhagic (determined by their minimal hemorrhagic dose – MHD) than PI SVMPs, lower molecular weight enzymes (Escalante et al., 2006). PIIIa SVMPs are known to interact with von Willebrand factor A domains found in some extracellular matrix proteins through their DC domain, which by their affinity/interaction may enhance their proteolytic activity (Serrano et al., 2007). When illustrating the presence of different amounts of PIIIa SVMPs in both venoms by antibody recognition, it was clear that the abundance of this subgroup is greater in Jararaca than in Fer-de-Lance. In addition, antibody recognition of these enzymes could provide insights regarding their activity since it has been suggested that there is a correlation between recognition and hemorrhagic activity (Rafael et al., 2008).

B. jararaca venom has a 52 kDa enzyme, Jararhagin, which is well studied and known to cause the major effects on tissue of envenomed victims (Laing and Moura-da-Silva, 2005). Jararhagin is also known to promote neutrophil migration, to directly interact with von Willebrand factor and to degrade many ECM proteins (Costa et al., 2002; Serrano et al., 2007). Comparison of the electrophoretic profiles of both venoms revealed in Fer-de-Lance venom a major band in the 40–50 kDa mass range which was identified as a PIIIa SVMP and assumed to be the molecular mass counterpart of Jararhagin in this venom. Although sequencing of this band gave 13 unique peptides similar to other hemorrhagic PIIIa SVMPS and was recognized by the Atrolysin A antibody, this enzyme has minimal gelatinolytic activity. This is relevant when considering that gelatinolytic activity and hemorrhagic potential have been shown to be associated (de Roodt et al., 2003), indicating a low hemorrhagic potential of this enzyme. This observation is also supported by the fact that cases of tissue necrosis and local or systemic hemorrhage are rarely reported in *B. lanceolatus* envenomation (Thomas et al., 1995). Still, this is not the first PIIIa SVMP with high sequence identity with other SVMPs and with no hemorrhagic activity.

Only a few non-hemorrhagic PIII SVMP enzymes have been isolated and characterized from *Bothrops* sp. venoms. PIIIa SVMPs with prothrombin activating activity and no hemorrhagic capability were identified and characterized in *Bothrops erythromelas* and *Bothrops neuwiedi* venoms (Govers-Riemslag et al., 1987; Silva et al., 2003). Despite the prothrombin activators presence, none of these bothropic venoms present a procoagulant envenomation profile. Furthermore, prothrombin activators are attributed to cause hemorrhage by enhancing consumption coagulation pathology (Berger et al., 2008). When we compared gelatinolytic activity between different *Bothrops* sp. snakes and two *Crotalus* sp. snakes we observed a correlation between the presence of a high proteolytic band in the PIIIa mass range (around 50 kDa) and the hemorrhagic pattern of the envenomation. Highly proteolytic and, therefore, hemorrhagic venoms have at least one major band in this mass range – e.g. *B. jararaca* and *C. atrox* – while low hemorrhagic

venoms are completely or partially devoid of these bands – e.g. *B. lanceolatus* and *Crotalus durissus* sp.

The proteomic approach we utilized in this investigation allowed for the generation of a distribution pattern of SVMPs classes in both venoms. Using a different approach, a recent proteomic study (Gutierrez et al., 2008) reported that Fer-de-Lance venom has 48.4% of the total protein composition being PIII SVMPs and only 25.8% being PIs. Considering the total composition, it was reported that SVMPs represents 74% of Fer-de-Lance venom. Our comparative methodology was designed based on the amount of unique peptides and their relative quantitation on different molecular mass ranges. Here we identified only 42% of the total *B. lanceolatus* venom composition being SVMPs and that this percentage is almost the same for *B. jararaca* venom (43%). We also showed that *B. lanceolatus* venom has relatively more PI SVMPs peptides identified in the above 25 kDa mass range (23%) than *B. jararaca* venom (16%). To date the only SVMP isolated and characterized from *B. lanceolatus* venom was a low molecular weight PI. The discrepancy in quantitation between our data and those Gutierrez and colleagues (Gutierrez et al., 2008) could be explained by the different approaches used. However, it is important to emphasize that our approach allowed the differentiation of SVMPs subgroups and, highlighted useful ways to correlate their presence with the activity of the venom.

With regard to the presence of other proteins, here we identified the major venom protein classes such as SVSPs, LAAOs, PLA2s and CLPs. Serine proteinases are the second most abundant protein class in both venoms. Interestingly, Jararaca venom has more CLP and PLA2 than Fer-de-Lance venom. The difference in composition of these other protein families could give insights for further investigations. Also, our approach was able to identify less abundant protein classes that were not identified by the previous *B. lanceolatus* proteomic work (Gutierrez et al., 2008). Besides the high abundant venom protein classes, *B. lanceolatus* venom has vNGF, VEGF, 3FTx, CNF-like, BPP, BPTI, Cu-oxidase, myotoxin and disintegrin proteins. Moreover, *B. jararaca* minor protein families identified in this work were in accordance with what has been published in previous proteomics and transcriptomics investigations (Cidade et al., 2006; Fox et al., 2006).

Although we were able to identify and compare the distribution of SVMPs subclasses and hypothesize their role in toxicological function in the two venoms, the envenomation process is a balance of many different enzymes activities as well as the host response. Coagulation disturbances are caused by the relationship of different enzymatic pathways. Prothrombin activation associated with an efficient fibrinolytic system leads to a consumption hemorrhage, as seen in Jararaca envenomation (Santoro and Sano-Martins, 2004), instead of a predominant procoagulant response expected by thrombin generation. Moreover, considering only the coagulation aspect of envenomation, one can observe at least five families of venom proteins (SVMPs, SVSPs, CLPs, PLA2 and 5′-NUCs) that could interact and contribute to those different clinical profiles. In addition, it could not be excluded the possibility of novel, unidentified toxins in the venom of Fer-de-lance. Thus, the study of the association of more than one protein family as well as the whole venom is crucial to describe all aspects of the clinical response to a typical envenomation. Unfortunately, to date, there is no successful animal model describing the thrombotic clinical manifestation observed in *B. lanceolatus* human envenomation (Gutierrez et al., 2008), while accidents involving *B. jararaca* have been well reproduced in more than one model (Sano-Martins et al., 1995; Santoro and Sano-Martins, 2004).

In summary, the present work reports the distinct protein distribution in the venoms of *B. jararaca* and *B. lanceolatus* by a combined approach of classical biochemical assays and proteomics. Of particular importance is the description of the different relative distribution of snake venom metalloproteinases (SVMP) subgroups as associated with different gelatinolytic potential. The absence of hemorrhagic events in Fer-de-Lance envenomation may be partially attributed to the presence of SVMPs primarily from the subgroup PI – generally considered to be weak hemorrhagins – while Jararaca venom is predominantly composed of high hemorrhagic PIII SVMPs. Our combined methodological approach gives rise to a useful tool in the investigation of presence and activity of SVMPs. In addition, the venoms proteomic profiles elucidated by these studies give rise to insights about other important protein families that could synergistically contribute to the disparate envenomation pathologies.

Acknowledgements

The authors thank the Brazilian agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, Brazil, for the PDEE fellowship to R.M.S. Terra. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) (J.A.G.), CAPES (R.M.S.T.) and the University of Virginia Office of Research Core Administration (J.W.F.).

Conflict of interest

The authors declare that there are no conflict of interest.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxicon.2009.06.010.

References

Angulo, Y., Escolano, J., Lomonte, B., Gutierrez, J.M., Sanz, L., Calvete, J.J., 2008. Snake venomics of Central American pitvipers: clues for rationalizing the distinct envenomation profiles of *Atropoides nummifer* and *Atropoides picadoi*. J. Proteome Res. 7, 708–719.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B., Fox, J.W., 1989. Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. Arch. Biochem. Biophys. 275, 63–71.

Bazaa, A., Marrakchi, N., El Ayeb, M., Sanz, L., Calvete, J.J., 2005. Snake venomics: comparative analysis of the venom proteomes of the Tunisian snakes *Cerastes cerastes*, *Cerastes vipera* and *Macrovipera lebetina*. Proteomics 5, 4223–4235.

Berger, M., Pinto, A.F., Guimaraes, J.A., 2008. Purification and functional characterization of Bothrojaractivase, a prothrombin-activating

metalloproteinase isolated from *Bothrops jararaca* snake venom. Toxicon 51, 488–501.
Bjarnason, J.B., Fox, J.W., 1994. Hemorrhagic metalloproteinases from snake venoms. Pharmacol. Ther. 62, 325–372.
Bjarnason, J.B., Fox, J.W., 1995. Snake venom metalloendopeptidases: reprolysins. Meth. Enzymol. 248, 345–368.
Calvete, J.J., Escolano, J., Sanz, L., 2007. Snake venomics of *Bitis* species reveals large intragenus venom toxin composition variation: application to taxonomy of congeneric taxa. J. Proteome Res. 6, 2732–2745.
Cidade, D.A., Simao, T.A., Davila, A.M., Wagner, G., Junqueira-de-Azevedo, I.L., Ho, P.L., Bon, C., Zingali, R.B., Albano, R.M., 2006. *Bothrops jararaca* venom gland transcriptome: analysis of the gene expression pattern. Toxicon 48, 437–461.
Costa, E.P., Clissa, P.B., Teixeira, C.F., Moura-da-Silva, A.M., 2002. Importance of metalloproteinases and macrophages in viper snake envenomation-induced local inflammation. Inflammation 26, 13–17.
de Roodt, A.R., Litwin, S., Vidal, J.C., 2003. Hemorrhagic activity of *Bothrops* venoms determined by two different methods and relationship with proteolytic activity on gelatin and lethality. Toxicon 41, 949–958.
Escalante, T., Shannon, J., Moura-da-Silva, A.M., Gutierrez, J.M., Fox, J.W., 2006. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. Arch. Biochem. Biophys. 455, 144–153.
Fernandez, P., Gutierrez, J.M., 2008. Mortality due to snakebite envenomation in Costa Rica (1993–2006). Toxicon 52, 530–533.
Fox, J.W., Ma, L., Nelson, K., Sherman, N.E., Serrano, S.M., 2006. Comparison of indirect and direct approaches using ion-trap and fourier transform ion cyclotron resonance mass spectrometry for exploring viperid venom proteomes. Toxicon 47, 700–714.
Fox, J.W., Serrano, S.M., 2005. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon 45, 969–985.
Fox, J.W., Serrano, S.M., 2008a. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. Proteomics 8, 909–920.
Fox, J.W., Serrano, S.M., 2008b. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. FEBS J. 275, 3016–3030.
Georgieva, D., Risch, M., Kardas, A., Buck, F., von Bergen, M., Betzel, C., 2008. Comparative analysis of the venom proteomes of *Vipera ammodytes ammodytes* and *Vipera ammodytes meridionalis*. J. Proteome Res. 7, 866–886.
Govers-Riemslag, J.W., Knapen, M.J., Tans, G., Zwaal, R.F., Rosing, J., 1987. Structural and functional characterization of a prothrombin activator from the venom of *Bothrops neuwiedi*. Biochim. Biophys. Acta 916, 388–401.
Gutierrez, J.M., Sanz, L., Escolano, J., Fernandez, J., Lomonte, B., Angulo, Y., Rucavado, A., Warrell, D.A., Calvete, J.J., 2008. Snake venomics of the lesser antillean pit vipers *Bothrops caribbaeus* and *Bothrops lanceolatus*: correlation with toxicological activities and immunoreactivity of a heterologous antivenom. J. Proteome Res. 7, 4396–4408.
Gutierrez, J.M., Theakston, R.D., Warrell, D.A., 2006. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. PLoS Med. 3, e150.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Laing, G.D., Moura-da-Silva, A.M., 2005. Jararhagin and its multiple effects on hemostasis. Toxicon 45, 987–996.
Malbranque, S., Piercecchi-Marti, M.D., Thomas, L., Barbey, C., Courcier, D., Bucher, B., Ridarch, A., Smadja, D., Warrell, D.A., 2008. Fatal diffuse thrombotic microangiopathy after a bite by the "Fer-de-lance" pit viper (*Bothrops lanceolatus*) of Martinique. Am. J. Trop. Med. Hyg. 78, 856–861.
Pinho, F.M., Oliveira, E.S., Faleiros, F., 2004. Snakebites in the state of Goias, Brazil. Rev. Assoc. Med. Bras. 50, 93–96.
Rafael, A., Tanjoni, I., Fernandes, I., Moura-da-Silva, A.M., Furtado, M.F., 2008. An alternative method to access in vitro the hemorrhagic activity of snake venoms. Toxicon 51, 479–487.
Sano-Martins, I.S., Santoro, M.L., Morena, P., Sousa-e-Silva, M.C., Tomy, S.C., Antonio, L.C., Nishikawa, A.K., Goncalves, I.L., Larsson, M.H., Hagiwara, M.K., et al., 1995. Hematological changes induced by *Bothrops jararaca* venom in dogs. Braz. J. Med. Biol. Res. 28, 303–312.
Santoro, M.L., Sano-Martins, I.S., 2004. Platelet dysfunction during *Bothrops jararaca* snake envenomation in rabbits. Thromb. Haemost. 92, 369–383.
Santoro, M.L., Sano-Martins, I.S., Fan, H.W., Cardoso, J.L., Theakston, R.D., Warrell, D.A., 2008. Haematological evaluation of patients bitten by the Jararaca, *Bothrops jararaca*, in Brazil. Toxicon 51, 1440–1448.
Serrano, S.M., Shannon, J.D., Wang, D., Camargo, A.C., Fox, J.W., 2005. A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. Proteomics 5, 501–510.
Serrano, S.M., Wang, D., Shannon, J.D., Pinto, A.F., Polanowska-Grabowska, R.K., Fox, J.W., 2007. Interaction of the cysteine-rich domain of snake venom metalloproteinases with the A1 domain of von Willebrand factor promotes site-specific proteolysis of von Willebrand factor and inhibition of von Willebrand factor-mediated platelet aggregation. FEBS J. 274, 3611–3621.
Silva, M.B., Schattner, M., Ramos, C.R., Junqueira-de-Azevedo, I.L., Guarnieri, M.C., Lazzari, M.A., Sampaio, C.A., Pozner, R.G., Ventura, J.S., Ho, P.L., Chudzinski-Tavassi, A.M., 2003. A prothrombin activator from *Bothrops erythromelas* (jararaca-da-seca) snake venom: characterization and molecular cloning. Biochem. J. 369, 129–139.
Stroka, A., Donato, J.L., Bon, C., Hyslop, S., de Araujo, A.L., 2005. Purification and characterization of a hemorrhagic metalloproteinase from *Bothrops lanceolatus* (Fer-de-Lance) snake venom. Toxicon 45, 411–420.
Thomas, L., Chausson, N., Uzan, J., Kaidomar, S., Vignes, R., Plumelle, Y., Bucher, B., Smadja, D., 2006. Thrombotic stroke following snake bites by the "Fer-de-Lance" *Bothrops lanceolatus* in Martinique despite antivenom treatment: a report of three recent cases. Toxicon 48, 23–28.
Thomas, L., Tyburn, B., Bucher, B., Pecout, F., Ketterle, J., Rieux, D., Smadja, D., Garnier, D., Plumelle, Y., 1995. Prevention of thromboses in human patients with *Bothrops lanceolatus* envenoming in Martinique: failure of anticoagulants and efficacy of a monospecific antivenom. Research group on snake bites in Martinique. Am. J. Trop. Med. Hyg. 52, 419–426.
Wang, W.J., Shih, C.H., Huang, T.F., 2004. A novel P-I class metalloproteinase with broad substrate-cleaving activity, Agkislysin, from *Agkistrodon acutus* venom. Biochem. Biophys. Res. Commun. 324, 224–230.

3.2 CAPÍTULO II

ENVENENAMENTOS POR LAGARTAS: ANÁLISE PROTEÔMICA DAS SECREÇÕES VENENOSAS DE *Lonomia obliqua*

3.2.1 Acidentes com lagartas

Apesar de comporem um grupo grande de cerca de 125.000 -150.000 espécies e estarem distribuídos mundialmente, poucos Lepidópteros são capazes de causar reações adversas em seres humanos (Hossler, 2010, Hossler, 2009). Porém, Lepidópteros da família Saturnidae são clinicamente importantes por gerarem acidentes graves causando efeitos tóxicos locais e sistêmicos. Espécies do gênero *Lonomia* são de especial interesse por ocasionarem uma síndrome hemorrágica que pode levar ao óbito, relatada desde o final da década de 1960 (Arocha-Pinango & Layrisse, 1969). O gênero é distribuído na América Latina em duas espécies principais, *Lonomia achelous* e *Lonomia obliqua*, que possuem alta incidência na Venezuela e no Brasil, respectivamente. No Sul do Brasil, a espécie *L. obliqua* (Figura 6) é causadora de acidentes graves resultando em óbitos desde a década de 1980 (Duarte et al., 1990). Segundo dados do SINAN, em 2008 foram notificados 592 casos de envenenamento lonômico, sendo a taxa de letalidade de 0,3%.

3.2.1.1 *Lonomia obliqua*

A lagarta *L. obliqua*, popularmente conhecida como taturana, é venenosa apenas na fase larval (do 1º ao 6º instar) quando possui o corpo recoberto por espículas quitinosas (Pinto et al., 2010). Diferentemente de serpentes e outros animais venenosos, a taturana não possui uma glândula especializada na produção de veneno e este tem apenas o propósito de defesa, não sendo utilizado para caça e alimentação. A secreção venenosa é produzida por um epitélio secretor (Figura 7),

sendo o veneno injetado nas vítimas pelas espículas que se quebram ao contato (Veiga et al., 2001).

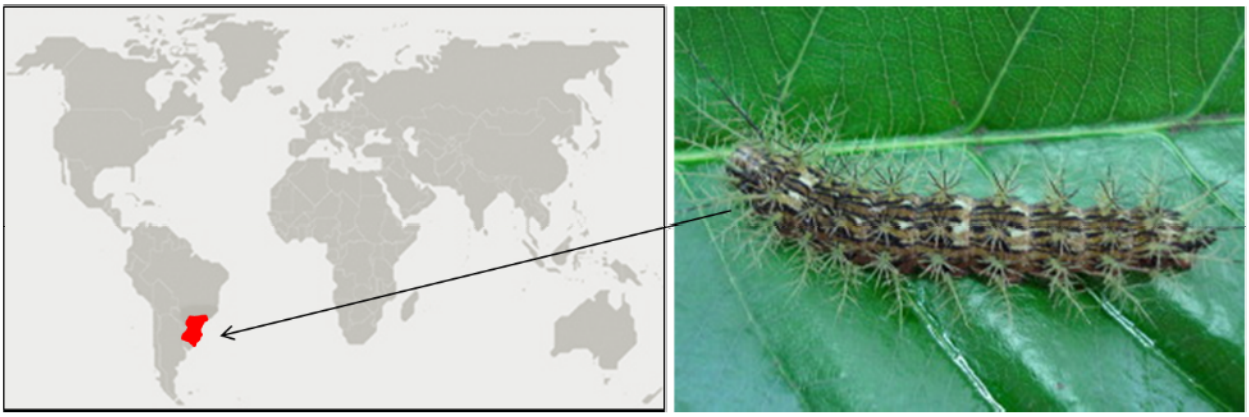


Figura 6 - Distribuição geográfica da lagarta *Lonomia obliqua*.

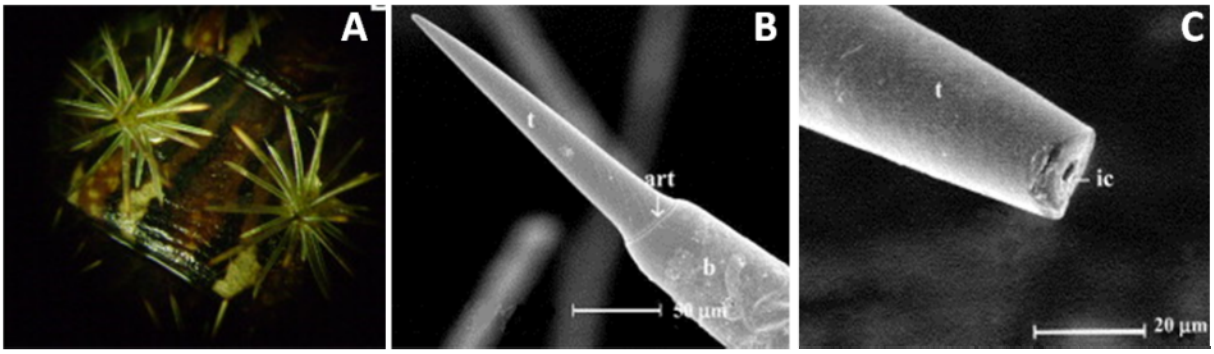


Figura 7 - A. Detalhe macroscópico das espículas de *L. obliqua* **B.** Microscopia eletrônica de uma espícula intacta e **C** partida, evidenciando o canal interno (ic) (Pinto et al., 2010, Veiga et al., 2001).

Os sintomas clínicos do envenenamento com lagartas *L. obliqua* são dor intensa e inflamação no ponto de contato, seguidos de sintomas sistêmicos como cefaléia e vômitos. O grave quadro hemorrágico que se instala (Figura 8), inclui equimose, gengivorragia, hemorragia pulmonar e cerebral, hematúria e falência renal (Kowacs et al., 2006, Garcia & Danni-Oliveira, 2007, Pinto et al., 2010).

Análise laboratorial do sangue das vítimas demonstram prolongamento dos tempos de coagulação, diminuição plasmática de fibrinogênio, fator V e XIII, pre-calicreína, plasminogênio, proteína C e α-2 antiplasmina. Há ainda uma elevação nos níveis do complexo trombina-antitrombina, fragmentos 1+2 de protrombina e D-dímeros (Zannin et al., 2003). Adicionalmente aos distúrbios da coagulação, os pacientes apresentam hematúria que pode evoluir para a falência renal aguda (Burdmann et al., 1996). Segundo Gamborgi, 32% dos pacientes envenenados precisam de sessões de hemodiálise (Gamborgi et al., 2006). Porém, a causa mortis mais frequente (cerca de 50% dos casos) é por hemorragia cerebral (Kowacs et al., 2006).

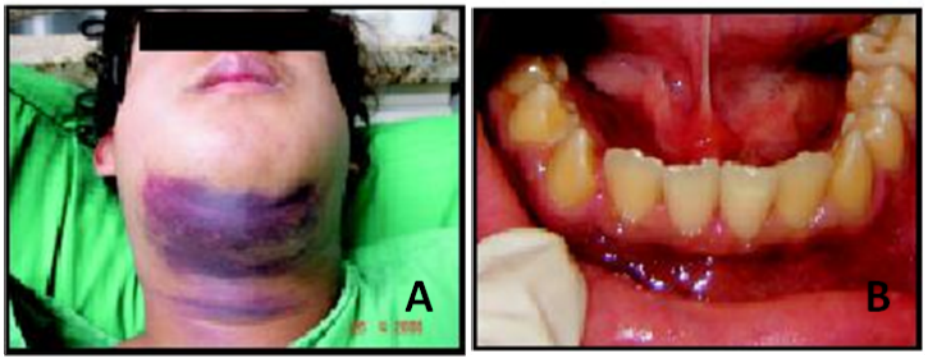


Figura 8 - A. Equimose e **B.** Gengivorragia após contato acidental com *L. obliqua* (Basualdo et al., 2008).

Experimentalmente, muitas das atividades tóxicas da secreção venenosa de *L. obliqua* são reproduzidas em modelos animais. Os distúrbios hemostáticos podem ser reproduzidos em ratos, coelhos e camundongos, com aumento do tempo de coagulação e diminuição da função plaquetária com diminuição discreta da contagem de plaquetas (Berger et al., 2010, Prezoto et al., 2002). Vale observar,

todavia, que nos ensaios *in vitro* o veneno bruto de *L. obliqua* apresenta potente ação pró-coagulante caracterizada por significativa redução dos tempos de protrombina e recalcificação (Pinto et al., 2006, Veiga et al., 2003). Certamente, *in vivo*, a ação pro-coagulante precede os efeitos pro-hemorrágicos do veneno. Assim, a partir dos dados experimentais e dos achados clínicos, é possível propor que o envenenamento com *L. obliqua* constitui também um quadro de coagulopatia de consumo, que se inicia com a ativação simultânea das cascatas da coagulação e da fibrinólise, depleção dos níveis de fibrinogênio, resultando numa pronunciada síndrome hemorrágica.

Adicionalmente, foi demonstrado que de maneira sinérgica aos efeitos diretos do veneno sobre a coagulação e a fibrinólise, existe um efeito indireto causado pela modulação da expressão gênica da vítima (Pinto et al., 2008). Há uma regulação positiva do receptor do ativador de plasminogênio tipo uroquinase (uPAR), comprovando uma ativação da fibrinólise, que consequentemente poderia afetar a matriz extracelular favorecendo o remodelamento e migração celular. A secreção venenosa é ainda capaz de induzir a regulação positiva de mediadores pró-inflamatórios, favorecendo a migração de monócitos, macrófagos, basófilos e células T e modulando a resposta inflamatória.

Dentre os componentes tóxicos identificados nas secreções venenosas de *L. obliqua* por métodos clássicos de purificação ou por análise transcriptômica estão lipocalinas, serinoproteases, fosfolipases, hialuronidases, lectinas e inibidores de proteases (Tabela III) (Pinto et al., 2010). Acredita-se que estes componentes estejam envolvidos diretamente com a ação pró-coagulante (ativador de protrombina, ativador de Fator X) e fibrinólise (fibrinogenase), com a atividade hemolítica (fosfolipase) e com a inibição dos fatores da coagulação das vítimas

(serpinas e outros inibidores de proteases) (Alvarez Flores et al., 2006, Gouveia et al., 2005, Pinto et al., 2004, Reis et al., 2001, Veiga et al., 2005).

Tabela III - Toxinas de *L. obliqua* com papel putativo no envenenamento (Pinto et al., 2010).

Toxina	Gen Bank	Mw predita / Mw observada (Da)	Método de identificação	Referência
Fibrinogenase	ND	35,000	Isolamento	**Pinto *et al*, 2004**
Ativador de Protrombina	AY908986	69,000	Isolamento	**Reis *et al*, 2001**
Ativador de Factor X	ND	45,000	Isolamento	**Alvarez-Flores *et al*, 2006**
Serino proteases	AY829844 AY829818 AY829819 AY829820 AY829821 AY829842 AY829843 AY829841	desconhecida 55,200 desconhecida desconhecida desconhecida desconhecida 30,100 desconhecida	Transcriptoma	**Veiga *et al*, 2005**
Hialuronidases	ND	53,000 49,000	Isolamento	**Gouveia *et al*, 2005**
Fosfolipase A2	ND	15,000	Isolamento	**Seibert *et al*, 2006**
Fosfolipase A2	AY829845	9,600	Transcriptoma	**Veiga *et al*, 2005**
Lectinas	AY829822 AY829836 AY829849 AY829846	33,700 33,900 desconhecida 16,300	Transcriptoma	**Veiga *et al*, 2005**
Lipocalinas	AY829833 AY829856 AY829809	20,600 13,500 desconhecida	Transcriptoma	**Veiga *et al*, 2005**
Serpinas	AY829814 AY829815 AY829816 AY829817 AY829847	50,200 41,600 desconhecida desconhecida desconhecida	Transcriptoma	**Veiga *et al*, 2005**
Outros inibidores de proteases	AY829810 AY829811 AY829812 AY829813 AY829839 AY829835 AY829837	14,600 41,800 8,600 7,300 7,200 8,000 4,100	Transcriptoma	**Veiga *et al*, 2005**

ND: não disponível

3.2.2 Secreções Venenosas de *Lonomia obliqua*: Proteômica Comparativa para a Identificação de Proteínas Envolvidas na Síndrome Hemorrágica

Lonomia obliqua venoumous secretions: comparative proteomics to the identification of proteins involved in the hemorrhagic syndrome

Renata M. S. Terra, Antônio F. M. Pinto, Nicholas E. Sheman, Jay W. Fox e Jorge A. Guimarães

Manuscrito em fase final de preparação

Resumo - Envenenamentos por contato acidental com a lagarta *Lonomia obliqua* são um problema de saúde pública emergente na região Sul do Brasil. Os envenenamentos são clinicamente caracterizados como uma coagulopatia de consumo com ativação exarcebada da coagulação sanguínea e fibrinólise acompanhada de inibição da agregação plaquetária, inflamação e vasodilatação. Um número pequeno de componentes das secreções venenosas da *L. obliqua* (usualmente uma mistura de veneno e hemolinfa) encontram-se purificados e caracterizados. Neste trabalho, descrevemos uma análise proteômica compreensiva das secreções venenosas de *L. obliqua*, veneno e hemolinfa, enfatizando os componentes tóxicos.

Serinoproteases, lipocalinas e inibidores de proteases são os grupos majoritários em ambas secreções. Neste trabalho, descrevemos pela primeira vez a presença de outras possíveis proteínas envolvidas no envenenamento, como metaloproteases e um domínio tipo fator Va da coagulação. Os dados deste trabalho podem ser utilizados na geração de novas hipóteses testáveis a respeito da progressão da síndrome hemorrágica, assim como basear o desenvolvimento de novas terapias adjuvantes ao soro anti-lonômico.

Este trabalho foi desenvolvido na Biomolecular Research Facility (University of Virginia) e no Laboratório de Bioquímica Farmacológica (UFGRS). Os dados aqui apresentados são parte de um manuscrito em fase final de preparação.

LONOMIA OBLIQUA VENOUMOUS SECRETIONS: PROTEOMIC APPROACH REVEALS NEW TOXIC PROTEINS INVOLVED IN THE HEMORRHAGIC SYNDROME

Renata M. S. Terra[1,2], Antônio F. M. Pinto[1,2], Nicholas E. Sheman[2], Jay W. Fox[2*] and Jorge A. Guimarães[1*]

[1]Laboratório de Bioquímica Farmacológica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, VA, USA

*Corresponding authors

J.A.G. guimar@cbiot.ufrgs.br

J.W.F. jwf8x@virginia.edu

Keywords: *L. obliqua*, caterpillar, venom, envenomation, proteome

Abstract

Lonomia obliqua accidental envenomations are an emerging health problem in South Brazil. Envenomations are clinically characterized as a consumption coagulopathy with an exacerbated activation of blood coagulation and fibrinolysis accompanied by inhibition of platelet aggregation, inflammation and vasodilatation. A few individual components of the venomous secretion (often a mixture of venom and hemolymph) have been fully purified and characterized. In the present study, we report a comprehensive proteomic analysis of *L. obliqua* venomous secretions emphasizing known and putative toxic components. Serine proteases and lipocalin were demonstrated to be the most abundant proteins along with protease inhibitors. Moreover, for the first time it was shown the presence of other possible toxic components such as metalloproteases and a factor Va-like domain. The findings from this work could be used in the generation of new testable hypothesis that could enhance the comprehension of envenomation pathology development and shed light on new adjuvant treatment to the anti-venom.

1. Introduction

The *Lonomia* genus of caterpillars are medically important animals known to possess a wide array of toxic components that affect the vascular system, blood coagulation, fibrinolysis and platelet function of the victims [1]. Accidents with *Lonomia* genus have been described in a wide area of Latin America, being *Lonomia achelous* found in Venezuela, Mexico, Colombia, French Guiana and in the Brazilian Amazon area while *Lonomia obliqua* occurrence has been registered in Argentina, Paraguay, Uruguay and the southern region of Brazil (Rio Grande do Sul, Santa Catarina, Paraná and São Paulo states) [2]. In south Brazil, victims of *Lonomia obliqua* envenomation with a typical profile of acquired hemorrhagic syndrome have been reported since the 1980s [3]. Initial symptoms include pain and burning sensation at the contact site, generally followed by more severe clinical manifestations, such as bleeding from skin and mucous membranes, epistaxis, hematuria, acute renal failure and melena. If the victim is not quickly treated, intracerebral bleeding may occur, leading to death [1].

The envenomation with *L. obliqua* occurs when the victim gets accidentally in contact with a colony of dozens or even hundreds of caterpillars camouflaged at tree trunks. Usually, at contact, the caterpillars are smashed and the venomous secretion (often a mixture of venom and hemolymph) is injected by the broken bristles. Unlike snakes and spiders, the venom of *L. obliqua* is not produced by a specialized gland, it is derived from a secretory epithelium and has the solely function of defense against predators [4]. A number of toxic components have been identified and purified from the venom (obtained from bristle maceration) and from the hemolymph [5-9]. In addition, a transcriptomic study identified several transcripts that could be related to envenomation symptomatology, especially regarding the hemostatic disturbances like serine proteases, phospholipases, lectins and protease inhibitors [10]. This comprehensive transcriptomic study allowed the formulation of testable hypotheses of venom's molecular actions. These hypotheses were tested both *in vitro* by gene expression analysis of cultured cells and *in vivo* by experimental envenomation in rats, giving hints in the direct and indirect effects of venomous secretion in the host system [11-12].

To date, it is known that *L. obliqua* venomous secretions are able to directly modulate the victim's hemostatic system by proteolytic activation of the coagulation cascade with generation of high concentrations of thrombin. In fact, *in vitro*

experiments with human plasma show that the crude venom presents potent pro-coagulant activity characterized by remarkable reduction of both pro-thrombin and re-calcification times [1, 13-14]. In addition, inhibition of platelet aggregation and activation of the fibrinolytic system, resulting in a consumption coagulopathy, were also described [7, 11, 13-14]. Moreover, gene expression analysis of envenomed cells showed that there is also an indirect activation of fibrinolytic system by up-regulation of urokinase plasminogen activator receptor (uPAR) together with a pro-coagulant response demonstrated as an increase in tissue factor (TF). Besides, the venom also triggers an acute inflammatory response and disturbance in the vascular system inducing increase in blood-brain barrier permeability, hypotension, pain and edema [11, 15-18]. Altogether the active principles of the venom so far described may not explain the whole processes involved in the envenomation, in order to produce such profound hemostatic disorder clinically observed in the victims of *Lonomia*'s envenomation. Even though there is a great amount of information regarding *Lonomia obliqua*'s tegument and bristles transcripts, until now proteomic data about the venomous secretions is limited to individual protein sequencing either by Edman degradation or mass spectrometry [10, 19]. More recently, the immunogenic proteins of the venom were identified by an immunoproteomic approach where it was possible to identified only three protein families: serpins, lipocalins and cuticle proteins [20]. Therefore, the knowledge regarding *L. obliqua* venomous secretions active principles is still deficient. So, here we present a comparative and comprehensive proteomic characterization of *L. obliqua* venomous secretions: bristle extract and hemolymph.

2. Material and Methods

2.1 *Lonomia obliqua* venomous secretions

L. obliqua caterpillars were kindly provided by Centro de Informações Toxicológicas (CIT-RS), Porto Alegre, Rio Grande do Sul, Brazil. The insects were collected directly from tree's trunks. *L obliqua* bristle extract (LOBE) and *L. obliqua* hemolymph (LOH) were obtained as previously described [13]. Briefly, bristles from last instar larvae were cut at the tegument insertion and homogenized in buffer (20 mM This-HCl, pH 7.5). Hemolymph was obtained by cutting the caterpillars' head and the abdominal and anal prolegs. The fluid expelled was collected with a syringe. Both bristle extract and hemolymph were centrifuged at 9600 g for 20 min

immediately after collection and the supernatants (denominated LOBE and LOH) stored at −20 °C until further use. The biological sample used was a preparation collected from 68 different individuals.

2.2 Samples preparation for proteomic analysis

2.2.1 SDS-PAGE

1D-SDS-PAGE (4-20 %) was performed according to Laemmli (1970) [21]. Protein bands were detected after staining with Coomassie brilliant blue. LOBE and LOH (triplicates of 50 µg each) gel lanes were cut in 5 slices, according to molecular weight (>75kDa, 75-50kDa, 50-37kDa, 37kDa, 37-25kDa and <25kDa).

2.2.2 In-gel digestion

Gel slices from SDS-PAGE were distained for 2 hours before reduction (10 mM DTT) and alkylation (50 mM iodoacetamide) at room temperature. Slices were washed with 100 mM ammonium bicarbonate and dehydrated with acetonitrile. Gel pieces were then rehydrated in modified trypsin solution (20 ng/mL, Promega) in 50 mM ammonium bicarbonate for 30 minutes on ice. Excess trypsin was removed and the digestion carried on overnight at 37 ºC. Tryptic peptides were extracted from gel pieces with 50 % acetonitrile/5 % formic acid solution. Tryptic peptide extracts were dried and reconstituted in 15 µL 1 % acetic acid for mass spectrometric analysis.

2.3 LC/MS/MS analysis

Samples were analyzed using a Thermo Electron LTQ ion-trap mass spectrometer. Samples were loaded into a capillary C18 analytical column (75 µm x 7.5 cm) at a flow rate of ~500 nL/min using an acetonitrile/0.1M acetic acid gradient (0-90 % acetonitrile). The instrument was programmed to acquire a cycle of one mass spectrum followed by MS/MS on the ten most abundant ions in a data-dependent mode. The electrospray voltage was set to 2.5 kV, and the capillary temperature was 210 °C. After MS/MS fragmentation was performed on a particular parent ion, the m/z was placed on an exclusion list for 120 seconds to enable greater dynamic range and prevent repeated analysis of the same peptide

The mass spectra were extracted and analyzed utilizing Bioworks Sequest 3.3.1 software. Searches were performed against a customized database containing sequences from insects, arachnids, amphibians and reptiles proteins deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/) from May, 2010. Spectra generated on the LTQ were searched using 1.5 Da parent tolerance and 1 Da fragment tolerance. All hits were required to be fully tryptic.

Scaffold (version 3.00.03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications and to concatenate the information acquired from gel slices. Peptide identifications were accepted if Sequest identifications had XCorr scores greater than 1.8, 2.2, 2.5 and 3.5 for singly, doubly, triply and quadruply charged peptides, respectively. Protein identifications were accepted if they contained at least 2 identified peptides. Relative quantifications were based on number of spectral counts. False positive rate was calculated at less than 2 % for both samples based on a reversed sequence database.

2.4. Western blot

L. obliqua bristle extract separated by SDS-PAGE was electrotransfered at 400 mA for 1 h onto PVDF membranes, and thereafter membranes were blocked with PBS-albumin 4 % buffer for 4 h. Membranes were incubated with anti-PIII SVMP serum (anti-jararhagin) kindly provided by Dr. Ana Maria Moura-da-Silva, Butatan Institute, São Paulo, Brazil (1:2000) overnight at 4 °C. Membranes were washed three times with PBS-tween 0.1 % and incubated with peroxidase-labeled anti-rabbit IgG (1:5000). The blot was developed according to manufacturer's recommendations using an ECL reagent (GE Healthcare, USA).

2.5 ECM gel degradation

In vitro degradation of extracellular matrix (ECM) gel was assayed. Briefly, ECM gel was incubated with LOBE in a 20 mM Tris-HCl pH 7.4 buffer for various times at 37 °C. Boiling for 5 minutes followed by acetone precipitation stopped the reaction. Degradation of ECM was evaluated by 10 % SDS-PAGE.

3. Results and Discussion

3.1 1D-SDS-PAGE analysis of *L. obliqua* venomous secretions

Unidimensional electrophoresis of LOBE and LOH followed by in-gel digestion was used as a decomplexation method (Figure 1). For LOBE, the in-gel analysis came up with 48 protein hits, with 293 unique peptides in 4901 spectral counts. LOH proteomic analyses produced 27 protein hits, with 202 unique peptides in 2540 spectral counts. Identified proteins from different protein families were divided in five groups according to their activity or role, named Lipocalin, Serine Proteases, Protease Inhibitors, Hypothetical Proteins and Housekeepers. A list of housekeepers

and hypothetical proteins is displayed in Table I; proteins with putative role in the pathological envenomation condition are shown in Table II.

The overall distribution of protein families that could contribute to the clinical manifestations of envenomation in LOBE and LOH are shown in Figure 2. For LOBE, protease inhibitors are the main group, representing 51 % of the proteins. LOBE is also abundant in lipocalin (34 %) and serine proteases (14 %). Additionally, LOBE has the presence of minoritary components such as metalloproteases, phospholipases and lectins. Lipocalin is a minor component of LOH, representing only 1 % of the toxic proteins. Metalloproteases and lectins are also minoritary components of LOH, which is, on the other hand, a rich source of protease inhibitors (62 %) and serine proteases (36 %).

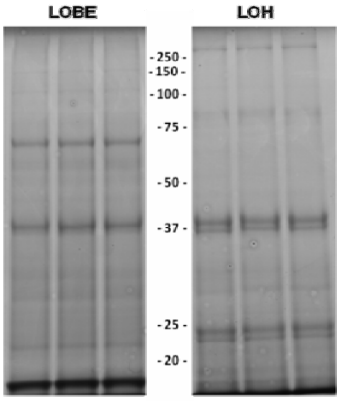


Figure 1 - *Lonomia obliqua* venomous secretions eletrophoretic profile. Bristle extract (LOBE) and hemolymph (LOH) were separated by SDS-PAGE. Six sections covering the full molecular weight range were cut and submitted to trypsin digestion prior to analysis by LC/MS/MS.

Table I – Housekeepers and hypothetical proteins identified by 1D-SDS-PAGE sections proteomic analysis

	Protein group	Protein name [*organism*]	Accession numbers	Protein MW (Da)	Unique peptides	Unique spectra	Spectral Count	Quantitative value	Sequence coverage
LOBE									
	Housekeepers	sensory protein 1 [*Lonomia obliqua*]	gi|56462366|gb|AAV91466.1|	14,475.10	4	4	15	12	37.80%
		actin [*Thrandina parocula*]	gi|187968807|gb|ACD44597.1|	26,117.00	2	3	7	5	9.32%
		alpha actin [*Atractaspis microlepidota*]	gi|15825436|gb|AAL09696.1|AF416707_1	42,044.20	4	4	16	12	14.60%
		60S ribosomal protein L40A [*Lycosa singoriensis*]	gi|161661039|gb|ABX75386.1|	17,988.60	4	4	15	12	25.60%
		reverse transcriptase [*Dugesiella sp.*]	gi|2736044|gb|AAB94039.1|	55,076.80	2	2	6	5	4.71%
		nucleoside diphosphate kinase [*Lycosa singoriensis*]	gi|161669226|gb|ABX75465.1|	17,263.40	2	2	2	2	14.90%
		histone H4 [*Citharischius crawshayi*]	gi|294653213|gb|ADF28514.1|	11,349.70	3	3	6	5	29.10%
		cytochrome b [*Typhlops platycephalus*]	gi|21429673|gb|AAM47766.1|	42,030.50	2	2	3	2	7.01%
		NADH dehydrogenase [*Amaurobioides sp.*]	gi|86559320|gb|ABD04068.1|	15,142.80	2	2	2	2	43.20%
		ribosomal protein 27 [*Lonomia obliqua*]	gi|56462232|gb|AAV91399.1|	23,489.20	2	2	2	2	12.50%
		elongation factor 1 [*Lonomia obliqua*]	gi|56462146|gb|AAV91356.1|	46,697.30	7	8	31	24	22.00%
		beta tubulin [*Ornithoctonus huwena*]	gi|197210385|gb|ACH48200.1|	17,855.00	4	4	21	16	27.10%
		NADH dehydrogenase [*Imantodes cenchoa*]	gi|189164481|gb|ACD77417.1|	65,769.60	2	2	4	3	4.75%
		actin 1 [*Lonomia obliqua*]	gi|56462250|gb|AAV91408.1|	32,940.90	6	8	59	46	36.10%
		NADH dehydrogenase [*Ramphotyphlops braminus*]	gi|164420890|ref|YP_001648393.1|	66,479.80	2	2	2	2	5.36%
		cobra serum albumin [*Naja naja*]	gi|469861|emb|CAA55333.1|	69,781.40	2	2	2	2	5.54%
		ferritin 1 [*Lonomia obliqua*]	gi|56462148|gb|AAV91357.1|	25,675.30	9	10	31	24	39.40%
		Hsp90 [*Opistophthalmus carinatus*]	gi|37594780|gb|AAQ94359.1|	83,105.70	8	8	34	26	13.40%
		hemolin [*Lonomia obliqua*]	gi|94471609|gb|ABF21071.1|	45,571.10	14	21	162	126	36.80%
		hemolin [*Lonomia obliqua*]	gi|94471613|gb|ABF21073.1|	45,095.90	28	48	702	544	71.40%
		hemolin [*Lonomia obliqua*]	gi|94471607|gb|ABF21070.1|	45,121.80	2	3	144	112	6.78%
		heat shock protein 4 [*Lonomia obliqua*]	gi|56462364|gb|AAV91465.1|	71,587.10	15	21	90	70	27.40%
		defense protein 1 [*Lonomia obliqua*]	gi|56462134|gb|AAV91350.1|	17,782.80	4	7	23	18	47.60%
		sonic hedgehog [*Python sebae*]	gi|171904016|gb|ACB56635.1|	32,812.80	2	2	2	2	10.80%
		putative dopa decarboxylase protein [*Lonomia achelous*]	gi|158451473|gb|ABW39097.1|	46,074.60	2	2	10	8	9.49%
		eukaryotic translation inititation factor 4B [*Lapemis hardwickii*]	gi|18000293|gb|AAL54908.1|AF165225_1	71,170.80	2	2	2	2	5.87%
	Hypothetical Proteins	hypothetical protein 3 [*Lonomia obliqua*]	gi|56462176|gb|AAV91371.1|	24,348.10	2	2	3	2	14.30%
		hypothetical protein 31 [*Lonomia obliqua*]	gi|56462322|gb|AAV91444.1|	24,951.00	7	8	21	16	40.90%
		hypothetical protein 22 [*Lonomia obliqua*]	gi|56462308|gb|AAV91437.1|	19,103.50	9	16	528	409	62.30%
LOH									
	Housekeepers	Hsp90 [*Opistophthalmus carinatus*]	gi|37594780|gb|AAQ94359.1|	83,105.70	2	2	2	3	3.48%
		NADH dehydrogenase [*Pseudoleptodeira latifasciata*]	gi|291612527|ref|YP_003540757.1|	65,988.80	2	2	2	3	7.61%
		actin 1 [*Lonomia obliqua*]	gi|56462250|gb|AAV91408.1|	32,940.90	3	3	9	13	12.60%
		ribosomal protein l19 [*Lycosa singoriensis*]	gi|161670278|gb|ABX75491.1|	24,348.90	2	2	2	3	8.33%
		cytochrome b [*Liophelidophis dolichocercus*]	gi|118773110|gb|ABL14135.1|	42,112.50	2	2	2	3	14.60%
		NADH dehydrogenase subunit 5 [*Ramphotyphlops braminus*]	gi|164420890|ref|YP_001648393.1|	66,479.80	2	2	2	3	5.86%
		60S ribosomal protein L40A [*Lycosa singoriensis*]	gi|161661039|gb|ABX75386.1|	17,988.60	3	3	17	24	21.80%
		hemolin [*Lonomia obliqua*]	gi|94471609|gb|ABF21071.1|	45,571.10	16	21	119	168	91.70%
		hemolin [*Lonomia obliqua*]	gi|94471611|gb|ABF21072.1|	45,135.90	2	3	5	7	4.12%
		hemocyanin subunit 3b [*Pantherus imperator*]	gi|242388954|emb|CAZ66715.1|	72,700.20	2	2	2	3	5.38%
		hemolin [*Lonomia obliqua*]	gi|94471613|gb|ABF21073.1|	45,095.90	30	42	317	447	78.20%
		hemolin [*Lonomia obliqua*]	gi|94471607|gb|ABF21070.1|	45,121.80	2	3	69	97	6.78%
		At cadherin [*Achaearanea tepidariorum*]	gi|61162140|dbj|BAD91058.1|	333,799.80	2	2	10	14	1.28%
		defense protein 1 [*Lonomia obliqua*]	gi|56462134|gb|AAV91350.1|	17,782.80	6	10	61	86	45.20%
		ferritin 1 [*Lonomia obliqua*]	gi|56462148|gb|AAV91357.1|	25,675.30	9	10	41	58	40.30%
	Hypothetical Protein	hypothetical protein 22 [*Lonomia obliqua*]	gi|56462308|gb|AAV91437.1|	19,103.50	9	13	282	397	62.30%

Table II – *L. obliqua* venomous secretions toxic proteins identified by 1D SDS-PAGE sections proteomic analysis.

	Protein group	Protein name [*organism*]	Accession numbers	Protein MW (Da)	Unique peptides	Unique spectra	Spectral Count	Quantitative value	Sequence coverage
LOBE									
	Serine Proteases	serine protease 7 [*Lonomia obliqua*]	gi|56462344|gb|AAV91455.1|	30,485.10	3	3	6	5	16.10%
		achelase I=fibrinolytic proteinase [*Lonomia achelous*]	gi|236202|gb|AAB19941.1|	22,453.50	10	13	83	64	71.40%
		prophenoloxidase activating factor 1 [*Lonomia obliqua*]	gi|56462350|gb|AAV91458.1|	42,876.10	22	26	205	159	59.80%
		putative serine protease-like protein 2 [*Lonomia obliqua*]	gi|56462300|gb|AAV91433.1|	32,129.20	7	9	28	22	39.30%
		serine protease 6 [*Lonomia obliqua*]	gi|63207768|gb|AAV91457.2|	35,498.70	8	12	81	63	27.60%
	Protease Inhibitors	putative cystatin precursor 1 [*Lonomia obliqua*]	gi|56462274|gb|AAV91420.1|	13,204.00	2	2	5	4	20.80%
		serpin 2 [*Lonomia obliqua*]	gi|56462292|gb|AAV91429.1|	43,748.60	12	14	84	65	32.90%
		serpin 3 [*Lonomia obliqua*]	gi|56462294|gb|AAV91430.1|	43,177.50	7	11	99	77	17.00%
		serine protease inhibitor 4 [*Lonomia obliqua*]	gi|56462296|gb|AAV91431.1|	36,416.70	28	51	1321	1024	74.80%
	Lectin	lectin 3 [*Lonomia obliqua*]	gi|56462334|gb|AAV91450.1|	36,329.70	5	5	14	11	25.90%
	Lipocalin	lipocalin 1 [*Lonomia obliqua*]	gi|56462328|gb|AAV91447.1|	22,415.50	16	28	1000	775	68.20%
	Metalloproteases	zinc metalloproteinase-disintegrin VAP1 [*Crotalus atrox*]	gi|82220669|sp|Q9DGB9.1|VMV1_CROAT	67,941.90	2	2	2	2	4.43%
		zinc metalloproteinase/disintegrin trigamin alpha [*Trimeresurus gramineus*]	gi|118643|sp|P15503.3|DISA_TRIGA	53,476.10	2	2	3	2	6.04%
		snake venom metalloprotease [*Philodryas olfersii*]	gi|241995587|gb|ACS74988.1|	68,271.40	3	3	6	5	9.82%
		zinc metalloproteinase/disintegrin graminelysin I [*Trimeresurus gramineus*]	gi|172044536|sp|P0C6E8.1|VMGRA_TRIGA	48,185.80	2	2	2	2	6.67%
		zinc metalloproteinase-disintegrin HF3 [*Bothrops jararaca*]	gi|82219706|sp|Q98UF9.3|VMHF3_BOTJA	67,677.10	2	2	2	2	8.75%
	Phospholipase	phospholipase A2 isozyme 3 [*Naja nigricollis*]	gi|129444|sp|P00605.1|PA23_NAJNG	13,250.10	2	2	4	3	19.50%
	Calcium channel blocker	omega-theraphotoxin-Hh1a [*Ornithoctonus huwena*]	gi|114152898|sp|P68424.2|TXH10_HAPSC	7,502.60	3	3	3	2	44.10%
	Coagulation Factor-like	pseutarin C [*Pseudonaja textilis*]	gi|33578334|gb|AAO38805.1|	165,918.80	3	3	8	6	3.97%
LOH									
	Serine Proteases	**achelase II=fibrinolytic proteinase [*Lonomia achelous*]**	**gi|236201|gb|AAB19940.1|**	**22,707.90**	**4**	**6**	**60**	**85**	**33.20%**
		prophenoloxidase activating factor 1 [*Lonomia obliqua*]	**gi|56462350|gb|AAV91458.1|**	**42,876.10**	**21**	**25**	**176**	**248**	**51.30%**
		serine protease 3 [*Lonomia obliqua*]	**gi|56462302|gb|AAV91434.1|**	**29,655.60**	**2**	**2**	**5**	**7**	**9.19%**
		achelase I=fibrinolytic proteinase [*Lonomia achelous*]	**gi|236202|gb|AAB19941.1|**	**22,453.50**	**11**	**16**	**331**	**466**	**72.30%**
	Protease Inhibitors	**serpin 2 [*Lonomia obliqua*]**	**gi|56462292|gb|AAV91429.1|**	**43,748.60**	**16**	**18**	**114**	**161**	**39.20%**
		putative cystatin precursor 1 [*Lonomia obliqua*]	**gi|56462274|gb|AAV91420.1|**	**13,204.00**	**5**	**7**	**30**	**42**	**34.20%**
		serpin 3 [*Lonomia obliqua*]	**gi|56462294|gb|AAV91430.1|**	**43,177.50**	**8**	**13**	**112**	**158**	**21.10%**
		serine protease inhibitor 4 [*Lonomia obliqua*]	**gi|56462296|gb|AAV91431.1|**	**36,416.70**	**29**	**53**	**734**	**1034**	**68.00%**
	Lectin	**lectin 3 [*Lonomia obliqua*]**	**gi|56462334|gb|AAV91450.1|**	**36,329.70**	**4**	**5**	**18**	**25**	**18.70%**
	Lipocalin	**lipocalin 1 [*Lonomia obliqua*]**	**gi|56462328|gb|AAV91447.1|**	**22,415.50**	**6**	**8**	**16**	**23**	**42.30%**
	Metalloprotease	**zinc metalloproteinase-disintegrin brevilysin-H6 [*Gloydius blomhoffi brevicaudus*]**	**gi|190358877|sp|P0C7B0.2|VMH6_AGKHB**	**68,199.10**	**2**	**2**	**2**	**3**	**4.92%**

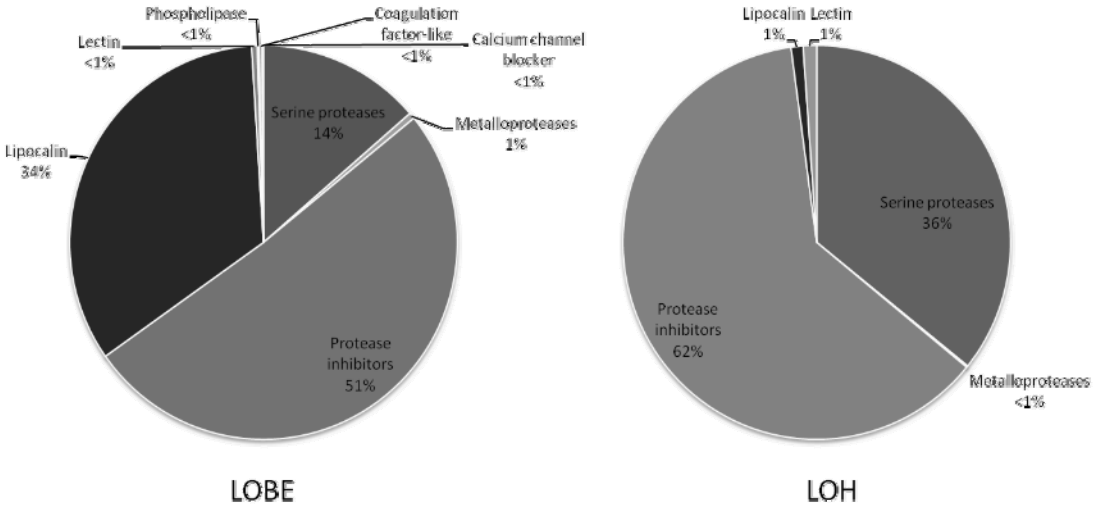


Figure 2 - Overall distribution of toxic components from *Lonomia obliqua* venomous secretions. Bristle extract (LOBE) and hemolymph (LOH) components abundance was calculated based on quantitative values.

3.1.1 Protease Inhibitors

As shown in Table II, among the most abundant proteins in the LOBE proteomic analysis is Serine Protease Inhibitor 4 (AAV91431.1), with 1321 spectral counts, 28 unique peptides and 75 % of coverage. Serpin 2 (AAV91429.1) and Serpin 3 (AAV91430.1) were two other identified proteins belonging to the Serine Protease Inhibitor family (Serpins). Besides, two other peptides from cystatin family of inhibitors were identified (Putative Cystatin Precursor, AAV91420.1).

In the proteomic analysis of LOH, Serine Protease Inhibitor 4 (AAV91431.1) is also the most abundant protein, with 734 spectral counts, 29 unique peptides sequenced and 68 % of coverage. Serpins 2 and 3 are also present in LOH, with higher spectral count and only slightly higher coverage than in LOBE. Five peptides from the Putative Cystatin Precursor (AAV91420-1) were also found.

Proteases inhibitors play essential role in physiological processes such as defense against infection and regulation of the prophenoloxidase cascade. Moreover, these inhibitors, especially Serpins, could directly interact with specific human coagulation factors and contribute to the coagulation disorders caused by envenomation [10].

This work's first attempt to do a mass spectrometric shotgun analysis used in-solution trypsin digestion. This approach yielded a very low amount of information

regarding the composition of the venom. A second attempt was made after boiling the venom for 10 minutes, resulting in complete digestion of the venom's proteins and a greater number of protein identification. Most likely, the high concentration of protease inhibitors in these samples, especially those from the Serpin family, caused inhibition of trypsin digestion.

3.1.2 Lipocalin

Lipocalin 1 is the second most abundant single protein in LOBE. Lipocalin 1 (AAV91447.1) has 1000 spectral counts, with 16 unique peptides sequenced and a total of 68 % of coverage, representing alone 34 % of the toxic proteins identified. In LOH, 6 unique peptides belonging to Lipocalin 1 were identified, with sequence coverage of 42 %. Lipocalins are well known binding proteins although in blood feeding arthopods, some proteins from this family present non-enzimatic anti-platelet agregation and anti-hemostatic roles [22-23]. In fact, a prothrombin activator purified from *L. obliqua* seems to be the only lipocalin with a serine protease catalytic activity described to date [24].

3.1.3 Serine Proteases

Serine proteases are the third group of abundant components of LOBE and Prophenoloxidase Activating Factor 1 (AAV91458.1) is the main protein, with 205 spectral counts. Serine proteases play essential role in hemolymph coagulation and prophenoloxidase activation and so are important tools against wound and infection in lepidopterans [10, 25]. Additionally, they are important components of many animals' venoms and, in snake envenomation, are known to interact with the victim's coagulation systems and cause coagulopathy [26]. Also, peptides from the fibrinolytic protease Achelase I (AAB19941.1) were identified.

LOH analysis revealed abundance of Achelase I (11 unique peptides and spectral count of 331) and Prophenoloxidase Activating Factor 1 (21 unique peptides and spectral count of 248). Four unique peptides of a serine protease named Achelase II were also identified. Achelase I and II are serine proteases with fibrinolytic activity well characterized in *Lonomia achelous* [27]. In *L. obliqua*, hemolymph has a high *in vitro* fibrinolytic activity and a fibrin(gen)olytic enzyme named Lonofibrase was also

isolated [7, 13]. During envenomation, these enzymes acting in the fibrinolytic system could exacerbate bleeding by directly enhancing fibrinolysis.

3.1.4 Metalloproteases

A previous work describing a transcriptome study performed in *L. obliqua* bristle and tegument have not found neither identified any metalloprotease or disintegrin-like/cysteine-rich domain sequences [10]. Surprisingly, in this proteomic characterization we were able to identify 11 unique peptides with sequence identity to snake venom metalloproteases (SVMP). The analysis against the venom database used in this work identified peptides related to five different snake proteins homologous to all three PIII SVMPS domains (metalloprotease, disintegrin-like and cysteine-rich domains) (Table III). Overall, we identified six unique peptides from the metalloprotease domain, two unique peptides containing the ECD sequence from the disintegrin-like domain and one peptide from the cysteine-rich domain. It was also possible to sequence two unique peptides from SVMP prepropeptide. Additionally, two metalloprotease domain peptides were sequenced in LOH sample.

Concerning the difference in findings in the transcriptome study and the one presented here, it is most likely that the proteins identified through proteomics are synthesized in a tissue other than the secretory epithelium in the tegument, most likely hemocytes. Due to the differences in sample collection and preparation between proteome and transcriptome techniques, such proteins were only identified here, once no hemocyte cDNA library has been constructed. Recently, a matrix metalloprotease from hemocytes of the Lepidopteran *Galleria mellonella* was identified with important role in collagen IV degradation during metamorphosis and activation of the innate immunity [28]. Actually, the venomous secretions studied here were obtained from last instar larvae that were about to start pupal morphogenesis. It is probable that *L. obliqua* metalloproteases are mainly involved in tissue remodeling, but is unavoidable to speculate a putative role in envenomation since they are major players in the development of snake envenomation hemorrhagic syndrome [29].

To confirm the presence of these enzymes in *L. obliqua* venomous secretions we performed a western blot against the major snake metalloprotease from *B. jararaca*, Jararhagin. In fact, as observed in Fig. 3A, the antibody recognized more than one

protein band in LOBE, all with high molecular weight. It is important to notice that the abundance of these proteins in LOBE is about 100 times less than in snake venoms, being a minoritary component, in accordance to what was identified in the proteome. In snake venom, these proteins are well characterized by their ability to degrade ECM proteins, facilitating vascular permeability and hemorrhage. To speculate the participation of metalloproteases in the facilitation of hemorrhage in *L. obliqua* envenomation we performed an *in vitro* ECM degradation assay (Fig. 3B). In fact, the venom is able to efficiently degrade ECM with short incubation times. Further investigation on ECM degradation and hemorrhage facilitation is being conducted at this time.

3.1.5 Other toxic proteins

Lectin 3 (AAV91450.1) was identified in both samples representing about 1 % of the total toxic components. Lectins are present in a wide variety of snake venoms and are known to interact with the victim's coagulation cascade, being relevant toxins to the development of the hemorrhagic syndrome [30]. In the same way, the phospholipase A2 identified herein may play essential role in myotoxicity, coagulation, platelet aggregation and hemolysis. Actually, there is a description of a purified phospholipase A2 from *L. obliqua* bristle extract with hemolytic activity [5]. Also, this enzyme seems to be involved in the *in vitro* venom-induced platelet aggregation since inhibition with p-bromophenacyl bromide seems to significantly decrease its biological activity [31].

Complementing these findings, there is also the identification of three unique peptides from a calcium channel blocker and three unique peptides from a coagulation factor Va-like domain. The presence of a factor Va-like domain points to the presence of another possible mechanism of prothrombin activation in *L. obliqua*. Similar to group C snake venom prothrombin activators, *L. obliqua* may possess a high molecular weight complex that has a serine protease catalytic subunit and a non-enzymatic factor Va-like domain [32].

Table III – *L. obliqua* metalloprotease peptides identified based on 1D SDS-PAGE sections proteomic analysis.

	Protein name	Protein accession numbers	Peptide sequence	Best SEQUEST XCorr score	Domain[1]
LOBE					
	Zinc metalloproteinase/disintegrin trigramin-alpha	gi|118643|sp|P15503.3|DISA_TRIGA	ALNIVTTLSVLEIWSEK	2.26	MP
			ASQLNVTPEQQR	2.29	propetide
	Snake venom metalloprotease	gi|241995587|gb|ACS74988.1|	NEADSTAVISACDGLK	2.39	propetide
			RNDNAQLITSIDFNGPTVGLGYVGTFCR	2.61	MP
			YLRSDPDNGMVEPGTK	2.23	Cys-rich
	Zinc metalloproteinase/disintegrin graminelysin	gi|172044536|sp|P0C6E8.1|VMGRA_TRIGA	ALNIVTTLSLLEIWSEK	2.35	MP
			LGIFVDHGMYTK	2.24	MP
	Zinc metalloproteinase-disintegrin HF3	gi|82219706|sp|Q98UF9.3|VMHF3_BOTJA	SECDIAESCTGQSADCPTDDFK	2.51	Dis *ECD
			SHDNAQLLTAIDFDGQTIGIANIASMCNQNK	3.04	MP
	Zinc metalloproteinase-disintegrin VAP1	gi|82220669|sp|Q9DGB9.1|VMV1_CROAT	DECDMADVCTGR	2.52	Dis *ECD
			YVKLFLVADYIMYLK	2.35	MP
LOH					
	Zinc metalloproteinase-disintegrin brevilysin-H6	gi|190358877|sp|P0C7B0.2|VMH6_AGKHB	HDNAQLLTAIDFNGPTIGYAYIASMCHPK	2.81	MP
			KHDNAQLLTAIDFNGPTIGYAYIASMCHPK	3.1	MP

[1] Domain assigned based on SVMP structures *ECD: peptide containing the ECD binding motif

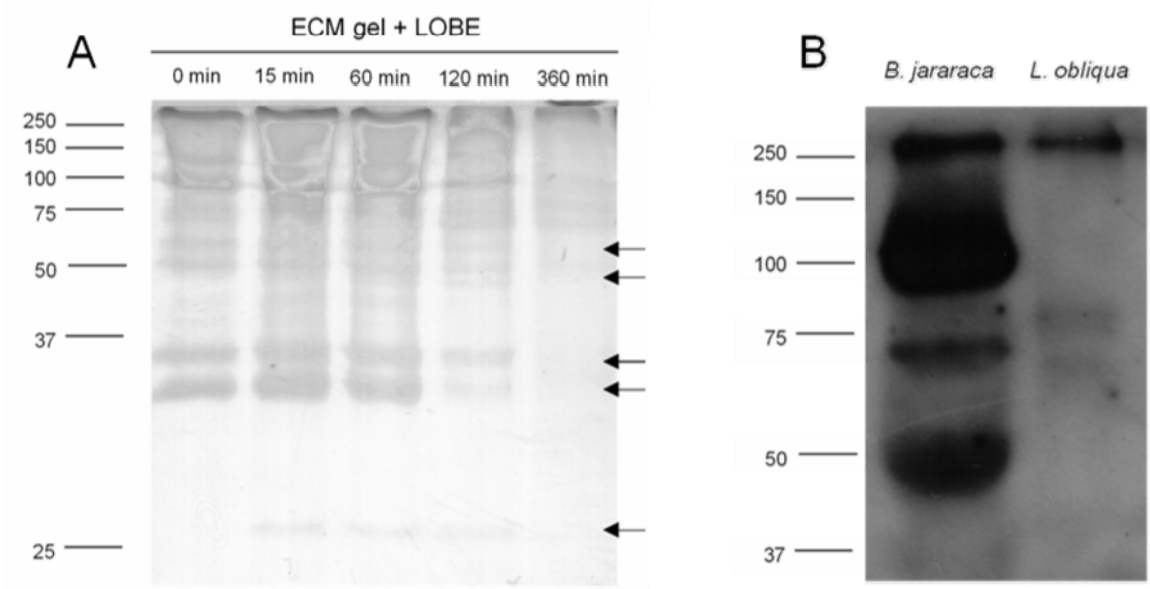


Figure 3 – Characterization of *L. obliqua* metaloproteases. A. Western blot identification of *L. obliqua* metalloproteases by an anti-PIII-SVMP antibody. *B. jararaca* venom – 10 μg; *L. obliqua* bristle extract 100 μg **B.** Extracellular matrix degradation by *L. obliqua* bristle extract was evaluated by SDS-PAGE at different incubation times.

4. Conclusion

Accidents with *Lonomia obliqua* are mostly characterized by bleeding as consequence of a severe hemorrhagic syndrome caused by a complex coagulopathic process that can easily evolve to death if not treated in the very beginning of the accident occurrence. Since the caterpillar venom is not synthesized by a gland, the toxic compounds are frequently derived from a mixture of a secretion produced by the specialized epithelium (*in vitro* obtained as a bristle extract and designated as the venom itself) and hemolymph. A few toxic components involved in the development of the hemorrhagic syndrome have been isolated from these toxic secretions and, together with the transcripts from bristles and tegument, have shed light about venom components participating in the pathological development of envenomation.

Here, we presented a comprehensive comparative proteomic analysis of the venom (bristle extract) and hemolymph highlighting, in a semi-quantitative manner, the components with known and putative biological effects. In fact, the results shown here corroborate with the observed clinical manifestations occurring after accidental

contact with *L. obliqua*. The hemorrhagic syndrome is a combination of direct and indirect action of venom proteins able to interact with coagulation factors, platelets and endothelium causing the exacerbated activation of blood coagulation and fibrinolysis, inhibition of platelet aggregation, vasodilatation and inflammation.

As well as in *L. obliqua* venomous secretions, serine proteases, lectins, phospholipases, metalloproteases, protease inhibitors are also biochemical tools used by many venomous and blood-feeding animals in the impairment of hemostasis. As a matter of fact, here, for the first time in an “omic” approach, proteins belonging to the metalloprotease family were identified in the *L. obliqua* secretions. Although metalloproteases are known to be essential in tissue remodeling during metamorphosis, the presence of these enzymes in the venomous secretions rise hypothesis about their toxic activity to be further tested. Moreover, the findings described here may give insights into adjuvant therapy to be administered together with the anti-venom to minimize envenomation progression and sequelae of victimized individuals.

5. Acknowledgements

The authors thank the Brazilian agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília, Brazil, for the PDEE fellowship to R.M.S. Terra. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) (J.A.G.), CAPES (R.M.S.T. and A.F.M.P.) and the University of Virginia Office of Research Core Administration (J.W.F. and A.F.M.P.).

6. References

[1] Pinto AF, Berger M, Reck J, Jr., Terra RM, Guimaraes JA. Lonomia obliqua venom: In vivo effects and molecular aspects associated with the hemorrhagic syndrome. Toxicon. 2010.

[2] Arocha-Pinango CL, Guerrero B. Lonomia genus caterpillar envenomation: Clinical and biological aspects. Haemostasis. 2001;31:288-93.

[3] Duarte AC, Caovilla J, Lorini I, Lorini D, Mantovani G, Sumida J, et al. Insuficiência renal aguda por acidentes com lagartas. Jornal Brasileiro de Nefrologia 1990;12:184-6.

[4] Veiga ABG, Blochtein B, Guimaraes JA. Structures involved in production, secretion and injection of the venom produced by the caterpillar Lonomia obliqua (Lepidoptera, Saturniidae). Toxicon. 2001;39:1343-51.

[5] Seibert CS, Tanaka-Azevedo AM, Santoro ML, Mackessy SP, Torquato RJS, Lebrun I, et al. Purification of a phospholipase A(2) from Lonomia obliqua caterpillar bristle extract. Biochemical and Biophysical Research Communications. 2006;342:1027-33.

[6] Reis CV, Portaro FCV, Andrade SA, Fritzen M, Fernandes BL, Sampaio CAM, et al. A prothrombin activator serine protease from the Lonomia obliqua caterpillar venom (Lopap) biochemical characterization. Thrombosis Research. 2001;102:427-36.

[7] Pinto AFM, Dobrovolski R, Veiga ABG, Guimaraes JA. Lonofibrase, a novel alpha-fibrinogenase from Lonomia obliqua caterpillars. Thrombosis Research. 2004;113:147-54.

[8] Gouveia AID, da Silveira RB, Nader HB, Dietrich CP, Gremski W, Veiga SS. Identification and partial characterisation of hyaluronidases in Lonomia obliqua venom. Toxicon. 2005;45:403-10.

[9] Alvarez Flores MP, Fritzen M, Reis CV, Chudzinski-Tavassi AM. Losac, a factor X activator from Lonomia obliqua bristle extract: its role in the pathophysiological mechanisms and cell survival. Biochem Biophys Res Commun. 2006;343:1216-23.

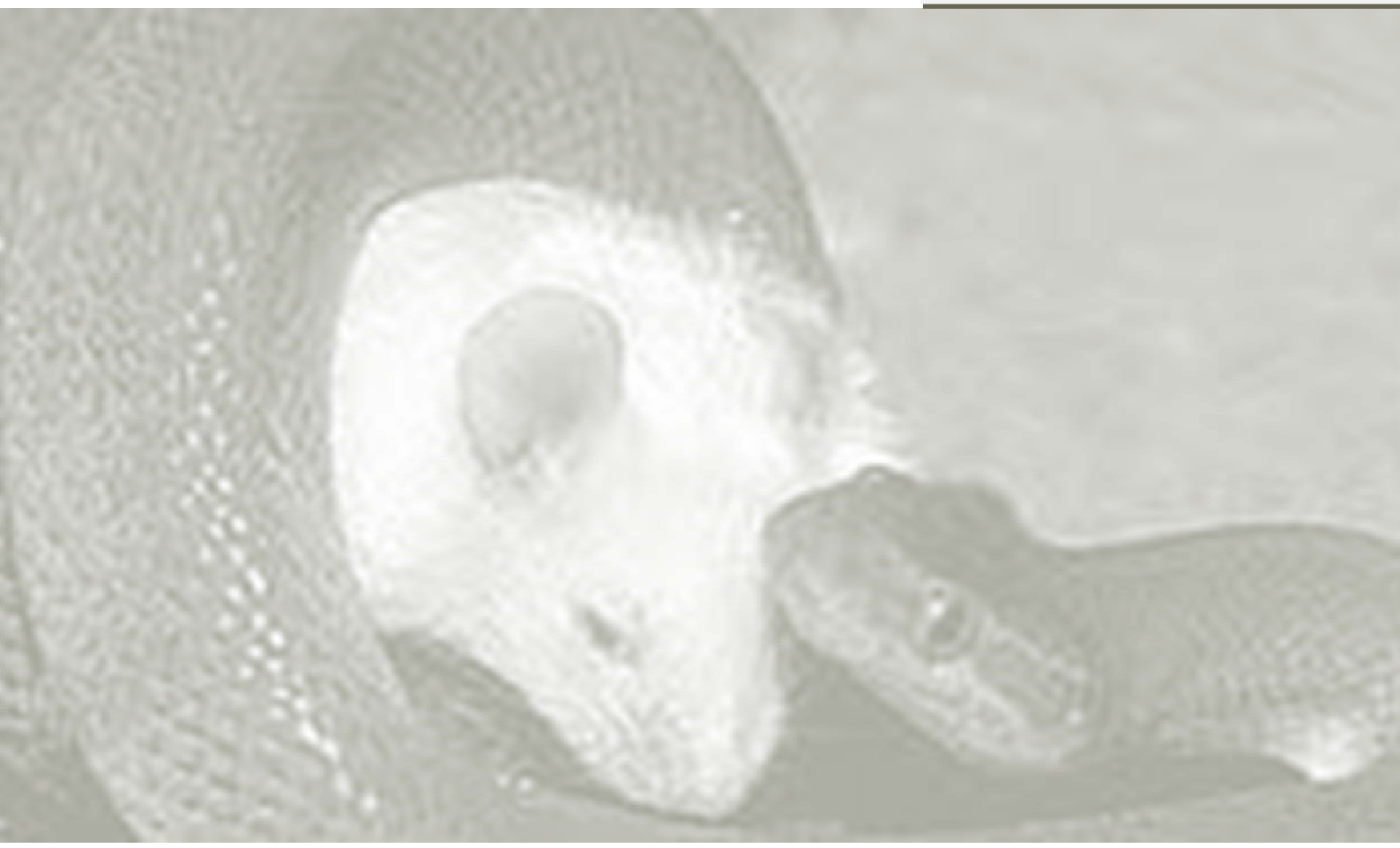
[10] Veiga AB, Ribeiro JM, Guimaraes JA, Francischetti IM. A catalog for the transcripts from the venomous structures of the caterpillar Lonomia obliqua: identification of the proteins potentially involved in the coagulation disorder and hemorrhagic syndrome. Gene. 2005;355:11-27.

[11] Pinto AF, Dragulev B, Guimaraes JA, Fox JW. Novel perspectives on the pathogenesis of Lonomia obliqua caterpillar envenomation based on assessment of host response by gene expression analysis. Toxicon. 2008;51:1119-28.
[12] Berger M, Reck J, Jr., Terra RM, Pinto AF, Termignoni C, Guimaraes JA. Lonomia obliqua caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats. Toxicon. 2010;55:33-44.
[13] Pinto AFM, Silva KRLM, Guimaraes JA. Proteases from Lonomia obliqua venomous secretions: Comparison of procoagulant, fibrin(ogen)olytic and amidolytic activities. Toxicon. 2006;47:113-21.
[14] Veiga ABG, Pinto AFM, Guimaraes JA. Fibrinogenolytic and procoagulant activities in the hemorrhagic syndrome caused by Lonomia obliqua caterpillars. Thrombosis Research. 2003;111:95-101.
[15] Ramos AT, Goncalves LRC, Ribeiro OG, Campos ACR, Sant'Anna OA. Effects of Lonomia obliqua (lepidoptera, saturniidae) toxin on clotting, inflammatory and antibody responsiveness in genetically selected lines of mice. Toxicon. 2004;43:761-8.
[16] de Castro Bastos L, Veiga ABG, Guimaraes JA, Tonussi CR. Nociceptive and edematogenic responses elicited by a crude bristle extract of Lonomia obliqua caterpillars. Toxicon. 2004;43:273-8.
[17] da Silva GH, Hyslop S, da Cruz-Hofling MA. Lonomia obliqua caterpillar venom increases permeability of the blood-brain barrier in rats. Toxicon. 2004;44:625-34.
[18] Bohrer CB, Reck Junior J, Fernandes D, Sordi R, Guimaraes JA, Assreuy J, et al. Kallikrein-kinin system activation by Lonomia obliqua caterpillar bristles: involvement in edema and hypotension responses to envenomation. Toxicon. 2007;49:663-9.
[19] Lilla S, Pereira R, Hyslop S, Donato JL, Le Bonniec BF, de Nucci G. Purification and initial characterization of a novel protein with factor Xa activity from Lonomia obliqua caterpillar spicules. J Mass Spectrom. 2005;40:405-12.
[20] Ricci-Silva ME, Valente RH, Leon IR, Tambourgi DV, Ramos OH, Perales J, et al. Immunochemical and proteomic technologies as tools for unravelling toxins involved in envenoming by accidental contact with Lonomia obliqua caterpillars. Toxicon. 2008;51:1017-28.
[21] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-5.

[22] Montfort WR, Weichsel A, Andersen JF. Nitrophorins and related antihemostatic lipocalins from Rhodnius prolixus and other blood-sucking arthropods. Biochim Biophys Acta. 2000;1482:110-8.
[23] Akerstrom B, Flower DR, Salier JP. Lipocalins: unity in diversity. Biochim Biophys Acta. 2000;1482:1-8.
[24] Reis CV, Andrade SA, Ramos OH, Ramos CR, Ho PL, Batista IF, et al. Lopap, a prothrombin activator from Lonomia obliqua belonging to the lipocalin family: recombinant production, biochemical characterization and structure-function insights. Biochem J. 2006;398:295-302.
[25] Kanost MR, Jiang H, Yu XQ. Innate immune responses of a lepidopteran insect, Manduca sexta. Immunol Rev. 2004;198:97-105.
[26] Kini RM. Serine proteases affecting blood coagulation and fibrinolysis from snake venoms. Pathophysiol Haemost Thromb. 2005;34:200-4.
[27] Amarant T, Burkhart W, LeVine H, 3rd, Arocha-Pinango CL, Parikh I. Isolation and complete amino acid sequence of two fibrinolytic proteinases from the toxic Saturnid caterpillar Lonomia achelous. Biochim Biophys Acta. 1991;1079:214-21.
[28] Altincicek B, Vilcinskas A. Identification of a lepidopteran matrix metalloproteinase with dual roles in metamorphosis and innate immunity. Dev Comp Immunol. 2008;32:400-9.
[29] Fox JW, Serrano SM. Timeline of key events in snake venom metalloproteinase research. J Proteomics. 2009;72:200-9.
[30] Morita T. Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. Toxicon. 2005;45:1099-114.
[31] Berger M, Reck J, Jr., Terra RM, Beys da Silva WO, Santi L, Pinto AF, et al. Lonomia obliqua venomous secretion induces human platelet adhesion and aggregation. J Thromb Thrombolysis. 2010.
[32] Kini RM. The intriguing world of prothrombin activators from snake venom. Toxicon. 2005;45:1133-45.

3.3 CAPÍTULO III

ANÁLISE PROTEÔMICA DE TECIDO ENVENENADO



3.3.1 Efeitos locais dos envenenamentos botrópicos

A injeção direta de venenos botrópicos no local da picada causa lesão celular através de danos proteolíticos que iniciam imediatamente após contato. Os indicativos clínicos - na forma de necrose tecidual, formação de bolhas, hemorragia e edema - são as manifestações mais dramáticas dos envenenamentos. De acordo com o conhecimento atual a respeito da composição destes venenos e o efeito de toxinas isoladas em modelos de envenenamento experimental, atribui-se às metaloproteases de venenos de serpentes (SVMPs) a ação proteolítica direta sobre matriz extracelular e liberação de mediadores endógenos de inflamação que irão, por fim, gerar a formação das bolhas, dermonecrose, hemorragia, dor e edema. Esses efeitos, associados à atividade de outras enzimas (fosfolipases, por exemplo), provocam, no tecido afetado, um estado isquêmico, contribuindo para a mionecrose (Gutierrez et al., 2009b).

Estudos *in vitro* demonstram claramente a degradação de diferentes proteínas da matriz extracelular por metaloproteases (Kamiguti et al., 1994, Baramova et al., 1989, Gutierrez et al., 2010, Pinto et al., 2007, Serrano et al., 2006, Escalante et al., 2006). *In vivo*, observações histológicas demonstram rápido dano à microvasculatura e desestabilização da membrana basal, levando ao extravasamento vascular (Gutierrez et al., 2009a, Baldo et al., 2010). De fato, células endoteliais tratadas *in vitro* com SVMPs sofrem desprendimento do substrato, alterações morfológicas e apoptose, porém este efeito possui uma janela temporal maior do que o observado *in vivo* (Lomonte et al., 1994, Tanjoni et al., 2005).

Na verdade, essas diferenças temporais entre os acontecimentos *in vivo* e *in vitro* são explicadas pela hipótese de que o mecanismo de ação das SVMPs seja resultante da ação proteolítica em conjunto com forças biofísicas provenientes do fluxo sanguíneo no tecido. Desta forma, o dano vascular é causado por atividade proteolítica direta sobre a membrana basal, que promove o enfraquecimento mecânico da estrutura dos capilares que rompem-se ao efeito das forças hemodinâmicas normais que operam sobre a vasculatura (Gutierrez et al., 2005). Experimentalmente, a interrupção do fluxo sanguíneo prévia à injeção de SVMP abole o dano vascular e consequente hemorragia (Gutierrez et al., 2006).

3.3.2 Proteômica de tecidos aplicada à investigação da patofisiologia dos envenenamentos por serpentes. Efeitos induzidos pela Jararagina.

Tissue proteomics in the investigation of local snakebite pathophysiology induced by jararhagin injection

Renata M. S. Terra, Antônio F. M. Pinto, Teresa Escalante, José M. Gutierrez, Jorge A. Guimarães e Jay W. Fox

Comunicação

Resumo - Envenenamentos por viperídeos causam drásticos efeitos locais, como hemorragia e necrose tecidual, pela ação de metaloproteases de venenos de serpentes (SVMPs). SVMPs são eficientes enzimas proteolíticas capazes de degradar proteínas de matriz extracelular causando desestabilização da microvasculatura e extravasamento sanguíneo. Apesar dos substratos das SVMPs estarem bem caracterizados *in vitro*, a identificação dos substratos *in vivo* é importante foco de pesquisa pela importância dos mesmos na geração de novas estratégias para prevenção do dano local. Neste trabalho apresentamos, através de uma abordagem de proteômica de tecidos, a avaliação dos efeitos locais diretos da Jararagina, uma metaloprotease do veneno da serpente *Bothrops jararaca*. Verificou-se um aumento em proteínas envolvidas no desenvolvimento local de hemorragia e inflamação. Mas, acima de tudo, identificamos possíveis novos alvos com importância na estabilização da matriz extracelular e na recuperação tecidual pós-dano. Os dados aqui apresentados permitem a geração de novas hipóteses

relativas às ações das SVMPs sobre a matriz extracelular a serem futuramente avaliadas.

Este trabalho foi desenvolvido na Biomolecular Research Facility (University of Virginia) em colaboração com o Laboratório de Bioquímica Farmacológica (UFGRS) e com o Instituto Clodomiro Picado da Costa Rica. Os dados aqui estão apresentados na forma de comunicação rápida e fazem parte de um manuscrito em fase de preparação.

TISSUE PROTEOMICS IN THE INVESTIGATION OF LOCAL SNAKEBITE PATHOPHYSIOLOGY INDUCED BY JARARHAGIN INJECTION

Renata M S Terra[1,2], Antônio F M Pinto[1], Teresa Escalante[3], José M Gutiérrez[3], Jorge A Guimarães[2] and Jay W Fox[1*]

[1]Laboratório de Bioquímica Farmacológica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, VA, USA

[3]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

[*]Corresponding author

Keywords: Proteomics, Jararhagin, envenomation, tissue damage, SVMP

Abstract

Viperid envenomation causes hemorrhage, edema and necrosis at the bite site mainly due to the action of snake venom metalloproteases (SVMPs). SVMPs are known to efficiently degrade extracellular matrix (ECM) proteins promoting destabilization of the microvasculature and blood extravasation. Although *in vitro* SVMP substrate specificity has been extensively reported, *in vivo* targets are a main research focus as their identification is important to generate strategies for local tissue damage prevention. In this work we demonstrate, from a tissue proteomics approach, the local direct effect upon mouse skin of Jararhagin, a metalloprotease isolated from *Bothrops jararaca* venom. Here we report that envenomed tissue presents increased levels of proteins involved in the setting up of hemorrhage and inflammation. We report the identification of new putative targets, implicated in ECM stabilization and wound healing. The data shown here promotes the generation of alternative hypothesis regarding SVMPs activities upon ECM to be further evaluated.

1. Introduction

Viperid snakebite accidents are characterized by local and systemic alterations such as tissue damage, edema, severe hemodynamic disturbances and hemorrhage. Snake venom metalloproteinases (SVMPs) are the main toxins involved in the development of a complex pathophysiology of snake envenomations. SVMPs are members of the M12 Reprolysin family and are structurally related to the ADAMs family of metalloproteinases [1]. All SVMPs have a very similar zinc-binding motif, however, not all are able to induce hemorrhage [2]. The snake venom metalloproteinases are classified according to the presence of non-catalytic subdomains. The PIII group of SVMPs shares a similar domain structure of some ADAM metalloproteinases, with the presence of a proteinase domain followed by disintegrin-like (Dis-like) and cysteine rich domains (Cys-rich) [3]. As a general rule, PIII SVMPs are more potent hemorrhagins then PI SVMPs, which are composed only by the proteinase domain [1].

The proteinase domain of all the SVMP hemorrhagic toxins is believed to function to degrade capillary basement membranes, endothelial cell surfaces, and stromal matrix ultimately causing extravasation of capillary contents into the surrounding stroma [4]. Alterations of the endothelium associated with hemorrhage from the microvasculature are observed in the first minutes after SVMP injection [5]. The following morphological changes include reduction in thickness of cells, drop in the number of pinocytotic vesicles and separation of the cell from their basement membrane, leading to disruption in the cellular integrity and extravasation [6]. Molecular observations include degradation of many extracellular matrix proteins (ECM) like nidogen and collagens [7]. The Dis-like and Cys-rich domains functions are not as clear, but they seem to facilitate enzyme-substrate interaction, mainly with

protein containing von Willebrand factor A1 domains, and to inhibit integrin binding to collagen, inducing specific proteolysis and enhancing hemorrhage [8-9]. Also, it has been shown that proteolytic degradation of basement membrane and subsequent local hemorrhage is not only based in the proteolytic action of SVMPs but, in fact, is a two-step event that connects enzymatic activity and biophysical forces associated with blood flow [5].

Jararhagin is a multifunctional hemorrhagic PIII SVMP isolated from the venom of *Bothrops jararaca*. Besides its action upon extracellular matrix proteins, disrupting capillary network and leading to hemorrhage, Jararhagin also compromises hemostasis by fibrinogen degradation and inhibition of platelet aggregation [10]. In addition, Jararhagin is able to interact with many other cell types, such as endothelial, melanoma, epithelial and neuroblastoma cells, causing detachment and apoptosis [11-14]. Moreover, Jararhagin is also able to provoke inflammatory reaction with induction of leukocyte accumulation and elevation of inflammatory mediators and cytokines [15-16].

In despite of the great amount of information regarding SVMPs proteolytic activity upon isolated proteins and cultured cells, the precise molecular mechanism by which Jararhagin and other SVMPs cause tissue damage provoking hemorrhage remains elusive. In this sense, we were able to successfully apply a proteomic approach to investigate wound exudate caused by snake toxins [17]. In the present study, we performed a tissue proteomic approach to illustrate the molecular mechanisms of snakebite local pathology caused by the PIII SVMP Jararhagin skin injection.

2. Experimental Procedures

2.1 Isolation of Jararhagin

Bothrops jararaca venom was obtained from Miami Serpentarium Laboratories (Florida, USA). Venom was kept lyophilized at – 20 °C until use. Jararhagin isolation was performed using a combination of hydrophobic interaction and ion exchange chromatography according to Paine et al., 1992 [18]. All protein determination was done with NanoOrange Protein Quantitation Kit (Invitrogen – Carlsbad, CA).

2.2 Experimental Local Envenomation

Experimental envenomation was performed in CD-1 mice (18 – 20 g body weight) with the approval of Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica. Mice were anesthetized with a mixture of xylazine and ketamine and divided in four groups. *Group 1*: injected intradermally with a sterile phosphate-buffered saline solution (PBS). *Group 2*: injected intradermally with 50 µg Jararhagin. *Group 3*: blood flow was interrupted as previous described [5] and mice were injected intradermally with PBS solution. *Group 4*: blood flow was interrupted in the same way as group 3 and mice were intradermally injected with 50 µg Jararhagin. Fifteen minutes after injection, mice were killed; the skin around the site of injection was dissected out and immediately placed into liquid nitrogen. After disruption with a pistile, 20 mM Tris.HCl, pH 7.6 buffer solution containing NaCl, Triton X-100, deoxycholate, SDS, EDTA and a cocktail of proteinase inhibitors was added. Samples were vortexed and then kept at 4 °C overnight. Then, samples were centrifuged and the supernatants were collected and freeze-dried.

2.3 SDS-PAGE and In-gel Enzymatic Digestion

Samples (100 µg of protein / group) were reconstituted in water and acetone-precipitated at 4 ˚C, overnight. Protein pellets were re-suspended in Laemmli buffer and SDS-PAGE gels (12 %) were performed according to Laemmli [19] under non-reducing conditions. Protein bands were detected after staining with Coomassie brilliant blue.

Gel lanes were cut in 10 equal-size sections. Gels sections were distained for 2 h and proteins were reduced (10 mM DTT) and alkylated (50 mM iodoacetamide) at room temperature. Gel sections were washed with 100 mM ammonium bicarbonate, dehydrated with acetonitrile and then, rehydrated in Promega modified trypsin solution (20 ng / mL) for 30 min on ice. Excess trypsin was removed and the digestion was carried on for 18 h at 37 °C. The tryptic peptides were extracted twice from gel sections with 30 µL of 50 % acetonitrile/5 % formic acid solution. The combined extracts were dried to a volume of 15 µL for mass spectrometric analysis.

2.4 Protein Identification by LC/MS/MS Analysis

LC/MS/MS analysis was performed using a Thermo Electron LTQ ion-trap mass spectrometer. Samples were loaded into analytical columns fabricated in-house by packing 7.5 cm Jupiter 10 µm C18 packing material (Phenomenex, Torrance, CA) into a 360 x 75 µm fused silica capillary (Polymicro Technologies, Phoenix, AZ) behind a bottleneck. Samples were injected into the mass spectrometer at 300 nL/min. Peptides were eluted from the analytical C18 column using an acetonitrile/0.1 M acetic acid gradient (2 - 80% acetonitrile). The instrument was programmed to acquire a cycle of one mass spectrum followed by MS/MS on the ten most abundant ions in a data-dependent mode. After MS/MS, fragmentation was carried out on a particular parent ion and the m/z was placed on an exclusion list for

2 minutes to enable greater dynamic range and prevent repeated analysis of the same ions. The electrospray voltage was set to 2.5 kV, and the capillary temperature was 210 ˚C.

The mass spectra were extracted and analyzed utilizing Bioworks Sequest 3.3.1 software. Searches were performed against an IPI Mouse database (http://www.ebi.ac.uk/IPI/). Spectra generated on the LTQ were searched using 1.5 Da parent tolerance and 1 Da fragment tolerance. All hits were required to be fully tryptic.

Scaffold (version Scaffold_2.02.01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if Sequest identifications had XCorr scores greater than 1.8, 2.3, 2.7 and 3.7 for singly, doubly, triply and quadruply charged peptides. Protein identifications were accepted if they contained at least 2 identified peptides. Quantifications were based on the calculated Quantitative values from the Scaffold software. Quantitative values extracted were used to calculate the ratios between samples. Proteins with a quantitative value lower than 5 were not considered; an arbitrary ratio cutoff of 2 was used to determine significantly changed proteins.

2.5 Histological evaluation

Group of four CD-1 mice (18 - 20 g) were injected intradermally, in the right gastrocnemius, with 50 µg of Jararhagin or 50 µL of PBS, as described above. One hour after injection, mice were sacrificed and the skin located around the injected region was immediately dissected out and cut into two halves. Tissue samples were rapidly fixed in zinc fixative (BD Pharmingen, San Jose, CA) and routinely processed for embedding in paraffin. Thick sections (4 µm) were prepared and stained with hematoxylin and eosin for histological assessment.

3 Results and Discussion

3.1 Macro and microscopic observations

Animals from group 1 and 3 had normal characteristic features of the skin. Group 2, animals injected with Jararhagin under normal blood flow condition, had prominent hemorrhage while group 4 had absence of blood extravasation. Under microscopic evaluation of group 2 animals, it was possible to observe the blood cells infiltration, presence of edemaciated areas and the dislodgement of the epidermis from the dermis contrasting with the normal skin appearance of group 1 (Figure 1). Histological evaluation showed the presence of hemorrhage, edema and extracellular matrix disruption only under normal blood flow conditions. These data is in accordance to what was previously shown [5], confirming once again the importance of blood flow biophysical forces to tissue damage.

3.2 Jararhagin-induced cell damage and extravasation under normal blood flow conditions.

Skin protein samples of mice injected with jararhagin (group 2) and control animals (group 1), under normal blood flow condition, were initially analyzed by 1D SDS-PAGE. LC/MS/MS analysis of gel slices gave an overview of the samples composition. Table I shows the increased and decreased proteins in envenomed tissue under normal blood flow.

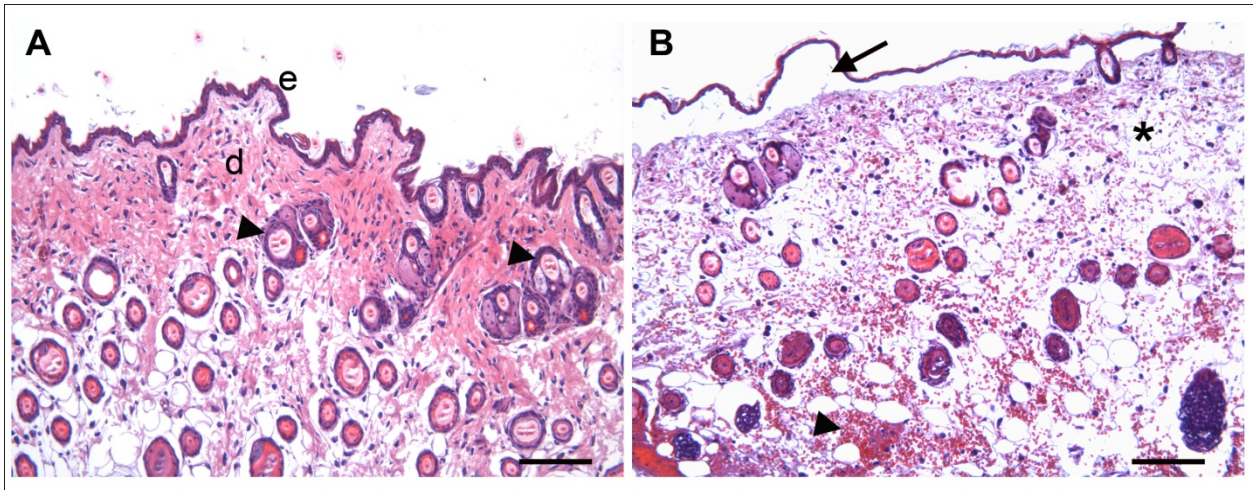


Figure 1: Histological alterations induced by jararhagin in the skin. Light micrographs of paraffin-embedded sections of mouse skin injected i.d. with either 100 μL of PBS **(A)** or 50μg of jararhagin in 100 μl of PBS **(B)**. Mice were sacrificed 15 min. after the injection. (A) Normal morphology of the skin, (e) epidermis, (d) dermis, (arrow head) hear follicle. (B) Notice the hemorrhage (arrow head), the separation of epidermis from dermis (arrow) and the loss of staining in the dermis due to edema (*). Bars = 100 μm.

Four pathologically important groups of proteins that are present in greater levels in the envenomed tissue can be highlighted: blood and erythrocytes proteins, oxidative stress proteins, acute-phase proteins and proteins released from the adjacent muscle tissue. As shown in table I, there is a 10- to 31-fold increase in erythrocytes proteins under flow conditions. Also, serum proteins are also in higher amounts in Jararhagin Flow, coherent with the well known hemorrhagic action of the toxin as well as the blood cells infiltration and edema observed in the histological evaluation. Capillary network disruption produces infiltrates in the damaged skin, increasing the amount of serum proteins in the area, as well as increasing enormously the amounts of erythrocytes in the damaged tissue. Moreover, the infiltrate causes an inflammatory reaction increasing the number of acute-phase proteins in the area, potentiating edema.

Table I - Difference in fold change abundance of proteins in Jararhagin injected tissue under normal blood flow. Fold change are shown between brackets

Up-regulated proteins	Down-regulated proteins
Blood and Erythrocytes	*Extracellular matrix*
Spectrin alpha chain, erythrocyte (IPI00323230) [14.0]	Collagen alpha-1(I) chain (IPI00329872) [3.5]
Spectrin beta 1 (IPI00131376) [10.0]	Collagen alpha-2(I) chain (IPI00222188) [5.2]
Bisphosphoglycerate mutase (IPI00221663) [17.0]	Periostin (IPI00120870) [3.5]
Carbonic anhydrase 1 (IPI00230320) [13.0]	
Carbonic anhydrase 2 (IPI00121534) [9.1]	*Extracellular matrix remodeling*
Erythrocyte of Band 3 anion transport protein (IPI00120761) [31.0]	Coagulation factor XIII A chain (IPI00402967) [4.0]
Hemoglobin subunit beta-1 (IPI00553333) [10.6]	
Hemoglobin subunit beta-2 (IPI00316491) [7.4]	
Hemoglobin, beta adult major chain (IPI00110658) [12.8]	
Oxidative stress	
Catalase (IPI00312058) [3.7]	
Glutathione peroxidase 1 (IPI00319652) [2.4]	
Acute-phase	
Alpha-2-macroglobulin (IPI00624663) [6.8]	
Ceruloplasmin (IPI00117831) [3.7]	
Inter alpha-trypsin inhibitor, heavy chain 4 (IPI00119818) [7.3]	
Murinoglobulin-1 (IPI00123223) [8.7]	
Serum amyloid P-component (IPI00309214) [2.8]	
Complement C3 long (IPI00323624) [3.6]	
Muscle-released	
Troponin I, fast skeletal muscle (IPI00223196) [7.0]	
Gelsolin (IPI00117167) [2.3]	

An increase of 3.7-fold in catalase and 2.4-fold in glutathione peroxidase I indicates oxidative stress in the damage tissue that may exacerbate the pathological response. Additionally, it was observed the presence of muscle tissue proteins evidencing adjacent damage.

On the other hand, decrease in fold-change of proteins is an indicative of degradation. In envenomed skin, we were able to identify a decrease in extracellular matrix proteins, periostin and alpha-1 and alpha-2 chains of collagen I. Although collagens are known substrate of SVMPs, degradation of periostin was observed here for the first time. Periostin is a matricellular protein involved in the modulation of cell-matrix interactions and matrix organization [20]. It has been characterized as an inducer of collagen fibrillogenesis after cell injury and as a reparative pathway in

wound repair [20-21]. The 3.5-fold decrease in periostin abundance may indicate a new substrate for SVMPs and an additional mechanism of matrix destabilization.

Also, we could detect a 4-fold decrease in blood coagulation factor XIII A chain. Coagulation factor XIII is a multimeric transglutaminase that, after activation, cross-links fibrin to promote clot stabilization preventing the elimination by the fibrinolytic system [22]. Moreover, FXIII is also responsible for cross-linking collagens I and III, laminin, fibronectin, vitronectin and tenascin enhancing extracellular matrix stability [23]. Once again, the decrease in a matrix-related protein could indicate alternative mechanisms of ECM degradation and destabilization to promote hemorrhage. In fact, it was demonstrated that factor XIII is able to counteracts the effects of a Clostridium *histolyticum* collagenase, promoting matrix resistance to proteolytic degradation and preventing fibroblast detachment and death [23].

In this sense, hemorrhage would be caused by the action of SVMPs by different, but synergistic mechanisms. First, direct proteolytic degradation of collagen and other ECM proteins as extensively demonstrated by *in vitro* and *in vivo* studies. Then, degradation of proteins involved in matrix secretion and stabilization, preventing resistance of ECM to proteolysis and impairing wound repair. Additionally, it may prevent FXIII-mediated clot stabilization enhancing local hemorrhage.

3.3 Jararhagin-induced cell damage in the absence of blood flow

When blood flow was experimentally interrupted, Jararhagin did not promote hemorrhage in the injected tissue. Proteomic analysis of skin demonstrated no degradation of ECM and the absence of proteins from blood, coherent with previously shown data [5]. Difference in fold change of proteins injected in the absence of blood flow (group 3) is shown in Table II.

Table II - Difference in fold change abundance of proteins in Jararhagin injected tissue under absent blood flow. Fold change are demonstrated between

Up-regulated proteins	Down-regulated proteins
Energetic metabolism	*Constitutive/ Housekeepers*
Glyceraldehyde-3-phosphate dehydrogenase (IPI00271869) [5.5]	Desmin (IPI00130102) [2.2]
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 1 (IPI00135284) [3.7]	Actin-related protein 2/3 complex subunit 2 (IPI00661414) [2.2]
Glyceraldehyde-3-phosphate dehydrogenase Pseudogene (IPI00123176) [2.0]	Tubulin beta-2C chain (IPI00169463) [2.3]
	Fibulin-5 (IPI00323035) [2.4]
	Isoform 1 of Filamin-A (IPI00131138) [2.8]
	Isoform 5g of Peripherin (IPI00129527) [3.2]
	40S ribosomal protein S14 isoform 1 (IPI00112407) [2.1]
	Proteasome subunit beta type-3 (IPI00314467) [2.1]
	Heterogeneous nuclear ribonucleoprotein R (IPI00128441) [2.2]
	60S ribosomal protein L7 (IPI00311236) [2.2]
	60S ribosomal protein L22 (IPI00222546) [2.2]
	Histone H2A.J (IPI00153400) [2.5]
	T-complex protein 1 subunit epsilon (IPI00116279) [2.6]
	Isoform 1 of Heterogeneous nuclear ribonucleoprotein M (IPI00132443) [2.9]

Mainly, it could be noticed an increase in GAPDH, indicating that the tissue is under hypoxia [24]. Actually, hypoxia was observed by previous experiments, denoting that under these experimental conditions the only effect perceived was the physiological response to absent blood irrigation. Additionally, it was observed the decrease in many constitutive proteins confirming absence of blood influx and cell damage.

4. Conclusions

The early time of tissue collection and processing used in this work gave us the possibility to evaluate the direct effect of the SVMP, giving the fact that later times would give us the secondary host response to envenomation. Although we decided to collect the tissue only 15 minutes after injection, we were able to identify critical tissue damage and pronounced hemorrhage.

Moreover, we identified putative new substrates to the proteolytic activity of SVMPs, generating new hypothesis on how tissue damage and hemorrhage may take place and evolve to necrosis. According to the data shown here, it is likely that

extracellular matrix destabilization occurs not only by direct degradation of its components, but also by impairing mechanisms underlying matrix resistance to proteolysis.

Together, these testable hypotheses generated here may contribute to the development of adjuvant snakebite therapies once antivenoms, although effective to systemic symptomatology, seem almost ineffective to local tissue injury.

6. References

[1] Bjarnason JB, Fox JW. Hemorrhagic metalloproteinases from snake venoms. Pharmacol Ther. 1994;62:325-72.

[2] Fox JW, Serrano SM. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon. 2005;45:969-85.

[3] Fox JW, Serrano SM. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. Febs J. 2008;275:3016-30.

[4] Baramova EN, Shannon JD, Bjarnason JB, Fox JW. Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. Arch Biochem Biophys. 1989;275:63-71.

[5] Gutierrez JM, Nunez J, Escalante T, Rucavado A. Blood flow is required for rapid endothelial cell damage induced by a snake venom hemorrhagic metalloproteinase. Microvasc Res. 2006;71:55-63.

[6] Gutierrez JM, Rucavado A, Escalante T, Diaz C. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. Toxicon. 2005;45:997-1011.

[7] Escalante T, Shannon J, Moura-da-Silva AM, Gutierrez JM, Fox JW. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. Arch Biochem Biophys. 2006;455:144-53.

[8] Serrano SM, Wang D, Shannon JD, Pinto AF, Polanowska-Grabowska RK, Fox JW. Interaction of the cysteine-rich domain of snake venom metalloproteinases with the A1 domain of von Willebrand factor promotes site-specific proteolysis of von

Willebrand factor and inhibition of von Willebrand factor-mediated platelet aggregation. Febs J. 2007;274:3611-21.

[9] Tanjoni I, Butera D, Bento L, Della-Casa MS, Marques-Porto R, Takehara HA, et al. Snake venom metalloproteinases: structure/function relationships studies using monoclonal antibodies. Toxicon. 2003;42:801-8.

[10] Laing GD, Moura-da-Silva AM. Jararhagin and its multiple effects on hemostasis. Toxicon. 2005;45:987-96.

[11] Costa EP, Del Debbio CB, Cintra LC, da Fontoura Costa L, Hamassaki DE, Santos MF. Jararhagin, a snake venom metalloprotease-disintegrin, activates the Rac1 GTPase and stimulates neurite outgrowth in neuroblastoma cells. Toxicon. 2008;52:380-4.

[12] Costa EP, Santos MF. Jararhagin, a snake venom metalloproteinase-disintegrin, stimulates epithelial cell migration in an in vitro restitution model. Toxicon. 2004;44:861-70.

[13] Correa MC, Jr., Maria DA, Moura-da-Silva AM, Pizzocaro KF, Ruiz IR. Inhibition of melanoma cells tumorigenicity by the snake venom toxin jararhagin. Toxicon. 2002;40:739-48.

[14] Moura-da-Silva AM, Marcinkiewicz C, Marcinkiewicz M, Niewiarowski S. Selective recognition of alpha2beta1 integrin by jararhagin, a Metalloproteinase/disintegrin from bBothrops jararaca venom. Thromb Res. 2001;102:153-9.

[15] Costa EP, Clissa PB, Teixeira CF, Moura-da-Silva AM. Importance of metalloproteinases and macrophages in viper snake envenomation-induced local inflammation. Inflammation. 2002;26:13-7.

[16] Gallagher P, Bao Y, Serrano SM, Laing GD, Theakston RD, Gutierrez JM, et al. Role of the snake venom toxin jararhagin in proinflammatory pathogenesis: in vitro and in vivo gene expression analysis of the effects of the toxin. Arch Biochem Biophys. 2005;441:1-15.

[17] Escalante T, Rucavado A, Pinto AF, Terra RM, Gutierrez JM, Fox JW. Wound exudate as a proteomic window to reveal different mechanisms of tissue damage by snake venom toxins. J Proteome Res. 2009;8:5120-31.

[18] Paine MJ, Desmond HP, Theakston RD, Crampton JM. Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from Bothrops jararaca venom. Insights into the disintegrin gene family. J Biol Chem. 1992;267:22869-76.

[19] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680-5.

[20] Hamilton DW. Functional role of periostin in development and wound repair: implications for connective tissue disease. J Cell Commun Signal. 2008;2:9-17.

[21] Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. J Mol Cell Cardiol. 2010;48:504-11.

[22] Muszbek L, Bereczky Z, Bagoly Z, Shemirani AH, Katona E. Factor XIII and atherothrombotic diseases. Semin Thromb Hemost. 2010;36:18-33.

[23] Zamboni P, De Mattei M, Ongaro A, Fogato L, Carandina S, De Palma M, et al. Factor XIII contrasts the effects of metalloproteinases in human dermal fibroblast cultured cells. Vasc Endovascular Surg. 2004;38:431-8.

[24] Graven KK, Farber HW. Endothelial cell hypoxic stress proteins. J Lab Clin Med. 1998;132:456-63.

4.
CONSIDERAÇÕES FINAIS

Os resultados aqui apresentados demonstram que, além de complexos, os venenos animais são capazes de interagir especificamente com diferentes moléculas, tecidos e organismos gerando quadros patológicos complexos.

No primeiro capítulo deste trabalho de tese as características individuais de dois venenos de serpentes evolutivamente relacionadas foram abordadas. As serpentes *B. jararaca* e *B. lanceolatus* apresentam, de maneira genérica, a mesma composição de toxinas, porém, são capazes de gerar perfis de envenenamento opostos. Numa análise mais detalhada dos venenos, é possível observar as diferenças na distribuição de toxinas da mesma família. O veneno hemorrágico de *B. jararaca* apresenta uma abundância maior de SVMPs da classe PIII, que são potentes hemorraginas, enquanto no veneno de *B. lanceolatus* prevalecem SVMPs da classe PI. Da mesma maneira, a atividade proteolítica dos dois venenos é discutida. Conclui-se, por fim, que as diferentes atividades biológicas são resultantes da combinação de uma distribuição desigual de subgrupos de SVMPs (tendo o grupo PIII atividade hemorrágica mais pronunciada), juntamente com as características individuais de cada enzima. Porém, o panorama é mais complexo, uma vez que existem outras famílias de proteínas com atividade sobre a hemostasia e a trombose e que também apresentam diferentes abundâncias relativas. Desta forma, surgem aqui questionamentos a respeito de como as toxinas agem sinergicamente, resultando no desenvolvimento de quadros patológicos distintos e

opostos que, neste caso, são a hemorragia, em *B. jararaca*, e a trombose, em *B. lanceolatus*. Também, especula-se como a abundância relativa de proteínas individuais é capaz de alterar a atividade biológica global dos venenos. Além disso, o veneno de *B. lanceolatus* representa mais um desafio na investigação destas atividades biológicas uma vez que tende a apresentar especificidade de espécie, causando acidentes pró-trombóticos em seres humanos e efeitos hemorrágicos em modelos animais.

No segundo capítulo, investigamos os componentes tóxicos das secreções venenosas de *L. obliqua* que participam no desenvolvimento da síndrome hemorrágica. Neste caso, inibidores de proteases e serinoproteases interagem diretamente com o sistema hemostático da vítima, gerando um quadro hemorrágico sistêmico com poucas repercussões locais, como necrose. Os dados obtidos aqui, por proteômica, identificaram ainda outros componentes minoritários e potencialmente tóxicos. A presença de diversos peptídeos homólogos a sequências de SVMPs inevitavelmente levanta hipóteses à respeito de um possível efeito tóxico.

Por fim, no terceiro e último capítulo avaliamos a atividade tóxica direta sobre o tecido afetado, como resultado da ação de uma SVMP, a Jararagina, usada como modelo experimental de envenenamento. Os dados aqui obtidos relatam, sob o ponto de vista molecular, as observações macro e microscópicas dos envenenamentos, como hemorragia e edema. Ademais, identificamos proteínas dotadas de atividade protetora na manutenção da integridade da matriz extracelular e na estabilização de coágulos que criam indícios de novos mecanismos de ação de SVMPs na geração direta do quadro patológico e na resposta fisiológica da vitima.

As abordagens proteômicas aqui apresentadas permitiram a geração de um grande número de dados à respeito da composição e efeitos tóxicos dos venenos.

Mais que isso, os dados apresentados geram novas hipóteses a serem testadas de maneira a contribuir e expandir significativamente o conhecimento atual no assunto.

5. REFERÊNCIAS

ALVAREZ FLORES, M. P., FRITZEN, M., REIS, C. V. & CHUDZINSKI-TAVASSI, A. M. 2006. Losac, a factor X activator from Lonomia obliqua bristle extract: its role in the pathophysiological mechanisms and cell survival. *Biochem Biophys Res Commun,* 343**,** 1216-23.

ANTUNES, T. C., YAMASHITA, K. M., BARBARO, K. C., SAIKI, M. & SANTORO, M. L. 2010. Comparative analysis of newborn and adult Bothrops jararaca snake venoms. *Toxicon, in press.*

AROCHA-PINANGO, C. L. & LAYRISSE, M. 1969. Fibrinolysis produced by contact with a caterpillar. *Lancet,* 1**,** 810-2.

BALDO, C., JAMORA, C., YAMANOUYE, N., ZORN, T. M. & MOURA-DA-SILVA, A. M. 2010. Mechanisms of vascular damage by hemorrhagic snake venom metalloproteinases: tissue distribution and in situ hydrolysis. *PLoS Negl Trop Dis,* 4**,** e727.

BARAMOVA, E. N., SHANNON, J. D., BJARNASON, J. B. & FOX, J. W. 1989. Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. *Arch Biochem Biophys,* 275**,** 63-71.

BASUALDO, A., DE OLIVEIRA, K. C., TREVISAN, T. & BARLETTA, F. B. 2008. Tratamento clínico e bucomaxilofacial de quadro hemorrágico-alérgico por Lonomia sp: relato de caso. *Stomatos,* 14**,** 46-54.

BERGER, M., RECK, J., JR., TERRA, R. M., PINTO, A. F., TERMIGNONI, C. & GUIMARAES, J. A. 2010. Lonomia obliqua caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats. *Toxicon,* 55**,** 33-44.

BJARNASON, J. B. & FOX, J. W. 1995. Snake venom metalloendopeptidases: reprolysins. *Methods Enzymol,* 248**,** 345-68.

BURDMANN, E. A., ANTUNES, I., SALDANHA, L. B. & ABDULKADER, R. C. 1996. Severe acute renal failure induced by the venom of Lonomia caterpillars. *Clin Nephrol,* 46**,** 337-9.

CALVETE, J. J. 2009. Venomics: digging into the evolution of venomous systems and learning to twist nature to fight pathology. *J Proteomics,* 72**,** 121-6.

CALVETE, J. J., JUAREZ, P. & SANZ, L. 2007. Snake venomics. Strategy and applications. *J Mass Spectrom,* 42**,** 1405-14.

CALVETE, J. J., SANZ, L., ANGULO, Y., LOMONTE, B. & GUTIERREZ, J. M. 2009. Venoms, venomics, antivenomics. *FEBS Lett,* 583**,** 1736-43.

CIDADE, D. A., SIMAO, T. A., DAVILA, A. M., WAGNER, G., JUNQUEIRA-DE-AZEVEDO, I. L., HO, P. L., BON, C., ZINGALI, R. B. & ALBANO, R. M. 2006. Bothrops jararaca venom gland transcriptome: analysis of the gene expression pattern. *Toxicon,* 48**,** 437-61.

DA SILVA, C. J., JORGE, M. T. & RIBEIRO, L. A. 2003. Epidemiology of snakebite in a central region of Brazil. *Toxicon,* 41**,** 251-5.

DE ARAUJO, A. L., DE SOUZA, A. O., DA CRUZ-HOFLING, M. A., FLORES, C. A. & BON, C. 2000. Bothrops lanceolatus (Fer de lance) venom induces oedema formation and increases vascular permeability in the mouse hind paw. *Toxicon,* 38**,** 209-21.

DE FARIA, L., ANTUNES, E., BON, C. & DE ARAUJO, A. L. 2001. Pharmacological characterization of the rat paw edema induced by Bothrops lanceolatus (Fer de lance) venom. *Toxicon,* 39**,** 825-30.

DE ROODT, A. R., LITWIN, S. & VIDAL, J. C. 2003. Hemorrhagic activity of Bothrops venoms determined by two different methods and relationship with proteolytic activity on gelatin and lethality. *Toxicon,* 41**,** 949-58.

DUARTE, A. C., CAOVILLA, J., LORINI, I., LORINI, D., MANTOVANI, G., SUMIDA, J., MANFRE, P. C., SILVEIRA, R. C. & MOURA, S. P. 1990. Insuficiência renal aguda por acidentes com lagartas. *Jornal Brasileiro de Nefrologia* 12**,** 184-186.

ESCALANTE, T., SHANNON, J., MOURA-DA-SILVA, A. M., GUTIERREZ, J. M. & FOX, J. W. 2006. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: a biochemical and immunohistochemical study. *Arch Biochem Biophys,* 455**,** 144-53.

FOX, J. W., MA, L., NELSON, K., SHERMAN, N. E. & SERRANO, S. M. 2006. Comparison of indirect and direct approaches using ion-trap and Fourier transform ion cyclotron resonance mass spectrometry for exploring viperid venom proteomes. *Toxicon,* 47**,** 700-14.

FOX, J. W. & SERRANO, S. M. 2005. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon,* 45**,** 969-85.

FOX, J. W. & SERRANO, S. M. 2008. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. *Proteomics,* 8**,** 909-20.

FOX, J. W. & SERRANO, S. M. 2009. Timeline of key events in snake venom metalloproteinase research. *J Proteomics,* 72**,** 200-9.

FRANCISCHETTI, I. M., CASTRO, H. C., ZINGALI, R. B., CARLINI, C. R. & GUIMARAES, J. A. 1998. Bothrops sp. snake venoms: comparison of some biochemical and physicochemical properties and interference in platelet functions. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol,* 119**,** 21-9.

GAMBORGI, G. P., METCALF, E. B. & BARROS, E. J. 2006. Acute renal failure provoked by toxin from caterpillars of the species Lonomia obliqua. *Toxicon,* 47**,** 68-74.

GARCIA, C. M. & DANNI-OLIVEIRA, I. M. 2007. [Occurrence of accidents caused by Lonomia obliqua Walker, in the State of Parana between 1989 and 2001]. *Rev Soc Bras Med Trop,* 40**,** 242-6.

GOUVEIA, A. I. D., DA SILVEIRA, R. B., NADER, H. B., DIETRICH, C. P., GREMSKI, W. & VEIGA, S. S. 2005. Identification and partial characterisation of hyaluronidases in Lonomia obliqua venom. *Toxicon,* 45**,** 403-410.

GUIMARAES, A. Q., CRUZ-HOFLING, M. A., FERREIRA DE ARAUJO, P. M., BON, C. & LOBO DE ARAUJO, A. 2004. Pharmacological and histopathological characterization of Bothrops lanceolatus (Fer de lance) venom-induced edema. *Inflamm Res,* 53**,** 284-91.

GUTIERREZ, J. M., ESCALANTE, T. & RUCAVADO, A. 2009a. Experimental pathophysiology of systemic alterations induced by Bothrops asper snake venom. *Toxicon,* 54**,** 976-87.

GUTIERREZ, J. M., NUNEZ, J., ESCALANTE, T. & RUCAVADO, A. 2006. Blood flow is required for rapid endothelial cell damage induced by a snake venom hemorrhagic metalloproteinase. *Microvasc Res,* 71**,** 55-63.

GUTIERREZ, J. M., RUCAVADO, A., CHAVES, F., DIAZ, C. & ESCALANTE, T. 2009b. Experimental pathology of local tissue damage induced by Bothrops asper snake venom. *Toxicon,* 54**,** 958-75.

GUTIERREZ, J. M., RUCAVADO, A., ESCALANTE, T. & DIAZ, C. 2005. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. *Toxicon,* 45**,** 997-1011.

GUTIERREZ, J. M., RUCAVADO, A., ESCALANTE, T., LOMONTE, B., ANGULO, Y. & FOX, J. W. 2010. Tissue pathology induced by snake venoms: how to understand a complex pattern of alterations from a systems biology perspective? *Toxicon,* 55**,** 166-70.

GUTIERREZ, J. M., SANZ, L., ESCOLANO, J., FERNANDEZ, J., LOMONTE, B., ANGULO, Y., RUCAVADO, A., WARRELL, D. A. & CALVETE, J. J. 2008. Snake venomics of the Lesser Antillean pit vipers Bothrops caribbaeus and Bothrops lanceolatus: correlation with toxicological activities and immunoreactivity of a heterologous antivenom. *J Proteome Res,* 7**,** 4396-408.

GUTIERREZ, J. M., WILLIAMS, D., FAN, H. W. & WARRELL, D. A. 2009c. Snakebite envenoming from a global perspective: Towards an integrated approach. *Toxicon*.

HOSSLER, E. W. 2009. Caterpillars and moths. *Dermatol Ther,* 22**,** 353-66.

HOSSLER, E. W. 2010. Caterpillars and moths: Part I. Dermatologic manifestations of encounters with Lepidoptera. *J Am Acad Dermatol,* 62**,** 1-10; quiz 11-2.

KAMIGUTI, A. S., SLUPSKY, J. R., ZUZEL, M. & HAY, C. R. 1994. Properties of fibrinogen cleaved by Jararhagin, a metalloproteinase from the venom of Bothrops jararaca. *Thromb Haemost,* 72**,** 244-9.

KOWACS, P. A., CARDOSO, J., ENTRES, M., NOVAK, E. M. & WERNECK, L. C. 2006. Fatal intracerebral hemorrhage secondary to Lonomia obliqua caterpillar envenoming: case report. *Arq Neuropsiquiatr,* 64**,** 1030-2.

LOBO DE ARAUJO, A., DONATO, J. L. & BON, C. 1998. Purification from Bothrops lanceolatus (fer de lance) venom of a fibrino(geno)lytic enzyme with esterolytic activity. *Toxicon,* 36**,** 745-58.

LOMONTE, B., GUTIERREZ, J. M., BORKOW, G., OVADIA, M., TARKOWSKI, A. & HANSON, L. A. 1994. Activity of hemorrhagic metalloproteinase BaH-1 and myotoxin II from Bothrops asper snake venom on capillary endothelial cells in vitro. *Toxicon,* 32**,** 505-10.

MERLE, H., DONNIO, A., AYEBOUA, L., PLUMELLE, Y., SMADJA, D. & THOMAS, L. 2005. Occipital infarction revealed by quadranopsia following snakebite by Bothrops lanceolatus. *Am J Trop Med Hyg,* 73**,** 583-5.

PICOLO, G., CHACUR, M., GUTIERREZ, J. M., TEIXEIRA, C. F. & CURY, Y. 2002. Evaluation of antivenoms in the neutralization of hyperalgesia and edema induced by Bothrops jararaca and Bothrops asper snake venoms. *Braz J Med Biol Res,* 35**,** 1221-8.

PINTO, A. F., BERGER, M., RECK, J., JR., TERRA, R. M. & GUIMARAES, J. A. 2010. Lonomia obliqua venom: In vivo effects and molecular aspects associated with the hemorrhagic syndrome. *Toxicon, in press.*

PINTO, A. F., DRAGULEV, B., GUIMARAES, J. A. & FOX, J. W. 2008. Novel perspectives on the pathogenesis of Lonomia obliqua caterpillar envenomation based on assessment of host response by gene expression analysis. *Toxicon,* 51**,** 1119-28.

PINTO, A. F., MA, L., DRAGULEV, B., GUIMARAES, J. A. & FOX, J. W. 2007. Use of SILAC for exploring sheddase and matrix degradation of fibroblasts in culture by the PIII SVMP atrolysin A: identification of two novel substrates with functional relevance. *Arch Biochem Biophys,* 465**,** 11-5.

PINTO, A. F. M., DOBROVOLSKI, R., VEIGA, A. B. G. & GUIMARAES, J. A. 2004. Lonofibrase, a novel alpha-fibrinogenase from Lonomia obliqua caterpillars. *Thrombosis Research,* 113**,** 147-154.

PINTO, A. F. M., SILVA, K. R. L. M. & GUIMARAES, J. A. 2006. Proteases from Lonomia obliqua venomous secretions: Comparison of procoagulant, fibrin(ogen)olytic and amidolytic activities. *Toxicon,* 47**,** 113-121.

PREZOTO, B. C., MAFFEI, F. H., MATTAR, L., CHUDZINSKI-TAVASSI, A. M. & CURI, P. R. 2002. Antithrombotic effect of Lonomia obliqua caterpillar bristle extract on experimental venous thrombosis. *Braz J Med Biol Res,* 35**,** 703-12.

REIS, C. V., FARSKY, S. H. P., FERNANDES, B. L., SANTORO, M. L., OLIVA, M. L. V., MARIANO, M. & CHUDZINSKI-TAVASSI, A. M. 2001. In vivo characterization of Lopap, a prothrombin activator serine protease from the Lonomia obliqua caterpillar venom. *Thrombosis Research,* 102**,** 437-443.

SANO-MARTINS, I. S., SANTORO, M. L., CASTRO, S. C., FAN, H. W., CARDOSO, J. L. & THEAKSTON, R. D. 1997. Platelet aggregation in patients bitten by the Brazilian snake Bothrops jararaca. *Thromb Res,* 87**,** 183-95.

SANO-MARTINS, I. S., SANTORO, M. L., MORENA, P., SOUSA-E-SILVA, M. C., TOMY, S. C., ANTONIO, L. C., NISHIKAWA, A. K., GONCALVES, I. L., LARSSON, M. H., HAGIWARA, M. K. & ET AL. 1995. Hematological changes induced by Bothrops jararaca venom in dogs. *Braz J Med Biol Res,* 28**,** 303-12.

SANTORO, M. L. & SANO-MARTINS, I. S. 2004. Platelet dysfunction during Bothrops jararaca snake envenomation in rabbits. *Thromb Haemost,* 92**,** 369-83.

SANTORO, M. L., SANO-MARTINS, I. S., FAN, H. W., CARDOSO, J. L., THEAKSTON, R. D. & WARRELL, D. A. 2008. Haematological evaluation of patients bitten by the jararaca, Bothrops jararaca, in Brazil. *Toxicon,* 51**,** 1440-8.

SERRANO, S. M., KIM, J., WANG, D., DRAGULEV, B., SHANNON, J. D., MANN, H. H., VEIT, G., WAGENER, R., KOCH, M. & FOX, J. W. 2006. The cysteine-rich domain of snake venom metalloproteinases is a ligand for von Willebrand factor A domains: role in substrate targeting. *J Biol Chem,* 281**,** 39746-56.

SERRANO, S. M., SHANNON, J. D., WANG, D., CAMARGO, A. C. & FOX, J. W. 2005. A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. *Proteomics,* 5**,** 501-10.

STROKA, A., DONATO, J. L., BON, C., HYSLOP, S. & DE ARAUJO, A. L. 2005. Purification and characterization of a hemorrhagic metalloproteinase from Bothrops lanceolatus (Fer-de-lance) snake venom. *Toxicon,* 45**,** 411-20.

TANJONI, I., WEINLICH, R., DELLA-CASA, M. S., CLISSA, P. B., SALDANHA-GAMA, R. F., DE FREITAS, M. S., BARJA-FIDALGO, C., AMARANTE-MENDES, G. P. & MOURA-DA-SILVA, A. M. 2005. Jararhagin, a snake venom metalloproteinase, induces a specialized form of apoptosis (anoikis) selective to endothelial cells. *Apoptosis,* 10**,** 851-61.

TERRA, R. M., PINTO, A. F., GUIMARAES, J. A. & FOX, J. W. 2009. Proteomic profiling of snake venom metalloproteinases (SVMPs): insights into venom induced pathology. *Toxicon,* 54**,** 836-44.

THOMAS, L., CHAUSSON, N., UZAN, J., KAIDOMAR, S., VIGNES, R., PLUMELLE, Y., BUCHER, B. & SMADJA, D. 2006. Thrombotic stroke following snake bites by the "Fer-de-Lance"Bothrops lanceolatus in Martinique despite antivenom treatment: a report of three recent cases. *Toxicon,* 48**,** 23-8.

THOMAS, L., TYBURN, B., BUCHER, B., PECOUT, F., KETTERLE, J., RIEUX, D., SMADJA, D., GARNIER, D. & PLUMELLE, Y. 1995. Prevention of thromboses in human patients with Bothrops lanceolatus envenoming in Martinique: failure of anticoagulants and efficacy of a monospecific antivenom. Research Group on Snake Bites in Martinique. *Am J Trop Med Hyg,* 52**,** 419-26.

VEIGA, A. B., BLOCHTEIN, B. & GUIMARAES, J. A. 2001. Structures involved in production, secretion and injection of the venom produced by the caterpillar Lonomia obliqua (Lepidoptera, Saturniidae). *Toxicon,* 39**,** 1343-51.

VEIGA, A. B., RIBEIRO, J. M., GUIMARAES, J. A. & FRANCISCHETTI, I. M. 2005. A catalog for the transcripts from the venomous structures of the caterpillar Lonomia obliqua: identification of the proteins potentially involved in the coagulation disorder and hemorrhagic syndrome. *Gene,* 355**,** 11-27.

VEIGA, A. B. G., PINTO, A. F. M. & GUIMARAES, J. A. 2003. Fibrinogenolytic and procoagulant activities in the hemorrhagic syndrome caused by Lonomia obliqua caterpillars. *Thrombosis Research,* 111**,** 95-101.

WILLIAMS, D., GUTIERREZ, J. M., HARRISON, R., WARRELL, D. A., WHITE, J., WINKEL, K. D. & GOPALAKRISHNAKONE, P. 2010. The Global Snake Bite Initiative: an antidote for snake bite. *Lancet,* 375**,** 89-91.

ZANNIN, M., LOURENCO, D. M., MOTTA, G., DALLA COSTA, L. R., GRANDO, M., GAMBORGI, G. P., NOGUTI, M. A. & CHUDZINSKI-TAVASSI, A. M. 2003. Blood coagulation and fibrinolytic factors in 105 patients with hemorrhagic syndrome caused by accidental contact with Lonomia obliqua caterpillar in Santa Catarina, southern Brazil. *Thromb Haemost,* 89**,** 355-64.

ZINGALI, R. B., CARLINI, C. R., FRANCISCHETTI, I. M. & GUIMARAES, J. A. 1990. Bothrops jararaca snake venom: effects on platelet aggregation. *Thromb Res,* 58**,** 303-16.

6. ANEXO I

CURRICULUM VITAE

TERRA, RMS

DADOS PESSOAIS

Nome: Renata Maria Soares Terra

Filiação: Lafaiete Oliveira Terra e Nadir Soares Terra

Nascimento: 23/10/1981, Porto Alegre/RS - Brasil

Endereço profissional: Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia, Laboratório de Bioquímica Farmacológica.

Av. Bento Gonçalves, 9500 - Prédio 43431- Laboratório 214 - Campos do Vale - Bairro Agronomia

91501970 Porto Alegre, RS - Brasil

Telefone: (51) 33086062

E-mail: reterra@cbiot.ufrgs.br

TÍTULOS ACADÊMICOS

2006-2010 Doutorado em Biologia Celular e Molecular.

Universidade Federal do Rio Grande do Sul, UFRGS, Rio Grande do Sul,Brasil.

Título: VENÔMICA: IDENTIFICAÇÃO DE PROTEÍNAS ENVOLVIDAS NA FISIOPATOLOGIA DE ENVENENAMENTOS ANIMAIS

Orientador: Jorge Almeida Guimarães.

Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil

2005-2006 Mestrado em Biologia Celular e Molecular.

Universidade Federal do Rio Grande do Sul, UFRGS, Rio Grande do Sul, Brasil.

Título: ANÁLISE CONFORMACIONAL DA MELITINA POR DINÂMICA MOLECULAR E CARACTERIZAÇÃO DOS EFEITOS DO PEPTÍDEO NA FUNÇÃO PLAQUETÁRIA

Orientador: Jorge Almeida Guimarães.

Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.

2000 - 2004 Graduação em Farmácia / Farmácia Industrial.

Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Rio Grande do Sul, Brasil.

Título: UTILIZAÇÃO DA TÉCNICA DE ESPECTROSCOPIA DE INFRAVERMELHO (FTIR-ATR) NO ESTUDO DE MECANISMOS DE AÇÃO DE FÁRMACOS ANTIMICROBIANOS.

Orientador: Mercedes Passos Geimba.

Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPQ, Brasil.

PRÊMIOS E DISTINÇÕES

2008 Outstanding student / Post-doc Poster Award, The Association of Biomolecular Research Facilities (ABRF)

ATIVIDADES CIENTÍFICAS

Publicações em Periódicos

1 Pinto, Antônio F.M. ; Berger, Markus ; Reck Jr., José ; Terra, Renata M.S. ; Guimarães, Jorge A. . Lonomia obliqua venom: In vivo effects and molecular aspects associated with the hemorrhagic syndrome. Toxicon (Oxford), 2010 *in press*.

2 Berger, Markus ; Reck, José ; Terra, Renata M. S. ; Beys da Silva, Walter O. ; Santi, Lucélia ; Pinto, Antônio F. M. ; Vainstein, Marilene H. ; Termignoni, Carlos ; Guimarães, Jorge A. . Lonomia obliqua venomous secretion induces human platelet adhesion and aggregation. Journal of Thrombosis and Thrombolysis, 2010 *in press*.

3 Berger, Markus ; Reck Jr., José ; Terra, Renata M.S. ; Pinto, Antônio F.M. ; Termignoni, Carlos ; Guimarães, Jorge A. . Lonomia obliqua caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats. Toxicon (Oxford), v. 55, p. 33-44, 2010.

4 Terra, Renata M.S. ; Pinto, Antônio F.M. ; Guimarães, Jorge A. ; Fox, Jay W. . Proteomic profiling of snake venom metalloproteinases (SVMPs): Insights into venom induced pathology. Toxicon (Oxford), v. 54, p. 836-844, 2009.

5 Reck Jr, José ; Berger, Markus ; Terra, Renata M. S. ; Marks, Fernanda ; Da Silva Vaz Jr, Itabajara ; Guimaraes, Jorge A. ; Termignoni, C . Systemic alterations of bovine hemostasis due to Rhipicephalus (Boophilus) microplus infestation. Research in Veterinary Science, v. 86, p. 56-62, 2009.

6 Escalante, Teresa ; Rucavado, Alexandra ; Pinto, Antonio F. M. ; Terra, Renata M. S. ; Gutierrez, Jose M. ; Fox, Jay W. Wound Exudate as a Proteomic Window to Reveal Different Mechanisms of Tissue Damage by Snake Venom Toxins. Journal of Proteome Research, v. 8, p. 5120-5131, 2009.

7 TERRA, Renata M. S.; GUIMARÃES, J. A.; VERLI, H. Structural and functional behavior of biologically active monomeric melittin. Journal of molecular graphics & modelling, V.25, p. 767-772, 2007.

8 PINTO, Antônio F. M.;TERRA, Renata M. S.; GUIMARÃES, Jorge A.; FOX, Jay W. Mapping von Willebrand factor A domain binding sites on a snake venom metalloproteinase cystein-rich domain. Archives of biochemistry and biophysics, V. 457, p. 41-46, 2007.

9 PINTO, Antônio F. M.; TERRA, Renata M. S.; GUIMARÃES, Jorge A.; KASHIWAGI, Masahide; NAGASE, Hideaki; SERRANO, Solange M. T.; FOX, Jay W. Structural features of the reprolysin atrolysin C and tissue inhibitors of metalloproteinases (TIMPs) interaction. Biochemical and Biophysical Research Communications, v. 347, p. 641-648, 2006.

10 BIZZANI, Delmar; MOTTA, Amanda S; MORRISSY, Juliana A. C.; TERRA, Renata M. S.; SOUTO, André A.; BRANDELLI, Adriano. Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by Bacillus cereus. International microbiology, v. 8, p. 125-131, 2005.

Trabalhos apresentados em Congressos e Simpósios

1 LI, L. ; TERRA, R. ; DRAGULEV, B. ; PINTO, A. F. M. ; ZIGRINO, P. ; MAUCH, C. ; FOX, J. W. . Genomic and Proteomic Evidence for Melanoma Microenvironment Remodeling and its Role in Metastasis . In: Keystone Symposia on Molecular and Cellular Biology - Omics Meets Cell Biology, 2009, Breckenridge, Colorado. Omics Meets Cell Biology, 2009.

2 TERRA, R. M. S. ; PINTO, A. F. M. ; ESCALANTE, T ; Gutierrez, J. M. ; GUIMARÃES, J. A. ; FOX, J. W. . Local Snakebite Pathophysiology Induced By Jararhagin Injection: A Tissue Proteomics Approach. In: Hupo 8th World Congress, 2009, Toronto. Clinical Proteomics, 2009. v. 5. p. 240-240.

3 TERRA, R. M. S. ; PINTO, A. F. M. ; ESCALANTE, T ; GUIMARÃES, J. A. ; Gutierrez, J. M. ; FOX, J. W. . Proteomics of snake envenomed hemorrhagic tissue using laser capture microdissection. In: ABRF (The Association of Biomolecular Resource Facilities) meeting, 2008, Salt Lake City. ABRF, 2008.

4 RECK JR., J. ; BERGER, M. ; TERRA, R. M. S. ; MARKS, F. ; DA SILVA VAZ JR., I. ; GUIMARÃES, J. A. ; TERMIGNONI, C. . Hemostatic disorders associated with Rhipicephalus (Boophilus) microplus infestation. In: VI International Conference on Ticks and Tick-borne Pathogens (TTP-6), 2008, Buenos Aires. VI International Conference on Ticks and Tick-borne Pathogens (TTP-6), 2008.

5 RECK JR., J. ; BERGER, M. ; TERRA, R. M. S. ; MARKS, F. ; DA SILVA VAZ JR., I. ; GUIMARÃES, J. A. ; TERMIGNONI, C. . Effects of the tick Rhipicephalus (Boophilus) microplus infestation on the host hemostatic system. In: 10th IUBMB Conference and XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2007, Salvador. XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2007.

6 BERGER, M. ; RECK JR., J. ; TERRA, R. M. S. ; PINTO, A. F. M. ; TERMIGNONI, C. ; GUIMARÃES, J. A. . Effects of Lonomia obliqua catterpillar venom on blood platelets. In: 10th IUBMB Conference and XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2007, Salvador. XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2007.

7 TERRA, R. M. S. ; PINTO, A. F. M. ; BERGER, M. ; DE OLIVEIRA, S. K. ; Juliano, M. A. ; Juliano, L. ; GUIMARÃES, J. A. . Melittin-induced platelet signaling and aggregation. In: 10th IUBMB Conference and XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2007, Salvador. XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2007.

8 TERRA, R. M. S. ; DE OLIVEIRA, S. K. ; GUIMARÃES, J. A. . Evaluation of

melittin effect over hemostasis. In: Reunião Anual da Sociedade Brasileira de Bioquímica, 2006, Águas de Lindóia, 2006.

9 PINTO, A. F. M. ; TERRA, R. M. S. ; NAGASE, H. ; GUIMARÃES, J. A. ; FOX, J. W. . Insight into the molecular interaction of snake venom metalloproteinase, atrolysin C, and tissue inhibitors of metalloproteinases. In: Reunião Anual da Sociedade Brasileira de Bioquímica, 2006, Águas de Lindóia, 2006.

10 RECK JR., J. ; BERGER, M. ; TERRA, R. M. S. ; MARKS, F. ; DA SILVA VAZ JR., I. ; GUIMARÃES, J. A. ; TERMIGNONI, C. . Avaliação de parâmetros hemostáticos de bovinos infestados pelo carrapato Rhipicephalus (Boophilus) microplus. In: Congresso Brasileiro de Parasitologia Veterinária, 2006, Ribeirão Preto. 2006.

11 TERRA, R. M. S. ; VERLI, H. ; GUIMARÃES, J. A. . Evaluation of Melittin Structure, Conformation and Oligomerization in Solution Using Molecular Dynamics Simulation. In: Reunião Anual da Sociedade Brasileira de Boquímica, 2005, Águas de Lindóia, 2005.

12 JAGER, A. ; TERRA, R. M. S. ; SOUTO, A. A. . Altas concentrações de trans-resveratrol em sucos de uva ecológicos. In: 26ª Reunião Anual da Sociedade Brasileira de Química, 2003, Poços de Caldas, 2003.

13 TERRA, R. M. S. ; CAZANOVA, R. F. ; JAGER, A. ; SOUTO, A. A. . Aplicação da Espectroscopia de Infravermelho (FT-IR ATR) na Investigação do Mecanismo de Ação da Cefalexina em E. coli. In: XV Salão de Inicição Científica - UFRGS, 2003, Porto Alegre, 2003.

14 TERRA, R. M. S. ; CAZANOVA, R. F. ; BORTOLOTTO, J. W. ; RAMOS, G. P. ; JAGER, A. ; SOUTO, A. A. . Espectroscopia de Infravermelho FT-IR ATR na avalição do mecanismo de ação do trans-Resveratrol em E. coli. In: IV Salão de Iniciação Científica - PUCRS, 2003, Porto Alegre, 2003.

15 JAGER, A. ; TERRA, R. M. S. ; SOUTO, A. A. . Aumento do tempo de conservação de frutas pela aplicação externa de trans-Resveratrol. In: IV Salão de Iniciação Científica - PUCRS, 2003, Porto Alegre, 2003.

16 BORTOLOTTO, J. W. ; TERRA, R. M. S. ; RAMOS, G. P. ; SOUTO, A. A. . Determinação de Tiocianato na Saliva Humana por Espectroscopia de FTIR-ATR. In: XV Salão de Iniciação Científica - UFRGS, 2003, Porto Alegre. XV Salão de Iniciação Científica, 2003.

7. ANEXO II

OUTROS TRABALHOS PUBLICADOS NO PERÍODO E RELEVANTES AO ASSUNTO DA TESE

Toxicon 56 (2010) 1103–1112

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Review

Lonomia obliqua venom: *In vivo* effects and molecular aspects associated with the hemorrhagic syndrome

Antônio F.M. Pinto, Markus Berger, José Reck Jr., Renata M.S. Terra, Jorge A. Guimarães*

Laboratório de Bioquímica Farmacológica, Centro de Biotecnologia (UFRGS), Av. Bento Gonçalves, 9500, CEP 91501-970, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 8 November 2009
Received in revised form 6 January 2010
Accepted 19 January 2010
Available online 28 January 2010

Keywords:
Lonomia obliqua
Caterpillar
Envenomation
Venom
Experimental model
Hemorrhagic syndrome

ABSTRACT

Caterpillar envenomation has been an emergent health issue. *Lonomia obliqua* is a medically important animal that causes a hemorrhagic syndrome that can progress to acute renal failure, intracranial hemorrhage and death. In the past few years the molecular characterization of *L. obliqua* venom in addition to experimental models has provided fundamental information to the understanding of the envenomation syndrome. Herein studies from several authors which characterized the complex toxic-pharmacological actions of whole venom are reviewed.

1. Introduction

Accidental contact with some lepidopteran caterpillars can inflict serious human injuries ranging from simple skin irritation to serious burns, allergic reactions, renal failure and hemorrhagic disturbances (Diaz, 2005). Like other venomous animals, such as snakes and spiders, these caterpillars produce a variety of toxic components that affect the vascular system, blood coagulation, fibrinolysis and platelet function (Arocha-Piñango et al., 2000). However, different from snakes and spiders, that use their venoms to immobilize and digest the prey, the caterpillar venomous components are useful for defense against predators.

From the medically important Saturniidae family, *Lonomia* genus has been attributed to cause human envenomations since late 1960s in Venezuela (Arocha-Piñango and Larysse, 1969). In Southern Brazil, *Lonomia obliqua* (Fig. 1a) caterpillar is becoming the most important venomous animal responsible for severe injuries, hemorrhagic disorders and often fatal outcome since the 1980s (Duarte et al., 1990). For instance, in the State of Rio Grande do Sul, located in this Brazilian region, more than a thousand accidents have been registered in the 1997 to 2005 period (Abella et al., 2006). In fact, based in the data for the year 2008, the Brazilian Ministry of Health registered an incidence of 8 lepidopteran envenomations per 100,000 inhabitants in Southern Brazil (SVS, 2009). Actually, this numbers are greatly underestimated due to the fact that most accidents are occurring in distant rural areas, where the cases are poorly reported. The emergent importance of *L. obliqua* accidents seems to be consequence to the extensive deforestation of rural areas and replacement of the native forest by fruit tree plantation, a rich source of food for this lepidopteran. The *L. obliqua* has gregarious habits, and this characteristic complicate the patient prognosis, since the accidents usually involve the contact of the victim with a caterpillar colony containing dozens or hundreds of caterpillars lying on the surface of tree trunks.

L. obliqua is venomous only in the larval stages (1st to 6th instars), when the body of the insect is covered by

* Corresponding author. Tel.: +55 51 33086068; fax +55 51 33087309.
E-mail address: guimar@cbiot.ufrgs.br (J.A. Guimarães).

0041-0101/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2010.01.013

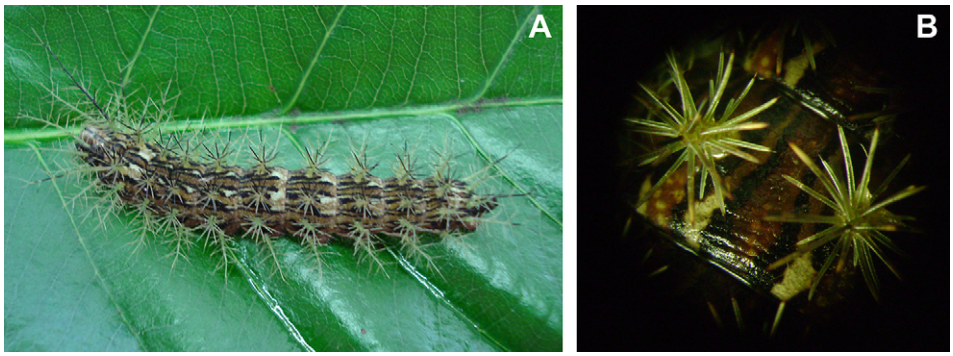


Fig. 1. The *Lonomia obliqua* caterpillar. A. *L. obliqua* (6th instar). B. Detail of *L. obliqua* bristles.

chitinous bristles (Fig. 1b). These structures are hard and spiny evaginations of the cuticle underneath. Contrarily to other venomous animals, there is no specialized venomous gland in *L. obliqua* and the venom is produced by a secretory epithelium localized under the cuticle (Veiga et al., 2001). The bristles have a hollow canal by which the venom is injected in the victim. The accident occurs when the whole animal is crushed by the victim; the insect's chitinous bristles are broken and the venomous secretions penetrate the human skin reaching blood circulation (Veiga et al., 2001).

In this review, we focused in studies that provided significant contributions to the comprehension of the biological effects of the whole venom in experimental models. In fact, these reports are responsible for identification and characterization of venom mechanisms capable of reproducing some of the main physiopathological aspects of the clinical envenomation.

2. Clinical aspects of human envenomation

Clinical symptoms of *L. obliqua* envenomation include local pain and inflammatory reaction, which starts immediately after contact; systemic reactions such as headache, fever, vomiting and asthenia, which appear a few hours after exposure; and bleeding diathesis characterized by hematomas and ecchymosis, hematuria, pulmonary and intracerebral hemorrhage and acute renal failure (Burdmann et al., 1996; Kowacs et al., 2006; Garcia and Danni-Oliveira, 2007).

Poisoned patients present a severe prolongation of the coagulation parameters such as prothrombin time (PT) and partial activated thromboplastin time (aPTT). Laboratory findings include a decrease in plasma levels of fibrinogen, factors V and XIII, pre-kallikrein, plasminogen, protein C and α2-antiplasmin, and an increase in the levels of thrombin-antithrombin complex (TAT), fragment $1+2$ from prothrombin activation (F1.2) and D-dimers. No alterations in von Willebrand Factor, factors X and II levels were found (Kelen et al., 1995; Zannin et al., 2003). Taken together, these clinical data indicate that a significant amount of intravascular thrombin is generated and the fibrinolytic system is activated in *L. obliqua* envenomation. The activation of blood coagulation and fibrinolysis causes the consumption of plasmatic factors leading to a consumption coagulopathy characteristic of this type of envenomation.

Despite this intense consumption coagulopathy, the platelet number appears to be diminished only in the most severe cases, been normal in mild ones (Zannin et al., 2003). However, the platelet function of these patients during envenomation has not been evaluated. Considering that platelets participate in several steps of the hemostatic process, including the amplification and propagation phases of the blood coagulation (Monroe and Hoffman, 2006; Hoffman and Monroe, 2001), these elements probably make a decisive contribution to the appearance of hemorrhagic syndrome during envenomation. Analogously, patients envenomed by *Bothrops jararaca* snakes presented a significant impairment of the platelet function that has been associated with the bleeding disorders observed after envenomation (Sano-Martins et al., 1997).

Besides the bleeding disorders, *Lonomia*-envenomed patients also develop renal problems. Hematuria is observed in most patients and may evolve to acute renal failure (Burdmann et al., 1996). A few histological reports of renal tissue of envenomed patients are consistent with tubular necrosis (Burdmann et al., 1996; Fan et al., 1998), but the pathogenesis of acute renal failure in *L. obliqua* envenomation is poorly understood. The difficulty of conducting early renal biopsies due the coagulation disturbances inherent to the incidents has contributed to this lack of knowledge. Although, the massive deposition of fibrin in the glomeruli capillaries due to intravascular coagulation and/or a direct action of the venom on the renal microcirculation cannot be discharged as causes of renal damage (Gamborgi et al., 2006).

Intracerebral hemorrhage is the main cause of deaths by *L. obliqua* envenomation. In the state of Paraná, also in Southern Brazil, hemorrhage of the central nervous system accounted for the death of 50% of *Lonomia*-envenomed patients registered from 1989 to 2005 (Kowacs et al., 2006). However, the mechanisms that lead to intracerebral hemorrhage in envenomed patients are also poorly understood.

3. Pharmacology and molecular aspects of experimental envenomation

The dramatic effects of *L. obliqua* venom in humans have been partially reproduced in a number of experimental

models. The biodistribution of venom components in animal models was studied by immunochemical and radiolabeling (Rocha-Campos et al., 2001; Da Silva et al., 2004b). Venom components could be detected in high quantities in kidneys, blood and urine as soon as one hour after intraperitoneal injection. Lower amounts of venom could be also detected in lungs, liver, spleen, heart, skeletal muscle and brain. Two hours after injection, most of the venom has been already eliminated by urine or remained in kidney tissue (Rocha-Campos et al., 2001). Furthermore, residual amounts of venom components could be detected up to 6 and 18 h after injection on the liver and kidneys, respectively (Da Silva et al., 2004b).

3.1. Coagulation and platelet aggregation disturbances

Various *in vivo* studies have been carried out to understand the mechanisms of hemostatic disturbances triggered by *L. obliqua* caterpillar venom (Kelen et al., 1995; Rocha-Campos et al., 2001; Prezoto et al., 2002; Berger et al., 2010). The coagulation disorders observed in humans after contact with *Lonomia* caterpillars can be reproduced in experimental animals. Administration of the crude extract of *L. obliqua* bristles to rats, rabbits and mice causes a dose-dependent increase of clotting time (PT, aPTT, thrombin time and whole blood clotting time) and can render the unclottable blood. Bristle extract also reduced plasma levels of fibrinogen and factor XIII and increased fibrin degradation products (Kelen et al., 1995; Prezoto et al., 2002; Berger et al., 2010). Furthermore, when crude bristle extract was administered in rats and the bleeding time assay was continuously monitored, a dose-dependent hemorrhagic effect of the venom was clearly observed (unpublished results). Envenomed rats also presented high levels of thrombin, plasmin and urokinase activities in plasma, indicating that coagulation and fibrinolysis are activated during *L. obliqua* envenomation (Berger et al., 2010).

In addition to the coagulation disorders, we have demonstrated that rats experimentally envenomed with bristle extract also presented an accentuated impairment of the platelet aggregation function. The aggregation response, induced by either ADP or collagen, is decreased in rats' plasma during the time course of envenomation. Maximum decrease in platelet aggregation occurred 6 h after venom injection for both agonists. At this time, even in the presence of thrombin (the most potent platelet aggregating agent), inhibition in aggregation was sustained in platelet rich plasma (PRP). On the other hand, it was found that envenomed animals presented only a slight reduction of approximately 26 % in the platelet count at 6 h, being normalized after 24 h of envenomation (Berger et al., 2010). These results are in accordance with those observed by Zannin et al. (2003), who analyzed a group of 105 patients envenomed with *L. obliqua*, in which only 9% presented thrombocytopenia (platelet counts $< 150{,}000/\mu L$).

The mechanisms involved in platelet aggregation inhibition also have been investigated. In our model of experimental envenomation, the platelet hypoaggregation was not related to the low levels of plasma fibrinogen as well, since fibrinogen replacement in PRP did not reestablished normal function. Interestingly, normoaggregation was observed when platelets were separated from plasma through a washing procedure. In addition, incubation of plasma from envenomed rats inhibits aggregation response of normal washed platelets, which indicates that an aggregation inhibitor is generated in plasma during envenomation. Moreover, experimentally envenomed animals presented an increase in nitric oxide (NO) plasmatic levels which coincided with the maximum inhibitory effect upon platelet aggregation. Indeed, the *in vivo* blockade of NO synthase activity by N^G-nitro-L-arginine-methyl-ester (L-NAME) pretreatment partially revert the platelet hypoaggregation response induced by envenomation, thus suggesting that NO can be one of the mediators that participate in platelet dysfunctions (Berger et al., 2010). The generation of fibrinogen/fibrin degradation products (FfDP) and D-dimers during envenomation can also participate in platelet hypoaggregation, since fragments deriving from fibrin and/or fibrinogen degradation could bind to the GPIIb–IIIa receptors and prevent normal platelet aggregation (Thorsen et al., 1986). In fact, envenomed patients presented high levels of FfDP (Zannin et al., 2003) and the plasmatic activities of plasmin and urokinase were significantly increased in envenomed animals (Berger et al., 2010).

Several toxins of *L. obliqua* venom could be directly or indirectly involved with the hemostatic disturbances observed in humans and experimental animals (Table 1). Indeed, the venom possesses high proteolytic, procoagulant and fibrin(ogen)olytic activities (Veiga et al., 2003; Pinto et al., 2006). As shown in Fig. 2, the active principles present in *L. obliqua* venom can interfere in several key points of the victims' hemostatic system. The enzymes responsible for these activities are mainly prothrombin and factor X activators and a fibrinogenase that have been already isolated and characterized from different venomous secretions of *L. obliqua* caterpillars (Donato et al., 1998; Reis et al., 1999; Pinto et al., 2004; Alvarez-Flores et al., 2006). The prothrombin and factor X activators generate significant amounts of intravascular thrombin, which leads to the activation of coagulation system and to consumption of fibrinogen and other coagulation factors. The fibrin(ogen)olytic enzymes degrade both fibrinogen and fibrin, contributing to the reduction of fibrinogen levels and to blood incoagulability. Furthermore, fibrin(ogen)olytic enzymes can also participate in generation of FfDP, which are probably involved in the platelet aggregation disturbances. Thus, these toxins acts synergistically to produce the hemostatic disturbances observed in humans and animal models.

3.2. Intravascular hemolysis

It has been reported that *L. obliqua* crude venom (bristle extract) causes intravascular hemolysis in rats, with a reduction in the number of circulating erythrocytes, increase in plasma hemoglobin in the first 6 h, with a decrease in total hemoglobin levels 24 h after envenomation, together with a decrease in haptoglobin levels (Seibert et al., 2004). Also, the urine of the animals was dark-red due to the presence of free hemoglobin (no erythrocytes were found), confirming the occurrence of hemoglobinuria

Table 1
Lonomia obliqua toxins which may be involved in envenomation.

Toxin	Gen Bank	Predicted Mw/observed Mw (Da)	Identification methology	Reference
Fibrinogenase	NA	35,000	Isolation	Pinto et al. (2004)
Prothrombin activator	AY908986	69,000	Isolation	Reis et al. (2001)
Factor X activator	NA	45,000	Isolation	Alvarez-Flores et al. (2006)
Serine proteinases	AY829844	Unknown	Transcriptome	Veiga et al. (2005)
	AY829818	55,200		
	AY829819	Unknown		
	AY829820	Unknown		
	AY829821	Unknown		
	AY829842	Unknown		
	AY829843	30,100		
	AY829841	Unknown		
Hyaluronidases	NA	53,000	Isolation	Gouveia et al. (2005)
		49,000		
Phospholipase A2	NA	15,000	Isolation	Seibert et al. (2006)
Phospholipase A2	AY829845	9,600	Transcriptome	Veiga et al. (2005)
Lectins	AY829822	33,700	Transcriptome	Veiga et al. (2005)
	AY829836	33,900		
	AY829849	Unknown		
	AY829846	16,300		
Lipocalins	AY829833	20,600	Transcriptome	Veiga et al. (2005)
	AY829856	13,500		
	AY829809	Unknown		
Serpins	AY829814	50,200	Transcriptome	Veiga et al. (2005)
	AY829815	41,600		
	AY829816	Unknown		
	AY829817	Unknown		
	AY829847	Unknown		
Other protease inhibitors	AY829810	14,600	Transcriptome	Veiga et al. (2005)
	AY829811	41,800		
	AY829812	8600		
	AY829813	7300		
	AY829839	7200		
	AY829835	8000		
	AY829837	4100		

in these animals. Such hemolytic activity presented by caterpillar's venom seems to be higher in animal models than in human. As a matter of fact, intravascular hemolysis has been observed only in cases where the victim gets in contact with a large number of caterpillars (Malaque et al., 2006).

A hemolytic activity has been described in crude bristle extract, which causes direct and indirect hemolysis of human and rat erythrocytes; such action was assigned to a phospholipase A2 (Table 1) present in this venomous preparation. Besides, degradation of cell membrane proteins was also detected, corroborating with the hemolytic effect observed in animal models (Seibert et al., 2006). In addition, hemolysis might be also due to deposits of microthrombi in the vasculature, as observed in thrombotic microangiopathy. In this case, the thrombi in capillaries create abnormally high levels of shear stress that fragments the red blood cells (Tsai, 2006).

3.3. Inflammation and vascular disorders

In addition to hemorrhagic syndrome, the *L. obliqua* envenomation could lead to many local effects at the contact site, such as burning sensation, pain, edema and erythema (Fan et al., 1998; Correa et al., 2004). These findings are probably related to the activation of the inflammation response elicited by the venom injection in the victim's body (Da Silva et al., 2004a; Bohrer et al., 2007).

The local pain due to the contact with *Lonomia* genus caterpillars is a common event. The pharmacological mechanisms of *L. obliqua* induced pain were studied by our group, using the rat paw model of nociception (De Castro Bastos et al., 2004). The nociception, evinced as nocifensive behavior, was more prominent 5–10 min after the venom injection, being however, significantly reduced by the pre-treatment of rats with indomethacin (nonselective inhibitor of cyclooxygenase), indicating that local pain induced by *L. obliqua* envenomation is, at least partially, mediated by prostaglandins (PG) (De Castro Bastos et al., 2004).

Despite the clear participation of PG in the nociception induced by *L. obliqua* envenomation, other mediators, namely bradykinin (BK), could contribute to the overall nociceptive/painful effect, since BK, one of the most powerful nociceptive agents known to date (Wang et al., 2006), is produced in *L. obliqua* envenomation (Bohrer et al., 2007). We have demonstrated that *L. obliqua* venom induces the activation of the kallikrein-kinin system (KKS) *via* plasma pre-kallikrein activation. It was also demonstrated, using guinea pig ileum bioassay, that the venom was able to generate BK directly from low molecular weight kininogen (LMWK) (Bohrer et al., 2007).

In this sense, the KKS seems to be the major responsible for edema formation induced by the contact with *L. obliqua* caterpillars (Bohrer et al., 2007). Using mice paw edema

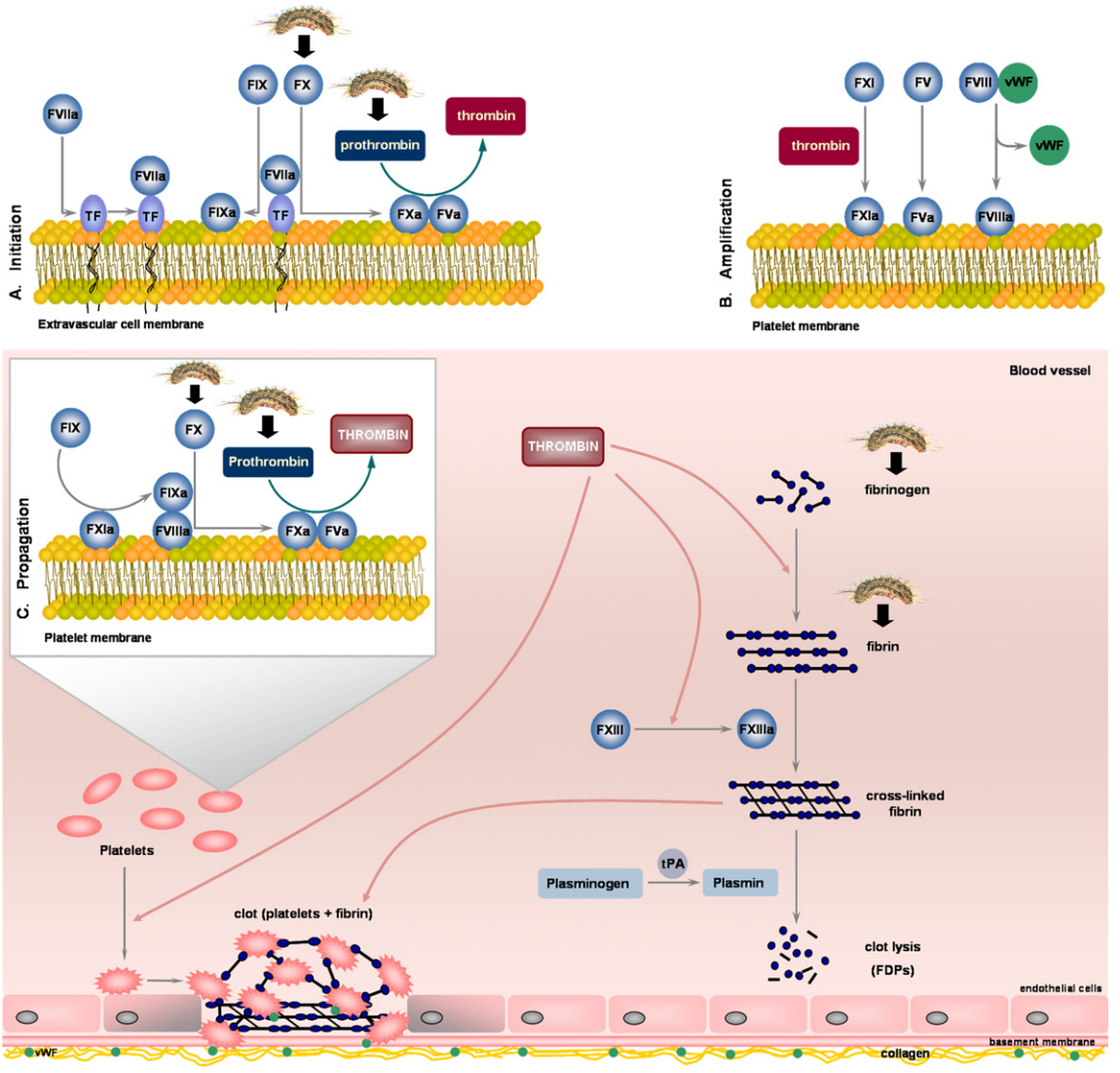


Fig. 2. Diagrammatic representation of the cell-based model of hemostasis. The coagulation reaction occurs in three different phases: initiation, amplification and propagation (for a review see Monroe and Hoffman, 2006). The sites of action of *L. obliqua* toxins are indicated by the presence of caterpillars. TF, tissue factor; vWF, Von Willebrand factor; tPA, tissue plasminogen activator.

bioassay, we have characterized the edematogenic activity of *L. obliqua* venom. The paw edema remains prominent up to 4–6 h post-envenomation (De Castro Bastos et al., 2004; Bohrer et al., 2007). Mice pre-treatment with HOE-140 (BK B_2 receptor antagonist) or aprotinin (kallikrein inhibitor) leads to 50–60% inhibition of the edematogenic response while loratadine (histamine H_1 receptor antagonist) pre-treatment slightly suppressed the edematogenic activity of the venom (De Castro Bastos et al., 2004; Bohrer et al., 2007). On the other hand, pre-treatment of mice with guanethidine (noradrenergic blocker) or indomethacin has no effect in *L. obliqua* induced edema (De Castro Bastos et al., 2004).

In spite of the local inflammatory effect, *L. obliqua* venom seems to be mainly dependent of the humoral factors (BK, PG, histamine); it also could be related with cellular responses. The migration and infiltration of inflammatory cells into the site of venom inoculation occurs mainly in a late phase (24 h post-envenomation), and this exudate is composed namely by neutrophils and monocytes (Ramos et al., 2004).

The pro-inflammatory properties of the venom are responsible not only for local effects, but also for systemic disturbances due to its ability to modulate the properties of the vascular system (Da Silva et al., 2004a; Bohrer et al., 2007). It is important to note that in some envenomed patients, mainly in severe cases, systemic vascular and inflammatory disorders were observed, such as the occurrence of drop in blood pressure followed by blood extravasation into the brain (Fan et al., 1998; Kowacs et al., 2006).

Experimental models showed that *L. obliqua* venom induces systemic alterations in the vascular tonus and in

vessel wall structure (Da Silva et al., 2004a; Bohrer et al., 2007). The occurrence of intra-cranial hemorrhages and neurological disturbances in some patients could be related with the disruption of the blood brain barrier (BBB). Experimental envenomation of rats leads to the increase of the BBB permeability, evinced by the observation of lanthanum ions deposition in tissues as seen by electron microscopy (Da Silva et al., 2004a). This increase in BBB permeability seems to be related with an alteration in microvessels diameter, probably *via* action of pro-inflammaory molecules, such as BK (Da Silva et al., 2004a). The main alterations in the central nervous system (CNS) are the presence of vacuoles (namely in hippocampus and cerebellum) and vasogenic edema, which could lead to blood leakage and hemorrhage into the CNS (Da Silva et al., 2004a).

In addition, we have shown that *L. obliqua* venom also induces a systemic transient drop in mean arterial blood pressure in experimental envenomation (Bohrer et al., 2007). This hypotensive effect is totally dependent of the KKS activation by venom, since the pre-treatment of the rats with HOE-140 or aprotinin completely abolishes the vasodilatatory effect (Bohrer et al., 2007).

All the inflammatory and vascular actions of the *L. obliqua* venom discussed above could not be related with only one specific toxin, and also seem to be independent of the activity of the procoagulant fraction of the venom (De Castro Bastos et al., 2004; Da Silva et al., 2004a; Ramos et al., 2004; Bohrer et al., 2007). This fact supports the hypothesis that the venom overall effect and the envenomation syndrome are the result of the synergistic action of a cocktail of compounds.

3.4. *Gene expression alterations induced by* Lonomia obliqua *envenomation*

In addition to venom direct actions, *L. obliqua* bristle extract produces indirect effects by triggering host cellular responses. We have demonstrated gene expression changes in envenomed fibroblasts through the microarray technology (Pinto et al., 2008). *L. obliqua* venom produces a cellular response through up-regulation of several genes that could be involved in the generation and/or amplification of some known clinical manifestations.

One of these effects is involved with activation and migration of inflammatory cells. Bristle extract induces up-regulation of pro-inflammatory mediators such as IL-8, IL-6, CCL2 and CXCL1. These mediators may be released by several cell types after inflammatory stimuli and are chemoattractants of monocytes, neutrophiles, basophiles and T-cells, thus modulating the inflammatory response. Additionally, up-regulation of the adhesive protein ICAM-3 stimulates the inflammatory cell infiltration in injured tissues. Moreover, prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2, a pro-inflammatory enzyme that triggers prostanoid biosinthesis, is also up-regulated. Together, these mediators probably play a key role in several inflammatory processes which occurs in the envenomation syndrome (Pinto et al., 2008).

Also, cellular response to envenomation could be partially involved in other manifestations such as edema, pain sensation and hypotension (De Castro Bastos et al., 2004; Bohrer et al., 2007), through up-regulation of dimethylarginine dimethylaminohydrolase 1 (DDAR), an enzyme involved in the biosynthesis of NO. In fact, we have demonstrated that NO level increases in experimental *in vivo* envenomation and participates in platelet aggregation disturbances (Berger et al., 2010).

Differently from *Lonomia achelous* (Arocha-Piñango et al., 2000), to date there is no report of a plasminogen activating activity on *L. obliqua* venom. However, our gene expression study revealed up-regulation of urokinase plasminogen activator receptor (uPAR). This data supports the hypothesis that indirect fibrinolytic system activation may occur *via* action of cell plasminogen activators (Pinto et al., 2008). This activation of the fibrinolytic system may generate a site of intense proteolytic activity which may be responsible not only for fibrin degradation but also can affect extracellular matrix proteins thus activating matrix metalloprotases, which facilitates cell migration and tissue remodeling. Moreover, it could be also capable of inactivating coagulation factors V and VIII (Vaughan and Declerck, 1998).

In addition, microarray results have shown an overexpression of tissue factor (TF) in envenomed cells. Indeed, we have demonstrated the cell culture supernatant of envenomed cells has a procoagulant activity that is not neutralized by anti-lonomic serum (ALS) suggesting an indirect venom effect in which cellular response would be responsible for the amplification of procoagulant stimulus (Pinto et al., 2008). Taken together, it is reasonable to propose that *L. obliqua* venom induces liberation of either procoagulant microparticles containing TF or a soluble form of TF (Bogdanov et al., 2003).

3.5. Lonomia obliqua *transcriptome*

According to a transcriptomic study performed by our group, it is estimated that there are at least four important protein families (Table 1) that could be related to the envenomation symptomatology, especially regarding hemostatic disturbances (Veiga et al., 2005). Serine proteinases are the most relevant protein family when considering their potential of interfering with blood coagulation. Moreover, serine proteases are an expressive group, representing 16.7 and 25% of the clusters derived from tegument and bristle transcriptome, respectively (Veiga et al., 2005). This protein group presents coagulation factors-like activities, so it is expected that these enzymes participate in the generation of thrombin, by activation of FX and prothrombin (Donato et al., 1998), and in the activation of the fibrinolytic system, contributing directly and indirectly to fibrinogen degradation (Pinto et al., 2004) and resulting in the hemorrhagic disorder. In fact, a fibrinogenolytic enzyme has been purified and characterized by our group (Pinto et al., 2004).

Phospholipases and lectins are other known groups of proteins that disturb hemostasis and can effectively affect blood coagulation and platelet aggregation. These proteins are important components of many other animals' venoms – such as snakes, bees and scorpions – and have been well characterized as important players in the modulation of the

hemostatic system. Phospholipases are enzymes that can directly modulate platelet aggregation and, also, they may destabilize coagulation complexes by degradation of phospholipids (Kerns et al., 1999; Kini, 2003). In addition, these enzymes are able to disrupt cell membranes and thus the hemolytic activity of *L. obliqua* bristle extract has been attributed to a phospholipase activity (Seibert et al., 2004). Lectins, particularly c-type lectins, are a relatively well-studied group of proteins in snake venoms that may exert an additional function in hemostasis modulation by interacting with coagulations factors and/or platelet receptors. Three lectin clusters were found in the bristle cDNA library, being LOqua-lect5 a protein with homology to many snake venom lectins being then another important candidate contributing to the hemorrhagic disorder.

Besides the enzymatic activities present in the venomous secretions, hemostasis can also be impaired by the action of proteinase inhibitors. Among this class of proteins, it is of special interest the serine proteases inhibitors (serpins). Serpins are known to have an important role in the physiology of insects but, more than that, they can function as key effectors in hemostasis, by inhibition of coagulation factors. They seem to be also an important component of the venom representing 27.8 and 10% of the clusters in tegument and bristle, respectively (Veiga et al., 2005).

The specific properties, functional characteristic and mechanisms of action of the great majority of the enzymes or protein classes that constitute *L. obliqua* venom is still poorly known and, more than that, the manner by which these proteins interact with each other and the way they affect the victim's physiology is unclear. The trancriptomic analysis has provided information to new and testable hypothesis (Veiga et al., 2005).

4. Treatment of *Lonomia obliqua* hemorrhagic syndrome

Anti-lonomic serum (ALS) has been produced by Instituto Butantan through successive intramuscular injections of bristle extract in horses. Initially, ALS consisted of whole IgG proteins purified from serum and sterilized by filtration (Dias da Silva et al., 1996). More recently, ALS preparation was refined by large scale production of horse serum rich in $F(ab')_2$ immunoglobulin fragments able to neutralize *L. obliqua* venom components (Rocha-Campos et al., 2001). One milliliter of ALS is capable of neutralizing the toxic effects present in 3 ID_{50} (incoagulation-inducing dose 50%, meaning the minimal dose of *L. obliqua* bristle extract that delays the blood clotting time in at least 15 min on 50% of mice tested) (Rocha-Campos et al., 2001).

From a therapeutic standpoint, ALS has been successfully used to re-establish the physiological coagulation parameters in poisoned patients (Ricci-Silva et al., 2008). A randomized, prospective controlled trial conducted in Southern Brazil between 2000 and 2002 with 44 patients in two distinct treatment groups showed that ALS was effective in reversing the clinical findings of *L. obliqua* envenomation syndrome with no significant adverse reactions. This study also demonstrated that approximately three ALS vials of 10 mL were effective for the treatment of typical envenomation cases (Caovilla and Barros, 2004).

Recently, through a proteomic approach, the ability of ALS to recognize specific proteins from the crude venom was used to evaluate the venom immunogenicity (Ricci-Silva et al., 2008). In some specific experimental conditions the bidimensional map (by 2D electrophoresis) of *L. obliqua* bristle extract exhibit 157 silver-stained spots, being about of 97% of the spots immunochemically revealed by immunoblotting analysis with ALS (Ricci-Silva et al., 2008). Although no significant novel relevant information about venom biochemical composition was obtained, since approximately only 20 spots were further evaluated for protein identification (Ricci-Silva et al., 2008), the immunoproteomic study support the hypothesis that the *L. obliqua* venom was a complex mixture of several largely immunogenic compounds with no specific(s) toxin(s) as major candidate(s) for ALS targeting. In fact, the low amount of *L. obliqua* venom used to immunize horses compared to other schemes of venom production and the high antibody titer obtained also suggests that the caterpillar's venom components are strong immunogens (Rocha-Campos et al., 2001).

Despite the clinical efficacy of the *L. obliqua* ALS, the promptly availability of this anti-venom in the regions of high incidence of accidents still remains a public health concern, namely in rural areas of Southern Brazil. In this sense, the use of synthetic drugs to treat the clinical syndrome induced by venomous animals always was a promising alternative to heterologous serotherapy, since these drugs, in general, seem to have more stability, a lower cost of production, and none of the collateral effects of heterologous therapy (Gutiérrez et al., 2007).

Before the development of ALS in Brazil, the use of antifibrinolytic drugs, such as ϵ-aminocaproic acid (EACA), was officially recommended as therapy against *L. obliqua* envenomation by the Brazilian Ministry of Health based on the clinical experience with *L. achelous* in Venezuela (FUNASA, 2001). Although the use of EACA has been a relative success in the cessation of clinical evidence of bleeding in envenomation by *L. achelous* (Arocha-Piñango et al., 1992), recent data suggest that its use to treat the *L. obliqua* envenomation should be dismissed (Gonçalves et al., 2007).

In experimental models of *L. obliqua* envenomation, the animals treated with EACA after 1 or 6 h post-envenomation, displayed neither fibrinogen level recovery nor normalization of coagulation parameters, similarly to animals treated only with saline. Contrarily, the hemostatic parameters of the rats treated with ALS return to the normal values. Moreover, a high death rate was observed in the group which received an early EACA treatment (15 min. post-envenomation) (Gonçalves et al., 2007).

Considering the severity of *L. obliqua* hemorrhagic syndrome, it is demanding urgent the search for new natural or synthetic venom inhibitors able to replace or, at least, be used as a complementary option to serotherapy. Moreover, this may allow significant advances in the field and thus, more deep research in the subject is needed. In fact, several components obtained predominantly from natural sources have been used to counteract the toxic effects of different venomous animals (Maiorano et al.,

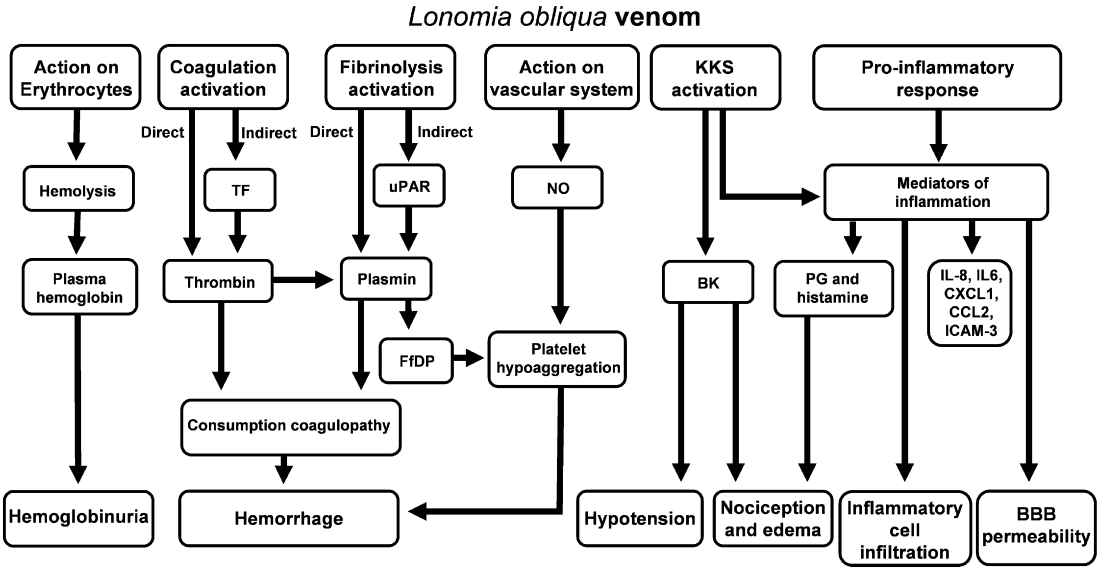


Fig. 3. Biological effects of *Lonomia obliqua* venom. A summary of the biological effects of *L. obliqua* venom discussed in this review are presented. KKS, kallikrein–kinin system; TF, tissue factor; uPAR, urokinase plasminogen activator receptor; NO, nitric oxide; BK, bradykinin; FfDP, fibrinogen/fibrin degradation product; PG, prostaglandin; BBB, blood brain barrier.

2005). For *L. obliqua* venom, we recently demonstrated that marine algae extracts of *Canistrocarpus cervicornis*, *Stypopodium zonale* and *Dictyota pfaffi* were able to antagonize the *in vitro* procoagulant effect of the venom. In addition, the extract of *D. pfaffi* was also able to inhibit the hemolytic activity (Domingos et al., 2009). Thus, marine algae may be used as antivenoms or may contribute to the development of compounds with antilonomic effects.

5. Final remarks

In spite of the emerging and crescent importance of *L. obliqua* envenomations, the biochemical mechanisms of action and the pathological basis behind the hemorrhagic syndrome still remains not fully understood. Fig. 3 summarizes all data known to date, concerning the interaction of the venom components with the hemostatic and inflammatory processes as well as with the vascular system. *L. obliqua* venom is able to directly and indirectly activate coagulation and fibrinolysis leading to a consumption coagulopathy. These events, in addition to platelet hypoaggregation phenomenon triggers the hemorrhagic syndrome observed in envenomed patients and experimental models. Besides, venom has a direct action upon erythrocytes, causing hemolysis and hemoglobinuria. Likewise, induction of inflammation and disturbances in the vascular system induces hypotension, pain, edema, cellular infiltration and increases BBB permeability. Altogether, this physiopathological manifestations account for the severe clinical profile observed in *L. obliqua* envenomation.

Taken together, the results discussed in this review support the concept that at least part of the physiopathological alterations in envenomation are due to indirect effects triggered by venom interaction with victims' proteins, cells, tissues and organs. Regardless of all information accumulated to date, important biochemical and pharmacological properties of the venom, including its composition and the mechanism of the envenomation itself, they all remain open research avenues to be explored. Prospectively it could be considered the need for a comprehensive proteomic characterization of *L. obliqua* venomous secretions; identification of the physiopathological mechanisms behind the acute renal failure and a complete pathological characterization of venom toxic effects in specific organs.

Acknowledgements

The authors are grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for funding and fellowships. Special thanks to Dr. Walter Beys (Centro de Biotecnologia -UFRGS) for his valuable suggestions and to Dr. Célia Carlini (Dep. de Biofísica – UFRGS) for her encouragement.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

Abella, H.B., Marques, M.G.B., Silva, K.R.L.M., Rossoni, M.G., Torres, J.B., 2006. Acidentes com lagartas do gênero Lonomia registrados no Centro de Informação Toxicológica do Rio Grande do Sul no período de 1997 a 2005. In: Nicolella, A. (Ed.), Toxicovogilância – toxicologia clínica: dados e indicadores selecionados. Secretaria da Saúde Fundação Estadual de Produção e Pesquisa em Saúde. Centro de Informação Toxicológica, Porto Alegre, pp. 29–34.

Alvarez-Flores, M.P., Fritzen, M., Reis, C.V., Chudzinski-Tavassi, A.M., 2006. Losac, a factor X activator from *Lonomia obliqua* bristle extract: its role in the pathophysiological mechanisms and cell survival. Biochem. Biophys. Res. Commun 343, 1216–1223.

Arocha-Piñango, C.L., de Bosch, N.B., Torres, A., Goldstein, C., Nouel, A., Argüello, A., Carvajal, Z., Guerrero, B., Ojeda, A., Rodriguez, A., et al., 1992. Six new cases of a caterpillar-induced bleeding syndrome. Thromb. Haemost. 67, 402–407.

Arocha-Piñango, C.L., Marval, E., Guerrero, B., 2000. Lonomia genus caterpillar toxins: biochemical aspects. Biochimie 82, 937–942.

Arocha-Piñango, C.L., Larysse, M., 1969. Fibrinolysis produced by contact with a caterpillar. Lancet 1, 810–812.

Berger, M., Reck Jr., J., Terra, R.M.S., Pinto, A.F.M., Termignoni, C., Guimarães, J.A., 2010. *Lonomia obliqua* caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats. Toxicon 55, 33–44.

Bogdanov, V.Y., Balasubramanian, V., Hathcock, J., Vele, O., Lieb, M., Nemerson, Y., 2003. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. Nat. Med. 9 (4), 458–462.

Bohrer, C.B., Reck Jr., J., Fernandes, D., Sordi, R., Guimarães, J.A., Assreuy, J., Termignoni, C., 2007. Kallikrein-kinin system activation by *Lonomia obliqua* caterpillar bristles: involvement in edema and hypotension responses to envenomation. Toxicon 49, 663–669.

Burdmann, E.A., Antunes, I., Saldanha, L.B., Abdulkader, R.C.M.R., 1996. Severe acute renal failure induced by the venom of *Lonomia* caterpillars. Clin. Nephrol. 46, 337–339.

Caovilla, J.J., Barros, E.J., 2004. Efficacy of two different doses of antilonomic serum in the resolution of hemorrhagic syndrome resulting from envenoming by *Lonomia obliqua* caterpillars: a randomized controlled trial. Toxicon 43, 811–818.

Correa, M.S., Batista, R.S., Gomes, A.P., Barbosa, A.F., Verzola, A.C., Oliveira, F., Squeff, F.A., Leal-Filho, J.M., Tavares, R.H., Amorim, D.S., Moreira, N.L., Santos, S.S., 2004. Erucismo por *Lonomia* spp em Teresópolis, RJ, Brasil: relato de um caso provável e revisão da literatura. Rev. Soc. Bras. Med. Trop 37, 418–421.

Da Silva, G.H., Hyslop, S., Cruz-Höfling, M.A., 2004a. *Lonomia obliqua* caterpillar venom increases permeability of the blood-brain barrier in rats. Toxicon 44, 625–634.

Da Silva, G.H., Panunto, P.C., Hyslop, S., Da Cruz-Höfling, M.A., 2004b. Immunochemical detection of *Lonomia obliqua* caterpillar venom in rats. Microsc. Res. Tech 65, 276–281.

De Castro Bastos, L., Veiga, A.B., Guimarães, J.A., Tonussi, C.R., 2004. Nociceptive and edematogenic responses elicited by a crude bristle extract of *Lonomia obliqua* caterpillars. Toxicon 43, 273–278.

Dias da Silva, W., Campos, C.M., Gonçalves, L.R., Sousa-e-Silva, M.C., Higashi, H. G., Yamagushi, I.K., Kelen, E.M., 1996. Development of an antivenom against toxins of *Lonomia obliqua* caterpillars. Toxicon 34, 1045–1049.

Diaz, J.H., 2005. The evolving global epidemiology, syndromic classification, management, and prevention of caterpillar envenoming. Am. J. Trop. Med. Hyg. 72, 347–357.

Domingos, T.F.S., Carvalho, C., Moura, L.A., Teixeira, V.L., Pereira, R.C., Bianco, E.M., Ferreira, W.J., Ramos, C.J.B., Miranda, A.L.P., Melo, P.A., Guimarães, J.A., Fuly, A.L., 2009. Antilonomic effects of Brazilian brown seaweed extracts. Nat. Prod. Commun. 4, 1075–1078.

Donato, J.L., Moreno, R.A., Hyslop, S., Duarte, A., Antunes, E., Le Bonniec, B. F., Rendu, F., de Nucci, G., 1998. *Lonomia obliqua* caterpillar spicules trigger human blood coagulation via activation of factor X and prothrombin. Thromb. Haemost. 79, 539–542.

Duarte, A.C., Caovilla, J., Lorini, D., Mantovani, G., Sumida, J., Manfre, P.C., Silveira, R.C., de Moura, S.P., 1990. Insuficiência renal aguda por acidentes com lagartas. J. Bras. Nefrol. 12, 184–186.

Fan, H.W., Cardoso, J.L.C., Olmos, R.D., Almeida, F.J., Viana, R.P., Martinez, A.P.P., 1998. Hemorrhagic syndrome and acute renal failure in a pregnant woman after contact with *Lonomia* caterpillars: a case report. Rev. Inst. Med. Top. S. Paulo 40, 119–120.

FUNASA, 2001. Acidentes por Lepidópteros. Manual de diagnóstico e tratamento de acidentes por animais peçonhentos. Fundação Nacional de Saúde (FUNASA), Ministério da Saúde, Brasil. Brasília, pp. 67–76.

Gamborgi, G.P., Metcalf, E.B., Barros, E.J.G., 2006. Acute renal failure provoked by toxin from caterpillars of the species *Lonomia obliqua*. Toxicon 47, 68–74.

Garcia, C.M., Danni-Oliveira, I.M., 2007. Occurence of accidents caused by *Lonomia obliqua* Walker, in state of Paraná between 1989 and 2001. Rev. Soc. Bras. Med. Trop. 40, 242–246.

Gonçalves, L.R., Sousa-e-Silva, M.C., Tomy, S.C., Sano-Martins, I.S., 2007. Efficacy of serum therapy on the treatment of rats experimentally envenomed by bristle extract of the caterpillar *Lonomia obliqua*: comparison with epsilon-aminocaproic acid therapy. Toxicon 50, 349–356.

Gutiérrez, J.M., Lomonte, B., León, G., Rucavado, A., Chaves, F., Angulo, Y., 2007. Trends in snakebite envenomation therapy: scientific, technological and public health considerations. Curr. Pharm. Des. 13, 2935–2950.

Hoffman, M., Monroe, D.M., 2001. A cell-based model of hemostasis. Thromb. Haemost. 85, 958–965.

Kelen, E.M.A., Picarelli, Z.P., Duarte, A.C., 1995. Hemorrhagic syndrome induced by contact with caterpillars of the genus *Lonomia* (Saturniidae, Hemileucinae). J. Toxicol. Toxin Rev. 14, 283–308.

Kerns, R.T., Kini, R.M., Stefansson, S., Evans, H.J., 1999. Targeting of venom phospholipases: the strongly anticoagulant phospholipase A2 from *Naja nigricollis* venom binds to coagulation factor Xa to inhibit the prothrombinase complex. Arch. Biochem. Biophys. 369, 107–113.

Kini, R.M., 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. Toxicon 42, 827–840.

Kowacs, P.A., Cardoso, J., Entres, M., Novak, E.M., Werneck, L.C., 2006. Fatal intracerebral hemorrhage secondary to *Lonomia obliqua* caterpillar envenoming, a case report. Arq. Neuropsiquiatr. 64, 1030–1032.

Maiorano, V.A., Marcussi, S., Daher, M.A.F., Oliveira, C.F., Couto, L.B., Gomes, O. A., Franc, S.C., Soares, A.M., Pereira, P.S., 2005. Antiophidian properties of the aqueous extract of *Mikania glomerata*. Toxicon 102, 364–370.

Malaque, C.M.S., Andrade, L., Madalosso, G., Tomy, S., Tavares, F.L., Seguro, A.C., 2006. Short report: a case of hemolysis resulting from contact with a *Lonomia* caterpillar in southern Brazil. Am. J. Trop. Med. Hyg. 74, 807–809.

Monroe, D.M., Hoffman, M., 2006. What does it take to make the perfect clot? Arterioscler. Thromb. Vasc. Biol. 26, 41–48.

Pinto, A.F.M., Dobrovolski, R., Veiga, A.B.G., Guimarães, J.A., 2004. Lonofibrase, a novel α-fibrinogenase from *Lonomia obliqua* caterpillars. Thromb. Res. 113, 147–154.

Pinto, A.F.M., Dragulev, B., Guimarães, J.A., Fox, J.W., 2008. Novel perspectives in the pathogenesis of *Lonomia obliqua* caterpillar envenomation based on assessment of host response by gene expression analysis. Toxicon 51, 1119–1128.

Pinto, A.F.M., Silva, K.R.L.M., Guimaraes, J.A., 2006. Proteases from *Lonomia obliqua* venomous secretions: comparison of procoagulant, fibrin(ogen)olytic and amidolytic activities. Toxicon 47, 113–121.

Prezoto, B.C., Maffei, F.H.A., Mattar, L., Chudzinski-Tavassi, A.M., Curi, P.R., 2002. Antithrombotic effect of Lonomia obliqua caterpillar bristle extract on experimental venous thrombosis. Braz. J. Med. Biol. Res. 35, 703–712.

Ramos, A.T., Gonçalves, L.R., Ribeiro, O.G., Rocha Campos, A.C., Sant'Anna, O.A., 2004. Effects of *Lonomia obliqua* (lepidoptera, saturniidae) toxin on clotting, inflammatory and antibody responsiveness in genetically selected lines of mice. Toxicon 43, 761–768.

Reis, C.V., Kelen, E.M.A., Farsky, S.H.P., Portaro, F.C.V., Sampaio, C.A.M., Fernandes, B.L., Camargo, A.C.M., Chudzinski-Tavassi, A.M., 1999. A Ca2+ activated serine protease (LOPAP) could be responsible for the haemorrhagic syndrome caused by the caterpillar *Lonomia obliqua*. Lancet 353, 1942.

Ricci-Silva, M.E., Valente, R.H., León, I.R., Tambourgi, D.V., Ramos, O.H., Perales, J., Chudzinski-Tavassi, A.M., 2008. Immunochemical and proteomic technologies as tools for unravelling toxins involved in envenoming by accidental contact with *Lonomia obliqua* caterpillars. Toxicon 51, 1017–1028.

Rocha-Campos, A.C., Gonçalves, L.R., Higasshi, H.G., Yamagushi, I.K., Fernandes, I., Oliveira, J.E., Ribela, M.T., Sousa-e-Silva, M.C., Dias da Silva, W., 2001. Specific heterologous $F(ab')2$ antibodies revert blood incoagulability resulting from envenoming by *Lonomia oblique* caterpillars. Am. J. Trop. Med. Hyg. 64, 283–289.

Sano-Martins, I.S., Santoro, M.L., Castro, S.C.B., Fan, H.W., Cardoso, J.L.C., Theakston, R.D.G., 1997. Platelet aggregation in patients bitten by the Brazilian snake *Bothrops jararaca*. Thromb. Res. 87, 183–195.

Seibert, C.S., Oliveira, M.R.L., Gonçalves, L.R.C., Santoro, M.L., Sano-Martins, I.S., 2004. Intravascular hemolysis induced by *Lonomia obliqua* caterpillar bristle extract: an experimental model of envenomation in rats. Toxicon 44, 793–799.

Seibert, C.S., Tanaka-Azevedo, A.M., Santoro, M.L., Mackessy, S.P., Torquato, R.J.S., Lebrun, I., Tanaka, A.S., Sano-Martins, I.S., 2006. Purification of a phospholipase A_2 from *Lonomia obliqua* caterpillar bristle extract. Biochem. Biophys. Res. Commun 342., 1027–1033.

Secretaria de Vigilância em Saúde, Ministério da Saúde SVS, 2009. Situação Epidemiológica das Zoonoses de Interesse à Saúde Pública. Boletim Eletrônico Epidemiológico 9 (1), 17. Brasília.

Thorsen, L.I., Brosstad, F., Gogstad, G., Sletten, K., Solum, N.O., 1986. Competitions between fibrinogen with its degradation products for interactions with the platelet-fibrinogen receptor. Thromb. Res. 44, 611–623.

Tsai, H.M., 2006. The molecular biology of thrombotic microangiopathy. Kidney Int. 70 (1), 16–23.

Vaughan, D.E., Declerck, P.J., 1998. Fibrinolysis and its regulation. In: Loscalso, J., Schafer, A.I. (Eds.), Thrombosis and Hemorrhage. Williams & Wilkins, Baltimore, pp. 155–170.

Veiga, A.B.G., Blochtein, B., Guimarães, J.A., 2001. Structures involved production, secretion and injection of the venom produced by the caterpillar *Lonomia obliqua* (Lepidoptera, saturniidae). Toxicon 39, 1343–1351.

Veiga, A.B.G., Pinto, A.F.M., Guimarães, J.A., 2003. Fibrinogenolytic and procoagulant activities in the hemorrhagic syndrome caused by *Lonomia obliqua* caterpillars. Thromb. Res. 111, 95–101.

Veiga, A.B.G., Ribeiro, J.M., Guimarães, J.A., Francischetti, I.M., 2005. A catalog for the transcripts from the venomous structures of the caterpillar *Lonomia obliqua*: identification of the proteins potentially involved in the coagulation disorder and hemorrhagic syndrome. Gene 355, 11–27.

Wang, H., Ehnert, C., Brenner, G.J., Woolf, C.J., 2006. Bradykinin and peripheral sensitization. Biol. Chem. 387, 11–14.

Zannin, M., Lourenço, D.M., Motta, G., Costa, L.R.D., Grando, M., Gamborgi, G. P., Noguti, M.A., Chudzinski-Tavassi, A.M., 2003. Blood coagulation and fibrinolytic factors in 105 patients with hemorrhagic syndrome caused by accidental contact with *Lonomia obliqua* caterpillar in Santa Catarina, Southern Brazil. Thromb. Haemost. 89, 355–364.

research articles Journal of proteome research

Wound Exudate as a Proteomic Window to Reveal Different Mechanisms of Tissue Damage by Snake Venom Toxins

Teresa Escalante,[†] Alexandra Rucavado,[†] Antonio F. M. Pinto,[‡] Renata M. S. Terra,[‡] José María Gutiérrez,[†] and Jay W. Fox*[,‡]

Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica, and Department of Microbiology, Health Sciences Center, University of Virginia, Jordan Hall, P.O. Box 800734, Charlottesville, Virginia 22908-0734

Received June 2, 2009

In light of the complexity of wound tissue, proteomic analysis may not clearly reveal the nature of the wound or the processes involved in healing. However, exudate associated with wounds may provide a "window" on cellular events leading to the development of the wound and/or its healing. In this investigation we performed proteomic analysis on wound exudates from muscular wounds in mice caused by two very different types of snake venom toxins: BaP1, a snake venom metalloproteinase and Mtx-I, a snake venom phospholipase A2. Proteomic analysis of the exudates associated with these wounds clearly differentiated them and offered new perspectives on functional mechanisms by which these toxins cause tissue damage. In the case of wounds caused by the metalloproteinase, there was evidence of degradation of nonfibrillar collagens whereas the phospholipase wound exudate was noted by the presence of fibrillar collagen type I, apolipoproteins A-I, A-IV, and E, and fibronectin. These results suggest that the hemorrhage caused by snake venom metalloproteinases may be associated with the degradation of specific extracellular matrix proteins which play a role in matrix/capillary stabilization and that release of apolipoproteins from their complexes may be involved with the dysfunctional hemostatsis observed following snake envenoming.

Keywords: snake venoms • snake venom metalloproteinases • SVMPs • extracellular matrix • nonfibrillar collagens • apolipoproteins • wound exudate • phospholipases A2 • myonecrosis • basement membranes

Introduction

Tissue injury caused by exogenous or endogenous agents is associated with the onset of an inflammatory reaction which, among other features, involves exudation of plasma.[1] In addition to possessing a high concentration of plasma-derived proteins, the composition of wound fluid is further influenced by the pathologic and inflammatory events occurring in the affected tissue, such as release of cytosolic components secondary to cellular damage, degradation of cellular and extracellular matrix proteins by proteinases present in exudates, generation of diverse inflammatory mediators, and reabsorption of exudate proteins by lymphatic vessels.[2−4] In turn, the composition of inflammatory exudate may also influence the reparative and regenerative events taking place in the tissues, since healing and nonhealing wounds have been characterized as showing variations in wound exudates composition.[5,6]

The field of proteomics provides highly useful methodologies to analyze complex protein mixtures and consequently can be used for the comparative analysis of a variety of fluids in different pathological conditions. Proteomic analyses have been performed in cerebrospinal fluid,[7,8] vitreous fluid,[9] ovarian cancer ascites,[10] peritoneal dialysate fluid,[11] synovial fluid,[12] sputum,[13] bronchoalveolar lavage fluid,[14] tear fluid,[15] and serum and plasma.[16,17] Recently, a systematic database for human fluid proteome research has been developed.[18] Proteomics has also been recently applied to the study of markers of healing in chronic wound fluid.[4] Thus, the possibility of using proteomic tools to characterize the wound environment through the analysis of exudate is a highly promising approach to gain a better understanding of the mechanisms underlying pathologic phenomena.

Snakebite envenomation, in particular by species of the family Viperidae, is characterized by prominent local tissue damage associated with a series of acute pathological alterations occurring in the anatomical area of venom injection.[19] Such alterations occur rapidly after the bite, and depending on the amount of venom injected and on the delay in antivenom treatment, they may result in permanent tissue damage and physical disability.[20−22] The pathogenesis of local tissue damage by viperid venoms has been investigated at the cellular level. Many of the effects of viperid snakebite envenoming are primarily the consequence of the direct action of

* To whom correspondence should be addressed. Jay W. Fox, Department of Microbiology, Health Sciences Center, University of Virginia, Jordan Hall, P.O. Box 800734, Charlottesville, VA 22908-0734. Fax: 1-804-982-2514. E-mail: jwf8x@virginia.edu.
[†] Universidad de Costa Rica.
[‡] University of Virginia.

10.1021/pr900489m CCC: $40.75
Published on Web 09/18/2009

Zn^{2+}-dependent snake venom metalloproteinases (SVMPs) and phospholipases A_2 (PLA_2s).[19,23,24] SVMPs, which belong to the M12 family of the reprolysin subgroup of metzincins,[25] induce hemorrhage through the degradation of proteins in the basement membrane surrounding capillaries.[26,27] In addition, they induce blistering and dermonecrosis,[28,29] myonecrosis,[30,31] and a prominent inflammatory reaction with release of diverse inflammatory mediators.[32,33] PLA_2s, on the other hand, are responsible for local myonecrosis due to their ability to disrupt the integrity of skeletal muscle fiber plasma membrane.[24,34] They also induce pain and edema,[35] affect the collecting lymphatic vessels,[36] and promote a complex inflammatory reaction.[37] Therefore, SVMPs and PLA_2s represent relevant models for the induction of acute tissue damage of varying pathogenesis. Owing to the structural similarity of these venom enzymes with their orthologs in mammals and in the toxic secretions of other organisms, the study of the mechanisms of tissue damage by venom SVMPs and PLA_2s may give rise to relevant insights into the understanding of tissue damage in many different pathological conditions beyond snakebite envenomation.

Proteomic analysis of wound tissue is problematic due to the extreme complexity of the sample; however analysis of wound exudate may provide a "window" on the cellular processes associated with specific wound types. In the present study, we performed a proteomic analysis of wound exudate collected from mice injected with either a hemorrhagic SVMP or a myotoxic PLA_2 from the venom of *Bothrops asper*, which from a medical standpoint is the most important snake from southern Mexico to the northern regions of South America. On the basis of previous knowledge gathered on the mechanisms of tissue damage inflicted by these toxins, a correlation between structural and cellular alterations and proteomic characteristics of wound fluid was performed. Our results give rise to novel information on the pathogenic processes underlying the tissue-damaging activity of these toxins and the subsequent inflammatory reaction.

Materials and Methods

Toxins. Both toxins were isolated from the venom of adult specimens of *Bothrops asper*, collected in the Pacific versant of Costa Rica and maintained at the serpentarium of Instituto Clodomiro Picado, Costa Rica. The venom used was a pool obtained from more than 40 individual specimens. After collection, venom was freeze-dried and stored at −20 °C. BaP1, a P−I SVMP exerting multiple tissue damaging activities, was isolated by a combination of ion-exchange chromatography on CM-Sephadex and affinity chromatography of Affi-gel Blue.[28,38] BaP1 is a 22.7 kDa protein whose sequence, crystal structure, and mechanism of tissue damage have been determined.[29,30,39] Myotoxin I (Mtx-I), an Asp49 myotoxic PLA_2, was isolated from this venom by ion-exchange chromatography on CM-Sephadex.[40] The pathogenesis of myonecrosis and the toxicological profile of this enzyme have been studied previously.[40−42]

Collection of Wound Exudate. Solutions of these toxins, at 1 mg/mL, were prepared using 0.14 M NaCl, 0.04 M phosphate, pH 7.2, solution (PBS) as solvent. Aliquots of 50 μL, containing 50 μg toxin, were injected intramuscularly, in the right gastrocnemius, of groups of 5 CD-1 mice (18−20 g). This dose was selected because previous studies had shown that it induces prominent myonecrosis in the case of Mtx-1[41] and hemorrhage and blistering, in the case of BaP1.[28,30] Control mice were injected with PBS alone. One hour after injection, mice were sacrificed by CO_2 inhalation, and a 5 mm section was cut with a scalpel in the skin overlying the injected muscle. Immediately, the sectioned skin was opened and a nonheparinized capillary tube was introduced under the skin to collect the wound fluid. An approximate volume of 20−50 μL was collected from each mouse. Exudate samples were then pooled and lyophilized. The animal experimental protocols followed were approved by the Institutional Committee for the Care and Use of Animals (CICUA) of the University of Costa Rica.

Mass Spectrometry Data Analysis and Presentation. Lyophilized wound exudate samples were resuspended in water and protein quantification was performed using NanoOrange protein kit (Invitrogen). Sixty micrograms of protein was acetone precipitated, resuspended in Laemmli buffer, applied to a 4−20% precast electrophoresis gel (Bio-Rad) and stained with Coomassie Blue. Gel lanes were cut in 10 equal size slices.

Gel slices were destained for 2 h and the proteins were reduced (10 mM DTT) and alkylated (50 mM iodoacetamide) at room temperature. Gel slices were washed with 100 mM ammonium bicarbonate, dehydrated with acetonitrile and dried in a speed vac. Hydration of the slices was performed with a solution of Promega modified trypsin (20 ng/mL) in 50 mM ammonium bicarbonate for 30 min on ice. Excess trypsin solution was removed and the digestion was carried on for an additional 18 h at 37 °C. Tryptic peptides were twice extracted from gel slices with 30 μL of a 50% acetonitrile/5% formic acid solution. The combined extracts were dried to a volume of 15 μL for mass spectrometric analysis.

LC−MS/MS was performed using a Thermo Electron LTQ ion-trap mass spectrometer. Analytical columns were fabricated in-house by packing 7.5 cm Jupiter 10 μm C18 packing material (Phenomenex, Torrance, CA) into a 25 cm length of 360 × 75 μm fused silica (Polymicro Technologies, Phoenix, AZ) behind a bottleneck. Samples were loaded directly onto these columns for the C18 analytical runs. In-gel digests (50% of each sample) were injected into the mass spectrometer at 300 nL/min. Peptides were eluted from the C18 column using an acetonitrile/0.1 M acetic acid gradient (2−90% acetonitrile). The instrument was programmed to acquire a cycle of one mass spectrum followed by MS/MS on the ten most abundant ions in a data-dependent mode. After MS/MS, fragmentation was carried out on a particular parent ion and the m/z was placed on an exclusion list for 2 min to enable greater dynamic range and prevent repeat analysis of the same ions. The electrospray voltage was set to 2.5 kV, and the capillary temperature was 230 °C.

The mass spectra were extracted and analyzed utilizing Bioworks Sequest 3.11 software. Searches were performed against a mouse IPI nonredundant database (http://www.ebi.ac.uk/IPI/). Spectra generated on the LTQ were searched using 1.5 Da parent tolerance and 1 Da fragment tolerance. All hits were required to be fully tryptic. The results from the searches were exported to Scaffold (version 2.2.03, Proteome Software Inc., Portland, OR). Scaffold was used to validate MS/MS based peptide and protein identifications and to visualize multiple data sets in a comprehensive manner. Confidence of protein identification in Scaffold is displayed as a Probability Legend with green coloration indicative of over 95% confidence and yellow as 80−94% confidence. Relative quantization of proteins was accomplished by summing all data from the 10 gel slices for a particular sample in Scaffold and then displaying the Quantitative Value from the program. This number gives an average total of nongrouped spectral counts for a protein

divided by the total nongrouping spectral counts for the 10 mass spectral runs from the gels slices from each lane (http://www.proteomesoftware.com/). This format of presentation allows for a relative quantitative comparison between a specific protein from different samples and to a certain degree gives some measure of relative abundance between proteins generated from the mass spectrometric analysis of the 10 gel slices for a particular exudate sample. Some of the data were further analyzed manually to determine if mass spectra were derived from proteins migrating in the gel at their expected molecular mass or at a lower mass. Proteins identified in gel slices representing molecular masses lower than expected masses were considered to be degraded. Degradation was quantitatively expressed as the percentage of the total amount of a given protein (the amount of that protein in all bands) showing molecular masses lower than the expected molecular mass of that particular protein.

Histology and Immunocytochemistry. Groups of four CD-1 mice (18−20 g) were injected by the intramuscular route, in the right gastrocnemius, with 50 μg of each toxin, dissolved in 50 μL PBS, as described above. Other groups received 50 μL PBS, as controls. One hour after injection, mice were sacrificed by CO_2 inhalation and the skin located around the injected region was immediately dissected out and cut into two halves. Tissue samples were rapidly fixed in zinc fixative (BD Pharmingen, San Jose, CA) and routinely processed for embedding in paraffin. Thick sections (4 μm) were prepared and stained with hematoxylin and eosin for histological assessment. In addition, sections were incubated with either a rabbit anti-mouse laminin 1 polyclonal antibody (Fitzgerald Industries International, Concord, MA) which should bind to the laminin β1 and γ1 chains present in mouse skin or a rabbit antihuman type VI collagen (generous gift from D. Mon-Li Chiu) which should bind to collagen VI α1, α2, and α3 chains of mouse skin collagen VI, followed by a goat antirabbit biotinylated polyclonal antibody (Vector Laboratories, Burlingame, CA). The reaction was amplified with a tyramide signal amplification kit (Perkin-Elmer, Boston, MA) and visualization was performed with streptavidin-Cy3 (Zymed Laboratories Inc., South San Francisco, CA). Subsequently the sections were incubated with Hoechst 33258 for nuclei staining.

Quantification of Creatine Kinase (CK). Groups of five CD-1 mice were injected intramuscularly with either 50 μg of Mtx-1 or BaP1, dissolved in 50 μL of PBS. Controls were injected with 50 μL of PBS alone. Wound exudates were collected as previously described in nonheparinized capillary tubes. After centrifugation at 5000× g for 3 min, the CK activity of supernatants was determined by using a commercial kit (CK-NAc, Biocon Diagnostik, Germany).

Results

Macroscopic Observations. Exudate collected from mice injected with the SVMP BaP1 was reddish in color, an expected consequence of hemorrhage induced by this toxin. In contrast, exudate from mice injected with Mtx-1 did not show visual evidence of hemorrhage. No exudate could be collected from mice injected with PBS alone, in agreement with the lack of pathological and inflammatory alterations in these animals.

Proteomic Analysis of Wound Exudates from BaP1 and Mtx-1 Treated Mice. The 1D SDS-PAGE is shown in Figure 1. Although some differences are observable in the gel patterns between the exudates of BAP1 and Mtx-1 treated mice, little less can be deduced from the image. From the mass spectral analysis of the gel bands of the BaP1-treated mouse wound exudate, 442 proteins were identified with XCorr Scores equal of greater than 1.8, 2.3, 2.7, and 3.7 for +1, +2, +3, and +4 ions, respectively, and protein identification probability above 95%. In the case of the Mtx-1 treated mouse wound exudates 1191 proteins were identified using the same parameters described above (for full reporting of mass spectrometry data see Supporting Information).

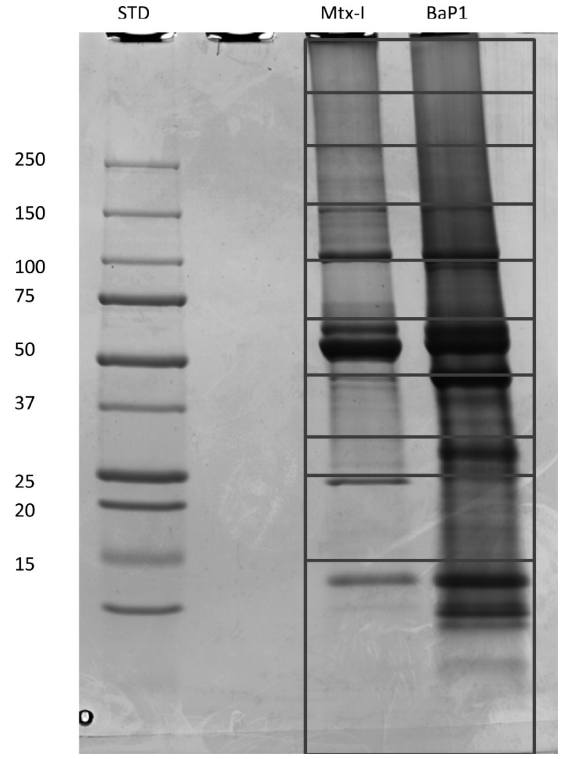


Figure 1. 1D SDS PAGE of wound exudates. Sixty micrograms of exudate from mice treated with either the myotoxin Mtx-I or the snake venom metalloproteinase BaP1 was electrophoresed on a 4−20% gradient gel followed by staining with Coomassie Blue. The grid depicts the gel slice positions for mass spectrometry analysis processing.

The most abundant proteins identified based on their Quantitative Value (see http://www.proteomesoftware.com/ for full description of term) were sorted into five categories based on normal tissue localization relevant to this study: (1) Serum (Table 1); (2) Serum Proteinase Inhibitors (Table 2); (3) Cytosol (Table 3); (4) Keratins (Table 4); and (5) Extracellular matrix (Table 5). Of the most abundant serum proteins identified in the wound exudates, several showed significant differential amounts between the exudates (>2 fold); these include apolipoprotein A-I, hemoglobin subunit beta-1, apolipoprotein A-IV, fibrinogen beta chain, apolipoprotein E and Ig gamma-2A chain C. Of these, only hemoglobin subunit beta-1 and fibrinogen beta chain were in greater abundance in the BaP1-induced wound sample compared to the Mtx-I sample. With the exception of fibrinogen, the largest differences in abundance in the identified serum proteins were observed in the apolipoproteins (Table 1).

Table 2 shows a subset of serum proteins that have been identified as proteinase inhibitors. Although there is little difference in the quantitative value observed for these proteins between the two exudates, it is interesting to note that the proteinase inhibitors from the BaP1 wound exudate show a

Table 1. Most Abundant Serum Proteins Identified in Mouse Wound Exudates

Protein	Accesion #	Mol. Weight	Quantitative Value		Fold change
			BaP1	Mtx-I	
Serum albumin	IPI00131695	69 kDa	3316	2596	-
Serotransferrin	IPI00139788	77 kDa	1434	1661	-
Isoform Long of Complement C3 (Fragment)	IPI00323624	186 kDa	639	735	-
Serpina3k Serine protease inhibitor A3K	IPI00131830	47 kDa	656	791	-
Alpha-2-macroglobulin	IPI00624663	167 kDa	726	565	-
Alpha-1-antitrypsin 1-6	IPI00117857	46 kDa	396	441	-
Apolipoprotein A-I	IPI00121209	31 kDa	48	357	7.44
Hemoglobin subunit beta-1	IPI00553333	16 kDa	625	209	2.99
Hemopexin	IPI00128484	51 kDa	217	256	-
Murinoglobulin-1	IPI00123223	165 kDa	134	348	2.60
Hemoglobin subunit beta-2	IPI00316491	16 kDa	465	128	3.63
Ceruloplasmin	IPI00117831	121 kDa	127	230	-
Transthyretin	IPI00127560	16 kDa	80	150	-
Vitamin D-binding protein	IPI00126184	54 kDa	131	114	-
Apolipoprotein A-IV	IPI00377351	45 kDa	14	172	12.29
Alpha-2-HS-glycoprotein	IPI00128249	37 kDa	49	147	3.00
Beta-globin	IPI00762198	16 kDa	169	98	-
Fibrinogen beta chain	IPI00279079	55 kDa	287	19	15.11
Isoform HMW of Kininogen-1	IPI00114958	73 kDa	61	88	-
Plasminogen	IPI00322936	91 kDa	48	97	2.02
Fibronectin	IPI00113539	272 kDa	100	61	-
Haptoglobin	IPI00409148	39 kDa	74	87	-
Alpha-1-antitrypsin 1-2	IPI00129755	46 kDa	117	62	-
Serine protease inhibitor A3C	IPI00128076	47 kDa	100	119	-
Apolipoprotein E	IPI00323571	36 kDa	8	119	14.88
Antithrombin-III	IPI00136642	52 kDa	46	76	-
Ig gamma-2A chain C region, A allele	IPI00420972	36 kDa	28	108	3.86

Table 2. Most Abundant Serum Proteinase Inhibitors Identified in Mouse Wound Exudate

Serpins	Accession #	Mol. Weight	Quantitative Value		Fold change	% Degradation	
			BaP1	Mtx-I		BaP1	Mtx-I
Serpina3k Serine protease inhibitor A3K	IPI00131830	47 kDa	656	791	-	11.3	0
Serpina1c Alpha-1-antitrypsin 1-6	IPI00117857	46 kDa	396	441	-	10.2	1.57
Serpina1b Alpha-1-antitrypsin 1-2	IPI00129755	46 kDa	117	62	-	14.6	0
Serpina3c Serine protease inhibitor A3C	IPI00128076	47 kDa	100	119	-	12.96	0
Serpinc1 Antithrombin-III	IPI00136642	52 kDa	46	76	-	11.11	5.95
Macroglobulins							
Alpha-2-macroglobulin	IPI00624663	167 kDa	726	565	-	25.7	6.45
Murinoglobulin-1	IPI00123223	165 kDa	134	348	2.5	31.8	2.9
Alpha-2-macroglobulin-P	IPI00454052	164 kDa	9	4	2.2	0	0

consistent level of degradation compared to the inhibitors from the Mtx-I wound exudate. The two macroglobulin proteinase inhibitors identified, alpha-2-macroglobulin and murinoglobulin-1, were found in greater abundance in the BaP1 wound exudate and also displayed the highest level of degradation among all the proteinase inhibitors identified.

In Table 3 we show the most abundant proteins normally having a cytosolic compartmentalization that were identified in the two samples. Of these proteins, essentially all were seen to have a greater differential abundance in exudate from Mtx-I wounds, with the greatest relative difference observed for myosin-8 and myosin-4. In that all these proteins are normally cytosolic, their presence in the exudate suggests cell lysis and this occurs to a much greater extent in the wounds from Mtx-I treated mice.

A number of keratins were identified in the wound exudates (Table 4) with all except keratin Kb40 having a greater abundance in the wound exudate from BaP1 treated mice.

Extracellular matrix proteins detected in the wound exudates are shown in Table 5. Of the most abundance extracellular matrix proteins identified, 16 showed differential abundances of 2 fold or greater. Of these, the fibrillar collagens I and III are more abundant in the exudate from the Mtx-I induced wound and show little evidence of proteolytic degradation, suggesting they are released essentially intact into the exudate. Of the collagens, which appear more abundant in the BaP1 wound exudates, there are 6 nonfibrillar collagens (Col VI, Col XIX, Col XII, Col XIV, Col XV, and Col XVI) and one fibrillar collagen, Col V. In general, the nonfibrillar collagens showed greater degradation, particularly in the BaP1 wound exudates,

Table 3. Most Abundant Cytosolic Proteins Identified in Mouse Wound Exudates

Protein	Accesion #	Mol. Weight	Total Amount		Fold change
			BaP1	Mtx-I	
Creatine kinase M-type	IPI00127596	43 kDa	95	438	4.6
Actin, alpha skeletal muscle	IPI00110827	42 kDa	57	134	2.4
Beta-enolase	IPI00228548	47 kDa	20	134	6.7
Fructose-bisphosphate aldolase A	IPI00221402	39 kDa	17	125	7.4
Glycogen phosphorylase, muscle form	IPI00225275	97 kDa	15	100	6.7
Phosphoglycerate mutase 2	IPI00230706	29 kDa	11	85	7.7
L-lactate dehydrogenase A chain	IPI00319994	36 kDa	3	82	27.3
Isoform M2 of Pyruvate kinase isozymes M1/M2	IPI00407130	58 kDa	9	76	8.4
Triosephosphate isomerase	IPI00467833	27 kDa	15	69	4.6
Myosin-8	IPI00265380	223 kDa	1	67	67.0
Fructose-bisphosphate aldolase	IPI00133580	39 kDa	14	58	4.1
Myosin-4	IPI00404837	223 kDa	1	56	56.0
Actin, cytoplasmic 1	IPI00110850	42 kDa	25	55	2.2
Alpha-actinin-2	IPI00331664	104 kDa	26	48	-
Parvalbumin alpha	IPI00230766	12 kDa	6	42	7.0
Malate dehydrogenase, cytoplasmic	IPI00336324	37 kDa	8	39	4.9
Enolase	IPI00122684	35 kDa	6	31	5.2
Myosin-6	IPI00129404	224 kDa	2	30	15.0
Myosin-1	IPI00380896	223 kDa	1	25	25.0
Myosin-binding protein C, fast-type	IPI00169994	127 kDa	5	24	4.8
Alpha-enolase	IPI00462072	47 kDa	6	23	3.8
Isoform 1 of Adenylate kinase isoenzyme 1	IPI00128209	22 kDa	0	23	
Phosphoglycerate kinase 1	IPI00555069	45 kDa	1	19	19.0
Tubulin alpha-1A chain	IPI00110753	50 kDa	2	16	8.0
Tubulin beta-2B chain	IPI00109061	50 kDa	3	16	5.3
Similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 1	IPI00135284	36 kDa	2	15	7.5
Actinin alpha 2	IPI00387557	104 kDa	5	14	2.8
Phosphoglucomutase-1	IPI00555140	62 kDa	2	14	7.0
Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	IPI00108939	47 kDa	1	13	13.0
6-phosphofructokinase, muscle type	IPI00331541	85 kDa	1	12	12.0
Alpha-actinin-4	IPI00118899	105 kDa	5	11	2.2
Glucose-6-phosphate isomerase	IPI00228633	63 kDa	1	10	10.0
Alpha-actinin-3	IPI00136701	103 kDa	3	9	3.0
Myosin-7	IPI00130653	223 kDa	1	8	8.0

whereas fibrillar collagens I and III in the Mtx-I exudate appeared to be relatively intact. The laminin α3 chain, associated with laminins 5, 5B, and 6, in mouse skin and laminin β1 chain, associated with laminins 6, 8, and 10 found in mouse skin were identified in the wound exudates and present with greater abundance in the BaP1-treated samples. These were observed to have undergone degradation. The small leucine-rich proteoglycan family members, lumican and decorin, and the glycoprotein vitronectin were observed to be in greater abundance in the Mtx-1 wound exudates and did not appear to be significantly degraded.

Histological and Immunocytochemical Observations. Tissue sections from control mice injected with PBS showed a structure typical of normal skin, with epidermis, dermis and hypodermis, with intact skin appendages (hair follicles, sebaceous glands) and vascular structure (Figure 2A). Tissue sections from mice injected with Mtx-1 showed a normal integrity of skin layers, i.e. no blisters, no damage of skin appendages and a normal vascular structure (Figure 2B). The only pathological effect noticed was necrosis of muscle fibers at the panniculus carnosus layer of the skin (not shown). In contrast, tissue from mice injected with BaP1 showed hemorrhage in the dermis and hypodermis, evidenced by the presence of abundant erythrocytes (Figure 2C) which correlates to the increase abundance of hemoglobin beta-2 chain detected in exudate from BaP1 wounds. In addition, edema and blistering were observed (Figure 2C). Moreover, hair follicles were affected, as some of them were lost from the dermis in areas of blister formation. Areas denuded of epidermis were also observed.

Immunodetection of various laminins using the polyclonal antibody against laminin 1 and of type VI collagen in PBS-injected samples revealed a typical distribution of these ECM proteins in the dermal-epidermal interface, as well as surrounding endothelial cells in capillary vessels and in the periphery of hair follicles (Figures 3A and 4A). A similar pattern was noticed in tissue from mice injected with Mtx-1, as no loss in immunostaining was observed for laminins and type VI collagen (Figures 3C and 4C). In contrast, tissue from mice injected with BaP1 showed evident alterations in the immunostaining to these antigens. An apparent reduction in the number of capillary vessels positive for these antigens was noticed in the dermis (Figures 3E and 4E). At the dermal-epidermal junction, in areas of blister formation, staining for laminins was observed at both sides of the blisters, whereas in the case of type VI collagen the staining was observed predominantly at the base of the blister. However, in some areas, the pattern of immunostaining in the dermal-epidermal junction was focally interrupted for both antigens (Figures 3E and 4E). A partial loss of staining, especially in the case of laminin,

Table 4. Most Abundant Keratins Identified in Mouse Wound Exudate

Protein	Accesion #	Mol. Weight	Quantitative Value		Fold change
			BaP1	Mtx-I	
Keratin complex 1, acidic, gene 10	IPI00755181	57 kDa	48	26	-
Keratin, type II cytoskeletal 1	IPI00625729	66 kDa	41	20	2.05
Keratin, type II cytoskeletal 73	IPI00347110	59 kDa	35	29	-
Keratin, type II cytoskeletal 6B	IPI00131366	60 kDa	32	16	2.00
Keratin, type II cytoskeletal 5	IPI00139301	62 kDa	20	1	20.00
Keratin, type I cytoskeletal 14	IPI00227140	53 kDa	18	1	18.00
Keratin, type II cytoskeletal 79	IPI00124499	58 kDa	14	10	-
Keratin, type I cytoskeletal 19	IPI00112947	45 kDa	12	2	6.00
Keratin (Fragment)	IPI00463282	25 kDa	12	3	4.00
Keratin, type II cytoskeletal 72	IPI00347096	57 kDa	8	5	-
Keratin, type I cytoskeletal 42	IPI00468696	50 kDa	6	1	6.00
Keratin intermediate filament 16a	IPI00131209	52 kDa	5	1	5.00
Isoform 1 of Keratin, type I cytoskeletal 13	IPI00136056	48 kDa	5	1	5.00
Keratin, type II cytoskeletal 2 epidermal	IPI00622240	71 kDa	3	5	-
Keratin Kb40	IPI00348328	112 kDa	3	10	3.33
Keratin, type I cytoskeletal 24	IPI00137658	54 kDa	3	1	3.00
Keratin, type I cytoskeletal 23	IPI00121462	48 kDa	3	1	3.00
Keratin, type II cytoskeletal 6A	IPI00131368	59 kDa	3	1	3.00
G Keratin type II cuticular Hb5	IPI00331459	56 kDa	3	1	3.00
Keratin, type I cytoskeletal 17	IPI00230365	48 kDa	3	1	3.00
Keratin type II	IPI00124800	53 kDa	2	1	2.00
Novel member of the keratin associated protein 4 (Krtap4) family	IPI00648917	28 kDa	0	1	

Table 5. Most Abundant Extracellular Matrix Proteins Identified in Mouse Wound Exudates

	Accesion #	Mol. Weight	Quantitative Value		Fold change	% Degradation	
			BaP1	Mtx-I		BaP1	Mtx-I
Collagen							
Collagen alpha-3 (VI) chain	IPI00830749	289 kDa	31	1	31.0	94.4	40.0
Collagen alpha-1(XIX) chain	IPI00126387	114 kDa	11	5	2.2	80.0	50.0
Isoform 1 of Collagen alpha-1(XII) chain	IPI00121430	340 kDa	6	1	6.0	100.0	0.0
Isoform 1 of Collagen alpha-1(XIV) chain	IPI00330632	193 kDa	3	1	3.0	33.0	0.0
Collagen alpha-1(XV) chain	IPI00409035	140 kDa	3		-	100.0	-
Collagen alpha-1(III) chain	IPI00129571	139 kDa	2	7	3.5	0.0	0.0
Isoform 1 of Collagen alpha-1(I) chain	IPI00329872	138 kDa	2	11	5.5	0.0	0.0
Collagen alpha-2(I) chain	IPI00222188	130 kDa	2	5	2.5	0.0	42.8
Isoform 2 of Collagen alpha-1(II) chain	IPI00471183	139 kDa	2	1	2.0	*	*
Isoform 1 of Collagen alpha-1(XVI) chain	IPI00648306	156 kDa	2	1	2.0	*	*
Collagen alpha-1(V) chain	IPI00128689	184 kDa	2	1	2.0	*	*
Isoform 7 of Collagen alpha-2(XI) chain	IPI00138069	162 kDa	1	1	-	*	*
Isoform 2 of Collagen alpha1 (XXII) chain	IPI00757330	161 kDa	1	1	-	*	*
Isoform 1 of Collagen alpha-1(XXVIII) chain	IPI00357842	119 kDa		1	-	-	100.0
Collagen alpha-1(XXVII) chain	IPI00408491	186 kDa		1	-	-	*
Laminin							
Isoform B of Laminin subunit alpha-3	IPI00125058	366 kDa	3	1	3.0	100.0	100.0
Laminin subunit beta-1	IPI00338785	202 kDa	2	1	2.0	*	*
Tetranectin	IPI00320239	22 kDa	1	1	-	0.0	0.0
Vitronectin	IPI00129240	55 kDa	18	39	2.2	25.0	3.3
Fibronectin	IPI00113539	272 kDa	100	61	-	81.0	17.2
Lumican	IPI00313900	38 kDa	5	24	4.8	0.0	0.0
Decorin	IPI00123196	40 kDa	1	11	11.0	0.0	0.0
Dystroglycan	IPI00122273	97 kDa	2	1	2	0.0	0.0

was also observed in hair follicles, which showed disorganization and diffuse staining (Figure 3E). Overall, these results corroborate the proteomic finding of an increased abundance of laminin and type VI collagen chains in the BaP1 wound

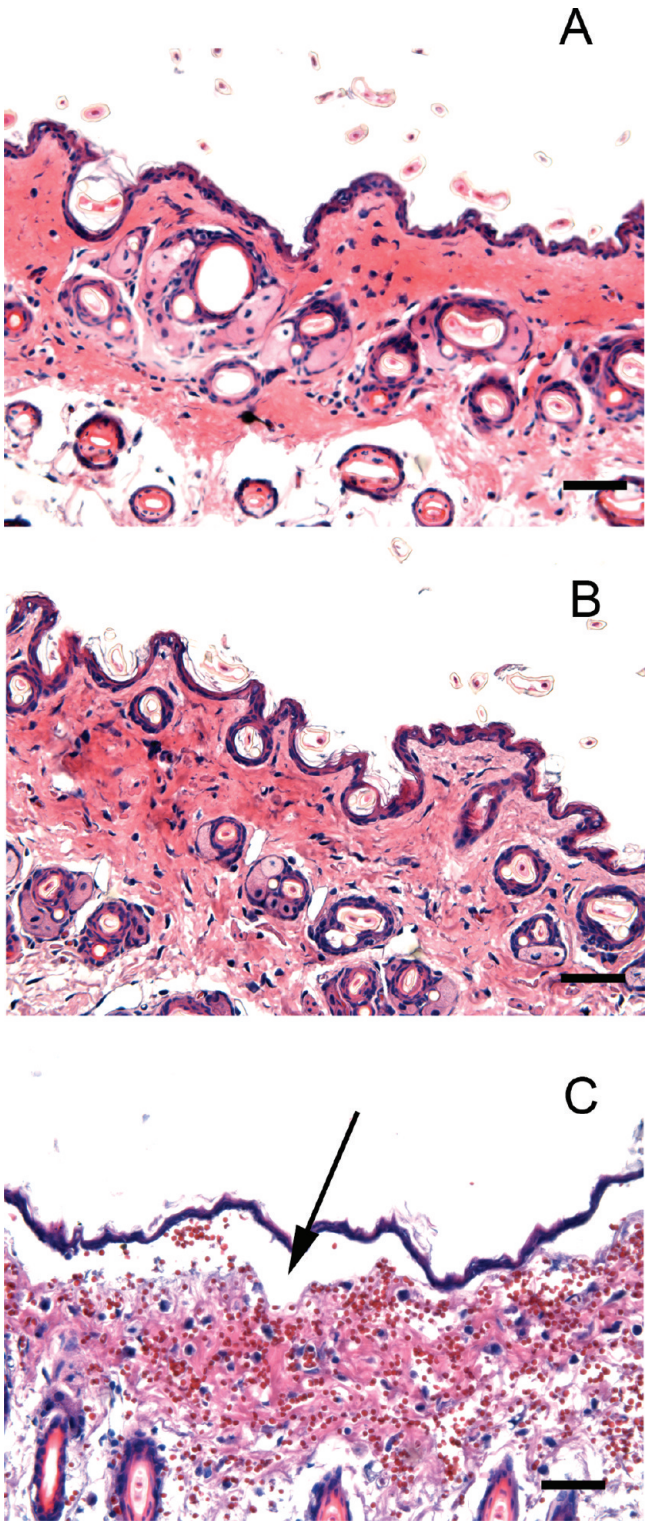


Figure 2. Light micrographs of histological sections of the skin in mice injected intramuscularly with either (A) PBS, (B) 50 μg of Mtx-1 or (C) 50 μg of BaP1. No alterations in the histological appearance of the skin were observed after Mtx-1 injection. In contrast, tissue injected with BaP1 showed abundant erythrocytes in the dermis, as a consequence of hemorrhage, and the formation of blisters (arrow).

exudates as well as the fact that they showed significant degradation and thus the loss of focal resolution of the antigen in immunostaining.

Quantification of Creatine Kinase (CK). A marked difference was observed in CK activity of exudate samples collected one hour after injection of the toxins. Exudate from animals receiving Mtx-1 had a CK activity of 178 600 $\pm$ 17 590 U/L, whereas exudates from mice injected with BaP1 had a CK activity of 8974 $\pm$ 1043 U/L ($P < 0.001$). Mice injected with PBS did not show any exudate formation and, consequently, samples could not be obtained for CK activity quantification. In that CK activity is representative of cell lysis, it appears that the cytolytic activity of Mtx-I, especially in muscle fibers, is significantly greater than BaP1 and thus this could explain the greater abundance of cytosolic proteins identified in the Mtx-I wound exudate compared to that of the BaP1 exudate.

Discussion

Proteomic analysis of wound exudates is a powerful tool to discern pronounced as well as subtle pathological events occurring in various models of tissue damage. In order to maximize the identification of proteins present in low concentration, some investigators have developed methods aimed at depleting highly abundant proteins, especially plasma proteins, from exudates by using immunoaffinity columns.[4,43] Others have developed effective protocols where no depletion of abundant proteins produces good results.[44] In our case, we chose the no depletion of plasma proteins approach which we believe give a reasonable, general view of wound exudates composition. In spite of this methodological choice, we were able to detect low amounts of a number of proteins, owing to the relatively high resolution of our methodological approach as evidenced by the large number of proteins identified in the exudates.

The proteomic analyses of wound exudates collected under the skin of mice injected with a myotoxic PLA_2 and a hemorrhagic and dermotoxic SVMP, isolated from the venom of *Bothrops asper*, show both similarities and differences which highlight the pathogenic mechanisms underlying the action of these toxins. As expected, the most abundant components in both types of exudates correspond to typical plasma proteins, as a consequence of exudation being associated with the characteristic acute inflammation induced by these types of toxins.[45−48] In the case of BaP1, inflammation-promoted exudate formation is also associated with direct extravasation of blood due to the hemorrhagic activity of this toxin.[30] Some of the plasma proteins detected are acute-phase proteins, such as proteinase inhibitors, fibrinogen, plasminogen and hemopexin, among others.[49] However, it is likely that, due to the early time interval when samples were collected, the full acute phase response has not developed, as suggested by the lack of detection of C-reactive protein, the component of acute-phase response that shows highest increments in inflammatory conditions.[49]

The relative abundance of some plasma-derived proteins was significantly higher in exudates collected from Mtx-1 injected mice compared to those from BaP1-injected mice. This is particularly remarkable in the case of the apolipoproteins. For example, in the Mtx-I generated wound exudate, apolipoproteins A-1, A-IV, and E were present at significantly greater abundance compared to BaP1 generated wound. It has been described that, during the acute phase response, a remodeling of high density lipoproteins (HDL) occurs. Such remodeling is mediated by the increase in serum amyloid A content and by the action of cholesteryl ester transfer protein and inflammatory group II secretory PLA_2.[50,51] Inflammatory PLA_2 and bee

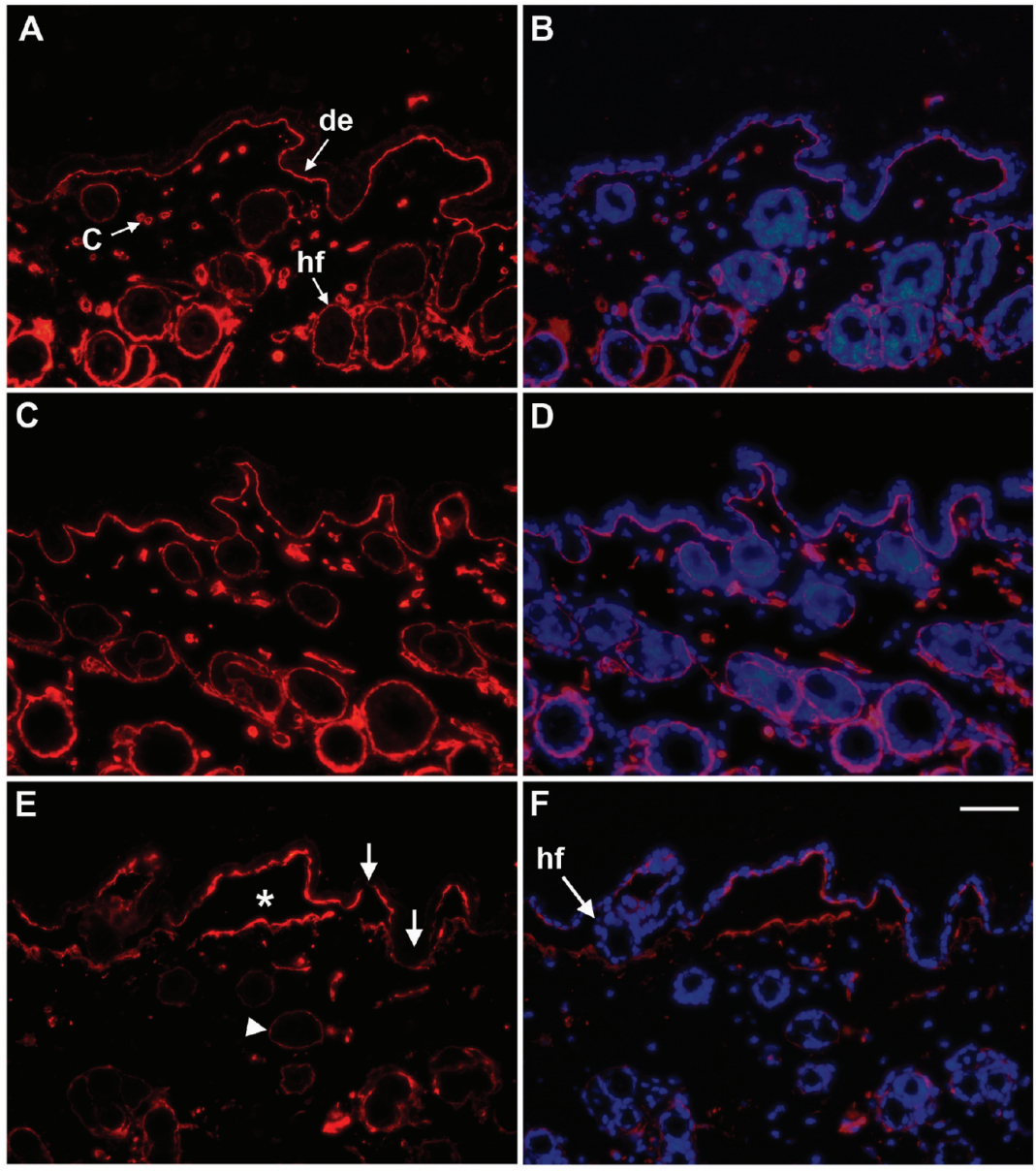


Figure 3. Detection of laminin by inmmunohistochemical staining. Paraffin-embedded sections of mouse skin 1 h after i.m. injection of PBS (A and B), 50 μg of Mtx-1 (C and D) or 50 μg BaP1 (E and F) stained with antilaminin 1 polyclonal antibody (detecting β1 and γ1 laminin chains in skin) and Hoechst 33258 for nuclei visualization. Detection was performed with a tyramide signal amplification kit using streptavidin-Cy3. (A, C, and E) Immunostaining for laminin β1 and/or γ1 chains; (B, D, and F) merged images of laminin staining and Hoechst. Observe the normal appearance of the epidermis in samples injected with PBS and Mtx-1, with positive staining for laminin in the basement membrane of the dermal-epidermal junction (de), hair follicles (hf) and capillaries (c). Notice the blister formation in samples injected with BaP1 (asterisk) with positive staining at both sides of the blister. In some areas there is interruption of laminin staining in the dermal-epidermal junction (arrows) and hair follicles (arrowhead), and detachment of a hair follicle (hf).

venom PLA_2 are known to promote the release of apolipoprotein apo-AI from HDL.[51,52] Since *B. asper* Mtx-1 is a PLA_2, we proposed that the increase in apolipoproteins in our proteomics analysis is the consequence of the Mtx-mediated release of apolipoproteins from HDL in the tissue and serum, making it more detectable in the wound exudate. The pathophysiological significance of this in the context of snake envenomation is unclear but could play a role in the local hemostatic dysfunction.

The results on fibrinogen are interesting because, in contrast with most plasma-derived proteins, the concentration of peptides bearing fibrinogen sequences is higher in exudates collected from BaP1-injected mice. The presence of fibrin(ogen)-degradation products in the BaP1 wound exudate can be interpreted as the result of BaP1's (a hemorrhagic SVMP) disruption of capillary integrity with the result of the formation of fibrin being deposited in the damaged vessel wall and interstitium[53,54] followed by fibrinolysis by both BaP1, a know fibrinolytic agent, as well as by endogenous plasmin activated as a consequence of fibrin formation.[38,55] It is therefore proposed that the fragments bearing fibrinogen sequences detected in our proteomic analysis represent fibrin-degradation products present in the exudate. Proteolytic degradation products of fibrinogen have been correlated with local increase in fibrinolysis following central nervous system traumatic injury.[7] These processes are absent in the case of tissue injected

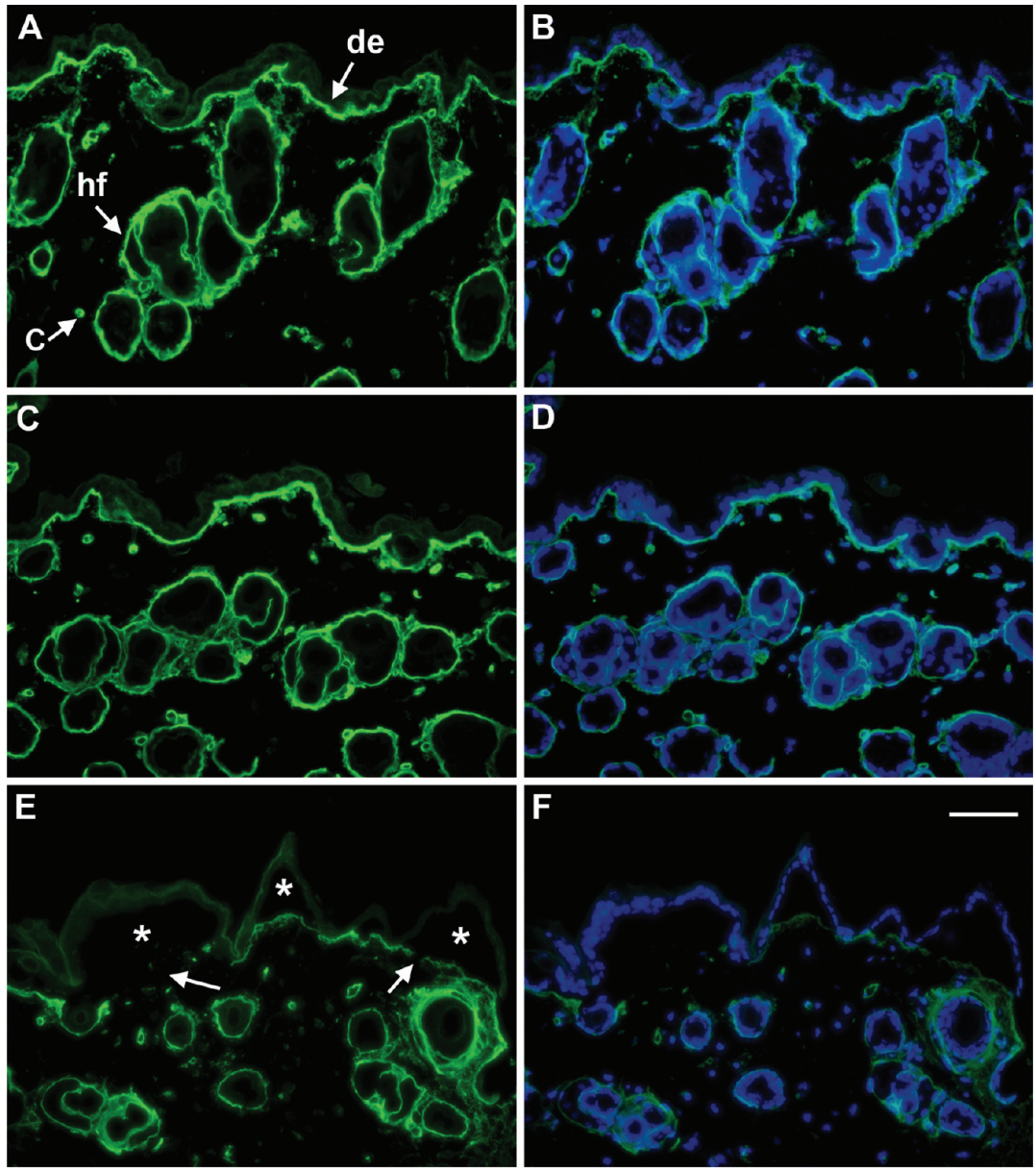


Figure 4. Detection of type VI collagen by inmmunohistochemical staining. Paraffin-embedded sections of mouse skin 1 h after i.m. injection of PBS (A and B), Mtx-1 (C and D) or BaP1 (E and F) stained with antitype VI collagen antibodies and Hoechst 33258 for nuclei visualization. Detection was performed with a tyramide signal amplification kit using streptavidin-Alexa 488. Immunostaining for type VI collagen (A, C and E) and merge images of type VI collagen and Hoechst (B, D and F). Observe the normal appearance of the epidermis in samples injected with PBS and Mtx-1, with positive staining for type VI collagen in the basement membrane of the dermal-epidermal junction (de), hair follicles (hf) and capillaries (c). Notice the blister formation in samples injected with BaP1 (asterisks) with positive staining only at the base of the blister. In some areas there is interruption of the staining at the dermal-epidermal junction (arrows).

with Mtx-1, since no blood vessel disruption occurs and, consequently, no fibrin clot formation takes place.

In agreement with previous pathological studies, wound exudate collected from mice receiving the myotoxic PLA_2 presented a greater abundance, compared to BaP1 wound exudates, of proteins normally found in the cytosol of muscle fibers, i.e. creatine kinase (CK) (M-type), α-actin (muscle), various myosins, α-actinin, and α-actinin-binding protein, as well as a number of cytosolic enzymes associated with glycolysis and other metabolic pathways occurring in the cytosol. Although these enzymes may have a diverse cellular origin, it is suggested that most are likely derived from skeletal muscle fiber cytosol. Myotoxic PLA_2s are known to induce a rapid disruption of the integrity of muscle cell plasma membrane, through catalytic and noncatalytic mechanisms.[24,34,41] This results in the release of cytosolic enzymes and other cellular markers. The presence of CK in exudates was corroborated by enzymatic analysis which showed a 20-fold higher activity of CK in exudates from Mtx-1-injected mice than in that of BaP1-injected mice. In contrast, in the case of BaP1, this SVMP predominantly induces hemorrhage and skin damage,[28,30] being able to induce muscle necrosis only at later time intervals, most probably associated with the onset of ischemia.[30] As our samples were collected one hour after injection it is unlikely that ischemia-induced muscle damage has developed at a significant extent. Thus, the higher cytotoxic, and especially

myotoxic, potential of the PLA_2 is clearly reflected in the exudate compositions. These results are corroborated by our histological observations which illustrate MtxI's ability to induce myonecrosis in muscle fibers of the panniculus carnosus layer of the skin.

The presence of higher abundance of protein sequences derived from keratins in exudates from BaP1-injected mice than in those of PLA_2-injected animals is clearly compatible with our histological observations and with previous studies showing blistering and dermonecrosis induced by BaP1, with little pathological effects of myotoxic PLA_2s in the skin.[28,29,56] In addition to blister formation, which depends on the direct cleavage of ECM proteins at the dermal-epidermal junction, BaP1 induces widespread apoptosis of keratinocytes.[29] Thus, the observed increase in keratin-derived sequences in exudate from BaP1-injected mice likely reflects the cytotoxic action of this enzyme on keratinocytes. The mechanisms of such cytotoxicity remain unknown but it is interesting to speculate that it could be related to cleavage of proteins forming connections between basal keratinocytes and basement membrane, thus affecting survival signals mediated by these interactions.

Extracellular matrix (ECM) alterations *in vivo* represent one of the lesser known aspects of the pathogenesis of local tissue damage in snakebite envenomation. Numerous studies have documented that SVMPs, both hemorrhagic and nonhemorrhagic, are able to degrade a variety of ECM proteins *in vitro*.[26,30,57−59] However, evidence of *in vivo* ECM degradation is scarce, and mostly derives from immunohistochemical results.[59] Our proteomic analysis of wound exudates is the first to generate *in vivo* information showing the presence of degraded ECM-derived sequences from tissue injected with BaP1. This is supported by previous studies which demonstrated BaP1 as well as other SVMPs' ability to degrade ECM components *in vitro*.[30,57,59] The interesting difference in the proteomic results for ECM proteins in the wound exudates is that in the BaP1 exudate there was a preponderance of nonfibrillar collagens identified whereas in the Mtx-I exudate fibrillar collagens I and III were more abundant (Table 5). The observation that nonfibrillar collagens, notable collagens VI, XII, and XIV, are subject to degradation by SVMPs has been demonstrated *in vitro*[60,61] and corroborated by the current *in vivo* investigation along with the identification of heretofore unknown *in vivo* collagen substrates, V, VII, XV, XVI, XIX, and XXII. In agreement with the proteomic findings, partial loss in the immunostaining for laminin and type VI collagen was observed, especially in the BM at the dermal-epidermal junction. Interestingly, several other proteins relevant to SVMP pathogenesis including collagen IV, a component of capillary basement membranes and a known *in vitro* and *in vivo* substrate for hemorrhagic SVMPs as well as the extracellular proteins matrilins were not detected. This can possibly be explained by the low amounts of degradation products due to the low abundance of these extracellular matrix proteins compared to other extracellular matrix substrates. Immunohistochemical observations of type VI collagen in the skin evidenced loss of immunostaining, compatible with degradation, especially at the dermal-epidermal junction. Taken together these results suggest that degradation of the nonfibrillar collagens, which support the integrity of the fibrillar collagen networks, basement membranes and subsequently cellular adhesion and tissue structure,[62,63] by SVMPs may represent a central mechanism in SVMP-derived hemorrhage and tissue destruction.

The identification of laminin sequences in the exudates, albeit at low levels, is compatible with degradation of laminin in the hair follicles (laminin 10), vessels (laminins 8 and 10) and at the dermal-epidermal junction (laminin 10), contributing to the pathogenesis of blistering, which is one of the main pathological effects induced by BaP1 in our model.[29,59] Interestingly, the exudate from PLA_2-injected tissue showed higher abundance of sequences of the fibrillar collagen binding proteoglycans lumican, decorin and vitronectin.[64] This finding, in the absence of proteolytic activity of Mtx-1, can be interpreted as a consequence of the expression and activation of matrix metalloproteinases (MMPs), which increase in muscle injected with both BaP1 and a myotoxic PLA_2.[55] Expression of MMP-9 is higher one hour after injection of myotoxic PLA_2 than after injection of BaP1.[55] Thus it appears that both BaP1 and Mtx-I can promote the release of these proteins into the wound exudate, either directly, as is likely with BaP1, or indirectly via Mtx-I activation of MMP-9 which subsequently acts on these proteins.

In conclusion, the proteomic characterization of wound exudate collected from mice injected with two different types of snake venom toxins, which inflict distinct modes of tissue damage, represents a novel, insightful approach to decipher overt and subtle underlying pathogenic mechanisms which are difficult to ascertain by histological and other more traditional methodologies. In the present study, the proteomic analyses revealed differences in the action of these toxins concerning ECM degradation, myotoxic activity, fibrinolytic effect and keratinocyte cytotoxicity and identified novel targets of these toxins. Further studies are ongoing to assess the time-course of exudate composition and to study the proteomic profile of exudates from animals injected with additional types of tissue-damaging toxins.

Acknowledgment. This study was supported by Vicerrectoría de Investigación, Universidad de Costa Rica (projects 741-A7-502 and 741-A7-604), the International Foundation for Science (project F/4096-1), NeTropica (project 2-N-2008), and the University of Virginia School of Medicine (J.W.F.). This work was carried out in partial fulfillment of the requirements for the Ph.D. degree for T. Escalante at the University of Costa Rica. We also acknowledge the excellent consultation of Dr. Nicholas Sherman in the University of Virginia W. M. Keck Mass Spectrometry Laboratory and Dr. Roswitha Nischt, University of Cologne.

Supporting Information Available: Link to access mass spectrometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gallin, J. I.; Snyderman, R. Inflammation. *Basic Principles and Clinical Correlates*; Lippincot: Philadelphia, 1999.
(2) Yager, D. R.; Nwomeh, B. C. The proteolytic environment of chronic wounds. *Wound Repair Regen.* **1999**, *7*, 433–441.
(3) Cutting, K. F. Wound exudate: composition and functions. *Br. J. Community Nurs.* **2003**, *8* (Suppl. 9), 4–9.
(4) Fernandez, M. L.; Broadbent, J. A.; Shooter, G. K.; Malda, J.; Upton, Z. Development of an enhanced proteomic method to detect prognostic and diagnostic markers of healing in chronic wound fluid. *Br. J. Dermatol.* **2008**, *158*, 281–290.
(5) Mast, B. A.; Schultz, G. S. Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen.* **1996**, *4*, 411–420.

(6) Pollins, A. C.; Friedman, D. B.; Nanney, L. B. Proteomic investigation of human burn wounds by 2D-difference gel electrophoresis and mass spectrometry. *J. Surg. Res.* **2007**, *142*, 143–152.
(7) Conti, A.; Sanchez-Ruiz, Y.; Bachi, A.; Beretta, L.; Grandi, E.; Beltramo, M.; Alessio, M. Proteome study of human cerebrospinal fluid following traumetic brain injury indicates fibrin(ogen) degradation products as traume-associated markers. *J. Neurotrauma* **2004**, *21*, 854–863.
(8) Sinha, A.; Srivastava, N.; Singh, S.; Singh, A. K.; Bhushan, S.; Shukla, R.; Singh, M. P. Identification of differentially displayed proteins in cerebrospinal fluid of Parkinson's disease patients: A proteomic approach. *Clin. Chim. Acta* **2009**, *400*, 14–20.
(9) Shitama, T.; Hayashi, H.; Noge, S.; Uchio, E.; Oshima, K.; Haniu, H.; Takemori, N.; Komori, N.; Matsumoto, H. Proteome profiling of vitreoretinal diseases by cluster analysis. *Proteomics Clin. Appl.* **2008**, *2*, 1265–1280.
(10) Kuk, C.; Kulasingam, V.; Gunawardana, C. G.; Smith, C. R.; Batruch, I.; Diamandis, E. P. Mining the ovarian cancer ascites proteome for potential ovarian cancer biomarkers. *Mol. Cell. Proteomics* **2009**, *8*, 661–669.
(11) Lin, W. T.; Tsai, C. C.; Chen, C. Y.; Lee, W. J.; Su, C. C.; Wu, Y. J. Proteomic analysis of peritoneal dialysate fluid in patients with dialysis-related peritonitis. *Ren. Fail.* **2008**, *30*, 772–777.
(12) Tabushi, Y.; Nakanishi, T.; Takeuchi, T.; Nakajima, M.; Ueda, K.; Kotani, T.; Makino, S.; Shimizu, A.; Hanafusa, T.; Takubo, T. Detection of citrullinated proteins in synovial fluids derived from patients with rheumatoid arthritis by proteomics-based analysis. *Ann. Clin. Biochem.* **2008**, *45*, 413–417.
(13) Gray, R. D.; MacGregor, G.; Noble, D.; Imrie, M.; Dewar, M.; Boyd, A. C.; Innes, J. A.; Porteous, D. J.; Greening, A. P. Sputum proteomics in inflammatory and suppurative respiratory diseases. *Am. J. Resp. Crit. Care Med.* **2008**, *178*, 444–452.
(14) Wu, J.; Kobayashi, M.; Sousa, E. A.; Liu, W.; Cai, J.; Goldman, S. J.; Dorner, A. J.; Projan, S. J.; Kavuru, M. S.; Qui, Y.; Thomassen, M. J. Differential proteomic analysis of bronchoalveolar lavage fluid in asthmatics following segmental antigen challenge. *Mol. Cell. Proteomics* **2005**, *4*, 1251–1264.
(15) Zhou, L.; Beuerman, R. W.; Huang, L.; Barathi, A.; Foo, Y. H.; Li, S. F.; Chew, F. T.; Tan, D. Proteomic analysis of rabbit tear fluid: Defensin levels after an experimental corneal wound are correlated with wound closure. *Proteomics* **2007**, *7*, 3194–3206.
(16) Merrick, B. A.; Bruno, M. E.; Madenspacher, J. H.; Wetmore, B. A.; Foley, J.; Pieper, R.; Zhao, M.; Makusky, A. J.; McGrath, A. M.; Zhou, J. X.; Taylor, J.; Tomer, K. B. Alterations in the rat serum proteome during liver injury from acetaminophen exposure. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 792–802.
(17) Quian, W. J.; Jacobs, J. M.; Camp, D. G.; Monroe, M. E.; Moore, R. J.; Gritsenko, M. A.; Calvano, S. E.; Lowry, S. F.; Xiao, W.; Moldawer, L. L.; Davis, R. W.; Tompinks, R. G.; Smith, R. D. Comparative proteome analyses of human plasma following *in vivo* lipopolysaccharide administration using multidimensional separations coupled with tandem mass spectrometry. *Proteomics* **2005**, *5*, 572–584.
(18) Li, S. J.; Peng, M.; Li, H.; Liu, B. S.; Wang, C.; Wu, J. R.; Li, Y. X.; Zeng, R. Sys-Body Fluid: a systematic database for human body fluid proteome research. *Nucleic Acids Res.* **2009**, *37*, D907–D912.
(19) Gutiérrez, J. M.; Lomonte, B. Efectos locales en el envenenamiento ofídico en América Latina. In: *Animais Peçonhentos no Brasil. Biología, Clínica e Terapêutica dos Acidentes*; Cardoso, J. L. C., França, F. O. S., Wen, F. H., Málaque, C. M. S.;, Haddad, V., Eds.; Sarvier: Sao Paulo, 2003; pp 310−323.
(20) Otero, R.; Gutiérrez, J.; Mesa, M. B.; Duque, E.; Rodríguez, O.; Arango, J. L.; Gómez, F.; Toro, A.; Cano, F.; Rodríguez, L. M.; Caro, E.; Martínez, J.; Cornejo, W.; Gómez, L. M.; Uribe, F. L.; Cárdenas, S.; Núñez, V.; Díaz, A. Complications of *Bothrops*, *Porthidium* and *Bothriechis* snakebites in Colombia. A clinical and epidemiological study of 39 cases attended in a university hospital. *Toxicon* **2002**, *40*, 1107–1114.
(21) Warrell, D. A. Epidemiology, clinical features and management of snake bites in Central and South America. In *Venomous Reptiles of the Western Hemisphere*; Campbell, J., Lamar, W. W., Eds.; Cornell University Press: Ithaca, 2004; pp 709−761.
(22) Gutiérrez, J. M.; Theakston, R. D. G.; Warrell, D. A. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. *PLoS Med.* **2006**, *3*, e412.
(23) Gutiérrez, J. M.; Rucavado, A. Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage. *Biochimie* **2000**, *82*, 841–850.
(24) Gutiérrez, J. M.; Ownby, C. L. Skeletal muscle degeneration induced by venom phospholipases A_2: insights into the mechanisms of local and systemic myotoxicity. *Toxicon* **2003**, *42*, 915–931.
(25) Fox, J. W.; Serrano, S. M. T. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon* **2005**, *45*, 969–985.
(26) Bjarnason, J. B.; Fox, J. W. Hemorrhagic metalloproteinases from snake venoms. *Pharmac. Ther.* **1994**, *62*, 325–372.
(27) Gutiérrez, J. M.; Rucavado, A.; Escalante, T.; Díaz, C. Hemorrhage induced by snake venom metalloproteinases: biochemical and biophysical mechanisms involved in microvessel damage. *Toxicon* **2005**, *45*, 997–1011.
(28) Rucavado, A.; Núñez, J.; Gutiérrez, J. M. Blister formation and skin damage induced by BaP1, a hemorrhagic metalloproteinase from the venom of the snake *Bothrops asper*. *Int. J. Exp. Pathol.* **1998**, *79*, 245–254.
(29) Jiménez, N.; Escalante, T.; Gutiérrez, J. M.; Rucavado, A. Skin pathology induced by snake venom metalloproteinase: acute damage, revascularization, and re-epithelization in a mouse ear model. *J. Invest. Dermatol.* **2008**, *128*, 2421–2428.
(30) Rucavado, A.; Lomonte, B.; Ovadia, M.; Gutiérrez, J. M. Local tissue damage induced by BaP1, a metalloproteinase isolated from *Bothrops asper* (terciopelo) snake venom. *Exp. Mol. Pathol.* **1995**, *63*, 186–199.
(31) Gutiérrez, J. M.; Romero, M.; Núñez, J.; Chaves, F.; Borkow, G.; Ovadia, M. Skeletal muscle necrosis and regeneration after injection of BaH1, a hemorrhagic metalloproteinase isolated from the venom of the snake *Bothrops asper* (terciopelo). *Exp. Mol. Pathol.* **1995**, *62*, 28–41.
(32) Teixeira, C. F. P.; Fernandes, C. M.; Zuliani, J. P.; Zamuner, S. F. Inflammatory effects of snake venom metalloproteinases. *Mem. Inst. Oswaldo Cruz* **2005**, *100* (Suppl. 1), 181–184.
(33) Gallagher, P.; Bao, Y.; Serrano, S. M. T.; Laing, G. D.; Theakston, R. D. G.; Gutiérrez, J. M.; Escalante, T.; Zigrino, P.; Moura-da-Silva, A. M.; Nischt, R.; Mauch, C.; Moskaluk, C.; Fox, J. W. Role of snake venom toxin jararhagin in proinflammatory pathogenesis: in vitro and in vivo gene expression analysis of the effects of the toxin. *Arch. Biochem. Biophys.* **2005**, *441*, 1–15.
(34) Montecucco, C.; Lomonte, B.; Gutiérrez, J. M. Cellular pathology induced by snake venom phospholipase A_2 myotoxins and neurotoxins: common aspects of their mechanisms of action. *Cell. Mol. Life Sci.* **2008**, *65*, 2897–2912.
(35) Chacur, M.; Longo, I.; Picolo, G.; Gutiérrez, J. M.; Lomonte, B.; Guerra, J. L.; Teixeira, C. F. P.; Cury, Y. Hyperalgesia induced by Asp49 and Lys49 phospholipases A_2 from *Bothrops asper* snake venom: pharmacological mediation and molecular determinants. *Toxicon* **2003**, *41*, 667–678.
(36) Mora, J.; Mora, R.; Lomonte, B.; Gutiérrez, J. M. Effects of *Bothrops asper* snake venom on lymphatic vessels: insights into a hidden aspect of envenomation. *PLoS Neglec. Trop. Dis.* **2008**, *2*, e318.
(37) Teixeira, C. F. P.; Landucci, E. C. T.; Antunes, E.; Chacur, M.; Cury, Y. Inflammatory effects of snake venom myotoxic phospholipases A_2. *Toxicon* **2003**, *42*, 947–962.
(38) Gutiérrez, J. M.; Romero, M.; Díaz, C.; Borkow, G.; Ovadia, M. Isolation and characterization of a metalloproteinase with weak hemorrhagic activity from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **1995**, *33*, 19–29.
(39) Watanabe, L.; Shannon, J. D.; Valente, R. H.; Rucavado, A.; Alape-Girón, A.; Kamiguti, A. S.; Theakston, R. D. G.; Fox, J. W.; Gutiérrez, J. M.; Arni, R. K. Amino acid sequence and crystal structure of BaP1, a metalloproteinase from Bothrops asper snake venom that exerts multiple tissue-damaging activities. *Protein Sci.* **2003**, *12*, 2273–2281.
(40) Gutiérrez, J. M.; Ownby, C. L.; Odell, G. V. Isolation of a myotoxin from *Bothrops asper* venom: Partial characterization and action on skeletal muscle. *Toxicon* **1984**, *22*, 115–128.
(41) Gutiérrez, J. M.; Ownby, C. L.; Odell, G. V. Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Exp. Mol. Pathol.* **1984**, *40*, 367–379.
(42) Gutiérrez, J. M.; Lomonte, B.; Chaves, F.; Moreno, E.; Cerdas, L. Pharmacological activities of a toxic phospholipase A isolated from the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* **1986**, *84*, 159–164.
(43) Bjorhall, K.; Miliotis, T.; Davidsson, P. Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *Proteomics* **2005**, *5*, 307–317.
(44) Boschetti, E.; Righetti, P. G. The art of observing rare protein species in proteomes with peptide ligand libraries. *Proteomics* **2009**, *9*, 1492–510.
(45) Chaves, F.; León, G.; Alvarado, V. H.; Gutiérrez, J. M. Pharmacological modulation of edema induced by Lys-49 and Asp-49 myotoxic phospholipases A_2 isolated from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **1998**, *36*, 1861–1869.

(46) Zuliani, J. P.; Fernández, C. M.; Zamuner, S. R.; Gutiérrez, J. M.; Teixeira, C. F. P. Inflammatory events induced by Lys-49 and Asp-49 phospholipases A_2 isolated from *Bothrops asper* snake venom: role in catalytic activity. *Toxicon* **2005**, *45*, 335–346.
(47) Fernandes, C. M.; Zamuner, S. R.; Zuliani, J. P.; Rucavado, A.; Gutiérrez, J. M.; Teixeira, C. F. P. Inflammatory effects of BaP1, a metalloproteinase isolated from *Bothrops asper* snake venom: leukocyte recruitment and release of cytokines. *Toxicon* **2006**, *47*, 549–559.
(48) Fernandes, C. M.; Teixeira, C. F. P.; Leite, A. C.; Gutiérrez, J. M.; Rocha, F. A. The snake venom metalloproteinase BaP1 induces joint hypernociception through TNF-α and PGE_2-dependent mechanisms. *Br. J. Pharmacol.* **2007**, *151*, 1254–1261.
(49) Gabay, C.; Kushner, I. Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.* **1999**, *340*, 448–454.
(50) van der Westhuyzen, D. R.; de Beer, F. C.; Webb, N. R. HDL cholesterol transport during inflammation. *Curr. Op. Lipidology* **2007**, *18*, 147–151.
(51) Jahangiri, A.; de Beer, M. C.; Noffsinger, V.; Tannock, L. R.; Ramaiah, C.; Webb, N. R.; van der Westhuyzen, D. R.; de Beer, F. C. HDL remodeling during the acute phase response. *Arterioscler. Thromb. Vasc. Biol* **2009**, *29*, 261–267.
(52) Rye, K. A.; Duong, M. N. Influence of phospholipids depletion on the size, structure, and remodeling of reconstituted high density lipoproteins. *J. Lipid Res.* **2000**, *41*, 1640–1650.
(53) Ownby, C. L.; Geren, C. R. Pathogenesis of hemorrhage induced by hemorrhagic proteinase IV from timber rattlesnake (*Crotalus horridus*). *Toxicon* **1987**, *25*, 517–526.
(54) Moreira, L.; Borkow, G.; Ovadia, M.; Gutiérrez, J. M. Pathological changes induced by BaH1, a hemorrhagic metalloproteinase isolated from *Bothrops asper* (terciopelo) snake venom, on mouse capillary blood vessels. *Toxicon* **1994**, *32*, 977–987.
(55) Markland, F. S. Snake venoms and the hemostatic system. *Toxicon* **1998**, *36*, 1749–1800.
(56) Rucavado, A.; Escalante, T.; Teixeira, C. F. P.; Fernandes, C. M.; Díaz, C.; Gutiérrez, J. M. Increments in cytokines and matrix metalloproteinases in skeletal muscle after injection of tissue-damaging toxins from the venom of the snake *Bothrops asper*. *Med. Inflamm.* **2002**, *11*, 121–128.
(57) Ohsaka, A. Hemorrhagic, necrotizing and edema-forming effects of snake venoms. In: *Handbook of Experimental Pharmacology*, Vol. 52; Lee, C. Y., Ed.; Springer Verlag: Berlin, 1979; pp 480−546.
(58) Baramova, E. N.; Shannon, J. D.; Bjarnason, J. B.; Fox, J. W. Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. *Arch. Biochem. Biophys.* **1989**, *275*, 63–71.
(59) Escalante, T.; Shannon, J.; Moura-da-Silva, A. M.; Gutiérrez, J. M.; Fox, J. W. Novel insights into capillary vessel basement membrane damage by snake venom hemorrhagic metalloproteinases: A biochemical and immunohistochemical study. *Arch. Biochem. Biophys.* **2006**, *455*, 144–153.
(60) Pinto, A. F.; Ma, L.; Dragulev, B.; Guimaraes, J. A.; Fox, J. W. Use of SILAC for exploring sheddase and matrix degradation of fibroblasts in culture by the PIII SVMP atrolysin A: identification of two novel substrates with functional relevance. *Arch. Biochem. Biophys.* **2007**, *465*, 11–15.
(61) Serrano, S. M. T.; Kim, J.; Wang, D.; Dragulev, B.; Shannon, J. D.; Mann, H. H.; Veit, G.; Wagener, R.; Koch, M.; Fox, J. W. The cysteine-rich domain of snake venom metalloproteinases is a ligand for von Willebrand factor A domains: role in substrate targeting. *J. Biol. Chem.* **2006**, *281*, 39746–39756.
(62) Jimenez-Mallebrera, C.; Maioli, M. A.; Kim, J.; Brown, S. C.; Feng, L.; Lampe, A. K.; Bushby, K.; Hicks, D.; Flanigan, K. M.; Bonnemann, C.; Sewry, C. A.; Muntoni, F. A comparative analysis of collagen VI production in muscle, skin and fibriblasts from 14 Ullrich congenital muscular dystrophy patients with dominant and recessive *COLA6* mutations. *Neurom. Disord.* **2006**, *16*, 571–582.
(63) Kuo, H. J.; Maslen, C. L.; Keene, D. R.; Glanville, R. W. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. *J. Biol. Chem.* **1997**, *42*, 26522–26529.
(64) Nikitovic, D.; Katonis, P.; Tsatsakis, A.; Karamanos, N. K.; Tsanakakis, G. N. Lumican, a small leucine-rich proteoglycan. *IUBMB Life* **2008**, *60*, 818–823.

PR900489M