UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E

MOLECULAR

Mecanismos de Toxicidade Sistêmica no Envenenamento pela Taturana *Lonomia obliqua*: Alterações Fisiopatológicas Renais e Vasculares na Nefrotoxicidade Induzida pelo Veneno

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

Área de concentração: Biologia Celular e Molecular

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"VIVER como si fosse morrer amanhã—
ESTUDAR como si fosse viver sempre".

Sto. Isidoro de Sevilha

Para minha mãe

AGRADECIMENTOS

"Além dos laços que se formam entre um pesquisador e seus contemporâneos, há os que o ligam àqueles que, antes dele, tiveram igual interesse pela ciência. Ele continua o caminho desbravado pelos seus antecessores ... É como se olhasse para trás e se sentisse preso aos que o precederam, como se fizesse parte de uma família ligada não pelo sangue mas pelos liames afetivos do mesmo ideal ..."

Cannon, W.B. - **The way of an Investigator**, W.N. Norton & Co. New York, 1945 pag. 83.

"...In practical terms, the discovery of bradykinin had a strong bearing on the problem of mediation in acute inflammation, in shock by venoms and toxins, on the mechanism of anaphylaxis, and opened a new perspective on the therapeutics of circulatory affections".

Maurício Oscar da Rocha e Silva. **Kinin Hormones. With Special Reference to Bradykinin and Related Kinins**. Springfield, IL: Charles C. Thomas, 1970.

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LISTA DE ABREVIATURAS

$\alpha_2 AP$	α_2-Antiplasmina
Arg	Arginina
BK	Bradicinina (do inglês, *Bradykinin*)
DABK	des-Arg^9-BK
DALBK	des-Arg^9-Leu^8-BK
CIT/RS	Centro de Informações Toxicológicas do Rio Grande do Sul
CIT/SC	Centro de Informações Toxicológicas de Santa Catarina
CIVD	Coagulação intravascular disseminada
CTGF	Fator de crescimento do tecido conjuntivo (do inglês, *connective tissue growth factor*)
Da	Dalton
D-D	D-Dímeros
ECA	Enzima conversora de angiotensina
EROs	Espécies reativas de oxigênio
F_{1+2}	Fragmento 1+2 da ativação de protrombina
Fg	Fibrinogênio
Hb	Hemoglobina
HO-1	Heme-oxigenase 1
HOE-140	D-Arg^0-[Hyp^3,Thi^5, D-Tic^7, Oic^8]-BK, (Icatibant)
HMWK	Cininogênio de alto peso molecular (do inglês, *high molecular weight kininogen*)
Hpt	Haptoglobina
Hpx	Hemopexina
IL-1β	Interleucina-1β
IL-4	Interleucina-4
IL-8	Interleucina-8
IL-10	Interleucina-10
IRA	Insuficiência renal aguda
kDa	Quilodaltons
Leu	Leucina
LMWH	Cininogênio de baixo peso molecular (do inglês, *low molecular weight kininogen*)

L-NAME	Metiléster de N^G-nitro-L-arginina (do inglês, N^G-nitro-L-arginine-methyl-ester)
LOPAP	Ativador de protrombina de *Lonomia obliqua* (do inglês, *Lonomia obliqua Prothrombin Activator Protease*)
LOSAC	Ativador de fator X de *Lonomia obliqua* (do inglês, *Lonomia obliqua Stuart-factor Activator*)
LPS	Lipopolissacarídeo
MAPK	Proteína ativada por mitógeno (do inglês, *mitogen-activated protein kinase*)
Mb	Mioglobina
MCP-1	Quimiocina envolvida no recrutamento de monócitos (do inglês, *monocyte chemoattractant protein-1*)
MCP-3	Quimiocina envolvida no recrutamento de monócitos (do inglês, *monocyte chemoattractant protein-3*)
MM	Massa molecular
NO	Óxido nítrico (do inglês, *nitric oxide*)
NOS	Óxido nítrico sintase (do inglês, *nitric oxide synthase*)
OMS	Organização Mundial da Saúde
PA	Ativador de plasminogênio (do inglês, *plasminogen activator*)
PAI-1	Inibidor do ativador de plasminogênio-1 (do inglês, p*lasminogen activator inhibitor-1*)
PC	Proteína C
P_{CG}	Pressão no capilar glomerular
PDF	Produto de degradação de fibrina
PGI_2	Prostaglandina I_2
PK	Precalicreína (do inglês, p*re-kallikrein*)
Plg	Plasminogênio
RFG	Ritmo de filtração glomerular
SCC	Sistema calicreína-cininas
TAT	Complexo trombina-antitrombina (do inglês, *thrombin-antithrombin complex*)
TF	Fator tecidual ou tissular (do inglês, *tissue factor*)
TFPI	Inibidor da via do fator tecidual (do inglês, *tissue factor pathway inhibitor*)

TGF-β	Fator de crescimento transformante (do inglês, *transforming growth factor - β*)
TNF-α	Fator de necrose tumoral-α (do inglês, *tumor necrosis factor-α*)
TP	Tempo de Protrombina
tPA	Ativador de plasminogênio tipo tecidual (do inglês, *tissue type plasminogen activator*)
TTPa	Tempo de Tromboplastina Parcialmente ativada
TT	Tempo de Trombina
TXA$_2$	Tromboxana A$_2$
uPA	Ativador de plasminogênio tipo uroquinase (do inglês, *urokinase type plasminogen activator*)
vWF	Fator de von Willebrand (do inglês, *von Willebrand factor*)

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RESUMO

O envenenamento por animais peçonhentos é considerado uma doença ocupacional comum e grave, especialmente em áreas rurais de países tropicais em desenvolvimento. Sua importância para a saúde pública tem sido largamente ignorada pelas autoridades médicas em todo o mundo. Apesar dos soros antivenenos serem produzidos por vários laboratórios distribuídos em todos os continentes, o alto número de acidentes, a mortalidade e a morbidade associadas a eles continuam tendo um grande impacto sobre a população e os sistemas de saúde desses países. Especificamente nas regiões do sul do Brasil, os acidentes com lagartas venenosas da espécie *Lonomia obliqua* têm sido um problema crescente, não somente pelas altas taxas de incidência, mas também pela severidade das consequências clínicas observadas nos casos de envenenamento. Frequentemente, as vítimas envenenadas apresentam uma síndrome hemorrágica grave, que pode evoluir para insuficiência renal aguda (IRA), hemorragia intracraniana e morte. O presente trabalho foi desenvolvido com o objetivo de investigar os mecanismos fisiopatológicos induzidos pela toxicidade sistêmica do veneno de *L. obliqua*, dando atenção especial às alterações renais e vasculares que são descritas como as principais causas de morte neste tipo de envenenamento. Os resultados obtidos são aqui apresentados na forma de artigos científicos que abordam os seguintes aspectos: (*i*) o conhecimento atual sobre os componentes moleculares do veneno de *L. obliqua* e as características clínicas do envenenamento; (*ii*) a caracterização das principais alterações histopatológicas, hematológicas, bioquímicas e genotóxicas em diferentes órgãos e a capacidade da soroterapia antiveneno em neutralizar os efeitos tóxicos sistêmicos do veneno de *L. obliqua* em um modelo experimental *in vivo* em ratos; (*iii*) a epidemiologia, a fisiopatologia e o manejo terapêutico da IRA induzida por diferentes venenos nefrotóxicos; (*iv*) os mecanismos envolvidos na IRA induzida pelo envenenamento experimental com *L. obliqua*; e (*v*) a contribuição dos componentes do sistema calicreína-cininas (SCC) para a instabilidade hemodinâmica, a inflamação e o consequente comprometimento renal observado no envenenamento experimental com *L. obliqua*. Resumidamente, os dados obtidos permitiram-nos indicar que a injeção subcutânea do veneno de *L. obliqua* em ratos induz alterações hemostáticas severas e danos em múltiplos órgãos, principalmente nos pulmões, coração, rins e baço. Há também evidências de que o veneno possui atividade cardiotóxica, miotóxica e genotóxica. Além disso, o tratamento com soro só é eficaz em neutralizar os efeitos

fisiopatológicos sistêmicos se for administrado durante a fase inicial de envenenamento. Em relação ao mecanismo da IRA induzida pelo veneno, este parece ser complexo e multifatorial envolvendo três aspectos principais: a citotoxicidade tubular derivada da ação do heme; as alterações vasculares, incluindo hipotensão sistêmica, aumento de permeabilidade vascular e deposição de fibrina nos capilares glomerulares; e a ativação do SCC renal. Em conjunto, estes mecanismos estão diretamente relacionados com as alterações funcionais observadas em ratos envenenados, como a hipoperfusão renal, inflamação, necrose tubular e a perda súbita das funções básicas do rim, incluindo perda da capacidade de filtração e excreção, concentração de urina e manutenção do equilíbrio hídrico. As ativações da calicreína renal e do receptor B1 de bradicinina (B1R) desempenham papel fundamental na IRA induzida por *L. obliqua*, pois tanto o bloqueio farmacológico do B1R quanto a inibição da calicreína são capazes de prevenir as alterações funcionais e histopatológicas renais observadas nos ratos envenenados. Os principais mecanismos envolvidos nesses efeitos benéficos estão associados com uma diminuição da resposta inflamatória renal (redução da migração de células proinflamatórias e dos níveis de citocinas proinflamatórias) e da degeneração tubular. Portanto, o conjunto de todos os resultados aqui mostrados permitiram fornecer evidências consistentes que relacionam a ativação do SCC e a IRA induzida por *L. obliqua* e também indicar que a inibição de componentes do SCC, principalmente a inibição de calicreína ou o antagonismo do B1R, podem ser alternativas terapêuticas para o controle da progressão da lesão renal nos casos de envenenamento.

ABSTRACT

The envenomation by venomous animals is considered a common and serious occupational disease, especially in rural areas of tropical developing countries. Its public health relevance has been largely ignored by medical authorities worldwide. Although antivenoms are produced by various laboratories in every continent, the burden of envenomation cases, causing both morbidity and mortality, still has a major impact on the population and on health-care systems. Specifically in regions of southern Brazil, accidents with the venomous caterpillar *Lonomia obliqua* have been an emergent problem, not only for their high incidence rates, but also by the severity of the clinical consequences observed in envenomation cases. Frequently, envenomed victims present a severe hemorrhagic syndrome that can progress to acute kidney injury (AKI), intracranial hemorrhage and death. Through this work we aimed to investigate the physiopathological mechanisms induced by the systemic toxicity of *L. obliqua* venom, with particular attention to renal and vascular disorders that are described as the leading cause of death in this type of envenomation. Results are presented here as scientific articles that address the following aspects: (*i*) the current knowledge about the molecular components of *L. obliqua* venom and the clinical features of envenomation; (*ii*) the characterization of the main histopathological, hematological, biochemical and genotoxic alterations in different organs and the ability of antivenom serotherapy to counteract the systemic toxic effects of *L. obliqua* venom using an *in vivo* experimental model in rats; (*iii*) some aspects of epidemiology, physiopathology and management of AKI induced by different nephrotoxic venoms; (*iv*) the mechanisms involved in experimental *L. obliqua*-induced AKI; and (*v*) the contribution of kallikrein-kinin system (KKS) components to the hemodynamic instability, inflammation and the consequent renal functional impairment during *L. obliqua* experimental envenomation. In summary, the data indicate that the subcutaneous injection of *L. obliqua* venom in rats induce severe hemostatic abnormalities and multi-organ damage, mainly in the lungs, heart, kidneys and spleen. There are also evidences of cardiotoxic, myotoxic and genotoxic activities in *L. obliqua* venom. Moreover, treatment with antivenom is only effective at counteracting the systemic physiopathological effects if it is administered during the initial phase of envenomation. Regarding the mechanism of venom-induced AKI, it seems to be complex and multifactorial involving three main issues: the heme-derived tubular cytotoxicity; vascular alterations, including systemic hypotension,

increase in vascular permeability and glomerular fibrin deposition; and the activation of renal KKS. Acting together these mechanisms are directly related to the functional alterations observed in envenomed rats such as renal hypoperfusion, inflammation, tubular necrosis and the sudden loss of basic renal functions, including filtration and excretion capacities, urinary concentration and maintenance of body fluid homeostasis. The activation of renal kallikrein and the bradykinin receptor B1 (B1R) play a crucial role in *L. obliqua*-induced AKI, because both the pharmacological blockade of B1R or kallikrein inhibition are able to prevent the renal functional and histopathological alterations observed in the kidneys of envenomed rats. The main mechanisms underlying these beneficial effects are associated with a decrease in renal inflammatory response (reduction of pro-inflammatory cell migration and pro-inflammatory cytokine levels) and tubular degeneration. Thus, the set of all findings allowed us to show a consistent evidence linking KKS activation with the *L. obliqua*-induced AKI and indicate that the inhibition of KKS components, mainly kallikrein inhibition or B1R antagonism, could be a therapeutic alternative to control the progression of renal injury in the cases of such envenomation.

APRESENTAÇÃO

O fascínio e o interesse pelos venenos animais é milenar. No entanto, somente no século XIX a composição dos venenos começou a ser melhor compreendida. Importantes contribuições nessa área foram feitas por brasileiros como Vital Brasil, um dos grandes responsáveis pelo desenvolvimento da toxinologia em nosso país. Desde então, as pesquisas básicas sobre a bioquímica e a farmacologia dos venenos contribuíram significativamente para a descoberta de novas substâncias com potencial aplicação terapêutica e, também, para o entendimento da fisiopatologia de inúmeras doenças. Exemplo clássico é a bradicinina, descoberta por pesquisadores brasileiros quando estudavam o mecanismo de ação do veneno de *Bothrops jararaca* (ROCHA E SILVA *et al.*, 1949). Hoje se sabe que essa descoberta conduziu não só a um novo campo de investigação científica, mas também à compreensão de diferentes patologias e ao desenvolvimento de uma das classes de anti-hipertensivos mais utilizadas no mundo inteiro (FERREIRA, 1965; ONDETTI *et al.*, 1971; CUSHMAN *et al.*, 1973).

Dentro deste contexto e com a intenção de contribuir para a geração de conhecimento em toxinologia, a presente tese de doutorado versa sobre a fisiopatologia dos envenenamentos e, em particular, busca caracterizar os mecanismos de toxicidade sistêmica e aqueles envolvidos na disfunção renal induzida pelo veneno da taturana *Lonomia obliqua* em modelos de experimentação animal. Os trabalhos aqui apresentados fazem parte do projeto intitulado "Os Venenos Animais: do Diagnóstico ao Tratamento, da Estrutura ao Mecanismo de Ação das Toxinas", e foram desenvolvidos na vigência de auxílio concedido pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) do Ministério da Educação (Toxinologia/Processo 23038.006277/2011-85). Grande parte da etapa experimental foi realizada no Laboratório de Bioquímica Farmacológica do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul (UFRGS) e no Laboratório de Fisiologia Renal do Departamento de Fisiologia e Biofísica da Universidade Federal de Minas Gerais (UFMG). Os experimentos com animais seguiram as atribuições e competências definidas na Lei 11.794/08 e em resoluções do Conselho Nacional de Controle em Experimentação Animal (CONCEA). Os protocolos utilizados foram previamente aprovados pela comissão de ética no uso de animais em pesquisa de ambas as instituições onde os experimentos foram realizados.

A tese está dividida em três partes principais: introdução, resultados e discussão. A primeira delas é destinada a uma breve revisão acerca dos conhecimentos necessários para o entendimento da segunda parte, onde são apresentados os resultados obtidos na forma de artigos científicos. Dois desses artigos tratam-se de revisão da literatura científica. Um deles versa sobre aspectos tóxico-farmacológicos do veneno da taturana e o outro sobre os mecanismos da insuficiência renal aguda induzida por veneno de animais peçonhentos. Outros três artigos, contendo dados experimentais, também fazem parte da seção de resultados e versam sobre: (*i*) os mecanismos de toxicidade sistêmica no envenenamento por *L. obliqua*, descrição de novas atividades e efeitos do soro antiveneno; (*ii*) os mecanismos da insuficiência renal aguda induzida por *L. obliqua*; e (*iii*) a participação do sistema calicreína-cininas na disfunção renal induzida pelo veneno. Por fim, na terceira parte da tese, os resultados obtidos são melhor explorados e discutidos. Na proposição desta terceira parte, é objetivo auxiliar a integração dos resultados e a geração de perspectivas para futuras pesquisas.

1. INTRODUÇÃO

1. INTRODUÇÃO

1.1 Os acidentes com animais peçonhentos e sua relevância para a saúde pública

O envenenamento por animais peçonhentos constitui um importante problema de saúde pública em muitas regiões, particularmente em áreas tropicais e subtropicais (GUIMARÃES, 2011; GUTIÉRREZ *et al.*, 2006). Considerando somente os acidentes com serpentes, estima-se que cerca de 5,4 a 5,5 milhões de pessoas são vitimadas por ano no mundo, resultando em 400.000 amputações e entre 20.000 e 125.000 mortes (CHIPPAUX, 1998, KASTURIRATNE *et al.*, 2008; WILLIAMS *et al.*, 2010). Certamente, esses números são subestimados, pois, além dos problemas de notificação, eles não incluem os acidentes com outros animais peçonhentos e venenosos. Interessante notar que, apesar dos acidentes não possuírem um potencial epidêmico como o de doenças infecciosas ou parasitárias, as taxas de mortalidade chegam a superar as de algumas moléstias tropicais reconhecidas pela Organização Mundial da Saúde (OMS) como doenças negligenciadas **(Tabela 1)**. Em razão disso, a partir de 2009, a OMS incluiu os acidentes com animais peçonhentos na lista de doenças negligenciadas e, desde então, iniciativas globais de combate ao problema vêm sendo estimuladas (GUTIÉRREZ *et al.*, 2013).

A ocorrência dos acidentes é particularmente grave para os habitantes de áreas rurais que são a parte da população mais atingida devido ao seu ambiente específico de trabalho, o que lhes proporciona um contato íntimo de mãos e pés, usualmente desprotegidos, contra uma gama bastante variada de espécies de animais, inclusive os venenosos (MINISTÉRIO DA SAÚDE, 1998; GARCIA & DANNI-OLIVEIRA, 2007). Vale ressaltar que a população rural desempenha um exaustivo trabalho agrário, o que exige saúde e integridade física. Já em 1897, Vital Brazil, atendendo pacientes em fazendas no interior de São Paulo, impressionava-se com o número elevado de pacientes picados por serpentes venenosas. A partir desta época, buscando desenvolver medicação eficaz que pudesse ser aplicada a tais pacientes, Vital Brazil e outros pesquisadores deram início aos estudos básicos sobre a bioquímica e a farmacologia dos venenos animais, o que acabou gerando o conhecimento científico necessário ao desenvolvimento dos soros antivenenos para o tratamento das vítimas (BARRAVIERA, 1999).

Tabela 1. Comparação da incidência e mortalidade nos acidentes com serpentes com algumas doenças negligenciadas reconhecidas pela OMS.

	Incidência	Número de 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
Esquistossomose	5.733.000	15.000
Envenenamento por serpentes	420.000-2.682.000	20.000-125.000
Febre amarela	100-2.100	60-100

Fonte: WILLIAMS *et al.*, 2010. Lista completa de doenças negligenciadas pode ser encontrada em: http://www.who.int/neglected_diseases/diseases/en/.

Atualmente, a utilização dos antivenenos produzidos nos Institutos Butantan (São Paulo, SP), Vital Brasil (Niterói, RJ) e Fundação Ezequiel Dias (Belo Horizonte, MG), vem reduzindo significativamente o número de óbitos ocasionados por acidentes com animais peçonhentos. Desde a implantação do Programa Nacional de Controle dos Acidentes por Animais Peçonhentos em 1986, todos os soros produzidos no Brasil são adquiridos pelo Ministério da Saúde e distribuídos às secretarias estaduais de saúde que, por sua vez, definem os pontos estratégicos para atendimento dos acidentados e utilização correta e racional dos antivenenos (MINISTÉRIO DA SAÚDE, 2005). Todavia, por falta de um levantamento epidemiológico preciso e problemas de distribuição, nem sempre tais produtos estão disponíveis em localidades distantes onde mais frequentemente os acidentes ocorrem, sendo, também, ainda muito elevados os índices de sequelas decorrentes das lesões geradas por tais envenenamentos (FAN & CARDOSO, 1995).

Especificamente no Brasil, o Sistema de Vigilância Sanitária do Ministério da Saúde estimou a ocorrência de 107.364 acidentes com animais peçonhentos (incluindo casos de envenenamento por serpentes, aranhas, escorpiões, abelhas e lagartas) que resultaram em 290 mortes somente no ano de 2009 (BOLETIM ELETRÔNICO EPIDEMIOLÓGICO, 2010). No mesmo ano, o Centro de Informações Toxicológicas do estado do Rio Grande do Sul (CIT/RS) registrou 5.912 acidentes e 5 óbitos

(NICOLELLA *et al.*, 2006-2012). No período de 2008–2012, um total de 28.895 acidentes foram notificados no Rio Grande do Sul, sendo 34 % destes acidentes causados por aranhas, 20 % por lagartas e 16 % por serpentes **(Tabela 2)**. Armadeiras (*Phoneutria* sp.), aranhas-marron (*Loxosceles* sp.), taturanas (*Lonomia* sp., *Dirphia* sp. e *Hylesia* sp.) e jararacas (*Bothrops* sp.) foram os principais animais envolvidos.

Tabela 2. Número de acidentes com animais peçonhentos registrados nos últimos 5 anos no Rio Grande do Sul.

Animais	Ano				
	2008	2009	2010	2011	2012
Animais aquáticos	10	12	23	26	21
Aranhas	1.917	1.922	2.196	1.996	1.916
Escorpiões	337	410	403	402	495
Outros Insetos	299	275	279	279	410
Lagartas	1.375	1.068	1.166	962	866
Serpentes	977	1.136	977	863	807
Outros	262	237	284	307	382
Não identificados	596	852	848	652	650
TOTAL	5.773	5.912	6.176	5.487	5.547

Fonte: Relatório Anual de Atendimento do Centro de Informação Toxicológica do Rio Grande do Sul (CIT/RS), Secretaria de Estado da Saúde do Rio Grande do Sul. Dados disponíveis em: http://www.cit.rs.gov.br.

O registro de acidentes com lagartas começou a aumentar no Rio Grande do Sul a partir de 1989 quando casos de hemorragia sistêmica foram observados em várias vítimas que tiveram contato com animais da espécie *Lonomia obliqua* (Lepidoptera, Saturniidae). Na mesma época, vários casos semelhantes também foram registrados em Santa Catarina e Paraná (DUARTE *et al.,* 1990). Os acidentes foram particularmente preocupantes entre 1989-1995, período que antecedeu o desenvolvimento do soro antiveneno específico, quando os níveis de letalidade chegaram a ser de 3 a 6 vezes maiores do que os observados em envenenamentos por serpentes (DIAZ, 2005; ABELLA *et al.*, 2006). O quadro clínico resultante do contato com *L. obliqua* é

caracterizado por uma síndrome hemorrágica com a presença de equimoses, hematúria, sangramento das mucosas e, em casos graves, hemorragia intracraniana e insuficiência renal aguda (PINTO *et al.*, 2010; VEIGA *et al.*, 2009). Nas seções subsequentes, serão apresentados detalhes da epidemiologia dos acidentes, fisiopatologia do envenenamento e composição do veneno de *L. obliqua*.

1.2 Os acidentes com a taturana *Lonomia obliqua*

1.2.1 Dados epidemiológicos

Os insetos da ordem Lepidóptera compreendem as borboletas (animais de hábitos diurnos) e as mariposas (animais de hábitos noturnos), existindo no Brasil cerca de 50.000 espécies diferentes. Em seu desenvolvimento, estes insetos passam pelas fases de ovo, larva ou lagarta, pupa ou crisálida e adulto, ou seja, apresentam evolução completa ou holometabólica (CARDOSO & HADDAD, 2005).

Quando na fase larval, são conhecidas popularmente por taturanas, bichos-cabeludos, lagartas-de-fogo, entre outras denominações. Nesta fase, alguns Lepidópteros são responsáveis por envenenamentos em humanos, cujos sintomas mais comuns são reações cutâneas, urticária, dor e sensação de queimadura no local de contato, podendo ocorrer também distúrbios da coagulação sanguínea, hemorragias e insuficiência renal aguda (DIAZ, 2005). No Brasil, já na época da colonização, o padre Anchieta citou, na "Carta de São Vicente" de 1560, o medo dos índios frente a algumas lagartas que causavam tais acidentes, produzindo inúmeras reações e dor intensa após o contato físico (ROTBERG, 1971; COSTA, 1994). Esses animais eram chamados de "tatá-raná", nome que em Tupi-Guarani significa "como fogo" ou "semelhante ao fogo"; mais tarde este nome originou, na língua portuguesa, a palavra "taturana". Hoje se sabe que poucas das espécies encontradas no Brasil são potencialmente perigosas, sendo que apenas as famílias Saturniidae (gêneros *Automeris*, *Dirphia*, *Hylesia* e *Lonomia*), Megalopygidae (gêneros *Podalia* e *Megalopyge*) e Arctiidae (especialmente a espécie *Premolis semirufa*) estão envolvidas em acidentes e apresentam, portanto, interesse médico e preocupação de saúde pública (MINISTÉRIO DA SAÚDE, 1998).

Das diversas espécies existentes no Brasil, apenas duas da família Saturniidae e pertencentes ao gênero *Lonomia* têm chamado atenção devido ao grave quadro de síndrome hemorrágica que causam em vítimas que entram em contado com suas cerdas urticantes. O primeiro relato de acidente com essas espécies foi realizado pelo médico mineiro Zoroastro de Alvarenga em 1912, que descreveu o caso de um agricultor que apresentou saliva sanguinolenta e urina de coloração marrom escura durante 5 dias após o contato com uma colônia de lagartas (ZANNIN, 2002).

No período de 1978 a 1982, um estudo retrospectivo cobrindo as regiões do sudeste do Amapá ao oeste da Ilha de Marajó indicou a ocorrência de 36 casos de

síndrome hemorrágica resultantes do contato com taturanas. Neste relato, a mortalidade foi de 38% e as lagartas eram da espécie *L. achelous* (FRAIHA-NETO *et al.*, 1986; 1997). A mesma espécie é responsável por acidentes hemorrágicos registrados desde 1967 na Venezuela (AROCHA-PIÑANGO, 1967). Há, ainda, registros de acidentes com este inseto na Guiana Francesa, no Paraguai, no Peru e no Equador (AROCHA-PIÑANGO *et al.*, 1992).

A partir de 1989, acidentes semelhantes aos ocorridos no norte do Brasil e na Venezuela começaram a ser registrados também nos estados do sul do Brasil. Duarte *et al.* (1990) relatou dois casos atendidos em Passo Fundo (RS) em que as vítimas apresentaram, além dos episódios de sangramentos, insuficiência renal aguda, complicação essa até então desconhecida. Em Santa Catarina e no Rio Grande do Sul, foram identificadas como responsáveis pelos acidentes as lagartas da espécie *L. obliqua*. No período de 1997 a 2012, foram registrados pelo CIT/RS um total de 1.836 acidentes com a taturana *L. obliqua* somente no Rio Grande do Sul **(Figura 1)**. Deste total, 12 pacientes evoluíram para o óbito, resultando numa taxa de letalidade de 0,65% neste período (NICOLELLA *et al.*, 2006-2012; ABELLA *et al.*, 2006).
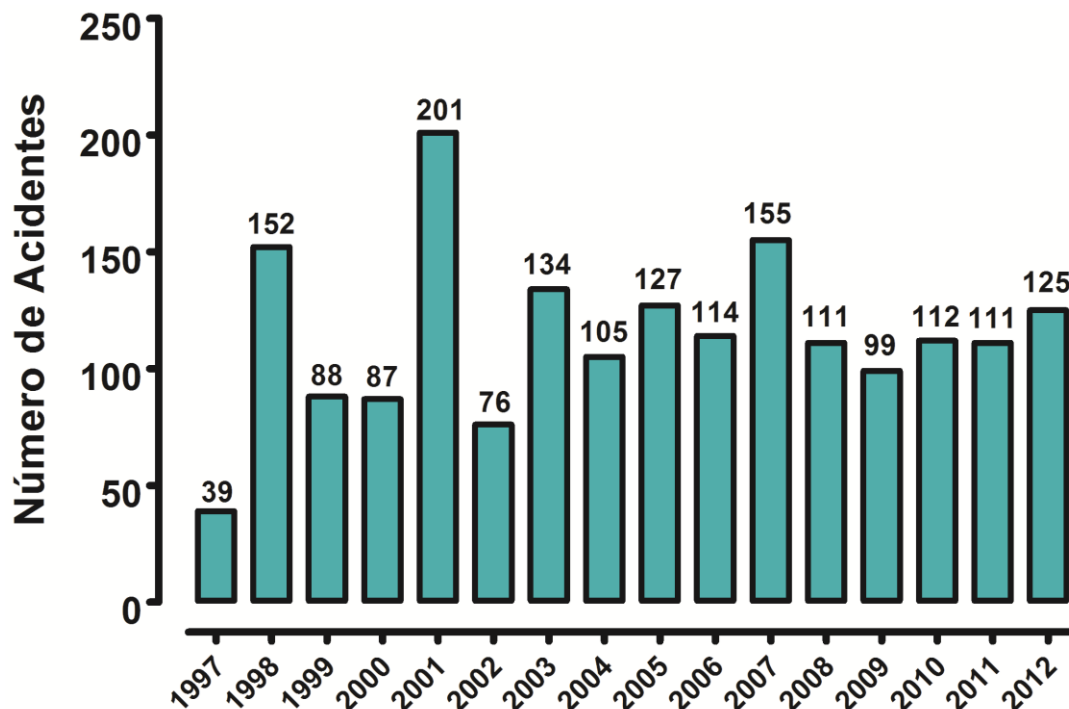
Figura 1. Números de acidentes causados por *Lonomia obliqua* **registrados pelo CIT/RS no período de 1997 a 2012**. Fonte: NICOLELLA *et al.,* 2006-2012; ABELLA *et al.,* 2006.

No Rio Grande do Sul, várias cidades **(Figura 2A)** têm registrado a ocorrência de acidentes ou identificação da presença da taturana. O somatório de dados retrospectivos (período de 1997 a 2005) permitiram demonstrar que 317 municípios do Estado são área de risco para acidentes com *Lonomia*, o que representa 64 % do total de municípios. Regiões do pampa e litoral são as de menor risco. Os maiores percentuais de notificações foram registrados em Passo Fundo, com 9,7 %, e em Bento Gonçalves, com 5,4 % dos casos **(Figura 2B)** (ABELLA *et al.*, 2006).
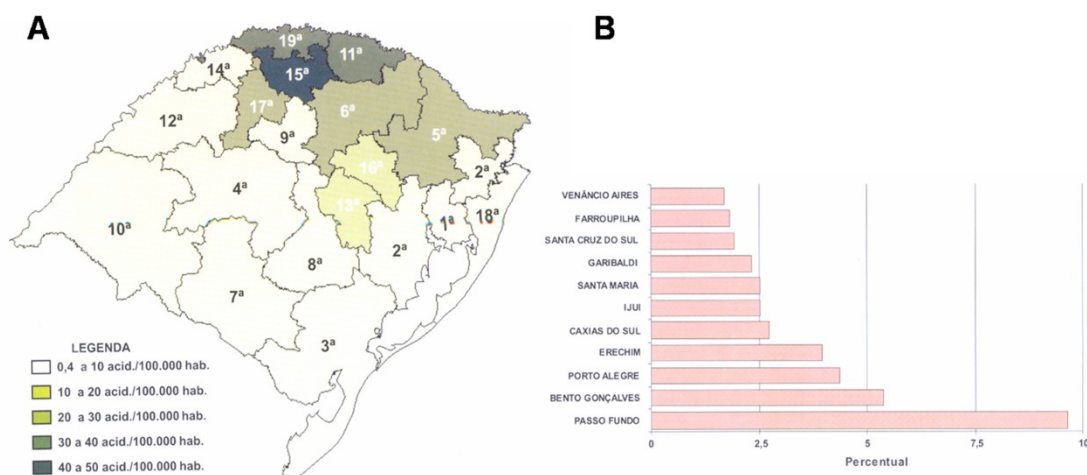
Figura 2. Localidade dos acidentes com *Lonomia obliqua* no Rio Grande do Sul. A. Incidência de acidentes com *L. obliqua* por 100.000 habitantes, nas respectivas Coordenadorias Regionais de Saúde. **B.** Percentual do número de acidentes causados por *L. obliqua* nas principais cidades do RS (1997-2005). Fonte: ABELLA *et al.*, 2006.

Já em Santa Catarina, no período de 1990 a 2001, foram registrados 1.851 acidentes que resultaram em seis óbitos (ZANNIN, 2002). No Paraná, entre 1989 e 2001, aconteceram 252 acidentes com o registro de cinco óbitos (taxa de letalidade de 1,9%) (GARCIA & DANNI-OLIVEIRA, 2007).

O perfil de sazonalidade dos acidentes com a lagarta *L. obliqua* é semelhante ao dos acidentes ofídicos. Um número maior de casos ocorre no verão, época em que o animal está na fase larval, o que coincide com a atividade agrícola mais intensa e com a época de férias, período em que as pessoas estão mais expostas e mantém maior contato com o ambiente (DIAZ, 2005). As regiões anatômicas mais frequentemente atingidas no contato com *Lonomia* são os membros superiores (78,1 %), principalmente as mãos, e membros inferiores (13,6 %), na sua maioria tornozelo e pé. Cabeça, tronco, dorso e

abdômen representam 8,3 % dos casos. Há predominância do sexo masculino (68 %) sobre o feminino (32 %) e os acidentes distribuem-se em todas as faixas etárias com um maior número de casos entre os jovens (até 20 anos). Os acidentes na idade adulta são principalmente ocorrências ocupacionais (ZANNIN *et al.*, 2003; ABELLA *et al.*, 2006; GAMBORGI *et al.*, 2006).

1.2.2 <u>Aspectos morfológicos da lagarta e do sistema de produção de veneno</u>

A *L. obliqua*, quando na sua fase larval, possui o corpo com coloração castanho-claro-esverdeado. Apresenta a região dorsal com uma linha longitudinal contínua marrom-escura, marginada de preto em toda a sua extensão. Outras duas faixas longitudinais, com manchas claras e levemente amareladas estão dispostas em alguns segmentos torácicos. Possuem cerdas esverdeadas em forma de espículas ou "espinhos" ramificados e pontiagudos de aspecto arbóreo simetricamente dispostos ao longo do dorso **(Figura 3A e B)**. São larvas gregárias que mimetizam o tronco de árvores silvestres e frutíferas tais como ipê, cedro, goiabeira, pereira, pessegueiro e ameixeira, onde se agrupam durante o dia e, à noite, sobem para as partes mais altas da árvore onde se localizam as folhas mais tenras das quais se alimentam **(Figura 3C)**. Habitam matas, parques e pomares domésticos (LORINI & CORSEUIL, 2001). Atualmente, acredita-se que o desequilíbrio ambiental causado pelo desmatamento e pelo cultivo de monoculturas forçaram a adaptação da *L. obliqua* aos pomares cultivados próximos às residências. Além disso, a utilização de agroquímicos nas grandes culturas pode ter exterminado inimigos naturais, desequilibrando o controle natural dessa espécie (VEIGA *et al.*, 2001).

Figura 3. A lagarta *Lonomia obliqua* **(taturana). A** e **B.** A lagarta evidenciando aspectos da morfologia externa. **C.** Colônia de lagartas sobre o tronco de uma árvore. Fotos: CIT/SC.

A *L. obliqua* apresenta um ciclo de vida médio de seis meses, desde a postura, eclosão dos ovos, fase larval, fase de pupa e animal adulto, incluindo, obrigatoriamente, o período do verão **(Figura 4)**. Na fase adulta, há um evidente dimorfismo sexual, no qual a mariposa macho apresenta coloração amarelada e a fêmea rosa-pardo. Esta fase tem duração de 7 a 10 dias. Após a cópula, a fêmea deposita os ovos nas folhas das plantas que servirão de alimento para as lagartas. Os ovos eclodem em aproximadamente 17 dias. Esta espécie apresenta seis ínstares larvais cujas diferenças são expressas em pequenas modificações estruturais que vão aumentando em complexidade a cada ínstar. A fase larval tem duração média de 85 dias, sendo que cada ínstar perdura por aproximadamente duas semanas (LORINI, 1997).
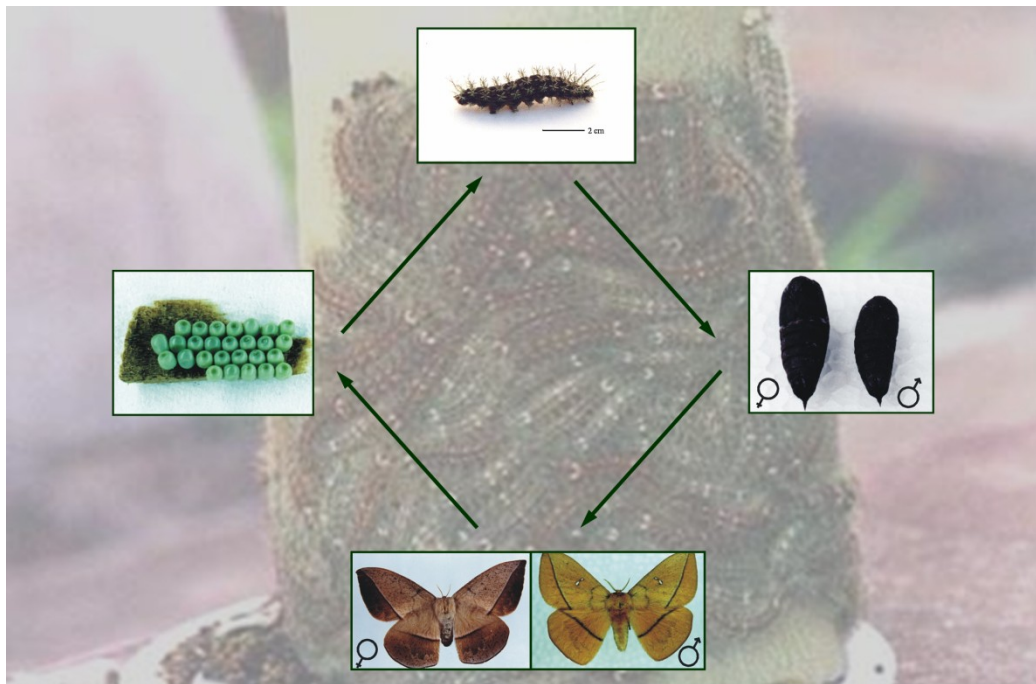
Figura 4. Ciclo de vida do lepidóptero *Lonomia obliqua*. Fotos: CIT/RS.

Em um estudo detalhado da morfologia da lagarta *L. obliqua* foi demonstrado que não há glândula produtora de veneno no animal e que o tegumento é revestido internamente por um epitélio secretor composto externamente por inúmeras especializações cuticulares **(Figura 5A)** (VEIGA *et al.*, 2001). Cada segmento do corpo da lagarta apresenta um conjunto de cerdas ou espículas de formato espinhoso, denominado *scolus* **(Figura 5B e C)**. Nos *scolus* dorsais, as pontas das cerdas são diminutas e, nos *scolus* subdorsais e subespiraculais, apresentam-se com pontas mais longas. As cerdas possuem um poro na extremidade que é a abertura de um canal interno onde o veneno é armazenado **(Figura 5D e 6C)**. Estas cerdas, por serem constituídas de quitina, são de fácil ruptura **(Figura 5D)**, o que acaba facilitando a injeção da secreção venenosa da taturana na pele ou mesmo no tecido subcutâneo da vítima, causando o envenenamento (VEIGA *et al.*, 2001; 2009).
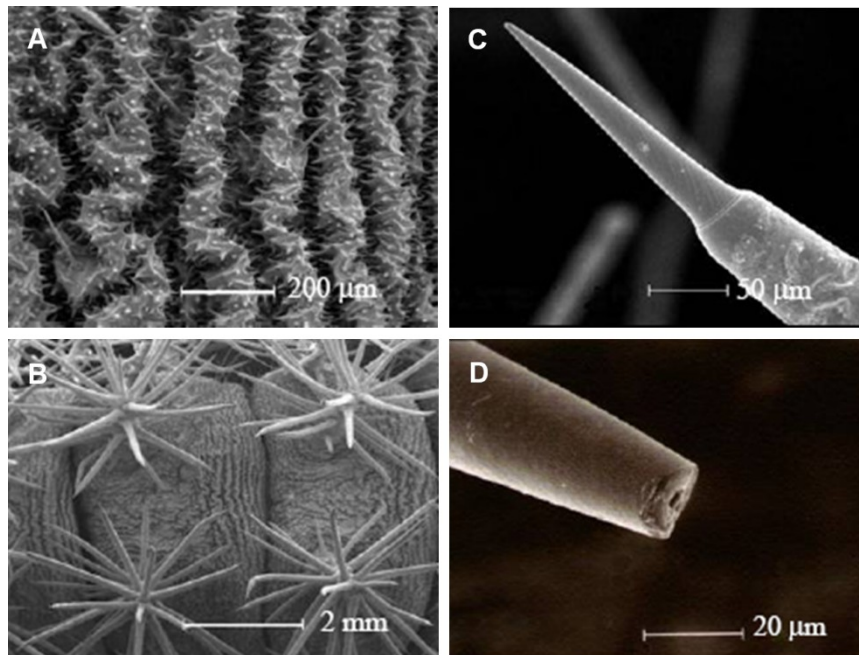
Figura 5. Ultraestrutura do tegumento de *Lonomia obliqua*. **A.** Estruturas quitinosas que compõem a base do tegumento. **B.** Região dorsal do tegumento com vários scoli contendo as espículas. **C.** Espícula íntegra. **D.** Espícula quebrada na extremidade com canal interno exposto. Fonte: VEIGA *et al.*, 2001.

O sistema de produção do veneno é formado por um epitélio subjacente ao tegumento da lagarta que reveste toda a parte interna das cerdas como uma evaginação contínua do corpo **(Figura 6)**. Em certas regiões deste epitélio, as células expandem-se e ficam mais cilíndricas do que cúbicas, formando um grupo de células especializadas onde se acumulam produtos de secreção contendo o veneno **(Figura 6A)**. Células irregulares da base dos *scolus* e mesmo aquelas que revestem o interior das cerdas também possuem atividade secretora intensa **(Figura 6B e C)**. Na região apical dessas células, os núcleos ficam evidenciados, a concentração de vesículas de secreção é mais alta e um depósito é formado **(Figura 6B)**. Essas vesículas e produtos de secreção acumulam-se e são armazenadas no espaço extracelular subcuticular entre a camada epitelial e a cutícula **(Figura 6B e C)**. Provavelmente, o veneno é liberado pela quebra das cerdas durante o contato físico, quando a lagarta sente-se pressionada contra a pele, e os produtos de secreção contendo as toxinas são drenados através do espaço subcuticular até o canal interno de onde é injetado **(Figura 6C)** (VEIGA *et al.*, 2001; 2009; PINTO *et al.*, 2010).
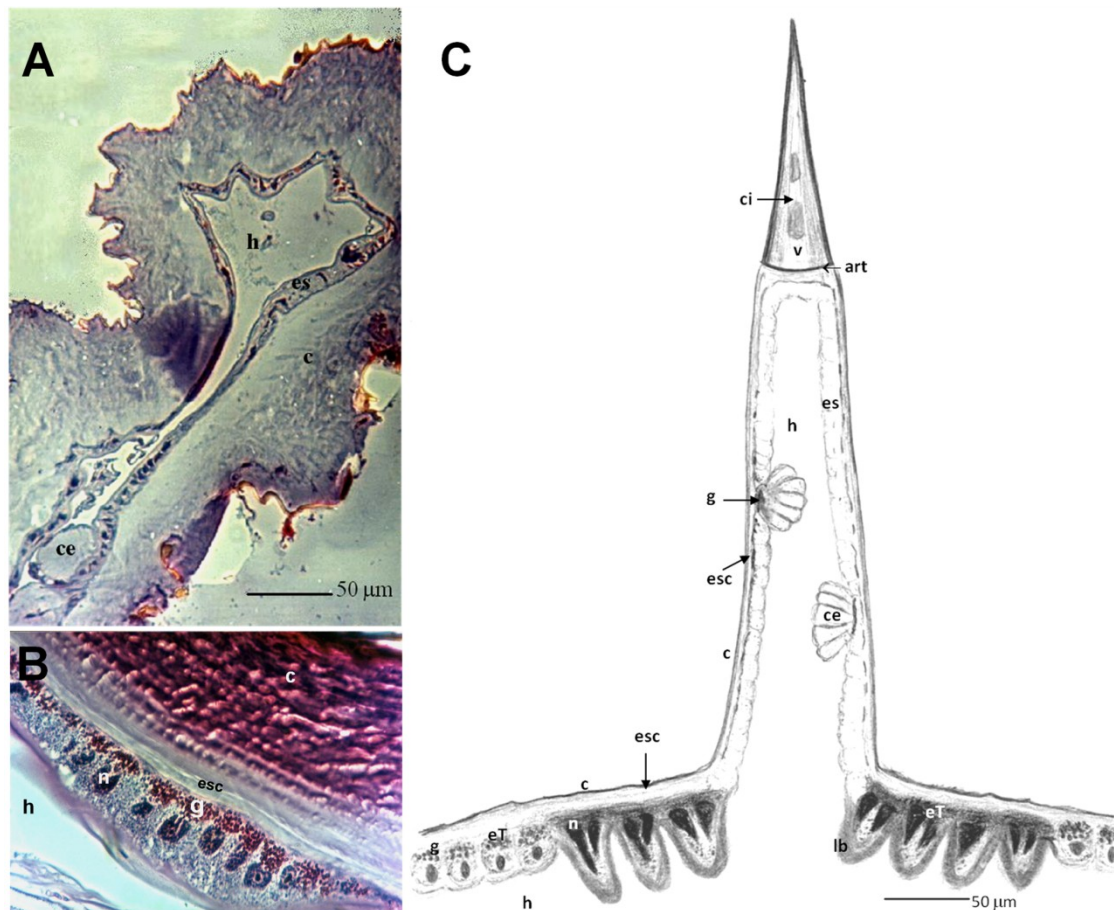
Figura 6. Sistema envolvido na produção e injeção do veneno de *Lonomia obliqua*. **A.** Seção longitudinal de uma cerda corada com fucsina básica-azul de metileno e visualizada por microscopia óptica. Notar a presença de um epitélio tipicamente secretor e células especializadas revestindo a parte interna da cerda. **B.** Detalhe do epitélio secretor mostrando o acúmulo de vesículas contendo grânulos com produtos de secreção no espaço subcuticular. **C.** Modelo esquemático da estrutura de produção e injeção do veneno. As toxinas são produzidas pelas células da base e interior das cerdas, direcionadas e armazenadas no espaço subcuticular e drenadas para o canal interno na ponta das cerdas durante o contato com a pele. h: hemolinfa, c: cutícula, ce: células especializadas, es: epitélio secretor, n: núcleo, g: grânulos, esc: espaço subcuticular, eT: epitélio tegumentar, lb: lâmina basal, art: articulação da ponta da cerda, ci: canal interno da ponta da cerda, v: veneno. Fonte: VEIGA *et al.*, 2001

1.2.3 A síndrome hemorrágica

O contato com as secreções venenosas de *L. obliqua* provoca uma reação imediata no local, sendo caracterizada por um ardor intenso (dor caracterizada por queimação), hiperemia, prurido, edema e bolhas sanguinolentas **(Figura 7A)**, manifestações comumente seguidas por sintomas gerais e inespecíficos do envenenamento, que podem surgir mais tardiamente como cefaléia holocraniana, mal-

estar geral, náuseas e vômitos; com menos frequência relata-se dores abdominais, mialgia, hipotermia e hipotensão. O desenvolvimento do quadro hemorrágico mais grave se segue com distúrbios de coagulação e sangramentos: gengivorragia **(Figura 7D)**, equimose **(Figura 7B e C)**, epistaxe, sangramento em feridas recentes ou já cicatrizadas, hemorragias intraarticulares, hematêmese, melena, hematúria, sangramento pulmonar e hemorragia cerebral (KELEN *et al.*, 1995; MINISTÉRIO DA SAÚDE, 1998). As manifestações clínicas podem agravar-se com hipotensão, choque e insuficiência renal, podendo evoluir para o óbito. As principais causas de morte já relatadas na literatura envolvem complicações renais e/ou hemorragia intracraniana (DUARTE *et al.*, 1990, 1996; KOWACS *et al.*, 2006).
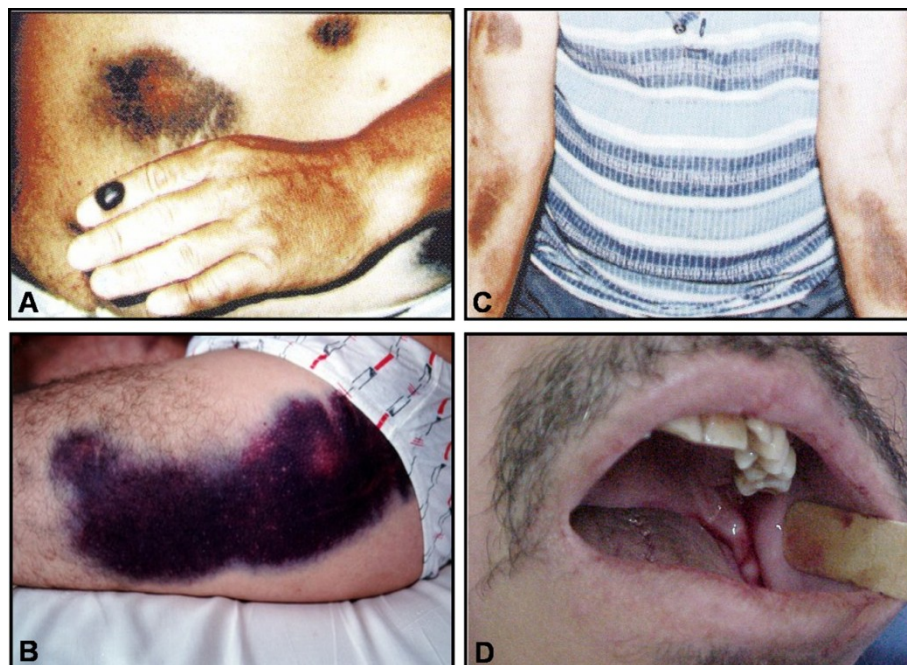
Figura 7. Sinais clínicos do envenenamento por *Lonomia obliqua*. **A.** Hematoma no dedo da mão de um paciente. **B** e **C.** Equimoses em diferentes regiões do corpo. **D.** Gengivorragia. Fotos: CIT/RS e ZANIN *et al.*, 2002.

Os dados laboratoriais indicam coagulopatia que se caracteriza pelo prolongamento acentuado do tempo de coagulação obtido pelos testes específicos como tempo de protrombina (TP), tempo de tromboplastina parcialmente ativada (TTPa) e tempo de trombina (TT) (AROCHA-PIÑANGO & GUERRERO, 2003). Verifica-se também queda nos níveis plasmáticos de fibrinogênio (Fg) e dos fatores V, XIII, precalicreína (PK), plasminogênio (Plg), proteína C (PC) e α_2-antiplasmina (α_2AP),

enquanto os níveis do complexo trombina-antitrombina (TAT), do fragmento 1+2 da ativação de protrombina (F_{1+2}) e de D-dímeros (D-D) encontram-se elevados (ZANNIN *et al.*, 2003). Os níveis dos fatores X, II, vWF e plaquetas estão normais na maioria das vítimas de acidentes, excetuando-se a contagem de plaquetas que está significativamente diminuída em casos graves de envenenamento (GAMBORGI *et al.*, 2006).

O intenso consumo dos fatores de coagulação observado nos pacientes envenenados é compatível com o quadro clínico de coagulação intravascular disseminada (CIVD). De fato, os elevados níveis de TAT e F_{1+2} indicam que há geração de trombina intravascular por meio da ativação de protrombina. Juntamente com componentes do veneno, a trombina formada seria responsável pelo consumo do fibrinogênio circulante e pela ativação do sistema de coagulação sanguínea, o que também levaria a uma diminuição de outros fatores como V, XIII, PK e à formação de fibrina.

O sistema fibrinolítico dos pacientes envenenados também parece estar ativado, já que os níveis de Plg e α_2AP estão reduzidos na maioria dos casos. Além disso, os níveis plasmáticos de D-D estão aumentados, confirmando que há intensa fibrinólise durante o envenenamento. Provavelmente, componentes do veneno são capazes de ativar direta ou indiretamente o Plg gerando quantidades significativas de plasmina intravascular. A plasmina assim formada, juntamente com enzimas fibrin(ogen)olíticas do veneno, atuam diretamente sobre a fibrina gerando os D-D. O excesso de plasmina é inibida pela serpina α_2AP, o que explica a redução na concentração plasmática desse inibidor.

Estudos *in vivo* em ratos ou mesmo *in vitro* em cultura de células confirmam as hipóteses de ativação da coagulação e fibrinólise e os dados indicativos de coagulopatia de consumo secundária à CIVD. Animais envenenados experimentalmente apresentam tempo de coagulação prolongado, níveis baixos de fibrinogênio circulante e alta atividade do tipo trombina e plasmina no plasma (BERGER *et al.*, 2010a). Além disso, fibroblastos tratados com veneno apresentam um perfil procoagulante com aumento de expressão de fator tecidual (FT) e do ativador de plasminogênio do tipo uroquinase (uPA) (PINTO *et al.*, 2008). Concomitante ao prolongamento do tempo de coagulação, há uma significativa diminuição da função plaquetária em ratos injetados com veneno por via subcutânea. Plaquetas de animais envenenados não agregam quando estimuladas por ADP, colágeno ou trombina, o que contribui sobremaneira para o quadro

hemorrágico final. Essa hipoagregação plaquetária não está associada com a queda dos níveis de fibrinogênio, mas sim com a geração de óxido nítrico (NO) intravascular, que é um potente inibidor da agregação plaquetária. De fato, foi confirmado que os níveis de NO aumentam no plasma, a expressão da óxido nítrico sintase (NOS) aumenta em fibroblastos e células endoteliais envenenadas e as plaquetas de animais envenenados previamente tratados com L-NAME (um inibidor da NOS) são capazes de agregar normalmente (PINTO *et al.*, 2008; BERGER *et al.*, 2010a; NASCIMENTO-SILVA *et al.*, 2013).

A gravidade da síndrome hemorrágica induzida por *Lonomia* sp. incentivou o desenvolvimento de um soro eficaz em neutralizar a ação do veneno. Disponível desde 1996, o soro antilonômico é produzido no Instituto Butantan a partir da imunização de eqüínos com o extrato bruto de espículas da lagarta (DIAS DA SILVA *et al.*, 1996; ROCHA-CAMPOS *et al.*, 2001). A correta utilização do soro antilonômico é capaz de solucionar ou reduzir a gravidade do quadro hemorrágico, sendo a terapia de escolha em casos de envenenamento pela taturana. O esquema de tratamento segue recomendação do Ministério da Saúde que indica a dose de soro antilonômico levando em consideração se o envenenamento é leve, moderado ou grave. Pacientes com envenenamento leve são aqueles que apresentam apenas manifestação de dor ou sensação de queimação no local do contato e sem alteração de coagulação ou sangramento até 72 horas após o acidente. Em geral, esses pacientes recebem apenas tratamento sintomático com analgésicos, evitando-se os salicilatos. Os casos de envenenamento moderado apresentam alterações de coagulação ou manifestações hemorrágicas (gengivorragia, equimose, hematoma ou hematúria), mas sem alterações hemodinâmicas (hipotensão, taquicardia ou choque) ou evidência de comprometimento renal. Esses pacientes recebem tratamento sintomático para dor e 5 ampolas de soro antilonômico via intravenosa (10 mL/ampola, sendo que cada ampola é capaz de neutralizar 3,5 mg do veneno). Pacientes graves são aqueles com alterações de coagulação, manifestações hemorrágicas em vísceras (hematêmese, hematúria, hipermenorragia, hemorragia pulmonar ou intracraniana) ou instabilidade hemodinâmica ou lesão renal. Para esses pacientes é recomendado além do tratamento sintomático e de suporte, 10 ampolas de soro antilonômico (MINISTÉRIO DA SAÚDE, 1998; CAOVILLA & BARROS, 2004). A administração de drogas anticoagulantes ou do ácido ε-aminocapróico (inibidor da fibrinólise) isoladamente ou em associação com a soroterapia não é recomendada, devido ao risco de se exacerbar o quadro hemorrágico

ou trombótico, conforme já demonstrado em modelos animais (GONÇALVES *et al.*, 2007). A infusão de sangue total ou plasma fresco para corrigir os níveis dos fatores de coagulação também é contraindicada, pois pode acentuar a coagulopatia de consumo. Apesar da eficácia, ainda permanece como desafio a produção do soro em quantidade suficiente para atender à crescente demanda devido ao aumento dos acidentes e a necessidade de disponibilizar o produto em tempo hábil para o tratamento em locais distantes onde os acidentes vêm ocorrendo com mais frequência.

1.2.4 <u>Composição bioquímica do veneno</u>

O veneno da lagarta *L. obliqua* é constituído por princípios ativos na sua maioria de natureza proteica ou peptídica com diferentes atividades tóxicas. Dados obtidos a partir de uma biblioteca de cDNA das cerdas da lagarta permitiram indicar que, do grupo de sequências expressas, cerca de 25 % codificam proteínas homólogas à serino-proteinases. Outros grupos de sequências codificam proteínas com homologia à lipocalinas (15 %), lectinas (15 %), serpinas 15 %), cisteíno-proteinases (10 %), inibidores do tipo Kazal (10 %), fosfolipases A2 (5 %) e cininogênios (5%) (VEIGA *et al.*, 2005, http://www.ncbi.nlm.nih.gov/projects/omes/). A análise do extrato das cerdas por espectrometria de massas também permitiu identificar serino-proteinases, lipocalinas e inibidores de proteases como proteínas majoritárias na secreção venenosa (RICCI-SILVA *et al.*, 2008). De fato, algumas toxinas pertencentes a essas e a outras classes vêm sendo isoladas com o objetivo de entender a contribuição de cada componente para o quadro clínico do envenenamento. Na **Tabela 3** estão listadas as toxinas isoladas do veneno até o momento; um breve comentário sobre o que se sabe a respeito do papel de cada uma para o envenenamento é apresentado a seguir. Maiores detalhes podem ser encontrados no primeiro artigo apresentado na seção "Resultados".

Ativadores de protrombina. Uma das primeiras atividades caracterizadas no veneno de *L. obliqua* foi o efeito procoagulante (DONATO *et al.*, 1998). O extrato das espículas da lagarta possui alta atividade proteolítica e é capaz de reduzir significativamente o tempo normal de coagulação do plasma pela ativação direta de protrombina e fator X (VEIGA *et al.*, 2003; PINTO *et al.*, 2006). Um desses componentes capaz de promover a ativação de protrombina foi isolado e denominado LOPAP ("*Lonomia obliqua prothrombin activator protease*") (REIS *et al.*, 2001a). A LOPAP nativa é descrita

como sendo uma lipocalina tetramérica de massa molecular igual a 69 kDa e não possui homologia de sequência com qualquer outro ativador já identificado em venenos. Tanto a LOPAP nativa (69 kDa) quanto a recombinante monomérica (20 kDa) são capazes de gerar trombina a partir de protrombina de maneira dose-dependente na presença de íons cálcio e reduzem o tempo de coagulação do plasma (REIS *et al.*, 2006). A atividade ativadora de protrombina é completamente abolida na presença de inibidores clássicos de serino-proteinases. Segundo esses autores, o mecanismo de ativação da protrombina pela LOPAP é semelhante ao do fator Xa do plasma, clivando a protrombina para formar pretrombina 2 e não mesotrombina como outros ativadores de veneno de serpentes que são tipicamente metaloproteinases (REIS *et al.*, 2001a; REIS *et al.*, 2006). Quando injetado por via intravenosa em ratos, a LOPAP reproduz o quadro de coagulopatia de consumo observado nos pacientes envenenados. Os animais apresentam incoagulabilidade sanguínea, baixos níveis de fibrinogênio, trombocitopenia, e depósitos de fibrina nos capilares glomerulares e pulmonares (REIS *et al.*, 2001b). Em células endoteliais tratadas com o ativador de protrombina há aumento na produção de NO e de prostaciclinas (PGI_2) e aumento na expressão de interleucina-8 (IL-8), o que sugere que a LOPAP pode estar envolvida na modulação do processo inflamatório durante o envenenamento (FRITZEN *et al.*, 2005).

Ativadores de fator X. Outra toxina pró-coagulante, atuando como ativador de fator X, também foi isolada a partir do extrato de espículas da taturana. LOSAC ("*Lonomia obliqua stuart-factor activator*") é uma serino-proteinase de cadeia polipeptídica única com massa molecular de 45 kDa. A enzima purificada é capaz de ativar o fator X de maneira dose-dependente na ausência de íons cálcio, tem ação pró-coagulante no plasma e induz produção de NO e aumento de expressão do ativador de plasminogênio tecidual (t-PA) em células endoteliais (ALVAREZ-FLORES *et al.*, 2006). Seus efeitos *in vivo* são desconhecidos até o momento.

Fibrinogenases. Além da ação procoagulante, o veneno de *L. obliqua* também possui atividade fibrinolítica, sendo capaz de degradar diretamente fibrinogênio e fibrina (PINTO *et al.*, 2006). A enzima responsável por essa atividade é uma serino-proteinase de 35 kDa denominada lonofibrase. A lonofibrase degrada as cadeias α e β do fibrinogênio, mostrando uma maior especificidade para a cadeia α. A enzima inibe a coagulação do fibrinogênio induzida pela trombina e também possui potente atividade

sobre fibrina, apresentando um padrão de degradação diferente do obtido para plasmina, o que sugere que esta fibrinogenase apresenta mecanismo de ação diferenciado e pode potencializar o quadro de ativação do sistema fibrinolítico observado durante o envenenamento (PINTO *et al.*, 2004).

Fosfolipases A$_2$. As fosfolipases estão presentes em praticamente todos os venenos animais. As fosfolipases A$_2$ hidrolisam fosfolipídeos gerando ácidos graxos livres e lisofosfolipídeos, podendo, assim, causar hemólise indireta. Também podem interferir diretamente no processo de coagulação sanguínea e agregação plaquetária (KINI, 2003). Seibert *et al.* (2003) encontraram uma atividade hemolítica no extrato de espículas da *L. obliqua*. Segundo estes autores, o veneno foi capaz de induzir hemólise direta e indireta em eritrócitos humanos e de ratos. Além disso, a injeção do extrato de espículas por via subcutânea em ratos leva a uma redução importante da contagem de eritrócitos, aumento dos níveis plasmáticos de hemoglobina e diminuição de haptoglobina (SEIBERT *et al.*, 2004). Casos de hemólise intravascular também já foram observados em pacientes envenenados (MALAQUE *et al.*, 2006). Sabe-se que uma fosfolipase A2 isolada do veneno contribui para esse efeito hemolítico. A enzima possui massa molecular de 15 kDa e homologia de sequência de aminoácidos com fosfolipases A2 encontradas em veneno de abelhas. A enzima purificada tem atividade hemolítica indireta e também causa hemólise intravascular em ratos (SEIBERT *et al.*, 2006). Adicionalmente, o veneno possui uma atividade tipo fosfolipase A2 capaz de induzir adesão e agregação plaquetária *in vitro* (BERGER *et al.*, 2010b). No entanto, a identidade da molécula responsável por esse efeito ainda permanece desconhecida.

Hialuronidases. As hialuronidases são enzimas bem caracterizadas nos venenos de serpentes, aranhas, abelhas e escorpiões. Atuam *in vivo*, degradando componentes da matriz extracelular dos tecidos e da parede dos vasos sanguíneos. Dessa forma, estas enzimas agem aumentando a distribuição de outras toxinas para os demais tecidos e órgãos, favorecendo as manifestações sistêmicas dos envenenamentos (SAMPAIO *et al.*, 1991; MARKOVI-HOUSLEY *et al.*, 2000; KUDO & TU, 2001; DA SILVA *et al.*, 2004). Especificamente no veneno de *L. obliqua* foram identificadas duas hialuronidases de 53 e 49 kDa, chamadas lonogliases. As enzimas são capazes de hidrolisar ácido hialurônico e sulfato de condroitina, sendo caracterizadas como β-endohexosaminidases. Estudos utilizando pele de coelhos demonstraram que as

lonogliases são capazes de hidrolisar o ácido hialurônico presente na matriz extracelular, o que facilita a penetração das toxinas na derme (GOUVEIA *et al.*, 2005).

Tabela 3. Toxinas isoladas do veneno de *Lonomia obliqua***.**

Toxina	Identificação	MM (Da)	Características	Referência
Fibrinogenase	Lonofibrase	35.000	Degrada cadeias α e β do fibrinogênio e fibrina. Inibe a coagulação do fibrinogênio induzida por trombina.	Pinto *et al.*, 2004; Veiga *et al.*, 2005
Ativador de protrombina	Lopap	69.000	Possui atividade pró-coagulante no plasma e ativa protrombina. Induz coagulopatia de consumo e trombocitopenia em ratos. Aumenta a produção de NO, PGI_2 e IL-8 em células endoteliais	Reis *et al.*, 2001; Fritzen *et al.*, 2005; Reis *et al.*, 2006
Ativador de fator X	Losac	45.000	Possui atividade pró-coagulante no plasma e ativa fator X. Em células endoteliais, induz produção de NO e tPA.	Alvarez-Flores *et al.*, 2006
Hialuronidases	Lonogliases	53.000 49.000	Atividade hidrolítica sobre componentes da matriz extracelular. Facilitam a biodistribuição das toxinas através da pele.	Gouveia *et al.*, 2005
Fosfolipase A2	-	15.000	Atividade hemolítica indireta *in vitro*. Induz hemólise intravascular *in vivo*.	Seibert *et al.*, 2006

MM: massa molecular; Lopap: *Lonomia obliqua* prothrombin activator protease; Losac: *Lonomia obliqua* stuart-factor activator.

1.3 Insuficiência renal aguda nos envenenamentos por animais peçonhentos

A insuficiência renal aguda (IRA) representa uma das principais complicações clínicas dos acidentes com animais peçonhentos. Os venenos de serpentes são, em sua maioria, altamente nefrotóxicos, porém casos mais graves e frequentes são observados em acidentes com serpentes asiáticas e africanas tais como *Daboia russelli siamensis* ("Russell's viper"), *Echis carinatus* ("Saw-scaled viper") e *Bitis arietans* ("Puff Adder"); e também com serpentes da América do Sul como as do gênero *Bothrops* (jararacas) e *Crotalus* (cascavéis) (RODRIGUES-SGRIGNOLLI *et al.*, 2011; PINHO *et al.*, 2008; SITPRIJA, 2006). Além das serpentes, aranhas do gênero *Loxosceles* (aranha-marron) e as abelhas africanizadas (*Apis mellifera*) são responsáveis por parcela significativa dos casos de IRA, principalmente no Brasil (ABDULKADER *et al.*, 2008). De maneira geral, as principais alterações renais descritas são necrose tubular aguda, redução do ritmo de filtração glomerular e oligúria. O mecanismo envolvido vem sendo alvo de intensa investigação e, ao que tudo indica, é multifatorial. Diferentes modelos experimentais indicam que pode haver tanto uma ação citotóxica direta do veneno sobre diferentes estruturas renais (como é o caso da toxina dermonecrótica de *Loxosceles* que se liga especificamente em células tubulares, induzindo alterações estruturais e morte celular), quanto uma resposta inflamatória secundária desencadeada em resposta ao envenenamento, que leva à liberação de citocinas e espécies reativas de oxigênio (BERGER *et al.*, 2012; PINHO *et al.*, 2008; CHAIM *et al.*, 2006). Há também uma contribuição importante decorrente da liberação de mioglobina e hemoglobina muito comum nos envenenamentos por *Crotalus* e *A. mellifera* (GRISOTTO *et al.*, 2006; PINHO *et al.*, 2005).

As complicações renais também são frequentes e usualmente graves nos envenenamentos por *L. obliqua*. Durante a década de 80, dados retrospectivos de acidentes ocorridos em Santa Catarina apontaram uma incidência de IRA de 18 %, sendo que a taxa de mortalidade chegou a 50 % entre os pacientes que desenvolveram IRA após o contato com a lagarta (DUARTE *et al.*, 1990; 1994). Em Passo Fundo (RS), de 134 vítimas de envenenamento atendidas no período de 1989 a 1998, 6 % desenvolveram IRA, dentre os quais 25 % foram à óbito devido ao dano renal (WALTER, 1999). Em outro estudo retrospectivo pelo qual foram analisados 2.067 pacientes envenenados também em Santa Catarina no período de 1989 até 2003, um total de 39 pacientes (1,9 %) desenvolveram IRA (GAMBORGI *et al.*, 2006). Dos

pacientes com IRA, cerca de 32 % (11) necessitaram de hemodiálise e 10,3 % (4) dos casos evoluíram para insuficiência renal crônica (IRC). Sete óbitos foram registrados nesse período devido às complicações renais. O uso do soro antilonômico a partir de 1995 não diminuiu a incidência de IRA (que permaneceu em torno de 2 % entre 1995-2003), mas diminuiu a gravidade dos casos e o número de pacientes que evoluíram para IRC. Os pacientes com IRA também apresentaram concomitantemente alterações hemorrágicas. Tempo de coagulação prolongado (10 a 30 minutos) foi detectado em 18 % e incoagulável (maior que 30 minutos) em 83 % dos pacientes com IRA. A contagem de plaquetas, hematócrito e hemoglobina também foram menores nesses pacientes (GAMBORGI *et al.*, 2006).

Outra característica clássica do envenenamento por *L. obliqua* e estreitamente relacionada ao desenvolvimento de IRA é a hematúria. A maioria dos estudos retrospectivos e relatos clínicos descrevem que 80 a 87 % dos pacientes com IRA apresentam hematúria macroscópica **(Figura 8A)** (GAMBORGI *et al.*, 2006; MALAQUE *et al.*, 2006; WALTER, 1999; BURDMANN *et al.*, 1996). O mesmo é observado em ratos injetados com o extrato de espículas da lagarta **(Figura 8B)**. Por exame direto do sedimento urinário é possível visualizar hemácias intactas e leucócitos **(Figura 8C)**.
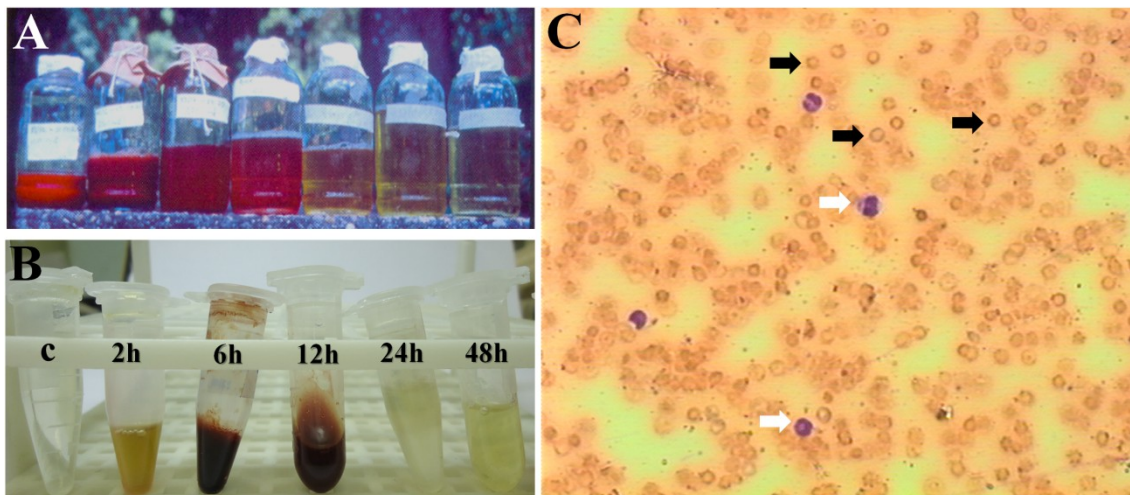
Figura 8. Hematúria. A. Amostras de urina de paciente envenenado por *L. obliqua*. **B.** Urina de ratos injetados com extrato de espículas de *L. obliqua* (1,5 mg/kg, via subcutânea) após diferentes tempos de envenenamento. c̲: urina de animal injetado com solução salina. **C.** Sedimento urinário de animal injetado com extrato de espículas após 6 horas de envenenamento. Notar a presença de hemácias intactas (setas pretas) e leucócitos (setas brancas). Coloração: May-Grünwald-Giemsa. Aumento: 40 X. Fonte: A. DUARTE *et al.*, 1994. B e C, M. BERGER (resultados experimentais).

O mecanismo da IRA induzida por *L. obliqua* e mesmo as alterações patológicas renais decorrentes do envenenamento são pouco conhecidas. Não existe qualquer estudo de investigação experimental e há apenas dois casos relatados na literatura em que foi realizada biópsia renal para exame de alterações histopatológicas. Em um deles, Burdmann *et al.* (1996) descreveram o caso de uma paciente com 67 anos que apresentou IRA do tipo anúrica após contato com inúmeras lagartas. A paciente foi submetida à biópsia renal depois de 17 dias do acidente e apresentou glomérulos de aparência normal, apesar de um espessamento da parede da cápsula de Bowman e atrofia tubular focal. Foi mantida em hemodiálise por 26 dias e recuperou a função renal. No outro relato é descrito o caso de uma paciente de 37 anos, grávida de 37 semanas, que teve contato com a palma da mão direita com aproximadamente 15 lagartas e apresentou gengivorragia, anúria e sangramento genital após 24 horas do contato. Devido ao descolamento precoce de placenta, o parto foi realizado e a paciente deu à luz a uma criança saudável. Os níveis de creatinina plasmática da paciente aumentaram de 4 mg/dL no 1º dia após o acidente para 10,1 mg/dL no 4º dia. Os níveis de ureia aumentaram de 41 mg/dL para 169 mg/dL no mesmo período. O tratamento por hemodiálise foi iniciado no 5º dia e os níveis de creatinina diminuíram para 3,8 mg/dL no 36º dia. Apenas no 27º dia (quando o quadro hemorrágico já havia sido revertido) uma biópsia renal foi realizada e se evidenciou a presença de necrose tubular aguda em regeneração (FAN *et al.*, 1998). Certamente, a impossibilidade de se fazer biópsia precoce, em função dos distúrbios de coagulação inerentes ao acidente, tem dificultado uma análise mais adequada das alterações anatomopatológicas renais. Portanto, este fato por si só e a gravidade dos casos de IRA justificam a importância do uso de modelos animais experimentais para o estudo dos mecanismos fisiopatológicos renais durante o envenenamento, o que também pode gerar conhecimento para o desenvolvimento de formas alternativas de tratamento.

1.4 O sistema calicreína-cininas

O sistema calicreína-cininas (SCC) é composto por enzimas e peptídeos biologicamente ativos que participam de diferentes processos fisiopatológicos. Além de estar envolvido na regulação direta de sistemas endógenos, o SCC está intimamente relacionado com vários mecanismos plasmáticos de ativação enzimática por proteólise limitada como coagulação sanguínea, fibrinólise, sistema renina-angiotensina e sistema complemento (MOVAT, 1979).

O mecanismo básico de funcionamento do SCC consiste na ativação da precalicreína (uma serinoprotease que pode ser encontrada sob duas formas: calicreína plasmática e tissular) que age sobre moléculas do precursor cininogênio gerando peptídeos genericamente conhecidos como cininas. A precalicreína pode ser ativada enzimaticamente pela ação de proteases como o fator XIIa e a tripsina (SHARIAT-MADAR *et al.*, 2002). Após ativação, a calicreína plasmática (que é sintetizada no fígado) age sobre o cininogênio de alto peso molecular (HMWK, do inglês, *high molecular weight kininogen*; 114 kDa), que circula conjugado a ela, gerando o nonapeptídeo ativo, a bradicinina (BK, do inglês, *bradykinin*; Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9). Já a calicreína tissular diferencia-se da plasmática quanto às características moleculares, físico-químicas e enzimáticas, e hidrolisa preferencialmente o cininogênio de baixo peso molecular (LMWK, do inglês, *low molecular weight kininogen*; 68 kDa) gerando calidina (Lys-BK) (PIERCE & GUIMARÃES, 1976; SCHMAIER *et al.*, 1999). A calicreína tissular é sintetizada e presente em órgãos como glândulas salivares, rins e pâncreas, e secretada em fluidos biológicos (urina, saliva e suor). No rim, a Lys-BK pode ser convertida à BK por aminopeptidases presentes no plasma e urina ou a BK é liberada diretamente pela calicreína renal (GUIMARÃES *et al.*, 1973; 1976; VIEIRA *et al.*, 1994). Além da BK e da calidina, diversas outras cininas existem como a T-cinina (Ile-Ser-BK), descrita pela primeira vez no plasma de ratos tratados com tripsina (OKAMOTO & GREENBAUM, 1983), e a des-Arg^9-BK, que atua exclusivamente em receptores B1 (ERDÖS, 1979).

As cininas possuem um tempo de meia-vida muito curto no organismo, sendo inferior a um minuto, pois logo após serem liberadas elas sofrem rápida inativação pela ação de enzimas conhecidas como cininases (FERREIRA & VANE, 1987). As cininases são metaloproteases zinco-dependentes encontradas nos tecidos e no plasma. Em estados não patológicos, são descritas sete metaloproteases responsáveis pela

clivagem de cininas em mamíferos: a mais conhecida é a enzima conversora de angiotensina (ECA) ou cininase II (uma dipeptidil-peptidase), a qual cliva entre os resíduos Pro^7-Phe^8 e logo após entre Phe^5-Ser^6. Contudo, carboxipeptidases (M e N), conhecidas como cininases do tipo I; aminopeptidases e endopeptidases (como a prolilendopeptidase) também estão envolvidas no metabolismo das cininas (ERDÖS, 1979).

As cininas exercem seus efeitos biológicos por meio do estímulo de, pelo menos, dois tipos de receptores transmembrana acoplados à proteína G, denominados receptores B1 (B1R) e B2 (B2R). BK e Lys-BK exercem a maioria de seus efeitos via B2R. Já a des-Arg^9-BK e a Lys-des-Arg^9-BK atuam preferencialmente por ligação ao B1R (REGOLI & BARABÉ, 1980). O B2R é constitutivamente expresso em diferentes tecidos e o B1R é normalmente ausente em tecidos não-traumatizados, tendo sua expressão induzida em condições específicas de estresse, lesão tissular, infecção ou inflamação (PESQUERO *et al.*, 1996; CALIXTO *et al.*, 2000). De fato, citocinas proinflamatórias como a interleucina-1β (IL-1β) e o fator de necrose tumoral-α (TNF-α) são capazes de regular a expressão do B1R através do fator de transcrição NF-κB (MEDEIROS *et al.*, 2001; 2004; CALIXTO *et al.*, 2004). Ao se ligarem a seus receptores, as cininas desencadeiam uma série de eventos intracelulares como mobilização de cálcio, ativação das fosfolipases A e C, produção de NO e prostaciclina (BHOOLA, 1992). A liberação de NO e prostaciclina leva a uma série de efeitos fisiológicos tais como vasodilatação periférica e aumento do fluxo sanguíneo, extravasamento vascular, quimiotaxia e ativação de mastócitos e macrófagos, contração da musculatura lisa visceral, liberação de autacóides e prostanóides, nocicepção (CALIXTO *et al.*, 2000) e de modo antagônico hipotensão sistêmica (ALVING *et al.*, 1978) e hipertensão portal hepática (BORGES *et al.*, 1976).

Diferentes toxinas presentes em venenos animais podem interferir com o funcionamento normal do SCC. Entre elas, destacam-se as enzimas semelhantes à calicreína, as cininogenases, as cininases, os potenciadores de cininas e os peptídeos que possuem efeitos biológicos semelhantes às cininas. Além disso, existem venenos com a capacidade peculiar de ativar o SCC mesmo sem apresentar qualquer enzima semelhante à calicreína, após desnaturação proteica (HAMBERG & ROCHA E SILVA, 1957) ou, mesmo, que podem ativar o SCC sem ter atividade proteolítica importante (DEUTSCH & DINIZ, 1955). No caso da lagarta *L. obliqua*, foi demonstrado que o veneno é capaz de induzir ativação de precalicreína plasmática apesar de ser desprovido

de significante atividade do tipo calicreína (BOHRER *et al.*, 2007). O mecanismo de ação permanece não esclarecido apesar depoder haver participação de mediadores liberados por células inflamatórias, visto a importante liberação de histamina mediada pelo veneno (DE CASTRO BASTOS *et al.*, 2004), de proteases pulmonares e de células endoteliais ativadas, e de moléculas de membrana basal, tipo colágeno, expostas em lesões vasculares. O veneno da taturana também possui atividade cininogenásica, sendo capaz de liberar BK a partir do LMWK. Quando injetado por via intravenosa, o extrato de espículas da lagarta (3 µg/kg) é capaz de induzir queda na pressão arterial média de ratos. O efeito observado é comparável ao produzido pela BK. A administração de aprotinina (um inibidor de calicreína plasmática) ou HOE-140 (um antagonista de B2R) previne a atividade hipotensora do veneno. Esses dados indicam que o efeito observado é dependente da ação da calicreína plasmática e mediado diretamente pela BK (BOHRER *et al.*, 2007). Da mesma forma, a ação edematogênica do veneno é inibida por aprotinina, HOE-140 e loratadina (um antagonista de receptor H1 de histamina), o que demonstra a importância da ativação do SCC para as diferentes alterações fisiopatológicas que ocorrem durante o envenenamento (DE CASTRO BASTOS *et al.*, 2004; BOHRER *et al.*, 2007).

Neste trabalho, levantamos a hipótese de que a ativação do SCC induzida pelo veneno de *L. obliqua* também está envolvida na disfunção renal descrita nos pacientes envenenados. Há três evidências principais que sustentam essa hipótese: (*i*) Todos os componentes do SCC são expressos no rim; (*ii*) o SCC participa do controle hemodinâmico, regulação da filtração glomerular e do equilíbrio hidro-eletrolítico e (*iii*) componentes do SCC estão envolvidos em diferentes patologias, incluindo a nefropatia diabética, a lesão renal por isquemia-reperfusão, a glomerulonefrite e a fibrose renal (KAKOKI *et al.*, 2004; WANG *et al.*, 2008; KLEIN *et al.*, 2009; KLEIN *et al.*, 2010).

2. OBJETIVOS

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Apesar do conhecimento adquirido até o momento sobre as toxinas e as disfunções hemostáticas, pouco se sabe sobre os mecanismos de toxicidade sistêmica no envenenamento pela taturana *L. obliqua*. Portanto, o presente trabalho foi desenvolvido com o objetivo geral de investigar as alterações fisiopatológicas induzidas pelo veneno em diferentes órgãos, dando particular atenção aos distúrbios renais e vasculares que são descritos como a principal causa de óbito neste tipo de envenenamento.

Para tanto, os objetivos específicos propostos foram:

❖ Caracterizar as principais alterações histopatológicas, hematológicas, bioquímicas e genotóxicas sistêmicas induzidas pelo veneno em diferentes órgãos utilizando um modelo experimental *in vivo* em ratos;

❖ Avaliar a capacidade do soro antiveneno (soro antilonômico) em neutralizar os principais efeitos tóxicos sistêmicos *in vivo*;

❖ Investigar os mecanismos envolvidos na disfunção renal induzida pelo veneno: Avaliar a função glomerular, tubular, equilíbrio hidroeletrolítico, hemodinâmica e as alterações histopatológicas renais de ratos experimentalmente envenenados por *L. obliqua*;

❖ Analisar a expressão diferencial de proteínas no rim de animais envenenados buscando identificar marcadores e vias de sinalização associadas à lesão renal;

❖ Avaliar os níveis de expressão dos componentes do sistema calicreína-cininas no rim durante o envenenamento;

❖ Investigar a contribuição de componentes do sistema calicreína-cininas para a lesão renal induzida pelo veneno.

3. RESULTADOS

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A seção de resultados apresentada a seguir está dividida em cinco capítulos que correspondem aos manuscritos produzidos ao longo do período de doutoramento. A formatação do texto utilizada nesta seção segue as normas exigidas pelos periódicos científicos internacionais. Cada capítulo inicia com um breve texto introdutório sobre o assunto abordado, em português, seguido de uma cópia do artigo ou do manuscrito, em inglês.

3.1 Capítulo I

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

Neste trabalho, uma revisão a respeito dos diferentes aspectos envolvidos na síndrome hemorrágica que ocorre nos envenenamentos pela taturana *L. obliqua* foi realizada. Ênfase foi dada para os efeitos do veneno *in vivo* em modelos animais e, também, para os aspectos clínicos extraídos de relatos de casos de vítimas envenenadas. A revisão permitiu a construção de uma visão geral e atualizada dos efeitos biológicos do veneno, bem como auxiliou a planejar as metas experimentais perseguidas nos demais trabalhos.

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Review

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

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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.

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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 on 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

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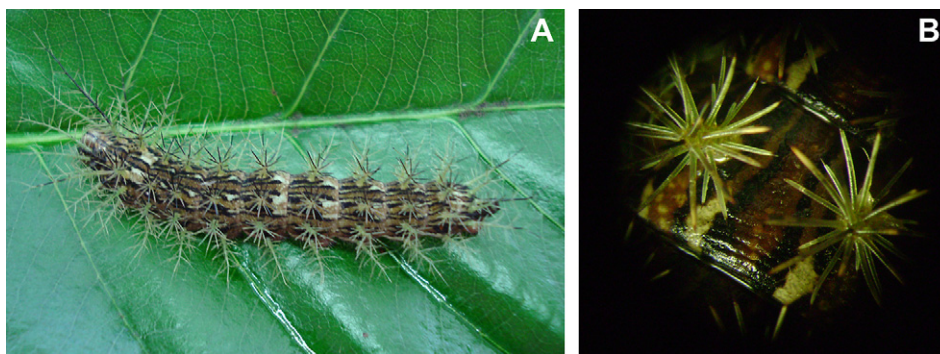
Fig. 1. The *Lonomia obliqua* caterpillar. A. *L. obliqua* (6th instar). B. Detail of *L. obliqua* bristles.

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 radio-labeling (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 intra-peritoneal 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 enve-nomed 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/μL).

The mechanisms involved in platelet aggregation inhibi-tion also have been investigated. In our model of experi-mental 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 aggrega-tion. 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 enveno-mation 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, procoagu-lant and fibrin(ogen)olytic activities (Veiga et al., 2003; Pinto et al., 2006). As shown in Fig. 2, the active princi-ples 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)o-lytic 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 enveno-mation, 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
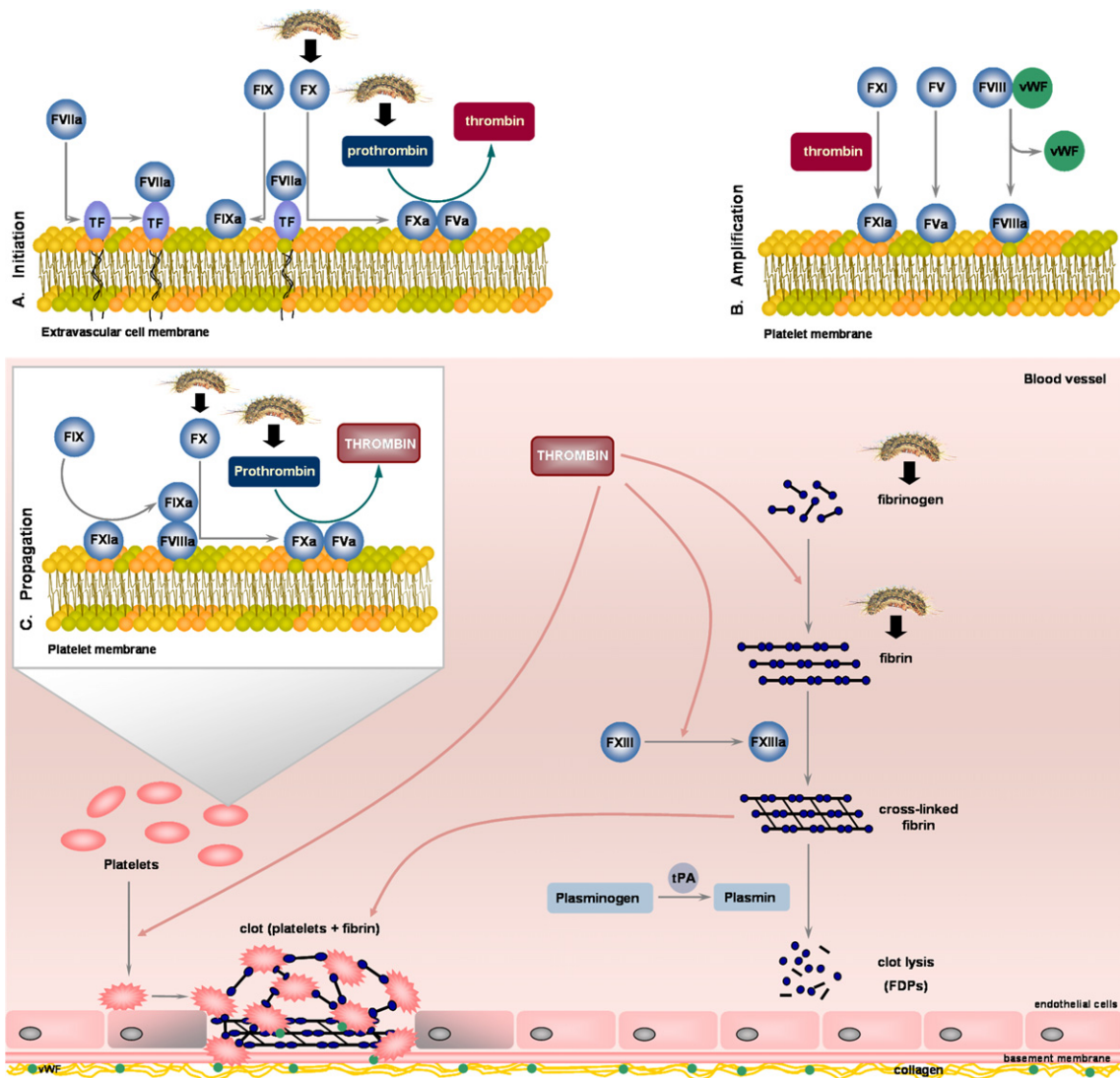
Fig. 2. Diagrammatic representation of the cell-based model of hemostasis. The coagulation reaction occurs in three different phases: initiation, amplification and propagation (for a review see Monroe and Hoffman, 2006). The sites of action of *L. obliqua* toxins are indicated by the presence of caterpillars. 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 biosynthesis, 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 over-expression 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 inter-acting 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 impor-tant role in the physiology of insects but, more than that, they can function as key effectors in hemostasis, by inhi-bition 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 con-sisted 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 success-fully 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 immu-noblotting 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 immu-noproteomic study support the hypothesis that the L. obli-qua 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 anti-fibrinolytic drugs, such as ε-aminocaproic acid (EACA), was officially recommended as therapy against L. obliqua enve-nomation 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 enve-nomation 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-envenoma-tion, 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.,

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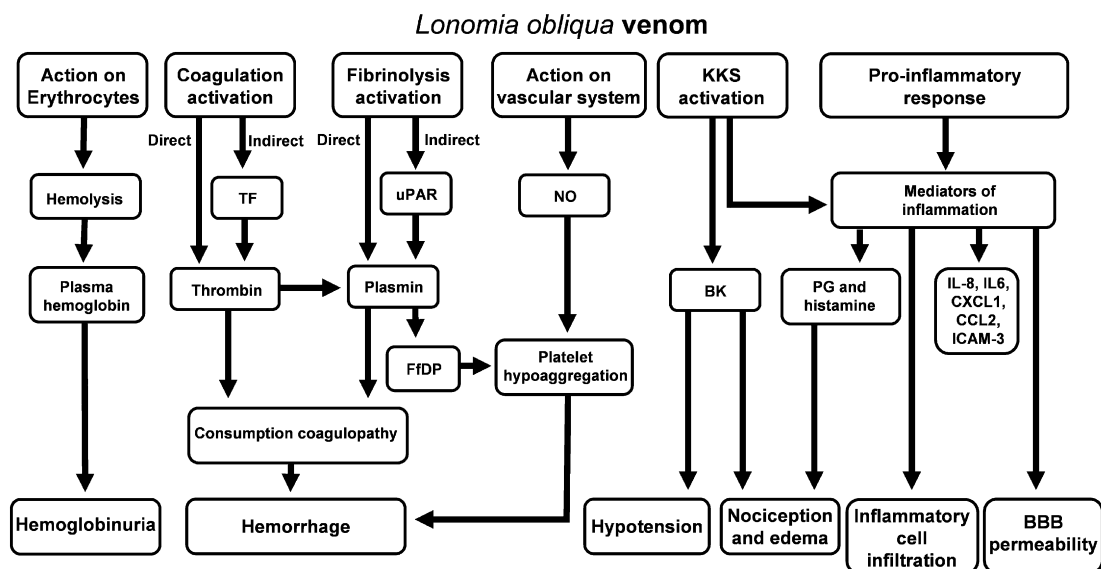
Fig. 3. Biological effects of *Lonomia obliqua* venom. A summary of the biological effects of *L. obliqua* venom discussed in this review are presented. KKS, kallikrein–kinin system; TF, tissue factor; uPAR, urokinase plasminogen activator receptor; NO, nitric oxide; BK, bradykinin; FfDP, fibrinogen/fibrin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

Conflicts of interest

The authors declare that there are no conflicts of interest.

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3.2 Capítulo II

Acute *Lonomia obliqua* **caterpillar envenomation-induced physiopathological alterations in rats: Evidence of new toxic venom activities and the efficacy of serum therapy to counteract systemic tissue damage**

Neste trabalho estão descritas as alterações de toxicidade aguda sistêmica do veneno de *L. obliqua* em ratos. Os principais resultados obtidos foram:

- Além dos distúrbios hemorrágicos clássicos já descritos, o veneno também é capaz de induzir lesão em múltiplos órgãos. Importantes alterações bioquímicas, hematológicas e morfológicas foram observadas e relacionadas principalmente às lesões cardíaca, renal, pulmonar e esplênica;
- Também há lesão muscular esquelética e o veneno induz dano genotóxico em diferentes órgãos incluindo fígado, pulmão, rim, coração e células sanguíneas como linfócitos. Há indicativo de que o dano genotóxico é mediado por espécies reativas de oxigênio;
- A administração do soro antilonômico na fase inicial do envenenamento (primeiras 2 h) é de fundamental importância para a neutralização dos efeitos tóxicos sistêmicos do veneno. Se administrado após 6 h de envenenamento, o soro é ineficaz em neutralizar os danos renal e cardíaco e o efeito hemolítico, apesar de reverter a coagulopatia de consumo;
- Os efeitos cardiotóxicos e genotóxicos do veneno foram descritos pela primeira vez neste trabalho.

O manuscrito a seguir foi submetido para publicação no periódico **Toxicon** em abril de 2013, revisado em julho de 2013 e aceito para publicação em agosto de 2013. Atualmente o artigo está disponível *on-line* (*in press*) em: http://www.sciencedirect.com/science/journal/aip/00410101.
DOI: 10.1016/j.toxicon.2013.08.061.

Decision Letter

Dear Prof. Jorge Almeida Guimarães,

I am pleased to confirm that your paper "Acute Lonomia obliqua caterpillar envenomation-induced physiopathological alterations in rats: Evidence of new toxic venom activities and the efficacy of serum therapy to counteract systemic tissue damage" has been accepted for publication in Toxicon.

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With kind regards,

Alan Harvey
Editor in Chief
Toxicon

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Acute *Lonomia obliqua* caterpillar envenomation-induced physiopathological alterations in rats: Evidence of new toxic venom activities and the efficacy of serum therapy to counteract systemic tissue damage

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ARTICLE INFO

ABSTRACT

The clinical manifestations of *Lonomia obliqua* caterpillar envenomation are systemic hemorrhage and acute kidney injury. In an effort to better understand the physiopathological mechanisms of envenomation, a rat model was established to study systemic tissue damage during *L. obliqua* envenomation. An array of acute venom effects was characterized, including biochemical, hematological, histopathological, myotoxic and genotoxic alterations. Rapid increases in serum alanine and aspartate transaminases, γ-glutamyl transferase, lactate dehydrogenase, hemoglobin, bilirubin, creatinine, urea and uric acid were observed, indicating that intravascular hemolysis and liver and kidney damage had occurred. Treatment with a specific antivenom (antilonomic serum) for up to 2 h post-venom injection neutralized the biochemical alterations. However, treatment after 6 h post-venom injection failed to normalize all biochemical parameters, despite its efficacy in reversing coagulation dysfunction. The hematological findings were consistent with hemolytic anemia and neutrophilic leukocytosis. The histopathological alterations were mainly related to hemorrhage and inflammation in the subcutaneous tissue, lung, heart and kidneys. Signs of congestion and hemosiderosis were evident in the spleen, and hemoglobin and/or myoglobin casts were also detected in the renal tubules. Increased levels of creatine kinase and creatine kinase-MB were correlated with the myocardial necrosis observed *in vivo* and confirmed the myotoxicity detected *in vitro* in isolated *extensor digitorum longus* muscles. Significant DNA damage was observed in the kidneys, heart, lung, liver and lymphocytes. The majority of the DNA lesions in the kidney were due to oxidative damage. The results presented here will aid in understanding the pathology underlying *Lonomia*'s envenomation.

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1. Introduction

Contact dermatitis and urticarial cutaneous reactions are well known signs of accidental contact with the hairs and spines of many lepidopterous larvae (Hossler, 2010). The consequences of these reactions are usually limited to local skin inflammation without any systemic tissue damage. However, contact with *Lonomia* spp. has been associated with potentially fatal systemic disorders, such as hemorrhage and acute kidney injury (AKI) (Arocha-Piñango et al., 2000; Pinto et al., 2010). One of these species is the moth *Lonomia obliqua* (Lepidoptera, Saturniidae), which is highly venomous in the larval stages. Larval forms occur during spring and summer in the southern regions of Brazil (mainly in the states of Rio Grande do Sul, Santa Catarina and Paraná) where envenomation by this animal is an important public health problem due to its high incidence (Veiga et al., 2009; Pinto et al., 2010; Guimarães, 2011). In fact, this caterpillar is responsible for severe and sometimes fatal accidents caused by skin contact with the bristles that cover the animal's body. Unlike snakes, spiders and scorpions, there is no specialized venomous gland in *L. obliqua*. The venom is produced by secretory epithelial cells of the tegument and stored in a hollow internal channel in each bristle. Because the bristles have weak articulations at their tips, only a slight contact with the skin is enough to break off these chitinous structures, injecting the venom into the subcutaneous tissue of victims (Veiga et al., 2001). Furthermore, accidents frequently involve colonies of dozens or hundreds of caterpillars that are camouflaged at tree trunks, which makes accidental contact more dangerous due to the venom quantities absorbed by the victim.

Clinical symptoms include local pain (burning sensation) and an inflammatory reaction, which starts immediately after contact, followed by systemic reactions, including headache, fever, vomiting and hypotension. Signs of bleeding diathesis, characterized by hematomas, ecchymosis, gross hematuria, hematemesis and melena are frequently observed between 6 and 72 h after contact. If the victim is not promptly treated, the clinical profile can evolve to intracerebral hemorrhage, AKI and death (Zannin et al., 2003; Kowacs et al., 2006; Garcia and Danni-Oliveira, 2007). Actually, the unique specific treatment available for *L. obliqua* envenomation is the early intravenous administration of anti-lonomic serum (ALS), an animal-derived antivenom. ALS is a concentrated pool of immunoglobulins (usually pepsin-refined F(ab′)2 fragments of whole IgG) that is purified from the plasma of a horse that has been immunized with the venom (obtained from bristle homogenates) (Rocha-Campos et al., 2001). In Brazil, ALS is produced by the Butantan Institute (São Paulo) and has been successfully used to re-establish physiological coagulation parameters in envenomed patients and experimental models (Caovilla and Barros, 2004). Despite its clinical efficacy, the prompt availability of ALS and a correct medical diagnosis in the regions of high incidence of accidents still remain public health concerns, namely, in rural areas of Southern Brazil. Another important problem is the fact that administration of ALS does not decrease the incidence of AKI, which is likely also related to the lack of knowledge about the mechanisms involved in kidney damage and its management (Gamborgi et al., 2006).

Recently, molecular biology and proteomic studies have contributed to the increasing number of toxins that have been identified in *L. obliqua* venomous secretions, providing valuable information regarding how this toxin cocktail acts on biological tissues (Veiga et al., 2005; Ricci-Silva et al., 2008). Toxins related to envenomation symptomatology, especially those that cause hemostatic disturbances, such as serine proteases, phospholipases A2, lectins and protease inhibitors, were identified. These toxins are able to directly modulate the victim's hemostatic system by proteolytic activation of the coagulation and fibrinolytic cascades, generating high concentrations of intravascular thrombin, plasmin, urokinase and kallikrein (Reis et al., 2006; Pinto et al., 2008; Berger et al., 2010a). As a consequence, consumption coagulopathy with decreased levels of fibrinogen, factors V and XIII, pre-kallikrein, plasminogen, protein C and α2-antiplasmin occurs (Zannin et al., 2003). Platelet aggregation function is also markedly impaired during envenomation, which contributes significantly to the bleeding disorders (Berger et al., 2010a,b). Moreover, the venom also triggers an acute inflammatory response and disturbances in the vascular system, inducing increases in blood–brain barrier permeability, hypotension and the nociceptive and edematogenic responses (Da Silva et al., 2004a; De Castro Bastos et al., 2004; Bohrer et al., 2007; Nascimento-Silva et al., 2012).

Despite understanding the mechanisms involved in the hemorrhagic syndrome, little is known about the systemic physiopathological effects induced by *L. obliqua* venom. Although venom components have been detected in several organs (including the kidneys, lungs, liver, spleen, heart and skeletal muscle) of rats following a single subcutaneous injection of the venom, the systemic tissue damage in these organs remains poorly characterized (Rocha-Campos et al., 2001; Da Silva et al., 2004b). For example, the current level of knowledge regarding the kidney damage is based only on a few clinical case reports in which hematuria and high levels of serum creatinine are described as the main features of *L. obliqua*-induced AKI (Burdmann et al., 1996). The venom-induced pathology in other organs remains completely unknown. In human patients, the impossibility of conducting early tissue biopsies, due to the coagulation disturbances inherent to the envenomation, has made it difficult to analyze the acute anatomopathological alterations. For these reasons, we believe that animal models of envenomation may be useful not only to characterize the underlying physiopathology but also to identify previously unknown toxic activities of the venom. Therefore, the aim of the present work was to develop a rat model to study systemic tissue damage during *L. obliqua* envenomation. An array of acute effects of the venom was characterized, including biochemical, hematological, histopathological, myotoxic and genotoxic alterations.

In summary, our data indicate that in addition to hemostatic abnormalities, there are also signs of multi-organ damage, mainly in the lungs, heart, kidneys and spleen. Treatment with ALS is only effective at counteracting the systemic physiopathological effects if it is administered during the initial phase of envenomation. In addition, this study

provides the first experimental evidence of the cardiotoxic, myotoxic and genotoxic activities of *L. obliqua* venom.

2. Materials and methods

2.1. Venom extraction and antivenom

L. obliqua caterpillars were kindly provided by the Centro de Informações Toxicológicas (CIT), Porto Alegre, Rio Grande do Sul, Brazil. The specimens used in this study were collected in the cities of Bom Princípio (Rio Grande do Sul, Brazil) and Videira (Santa Catarina, Brazil). *L. obliqua* venom was obtained by cutting the bristles at the caterpillar's tegument insertion, and the excised material was kept at 4 °C prior to the preparation of the extract, which occurred immediately after dissection. The bristles were macerated in cold phosphate-buffered saline (PBS), pH = 7.4, and centrifuged at 9600 × *g* for 20 min. The supernatant, designated **L**onomia **ob**liqua **B**ristle **E**xtract (LOBE), was used as the venomous secretion in all experiments. The protein content of the LOBE samples was determined using a BCA assay kit (Pierce, Rockford, Illinois, USA) and the aliquots were stored at −80 °C prior to use. The total number of caterpillars used for bristle extract preparation was 187 specimens and the protein concentration of the LOBE samples was 3.83 mg/mL. The total amount of venom extracted per caterpillar was 1.2 mg. All of the LOBE samples had similar *in vitro* pro-coagulant activities and the protein compositions were also similar, as monitored by electrophoresis and gel filtration chromatography (Pinto et al., 2006; Berger et al., 2010a,b).

L. obliqua antivenom (antilonomic serum – ALS) was provided by the Butantan Institute (São Paulo, Brazil). Each ampoule of ALS (10 mL/vial) is able to neutralize 3.5 mg of the LOBE. The ALS used here is the same one distributed to hospitals to treat envenomed patients.

2.2. Animals and ethical statements

Adult male Wistar rats, weighing 250–300 g, were supplied by the Central Animal Facility (CREAL), Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Brazil. They were housed in plastic cages (5 animals per cage) within a temperature controlled room (22–23 °C, on a 12 h light/dark cycle, with the lights on at 7:00 am) and had free access to water and food. All procedures involving animals were carried out in accordance with the Guiding Principles for the Use of Animals in Toxicology (International Society of Toxicology, http://www.toxicology.org) and the Brazilian College of Animal Experimentation (COBEA). The experimental protocol was approved by the ethical committee on research animal care of the Federal University of Rio Grande do Sul, Brazil (register number **2008177/2009**).

2.3. Experimental design

2.3.1. Venom treatment

To follow the time course of physiopathological alterations, we developed an experimental model of envenomation in rats. The animals were divided into two groups:

(*i*) *Control group* (*CTRL*) – Animals (*n* = 6 per sampling time) were injected subcutaneously (s.c.) with 100 μL of sterile PBS solution. (*ii*) *Experimental group* (*LOBE*) – Animals (*n* = 8 per sampling time) were injected s.c. with a solution containing 1.0 mg of the LOBE per kg of body weight in a final volume of 100 μL. At several time points post-venom injection (2, 6, 12, 24, 48 and 96 h), blood and various organs were collected for biochemical, hematological and histopathological analysis. This venom dose was selected based on the results of our previous experiments using rats as an animal model (Berger et al., 2010a) and was also based on other studies that have used similar doses to reproduce the consumption coagulopathy observed in humans (Dias da Silva et al., 1996; Rocha-Campos et al., 2001).

2.3.2. Antivenom treatment

The neutralizing ability of the antivenom was tested using the experimental model of envenomation. Rats that had previously been injected with the LOBE (1.0 mg/kg, s.c.) were treated 2 or 6 h after venom injection. At these time intervals, the animals (*n* = 8/group) were intravenously (i.v.) administered through the caudal vein with a sterile PBS solution (1 mL/100 g of body weight) or ALS (1 mL/100 g of body weight). Additional control groups (*n* = 6/group) were injected only with PBS or ALS under the same conditions. At 24 h after the treatments, blood was collected to measure biochemical and hematological markers of tissue damage. The dose of ALS used here is sufficient to completely neutralize the *in vitro* pro-coagulant activity of the LOBE. Moreover, the same dose was used in a previous study to compare the efficacy between ALS and antifibrinolytic drugs (Gonçalves et al., 2007).

2.4. Blood samples

After treatment, animals from the different groups were anesthetized intraperitoneally (i.p.) with a mixture of ketamine (75 mg/kg) (Syntec, São Paulo, Brazil) and xylazine (10 mg/kg) (Syntec, São Paulo, Brazil), and blood was collected by cardiac puncture. For the coagulation and hematological assays, the blood samples were collected in 1:10 (v/v) 3.8% trisodium citrate (Merck, Darmstadt, Germany) or 1:16 (v/v) 10% Na$_2$-EDTA (Merck, Darmstadt, Germany), respectively, while for the biochemical assays, no anticoagulants were used. All samples had 2% (v/v) ALS added to block the activity of the toxin after blood collection. Plasma and serum were obtained by centrifugation at 1500 × *g* for 10 min and stored at −80 °C prior to use.

2.5. Biochemical parameters

Serum samples were used to measure several biochemical markers of tissue injury. Blood urea nitrogen (BUN), creatinine (Cr), uric acid (UA), creatine kinase (CK), creatine kinase – MB fraction (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (γ-GT), lactate dehydrogenase (LDH), plasma free hemoglobin (Hb) and bilirubin (BIL) levels were determined using commercially available kits

(BioClin/Quibasa, Belo Horizonte, Brazil), following the manufacturer's recommended instructions. The absorbance was read using a SP-220 spectrophotometer (BioSpectro, Paraná, Brazil), or the protocol was adapted for use in 96-well plates and the reads were performed using a SpectraMAX microplate reader (Molecular Devices Co., Sunnyvale, USA). Free hemoglobin (Hb) was measured in the plasma samples that had been collected with Na_2-EDTA. In these cases, plasma Hb levels were determined directly by spectrophotometry using a standard curve made with known concentrations of purified Hb (Sigma–Aldrich, Saint Louis, MO, USA). Samples with levels of free Hb higher than 180 mg/dL due to LOBE-induced intravascular hemolysis were diluted to avoid interference during the determination of other parameters.

2.6. Hematological parameters

Complete blood cell counts were carried out on plasma samples containing the anticoagulant Na_2-EDTA. Hemoglobin concentration (Hb), hematocrit (Hct), red blood cell counts (RBC), white blood cell counts (WBC) and the hematimetric indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH] and mean corpuscular hemoglobin concentration [MCHC]) were determined using an automated cell counter (ABX Micros ESV 60, Horiba, São Paulo, Brazil). Platelet counts (PLT) were performed using a hemocytometer (Neubauer chamber) with 10% ammonium oxalate (Merck, Darmstadt, Germany) as diluent. Blood smears stained with May-Grünwald Giemsa (BioClin/Quibasa, Belo Horizonte, Brazil) were prepared for direct examination of red blood cell morphology, platelet morphology and leukocyte differential counts under light microscopy. Reticulocyte counts (Retic) were also determined in blood smears with Brilliant Cresyl Blue (Laborclin, Paraná, Brazil) staining immediately after blood collection. Retic values were expressed as the % of total RBC counts. Blood coagulation parameters (activated partial thromboplastin time [aPTT] and fibrinogen concentration [FBG]) were measured in plasma that had been collected with trisodium citrate by following a previously described protocol (Berger et al., 2010a).

2.7. Histopathological analyses

All animals from the control and envenomed groups (at each sampling time) were necropsied and gross macroscopic alterations were examined. The kidneys, spleen, liver, heart, lungs, brain, cerebellum and skin (at the site of venom injection) were then carefully removed and fixed in a 10% neutral buffered formaldehyde solution. The tissues were dehydrated in gradual alcohol from 50% to 100%, cleared in xylene and embedded in paraffin. Subsequently, the samples were sectioned and stained with hematoxylin and eosin (H&E) for further analysis by light microscopy.

2.8. In vitro myotoxic activity

The *in vitro* myotoxic activity assays were performed as previously described (Melo and Suarez-Kurtz, 1988; Fuly et al., 2003). Briefly, rats were anesthetized (as described above) and the *extensor digitorum longus* (EDL) muscles were carefully dissected, weighed and transferred to a bath chamber with a 2.5 mL capacity. The muscles were superfused continuously with a physiological solution (135 mM NaCl; 5 mM KCl, 2 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM $NaHPO_4$; 15 mM $NaHCO_3$ and 11 mM dextrose, pH = 7.35) that was equilibrated with 95% O_2/5% CO_2. During superfusion, different concentrations of the LOBE (40 and 80 μg/mL), or the LOBE (40 μg/mL) that had been previously incubated with ALS (800 μL), were added to the bath. *Bothrops jararaca* snake venom (Butantan Institute, São Paulo, Brazil), and Triton X-100 (Sigma–Aldrich, Saint Louis, MO, USA) were used as positive controls for muscle damage under the same conditions. Preincubation of the venom with antivenom was performed at room temperature 30 min prior to addition to the perfusion bath. Samples of the perfusate (0.4 mL) were collected at 30 min intervals over a total period of 120 min and replaced with fresh solution. The collected samples were stored at 4 °C and their creatine kinase (CK) activity was determined according to the procedure described above (Subsection 2.5). EDL preparations were mounted in the bath chambers for a period of 60 min prior to the experiment for measurements of basal CK release rates, which were defined as the amount of enzyme released from the muscles into the perfusion bath prior to the addition of venom. The increase in CK released from EDL muscles after addition of LOBE was considered to be indicative of direct myotoxic activity. CK activity was expressed as enzyme units released into the medium per gram of muscle (U/g). One enzyme unit was defined as the amount that catalyzes the transformation of 1 μmol of substrate per min at 25 °C.

2.9. Determination of DNA damage and oxidized bases

2.9.1. Sample preparation

The genotoxic activity was detected *in vivo* using the model of envenomation described in Subsection 2.3.1. The blood, liver, lungs, heart and kidneys were collected at 6, 12 and 48 h after LOBE injection (1 mg/kg, s.c.). The organs were gently homogenized in a cold PBS solution (2 mL) to obtain a cell suspension. Total blood was used for the detection of DNA damage in lymphocytes. Genotoxicity was then evaluated using the comet assay.

2.9.2. Comet assay

The alkaline comet assay was performed as described by Singh et al. (1988), with minor modifications (Azqueta et al., 2009; Tice et al., 2000). Briefly, 20 μL of homogenized organs and blood were mixed with 0.75% low-melting point agarose and immediately spread onto a glass microscope slide that had been pre-coated with a layer of 1% normal-melting point agarose. The slides were then incubated in an ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH = 10.0; Gibco BRL, Grand Island, NY) at 4 °C for at least 1 h to remove the cellular proteins and membranes, leaving the DNA as "nucleoids". In the modified version of comet assay, the slides were removed from the lysis solution and washed three times in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM Na_2-EDTA, and 0.2 mg/mL BSA,

pH = 8.0), were drained and were incubated at 37 °C in this buffer with one of the following: 70 μL of Fpg (New England Biolabs, Beverly, MA, USA) at 100 mU per gel for 45 min (for the detection of oxidized purines) or 70 μL of Endo III (New England Biolabs, Beverly, MA, USA) at 100 mU per gel for 30 min (for the detection of oxidized pyrimidines). After lysis, the slides were placed in a horizontal electrophoresis unit that had been filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13.0), which was left to cover the slides for 20 min at 4 °C to allow the DNA to unwind and reveal the expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V (78 V/cm) and 300 mA. All of the steps outlined above were performed under yellow light or in the dark to prevent additional DNA damage. The slides were then neutralized (0.4 M Tris, pH = 7.5), washed in double-distilled water and stained using a silver staining protocol, as described by Nadin et al. (2001). After the staining step, the gels were left to dry at room temperature overnight and were analyzed using an optical microscope. To ensure adequate electrophoresis conditions and efficiency, negative and positive internal controls (human blood with and without 40 μM methyl methanesulfonate [MMS, Sigma–Aldrich, Saint Louis, MO, USA] treatment) were included in each experiment. Test slides were scored only when the internal controls showed clearly positive or negative results (Greggio et al., 2009). One hundred cells (50 cells from each of two replicate slides of each organ) were selected and analyzed for DNA migration. When selecting the cells, cells around the edges or air bubbles were excluded (Azqueta et al., 2009). The cells were scored visually into five classes according to tail length: class 0: undamaged, without a tail; class 1: with a tail shorter than the diameter of the head (nucleus); class 2: with a tail length 1–2 times the diameter of the head; class 3: with a tail longer than 2 times the diameter of the head; and class 4: comets with no heads. International guidelines and recommendations for the comet assay consider visual scoring of the comets to be a well-validated evaluation method (Burlinson et al., 2007). The genotoxic effects were estimated based on two different parameters: damage index (DI) and damage frequency (DF). The damage index ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The damage frequency (%) was calculated based on the number of cells with tails compared to the number of cells with no tails. Levels of Endo III and Fpg-sensitive sites were calculated from the DI score obtained with enzyme treatment minus the score without enzyme treatment (buffered). The vehicle was used as a negative control, and treatment with 4×10^{-5} M MMS for 1 h was used as a positive control.

2.10. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as the means ± standard error (SE). All biochemical and coagulation parameters were measured in triplicate. The significant differences between the mean values of two experimental groups were determined using the Student's *t* test. When more than two groups were compared, an analysis of variance was used, followed by Bonferroni's post-hoc test to compare pairs of means. *P* values less than 0.05 were chosen to establish significance.

3. Results

3.1. Clinical features and post mortem findings

Between 2 and 6 h after LOBE administration (1 mg/kg, s.c.), the rats presented signs of acute toxicity, including progressive malaise, lethargy, dyspnea, tachycardia, prostration and high sensitivity at the venom injection site. Despite general weakness, the animals showed no clear signs of neuromuscular toxicity, such as muscle trembling, paralysis or convulsions. Most of the envenomed animals displayed hematuria (dark-brown urine at 6–12 h), but no signs of macroscopic skin hemorrhage, petechiae, ecchymosis, suffusions or nasal and eye bleeding were observed. After 48 h, all of the rats had gradually recovered from the clinical symptoms and returned to normal. Until the end of the experiments (96 h), no deaths were registered. The animals in the control group (injected s.c. with PBS solution) exhibited no ill effects.

Post-mortem examinations showed evidence of systemic effects of the venom. During the first 12 h period, the animals displayed blood in the abdominal cavity, signs of lung hemorrhage (hemorrhagic spots), spleen and kidney enlargement and congestion (Fig. 1). The kidneys also seemed to have darkened slightly and had black spots on their surface (Fig. 1). The bladder was often edematous and enlarged. The brain and gastrointestinal system appeared to be macroscopically normal (not shown).

3.2. Biochemical and hematological parameters

To evaluate the acute systemic physiopathological effects of the venom, several biochemical and hematological markers of tissue lesions were measured (Table 1). Subcutaneous injection of *L. obliqua* venom caused a marked increase in serum AST, peaking between 12 and 48 h. Although less markedly than AST, serum ALT also increased rapidly after the first 2 h, reaching a maximum at 12 h. Serum levels of γ-GT increased over the first 6 h and remained elevated until 48 h. In comparison to the controls, high levels of plasma free hemoglobin, LDH and bilirubin were detected at 6 and 12 h, indicating that intravascular hemolysis had occurred. Markers of renal damage, such as creatinine, BUN and uric acid, also displayed important alterations. Serum creatinine increased mainly between 6 and 96 h, reaching maximal values at 48 h, whereas BUN increased 12, 24 and 48 h after venom injection, returning to normal levels thereafter. The animals had hyperuricemia throughout the time of envenomation, with the levels of uric acid reaching 8 times the control values ($p < 0.001$) (Table 1).

Hematological parameters were evaluated at 6, 12 and 48 h post-envenomation. The obtained results are summarized in Table 2. LOBE injection caused a statistically significant decrease in red blood cell count and hemoglobin at 12 and 48 h, whereas the platelet count decreased slightly at 12 h and returned to normal after 48 h. Hematocrit values were lower when compared to the controls at
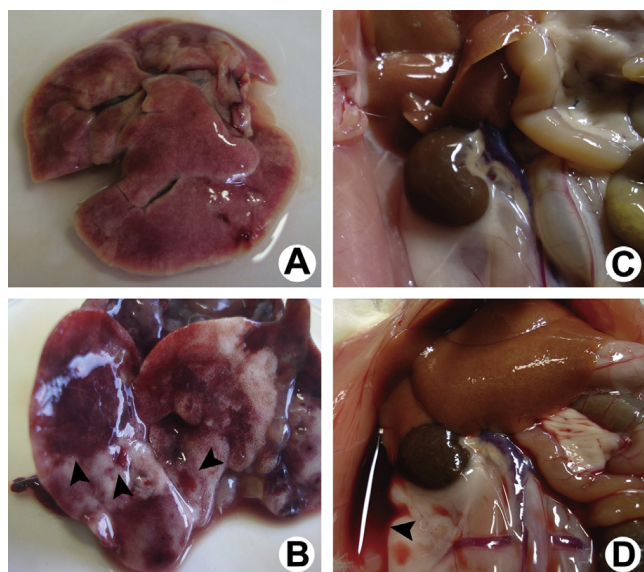
Fig. 1. Macroscopic organ alterations after *L. obliqua* envenomation in rats. Representative images of: **A**. The lungs from a control animal (one that had been injected with PBS solution, s.c.), showing normal morphology. **B**. Lungs from an animal that had been injected with LOBE (1 mg/kg, s.c.) after 6 h of envenomation, showing signs of petechial hemorrhage (arrowheads). **C**. Abdominal cavity of a control animal with normal appearance. **D**. Abdominal cavity of an animal 12 h post-envenomation, evidencing the occurrence of bleeding. Also note the kidneys, which appear to be slightly darkened and have black spots on their surface.

all of the time points evaluated. The reticulocyte number (immature red cells) increased in the blood stream as a result of hemolysis and anemia. The hematimetric indices, MCV and MCH, also increased at 48 h, whereas MCHC and total protein remained unchanged. Envenomed rats displayed leukocytosis between 6 and 12 h, mainly due to high neutrophil (6–48 h) and lymphocyte (6–12 h) counts. Compared to control values, a 15-fold increase was observed only in neutrophil numbers at 6 h. A less expressive increase in monocytes and eosinophil counts was also observed at the same time. Under light microscopy, the blood smears revealed fragmented erythrocytes, spherocytes and significant anisocytosis. The leukocytes appeared to have normal morphology (data not shown).

3.3. Histopathological alterations

Evidence of tissue damage was observed mainly between 6 and 48 h of envenomation. Skin microscopy, at the site of LOBE injection, showed hemorrhagic lesions, muscle necrosis and focal inflammatory infiltration that was associated with edema of varying intensities (Fig. 2A and B). At 6 h, the kidneys displayed red-brown and hyaline pigments, which formed intra-tubular cylinders and/or accumulated within the tubular epithelial cells (Fig. 2C and insert in 2D). At 48 h, an inflammatory infiltrate was observed in the medullar region of the kidneys (Fig. 2D). Marked diffuse congestion and erythrophagocytosis were observed in the spleens at 6 h, whereas large aggregates of hemosiderin engulfed macrophages were noted at 48 h (Fig. 2E and F). The lungs displayed intense hemorrhaging, which was evidenced by the presence of abundant erythrocytes in the bronchiolar and alveolar spaces mainly at 6 h. Lung sections also demonstrated mixed inflammatory infiltrate of polymorphonuclear and mononuclear cells that dilated the alveolar septa (48 h), edema in the pulmonary parenchyma and perivascular edema (12 h) (Fig. 3). No morphological changes were observed in the liver, brain and cerebellum at any of the time points evaluated. After 96 h of envenomation, all organs displayed normal morphology. The animals in the control group (those that had been injected with PBS) exhibited no alterations at all.

3.4. Myotoxicity

The myotoxicity of *L. obliqua* venom was evaluated both *in vivo* (Fig. 4) and *in vitro* (Fig. 5) by measuring the release of creatine-kinase (CK) and its cardiac isoform, creatine-kinase-MB (CK-MB), and was also evaluated by morphological examination. After subcutaneous injection of LOBE (1 mg/kg), the rats displayed high levels of serum CK, which was the first evidence of skeletal muscle damage. At 12 h, serum CK activity had increased 20-fold, reaching levels 40 times higher than control values at 48 h (Fig. 4A). There was also a significant increase in serum CK-MB activity, which reached a maximum at 12 h (53.6 ± 7.5 U/L) as compared to the control group (5.8 ± 0.4 U/L), indicating that cardiac

Table 1
Biochemical markers of tissue injury during the time course of *L. obliqua* envenomation in rats.

Parameter	Time after envenomation (h)						
	CTRL	2	6	12	24	48	96
AST (U/mL)	11.8 ± 0.2	32.3 ± 0.4	30.7 ± 2.6	119.1 ± 15.2^a	113.5 ± 6.8^a	139.5 ± 12.3^a	17.9 ± 1.4
ALT (U/mL)	22.4 ± 0.6	51.9 ± 0.9^a	43.9 ± 3.5^a	69.8 ± 3.9^a	67.3 ± 3.3^a	49.2 ± 2.4^a	29.9 ± 1.2
γ-GT (U/L)	9.2 ± 0.6	14.1 ± 0.9	23.4 ± 1.4^b	30.6 ± 2.0^a	36.6 ± 3.5^a	38.9 ± 3.0^a	15.8 ± 1.7
Hb (mg/dL)	57.7 ± 4.6	131.1 ± 4.4	399.9 ± 62.5^a	300.7 ± 8.9^b	135.5 ± 42.0	47.1 ± 15.4	44.1 ± 11.6
LDH (U/L)	431.8 ± 40	2288 ± 167	7374 ± 882^a	8578 ± 1484^a	1484 ± 216	928.8 ± 55	735.2 ± 123
BIL-T (mg/dL)	0.2 ± 0.08	0.6 ± 0.07	1.8 ± 0.2^a	1.7 ± 0.2^a	0.8 ± 0.05	0.2 ± 0.08	0.3 ± 0.07
BIL-D (mg/dL)	0.07 ± 0.03	0.4 ± 0.06	0.9 ± 0.08^a	1.0 ± 0.1^a	0.5 ± 0.04	0.07 ± 0.03	0.06 ± 0.01
BIL-I (mg/dL)	0.1 ± 0.05	0.2 ± 0.01	0.9 ± 0.09^a	0.7 ± 0.09^a	0.3 ± 0.03	0.1 ± 0.05	0.2 ± 0.01
Cr (mg/dL)	0.3 ± 0.03	0.7 ± 0.1	1.4 ± 0.3^c	2.5 ± 0.4^a	2.3 ± 0.1^a	3.5 ± 0.3^a	1.3 ± 0.1^c
BUN (mg/dL)	43.3 ± 0.6	53.8 ± 1.6	74.5 ± 2.3	104.7 ± 6.3^a	193.8 ± 9.6^a	211.0 ± 9.5^a	50.5 ± 0.9
UA (mg/dL)	0.6 ± 0.04	1.6 ± 0.06^c	1.8 ± 0.1^b	3.0 ± 0.1^a	3.6 ± 0.2^a	4.8 ± 0.2^a	3.8 ± 0.3^a

CTRL: control (animals injected with PBS); AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GT: γ-glutamyl transferase; Hb: plasma hemoglobin; LDH: lactate dehydrogenase; BIL-T: total bilirubin; BIL-D: direct bilirubin; BIL-I: indirect bilirubin; Cr: creatinine; BUN: blood urea nitrogen; UA: uric acid. Data are presented as means $\pm$ SE of 6 animals (controls) and 8 animals (LOBE, 1 mg/kg, via s.c). Values of $^a(p < 0.0001)$, $^b(p < 0.001)$ and $^c(p < 0.05)$ were considered statistically different when compared to the basal levels of control rats (ANOVA followed by Bonferroni's post hoc test).

Table 2
Hematological parameters during the time course of *L. obliqua* envenomation in rats.

Parameters	Time after envenomation (h)			
	CTRL	6	12	48
Erythrocytes				
RBC ($\times 10^{12}$/L)	8.6 ± 0.01	7.9 ± 0.1	7.2 ± 0.3[b]	4.4 ± 0.3[a]
Retic (%)	1.7 ± 0.2	2.8 ± 0.1	9.4 ± 0.9[a]	14.7 ± 1.1[a]
PLT ($\times 10^3$/μL)	465.7 ± 43.1	349.8 ± 16.1	316.3 ± 44.7[c]	506.6 ± 34.3
Hb (g/dL)	15.6 ± 0.07	14.5 ± 0.2	13.8 ± 0.1[b]	10 ± 0.6[a]
Hct (%)	48.5 ± 0.7	43 ± 0.7[c]	41 ± 0.4[a]	32 ± 1.7[a]
MCV (fL)	57.7 ± 0.3	56.5 ± 0.03	57 ± 2.1	72.1 ± 1.1[a]
MCH (pg)	18.2 ± 0.1	18.2 ± 0.01	19.3 ± 0.7	23.5 ± 0.2[a]
MCHC (g/dL)	31.9 ± 0.3	32.9 ± 0.4	33.7 ± 0.1	31.2 ± 0.3
TP (mg/dL)	67 ± 3.1	66.5 ± 1.5	71 ± 1.1	71 ± 1.2
Leukocytes				
WBC ($\times 10^9$/L)	3.8 ± 0.08	16.5 ± 1.3[a]	14.1 ± 0.5[a]	5.6 ± 0.5
Lymph ($\times 10^9$/L)	2.5 ± 0.1	4.6 ± 0.3[a]	7.5 ± 0.3[a]	2.4 ± 0.1
Neut ($\times 10^9$/L)	0.8 ± 0.1	11.9 ± 0.5[a]	5.3 ± 0.3[a]	2.8 ± 0.4[b]
Mono ($\times 10^9$/L)	0.2 ± 0.04	0.7 ± 0.07[c]	1.3 ± 0.1[a]	0.3 ± 0.04
Eosino ($\times 10^9$/L)	0.01 ± 0.001	0.2 ± 0.04[c]	0.1 ± 0.03	0.02 ± 0.001
Baso ($\times 10^9$/L)	0.03 ± 0.009	0.02 ± 0.008	0.02 ± 0.007	0.02 ± 0.008

CTRL: control (animals injected with PBS); RBC: red blood cells; Retic: reticulocytes; PLT: platelets; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; TP: total protein; WBC: white blood cells; Lymph: lymphocytes; Neut: neutrophils; Mono: monocytes; Eosino: eosinophils; Baso: basophils. Data are presented as means ± SE of 6 animals (controls) and 8 animals (LOBE, 1 mg/kg, via s.c.). Values of [a] ($p < 0.0001$), [b] ($p < 0.001$) and [c] ($p < 0.05$) were considered statistically different when compared to the basal levels of control rats (ANOVA followed by Bonferroni post hoc test).

damage had occurred. These values remained elevated at 24 h (45.6 ± 3.4 U/L) and 48 h (40.0 ± 0.9 U/L) (Fig. 4B). Heart histopathological analyses confirmed the cardiotoxicity of the venom. Necrosis of cardiomyocytes was associated with inflammation and myocardial hemorrhage between 6 and 48 h (Fig. 4C–E).

To investigate a possible direct myotoxic effect of the LOBE, an isolated muscle preparation was used. As shown in Fig. 5A, when two different concentrations of the LOBE were added to the *extensor digitorum longus* (EDL) preparations, dose- and time-dependent increases in CK release rates were observed in comparison to the controls (EDL treated with PBS). This result indicated that the venom has specific myotoxins that are able to act directly on muscle cells, which confirms the data obtained systemically in envenomed rats. When compared with the snake venom from *B. jararaca* (a well characterized myotoxic venom), the LOBE presented a myotoxic activity that was approximately 32.6% lower at the same dose (Fig. 5B). In addition, the previous incubation of the LOBE with antilonomic serum (ALS) resulted in a reduction of 70.6% in CK release rate from the EDL. The highest level of released CK was observed upon the addition of the cytotoxic compound Triton X-100 (0.01%), which was used as a non-specific, non-biological positive control. Taking the muscle injury induced by Triton X-100 to be 100%, the myotoxic damage of the *B. jararaca* and *L. obliqua* venoms reached 58.8% and 39.6%, respectively, in our experimental conditions.

3.5. Genotoxicity

Because the maintenance of genomic stability is essential for cellular function, we measured the genotoxic effects induced by *L. obliqua* experimental envenomation *in vivo* (Fig. 6). In the first set of experiments, DNA damage in the different organs and lymphocytes of rats 12 h after LOBE injection (1 mg/kg, s.c.) was assessed using the alkaline comet assay. For all samples, cell viability was evaluated using the trypan blue exclusion method and was found to be greater than 90% in every experiment. The internal controls for the comet assay, using human blood cells, showed low damage in the negative control (DI = 0–10) and high damage in the positive control (DI = 180–300), thus validating the test conditions. As expected, exposure of the lymphocytes, heart, lungs, liver and kidney cells that had been isolated from normal animals to methyl methanesulfonate (MMS), which was used as positive control, resulted in a significant increase in DNA damage (not shown). As shown in Fig. 6A, envenomed rats displayed high levels of DNA damage in the cells of all organs evaluated, as well as in the lymphocytes. The damage levels in the cells of the control animals (those that had been injected with PBS) did not change significantly. The damage index in lymphocytes and kidneys reached levels that were 6.4 and 5.4 times higher than the levels in their respective controls.

In another set of experiments, the kidneys were chosen to determine the temporal pattern of DNA damage at distinct time points after LOBE injection. In such cases, kidneys were selected because they had the highest damage index among the organs examined and also due to the high incidence of renal injury observed in human patients (Gamborgi et al., 2006). At 6 h, kidney DNA damage had increased, reaching a maximal level at 12 h. After 48 h, the damage index decreased but was still significantly different from the controls (Fig. 6B). In order to verify the oxidative nature of the DNA damage detected in the kidney cells of LOBE-injected rats, we carried out a modified comet assay. While the alkaline test normally detects primarily repairable DNA single- and double-strand breaks and alkali-labile sites, the modified version is more specific to oxidative damage than the standard method. The modified version includes an incubation step with lesion-specific endonucleases that recognize resultant abasic sites and convert them into single-strand breaks. In the present study, we
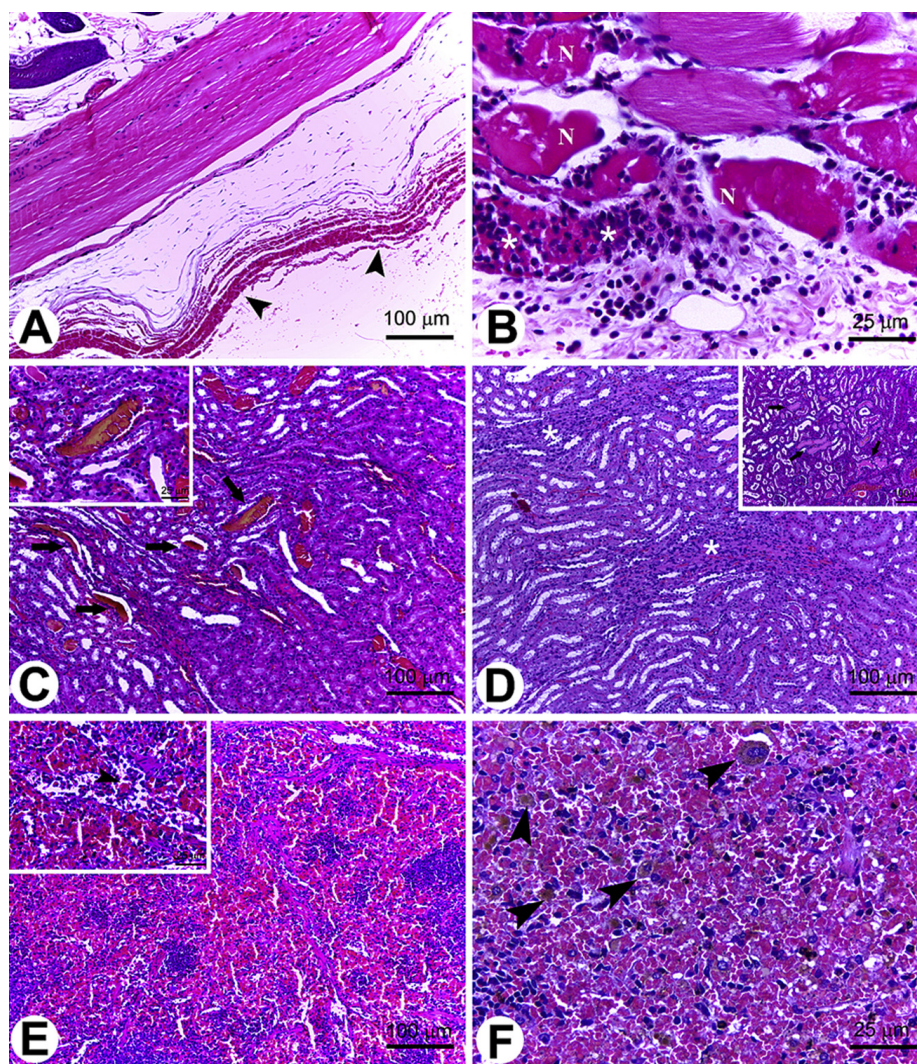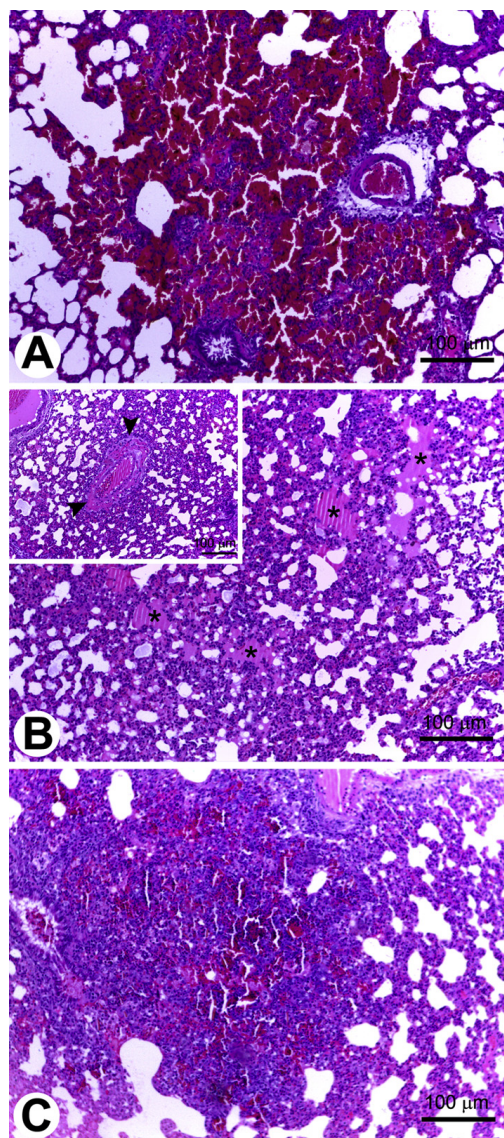
Fig. 2. Light micrographs showing the histopathological changes induced by *L. obliqua* venom in the vital organs of rats. **A**. Skin, 6 h post-envenomation, presenting subcutaneous hemorrhage at the site of venom injection (arrowheads). **B**. Skin, 12 h post-envenomation. Note the skeletal muscle necrosis (N) and inflammatory infiltrate (white asterisk). **C**. Kidney, 6 h post-envenomation. Cortical sections showing the deposits of a red-brown material in the tubules forming granular casts (arrows). *Insert*: Detail of an intra-tubular cast. **D**. Kidney, 48 h post-envenomation. Medullar section evidencing inflammatory infiltrate (white asterisk). *Insert*: Cortical section showing tubules with hyaline casts (arrows). **E**. Spleen, 6 h post-envenomation. Note the marked diffuse congestion and erythrophagocytosis. *Insert*: Detail of erythrophagocytosis (arrowhead). **F**. Spleen, 48 h post-envenomation. Note the large aggregates of hemosiderin engulfed macrophages (arrowheads). All sections were stained with H&E. Magnifications: 10× (Panels A, C, D, inserts in D and E); 40× (Panels B, inserts in C, E and F).

used Fpg, which is specific for oxidized purines, and Endo III, which targets oxidized pyrimidines. Thus, increases in the damage index after enzyme incubation specifically represent the extent of oxidative DNA damage. As shown in Fig. 6C and D, there was an increase in the oxidative damage score after incubation with Fpg and Endo III, indicating the presence of oxidized purines and pyrimidines. As the levels of ordinary and oxidatively generated DNA adducts were similar (mainly between 6 and 12 h), the majority of the DNA damage observed in the kidneys was likely due to oxidative insult (Fig. 6B–D).

3.6. Effects of antivenom treatment on markers of systemic tissue damage

Since the administration of antilonomic serum (ALS) is the only specific treatment actually available for *L. obliqua* envenomation, we decided to test its efficacy in neutralizing biochemical and coagulation abnormalities using our experimental model. For this purpose, ALS was intravenously administered at 2 or 6 h post-LOBE injection (1 mg/kg, s.c.). After 24 h of envenomation, different biochemical markers and coagulation parameters were determined (Table 3). Generally, treatment with ALS is able to neutralize LOBE-induced biochemical alterations only if administered within the first 2 h of envenomation. For example, animals treated with ALS at 2 h had a decrease of 3.6- and 2.5-fold in the levels of serum creatinine and urea, respectively, when compared with the group treated 6 h after LOBE injection. In addition, both the creatinine and urea levels of the envenomed animals that had been treated at 2 h with ALS were not significantly different from the values observed in non-envenomed rats that had been treated with PBS or ALS, indicating that these levels had returned to control values. Similar results were obtained for other parameters, such as CK, CK-MB, AST and ALT,

Fig. 3. Light micrographs showing the histopathological changes in the lungs of rats that had been injected with *L. obliqua* venom. Section of the lungs from envenomed animals presented extensive hemorrhage mainly 6 h post-venom injection (**A**), signs of inflammation such as edema (**B**) and perivascular edema (*insert* in **B**) at 12 h, and inflammatory cell infiltrate (**C**) at 48 h. All sections were stained with H&E. Asterisks and arrowheads in panel B indicate areas of edema and perivascular edema, respectively. Magnification: 10× (Panels A–C).

histological signs of hemorrhage were also absent in the envenomed groups that had received ALS injections at 2 or 6 h (results not shown).
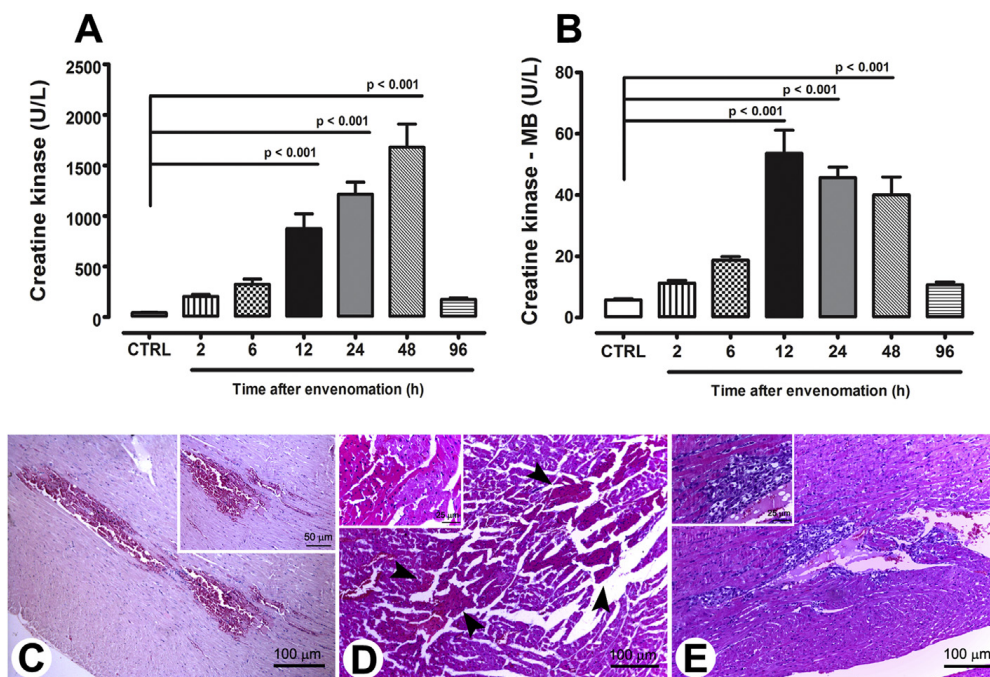
4. Discussion

In the present study, we used an experimental model in rats to investigate the acute physiopathological effects of *L. obliqua* venom. This model allowed for the broad characterization of venom-induced tissue damage, including biochemical, hematological, histopathological, myotoxic, cardiotoxic and genotoxic alterations.

Envenomed animals showed macroscopic and histological evidence of systemic hemorrhages, confirming the main clinical profile observed in human patients (Zannin et al., 2003). Bleeding in the abdominal cavity and subcutaneous tissue, hematuria and hemorrhages in the myocardium and pulmonary parenchyma were observed in our experimental animals. Actually it is known that different venom toxins are involved in these hemorrhagic alterations. Most of the toxins are serine proteases, an expressive group representing 16.7% and 25% of the clusters derived from the tegument and bristle transcriptomes, respectively (Veiga et al., 2005). This protein group displays coagulation factor-like activities, so these enzymes are expected to participate in the generation of thrombin by activation of factor X and prothrombin (Veiga et al., 2009; Berger et al., 2010a) and in the activation of the fibrinolytic system, contributing directly and indirectly to fibrinogen degradation (Pinto et al., 2006), resulting in consumption coagulopathy. In fact, serine proteases with fibrinogenolytic, prothrombin and factor X activating activities have been purified and characterized in this venom (Alvarez-Flores et al., 2006; Pinto et al., 2004; Reis et al., 2006). Rats injected intravenously with one of these enzymes, a purified prothrombin activator, displayed coagulopathy that was associated with reduced levels of fibrinogen, pulmonary hemorrhage and leukocyte infiltration in the lungs (Reis et al., 2001), which was similar to the observations presented here for whole venom.

In addition to the hemostatic abnormalities, the rats displayed intravascular hemolysis, as evidenced by alterations in several parameters, such as high levels of free hemoglobin, increased unconjugated bilirubin levels, high serum LDH activity, decreased RBC counts and hematocrit, and the presence of reticulocytes (immature RBCs), spherocytes and fragmented RBCs in the blood smears. The spleens of the envenomed animals also presented signs of erythrophagocytosis and deposits of hemosiderin, indicating high clearance of defective RBCs and the accumulation of hemoglobin metabolic products. An important contribution of this hemolytic process to venom-induced pathology is most likely related to the deposition of hemoglobin in the renal tubules. Hemolysis-related AKI is characterized by the formation of tubular hemoglobin casts, which are highly nephrotoxic (Zager, 1996). In the present study, envenomed animals presented red-brown and hyaline pigments with a granular appearance in their renal tubules which were most likely due to the formation of hemoglobin and/or myoglobin deposits. Reports describing a human case of hemolysis-related AKI, and also

which became normalized only in envenomed rats that had received ALS within the first 2 h. Likewise, plasma hemoglobin levels were also decreased in envenomed rats when ALS was injected at 2 h. However, this reduction was not statistically significant in comparison to envenomed animals that had been treated with PBS instead of ALS. Thus, ALS was not able to completely reverse intravascular hemolysis, even if given early after envenomation.

As expected, envenomed animals that were treated with PBS developed consumptive coagulopathy, with lower levels of fibrinogen and prolonged activated partial thromboplastin time (Table 3). In this case, the treatment with ALS both at 2 or 6 h after venom injection normalized the coagulation parameters. The macroscopic and

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Fig. 4. *In vivo* myotoxic and cardiotoxic activities. Serum levels of creatine kinase (**A**) and creatine kinase-MB (**B**) during the time-course of *L. obliqua* envenomation in rats. Histopathological analyses of the heart confirmed the cardiotoxicity of the venom. At 6 h of envenomation, there were extensive areas of myocardial hemorrhage (**C**). Necrosis of cardiomyocytes (**D**) and inflammation (**E**) were observed mainly at 12 and 48 h, respectively. These alterations are shown in detail in the insert of each image. Arrowheads in panel D indicate necrotic cells. All sections were stained with H&E. Magnifications: $10\times$ (Panels C, D and E); $20\times$ (Insert in C); $40\times$ (Inserts in D and E). Biochemical data are presented as the means $\pm$ SE. Each sample from the control (CTRL, $n = 6$, injected with PBS, s.c.) or envenomed animals ($n = 8$, injected with LOBE, 1 mg/kg, s.c.) was measured in triplicate. Values of $p < 0.001$ were considered to be significantly different from the controls.
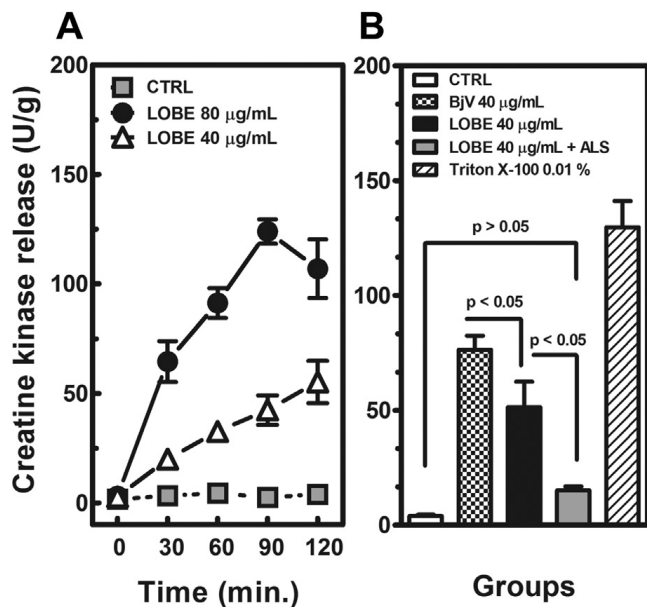
Fig. 5. *In vitro* myotoxic activity. The effects of *L. obliqua* venom were tested on isolated EDL muscles. **A.** At time $= 0$, PBS (control, CTRL) or different concentrations of LOBE were added to the perfusion bath containing the EDL muscles. Aliquots were taken at the indicated times to determine creatine kinase (CK) levels. Each time point represents the means $\pm$ SE of six independent experiments. The levels of CK that were released from EDL previously treated with LOBE differed from the controls at all time points ($p < 0.05$). **B.** The myotoxic activity of LOBE was also compared in the presence or absence of antilonomic serum (ALS). *Bothrops jararaca* venom (BjV) and Triton X-100 (0.01%) were used as positive controls for myotoxic activity. After 120 min, aliquots were taken for the CK determinations. Data are expressed as the means $\pm$ SE ($n = 6$/group). Values of $p < 0.05$ were considered to be statistically significant for the comparisons among the groups.

an experimental study confirming the occurrence of intravascular hemolysis, have already been published (Seibert et al., 2004; Malaque et al., 2006). However, little is known regarding the ability of ALS to neutralize hemolytic effects. Our data indicate that ALS was not able to completely reverse intravascular hemolysis, even if administered early in the envenomation. The case report of hemolysis-related AKI also indicated that treatment with ALS did not reduce hemoglobin levels to normal values and the patient did not completely recover renal function until 1 month after the accident, despite improvements in coagulation tests (Malaque et al., 2006). Until now, the main component with high *in vitro* hemolytic activity isolated from this venom was the phospholipase A2 enzyme, although the presence of proteolytic enzymes that act specifically on the membrane glycoproteins of erythrocytes cannot be ruled out (Seibert et al., 2006, 2010).

Since myotoxins are commonly described in several snake, spider and bee venoms, the presence of myotoxic activity in *L. obliqua* was investigated using specific biochemical markers, *in vitro* experiments and histological analyses. In this sense, elevations of serum CK and CK-MB activities were detected, indicating systemic damage to skeletal and cardiac muscles. CK is a dimer with M and B subunits that is found primarily in the muscle, myocardium, brain and lung tissues and exists as three dimeric isoenzymes: CK-MM, CK-MB and CK-BB. CK-MB accounts for 5%–50% of total CK activity in the myocardium and is well-established to be a clinical marker that can confirm acute myocardial infarction both in humans and experimental animals (Apple and Preese, 1994; Shashidharamurthy et al.,
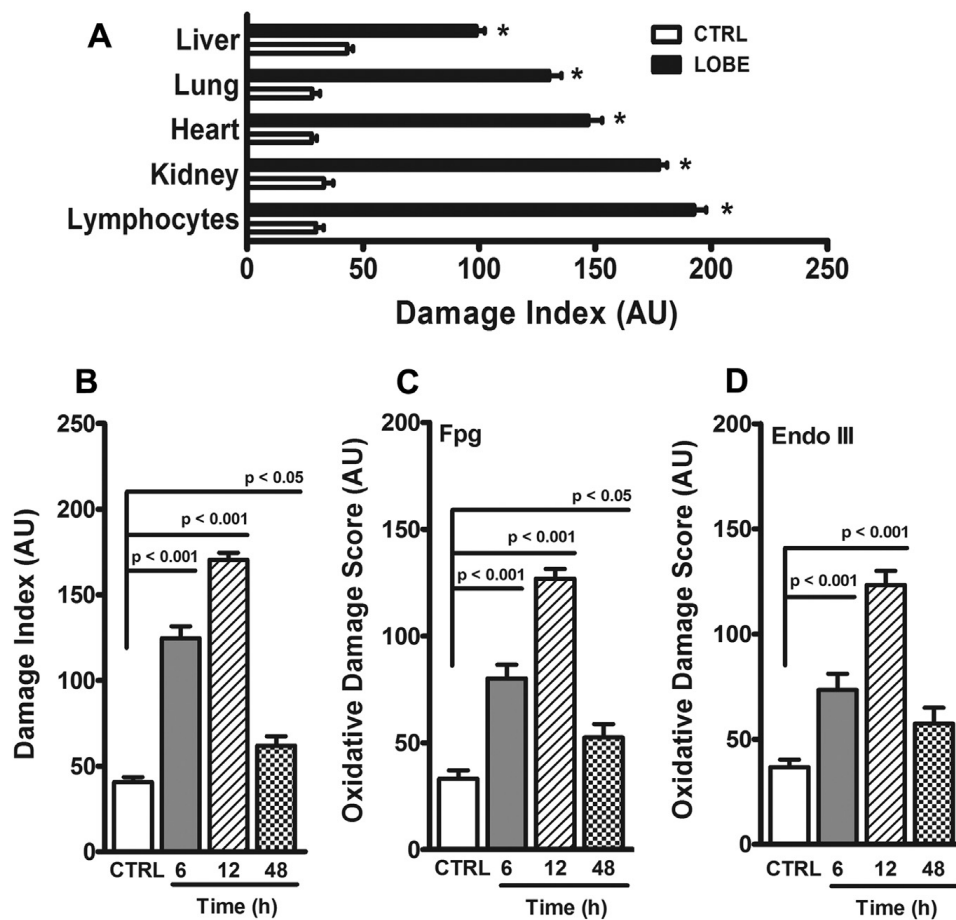
Fig. 6. Genotoxic activity. **A**. DNA damage in the different organs of the control (CTRL, injected with PBS, s.c.) and envenomed animals (injected with the LOBE, 1 mg/kg, s.c.) was assessed using the alkaline comet assay 12 h post-venom injection. **B**. The temporal pattern of DNA damage in the kidneys of the control and envenomed animals at different time points. **C** and **D**. Oxidative DNA damage in the kidneys was also determined at different times post-venom administration using the modified version of the comet assay with Fpg (C) and Endo III (D) enzymes. Data are expressed as the means $\pm$ SE ($n = 6$ rats/group). Values of $p < 0.05$ and $p < 0.001$ (*) were considered to be statistically significant in comparison to the control groups.

2010). Correlated with the increases in CK and CK-MB, histological analyses revealed extensive muscle damage mainly in the subcutaneous tissue (at the local site of venom injection) and myocardial necrosis. These observations support the idea that the LOBE has cardiotoxic activity, which was unknown up until now. Clinical reports of human envenomation that are available in the literature do not describe symptoms of cardiac dysfunction, and CK or CK-MB levels are rarely measured in these patients, making it difficult to make any comparisons with our experimental data. Our hypothesis is that the contribution of muscle damage observed herein is more related to myoglobin release from the myocytes or cardiomyocytes than to a mechanism that is associated with heart dysfunction. Indeed, similar to hemoglobin, myoglobin can also precipitate in renal tubules, after being filtered by the glomeruli, and forms obstructive casts. The direct myotoxic activity of LOBE was confirmed *in vitro* by the experiments with isolated EDL muscles. LOBE showed a dose- and time-dependent myotoxicity in isolated EDL, although its potency was lower when compared to *B. jararaca* venom. In fact, different myotoxins have been described in *B. jararaca* venom, including metalloproteinases and myotoxic

phospholipase A2 (Zelanis et al., 2011), while in *L. obliqua* the toxins responsible for this activity remain completely unknown. However, *L. obliqua* myotoxins seem to be recognized by ALS because treatment with this serum was able to reverse CK release *in vitro* (from EDL muscle) and *in vivo* if administered within 2 h of envenomation.

Though AKI is the leading cause of death following *L. obliqua* envenomation, the mechanisms involved in kidney disorders are poorly understood (Gamborgi et al., 2006). The current knowledge is based on clinical data from human victims in which hematuria, high levels of serum creatinine and acute tubular necrosis were described to be the main features of *L. obliqua*-induced AKI (Burdmann et al., 1996). In our experimental model, in addition to the high levels of serum creatinine, the rats also displayed uremia and hyperuricemia, suggesting impaired renal function. Generally, the mechanism underlying venom-induced AKI is complex and appears to be multifactorial. Until now, studies performed with a variety of nephrotoxic venoms have indicated that AKI is associated with both the direct cytotoxic action of the venom on renal structures and a secondary response of the whole organism resulting from systemic envenomation (Abdulkader et al., 2008; Berger

Table 3
Effects of treatment with anti-Ionomic serum (ALS) on markers of systemic tissue damage during *L. obliqua* envenomation.

Treatment group	Cr (mg/dL)	BUN (mg/dL)	CK (U/L)	CK-MB (U/L)	Hb (mg/dL)	AST (U/mL)	ALT (U/mL)	FBG (g/L)	aPTT (s)
PBS	0.3 ± 0.04	67.1 ± 2.9	16.7 ± 1.1	6.0 ± 0.5	65.2 ± 5.1	16.5 ± 1.6	14.9 ± 2.1	1.6 ± 0.04	31.7 ± 3.8
ALS	0.3 ± 0.07[b,c]	65.5 ± 3.7[b,c]	18.9 ± 1.5[b,c]	5.3 ± 0.4[b,c]	62.2 ± 2.3[c]	36.3 ± 3.1[b,c]	42.1 ± 1.9[a,b,c]	2.1 ± 0.4[b]	32.7 ± 2.8
LOBE + PBS	1.9 ± 0.2[a]	175.3 ± 18.8[a]	244.2 ± 26.6[a]	54.3 ± 7.1[a]	215.6 ± 72.6[a]	106.7 ± 20.8[a]	72.1 ± 5.1[a]	0.5 ± 0.04[a]	79.9 ± 17[a]
LOBE + ALS (2 h)	0.5 ± 0.05[b,c]	53.2 ± 2.0[b,c]	32.9 ± 4.7[b,c]	5.8 ± 0.8[b,c]	137.4 ± 19.8	30.3 ± 1.0[b,c]	26.3 ± 1.7[b,c]	2.3 ± 0.3[b]	22.5 ± 1.5[b]
LOBE + ALS (6 h)	1.8 ± 0.2[a]	133.3 ± 4.6[a,b]	278.9 ± 25.5[a]	41.1 ± 5.1[a,b]	271.1 ± 53.9[a]	90.9 ± 5.9[a]	67.0 ± 6.3[a]	1.6 ± 0.2[b]	36.4 ± 5.6[b]

Cr: creatinine; BUN: blood urea nitrogen; CK: creatine kinase; CK-MB: creatine kinase isoenzyme MB; Hb: plasma hemoglobin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; FBG: fibrinogen; aPTT: activated partial thromboplastin time. Envenomed animals ($n = 8$/group) were treated with PBS or ALS after 2 or 6 h of LOBE injection (1 mg/kg, via s.c). Control animals ($n = 6$/group) were injected only with PBS or ALS. After 24 h, samples of plasma and serum were collected. Data are presented as means ± SE. Statistical analyses were performed by ANOVA followed by Bonferroni's post hoc test. [a]($p < 0.05$ vs PBS), [b]($p < 0.05$ vs LOBE + PBS) and [c]($p < 0.05$ vs LOBE + ALS 6 h).

et al., 2012). The secondary response is usually triggered by renal inflammation, oxidative damage and the release of cytokines and vasoactive substances that lead to changes in renal function and hemodynamics. Hemolysis, rhabdomyolysis and/or the intravascular deposition of platelets and fibrin in the kidney microcirculation are also important contributors to this process (Sitprija, 2006). Recently, high levels of uric acid were observed to play an important role in AKI induced by *Crotalus* envenomation, since the treatment with allopurinol, a hypouricemiant agent, significantly reduced the lethality rate and ameliorated renal histopathological changes (Frezzatti and Silveira, 2011). Marked hyperuricemia is known to cause AKI by the supersaturation, crystallization and deposition of urate crystals, as well as by contributing to renal vasoconstriction, since soluble uric acid has been shown to inhibit endothelial NO bioavailability (Yamasaki et al., 2008; Ejaz et al., 2007). During *L. obliqua* envenomation, the rats also presented high levels of uric acid, tubular obstructive casts and inflammatory infiltrates in the kidneys. However, the actual contribution of these elements to AKI requires further study. Interestingly, antivenom serotherapy was able to reduce creatinine and urea levels only if administered within 2 h of LOBE injection. Antivenom treatment after 6 h was unable to fully correct the renal parameters, despite its ability to normalize coagulation abnormalities. Thus, it seems that the time elapsed between the accident and the administration of antivenom is crucial for a successful renal therapy. Confirming our observations, it was demonstrated that a time interval of more than 2 h between the accident and administration of the antivenom was associated with the development of AKI, as well as with the risk of death or permanent injuries after *Bothrops* and *Crotalus* envenomation (Otero et al., 2002; Pinho et al., 2005).

Unlike the lungs, kidneys, spleens and hearts, no morphological changes were observed in the livers, brains and cerebellums of rats that had been injected with *L. obliqua* venom. Nevertheless, biochemical markers of acute liver injury (AST, ALT and γ-GT) were increased in the serum of animals after envenomation. As it is known that some of these enzymes are not specific to the liver, it is possible that they were derived from other sources, such as the red blood cells or skeletal muscle. For instance, increases in AST activity are also associated with damage to cardiac and skeletal muscle and the kidneys (Prado et al., 2010; Shashidharamurthy et al., 2010). Despite these apparently conflicting observations, we cannot rule out the occurrence of liver injury, mainly because evidence of DNA damage was detected in liver cells using the comet assay. Probably, these findings indicate that the extent of acute hepatic injury in this model of envenomation was subtle and did not lead to gross histological alterations.

As mentioned above, *L. obliqua* envenomation may have triggered an intense inflammatory response, which may be involved in several of the clinical manifestations. The activation of the kallikrein-kinin system and the consequent release of vasoactive mediators (mainly bradykinin, histamine and prostaglandins) seems to play an essential role in the edematogenic, nociceptive and vascular effects (Bohrer et al., 2007). Accordingly, we have shown that during envenomation the animals experienced neutrophilic leukocytosis, indicating that a systemic inflammatory response had occurred. Histological sections also provided evidence of inflammatory cell infiltrates in the heart, lungs and kidneys. Corroborating these results, a clear activation in the vascular bed that was characterized by an increase in leukocyte rolling and adhesion to the endothelium was observed in hamster cheek pouch venules that had previously been incubated with low doses of LOBE (Nascimento-Silva et al., 2012). The up-regulated expression of genes from pro-inflammatory mediators and adhesion molecules, such as IL-8, IL-6, CCL2, CXCL1, E-selectin, VCAM-1 and ICAM-3, was also detected in endothelial cells and fibroblasts after incubation with LOBE. Once released, these mediators acted as chemoattractants, inducing inflammatory cell migration to the sites of injury (Pinto et al., 2008; Nascimento-Silva et al., 2012).

Recently, classical methods of genetic toxicology have been applied to the identification of potential therapeutic agents in animal venoms (mainly for the treatment of some types of cancer) and have also provided a better understanding of the toxic mechanisms of action of these venoms in the human body (Marcussi et al., 2011, 2013). During envenomation, genotoxic damage can occur directly due to the cytotoxic activity of the venom or indirectly through the production of cytotoxic mediators (such as free radicals, for example) in response to tissue injury. In both cases, the damage could lead to DNA fragmentation and eventually, cell death. In the case of cell survival, DNA lesions not corrected during the cell repair process might result in permanent mutations and changes to cellular morphology or physiology that could also be transmitted hereditarily to future generations. Therefore, it is possible that the genotoxic effects are involved not only in the acute toxicity, but

also in chronic diseases, and may even be involved in mutagenic and carcinogenic events resulting from envenomation. In this sense, it has been shown that some *Bothrops* toxins are able to induce genotoxic and mutagenic effects in isolated human lymphocytes, as evidenced by the comet and micronucleus assays, respectively (Marcussi et al., 2013). Here, various organs of animals that had been injected with *L. obliqua* venom presented DNA lesions, indicating the high genotoxic potential of this venom. DNA damage was detected in the kidneys, heart, lungs, liver and lymphocytes of envenomed rats. Specifically, DNA lesions in the kidneys were prominent 6, 12 and 48 h post-envenomation, and the majority of these lesions were due to oxidative damage because oxidized purines and pyrimidines were detected. In fact, the possible production of free radicals during envenomation should be considered in an effort to understand the complex mechanisms involved in kidney dysfunction. In this case, the presence of hemoglobin and/or myoglobin deposits in the renal tubules may contribute to kidney dysfunction, since the degradation of these molecules releases free iron and heme, which catalyze the production of free radicals and induce lipid peroxidation, respectively (Zager, 1996; Yamasaki et al., 2008). The participation of oxidative damage was confirmed in a model of *Crotalus*-induced AKI, in which treatment with antioxidant agents protects against venom-mediated nephrotoxicity (Alegre et al., 2010).

5. Conclusions

In this work, we characterized a series of acute physiopathological effects induced by the subcutaneous injection of *L. obliqua* venom in rats. Our data reveal important biochemical, hematological and histopathological alterations, suggesting the occurrence of multi-organ damage and confirming that the rat is a good animal model for studying hemorrhagic disturbances, as well as organ specific injuries, such as AKI. Interestingly, myotoxic, cardiotoxic and genotoxic activities were identified during our experiments. To our knowledge, this is the first study to show these activities of *L. obliqua* venom. Finally, the findings presented here emphasize the fact that a correct diagnosis and early treatment is essential for successful antivenom serotherapy, since the efficacy of serotherapy in neutralizing the physiopathological alterations is only observed if serotherapy is administered during the initial phase of envenomation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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3.3 Capítulo III

Acute kidney injury induced by snake and arthropod venoms

Considerando que a lesão renal aguda é a principal complicação clínica dos acidentes com animais peçonhentos, os principais fatores que contribuem para a gravidade desses casos são examinados e discutidos em detalhes no presente trabalho.

Os seguintes aspectos foram abordados:

- Epidemiologia geral e dos casos de lesão renal aguda causados por serpentes e artrópodos venenosos prevalentes no Brasil;
- Toxinas de venenos de serpentes e artrópodos venenosos com atividade nefrotóxica, dando ênfase para aquelas toxinas encontradas em animais que causam acidentes no Brasil;
- Fisiopatologia das alterações renais causadas por venenos animais;
- Formas de tratamento e manejo dos pacientes com insuficiência renal decorrente dos acidentes com animais peçonhentos.

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RENAL FAILURE – THE FACTS

Edited by **Momir Polenakovic**

9

Acute Kidney Injury
Induced by Snake and Arthropod Venoms

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1. Introduction

Snakebites and accidents caused by venomous arthropods (mainly spiders, scorpions, bees, wasps and caterpillars) are important public health problem. Despite of this, public health authorities, nationally and internationally, have given little attention to this problem worldwide (Warrell, 2010; Williams et al., 2010). As a consequence, the morbidity and mortality associated with snake and arthropod envenoming produce a great impact on the population and on the health-care systems. One of the most important and lethal effect of these animal venoms is nephrotoxicity (Sitprija, 2006). Specifically in South America and Brazil, the main snakes responsible for cases of acute kidney injury (AKI) are those from *Bothrops* and *Crotalus* genus. Among venomous arthropods, AKI has been reported after accidents with bees, spiders of the genus *Loxosceles* and caterpillars of the genus *Lonomia*.

Taking in account the importance of accidents with these venomous animals, in this chapter we reviewed the main mechanisms that play a role in AKI induced by the most common snakes and arthropods found in South America. The following key aspects are addressed: Epidemiology, clinical renal manifestations, renal pathophysiology, diagnosis, clinical management of AKI and the currently experimental models used to study the venom-induced AKI.

2. Epidemiology and prevalence of venomous snakes and arthropods in South America

Given the wide distribution of venomous animals, particularly in tropical and subtropical regions, the extensive number of accidents and the complexity of the clinical conditions it causes, the distint types of envenomation can be considered a global problem because they assume great public health importance, especially in the poorest areas of the world (World Health Organization [WHO], 2007). This environmental and occupational disease affects mainly agricultural workers and their children in some of the most impoverished rural

communities of developing countries in Africa, Asia, Latin America and Oceania. Populations in these regions experience high morbidity and mortality because of the poor access to health services, which are often suboptimal, and, in some instances, a scarcity of antivenom, which is the only specific treatment so far tecnically possible to be available. A large number of victims survive with permanent physical and psychological sequelae (Gutiérrez et al., 2010; Kasturiratne et al., 2008; Warrell, 2010).

A group of venomous animals is responsible for medically important accidents: snakes, scorpions, spiders, caterpillars, bees and wasps. Global epidemiological data on accidents with these different types of animals are scarce and often depend on the existence of country-specific estimates based on hospital admissions data and community-based population surveys. Unfortunately, in the low-income countries, where most accidents occur, there is not such a well organized health systems in order to correctly report the envenomation cases (Kasturiratne et al., 2008; Williams et al., 2010). Nevertheless, after the incorporation of snakebite envenomations on the World Health Organization list of neglected tropical diseases in 2009 (www.who.int/neglected_diseases/diseases/ snakebites/en/), more attention has been given to the lack of information on the true epidemiological impact of accidents, especially in the cases of snakebites. Current data indicate that 5.4 to 5.5 million people are bitten by snakes each year, resulting in near 400,000 amputations, and between 20,000 to 125,000 deaths (Chippaux, 1998, Kasturiratne et al., 2008; Williams et al., 2010). The highest burden of snakebite was identified in South and Southeast Asia, sub-Saharan Africa and Central and South America. Annually, Asia and Africa have incidence rates of 1.2 million and 1 million bites with 60,000 and 20,000 deaths, respectively. In Central and South America, epidemiological data indicate the occurrence of 300,000 snakebites per year which result in 4,000 deaths and approximately 12,000 cases of physical sequelae (Chippaux, 2011; Gutiérrez et al., 2010).

Specifically in Brazil, data from the System of Health Surveillance of the Ministry of Health indicate the ocorrence of 107,364 accidents with venomous animals in the year of 2009 (including cases of snake, scorpion, spider, caterpillar and bee envenomations) which resulted in 290 deaths. When compared to the 2008 year there were an increase of 12 % and 16 % in the total number of accidents and deaths, respectively (Boletim eletrônico epidemiológico, 2010). The majority of reported cases was caused by snakes and scorpions, which were also responsible for the highest rates of lethality (Table 1). Most snakebite (53 %) occurred from January to May, which reflect the influence of seasonal factors, such as an increase in temperature and humidity associated with the rainy season in some regions of Brazil. Human agricultural activities were also associated with envenomations, since 78 % of accidents occurred in rural areas. Snakes of *Bothrops* genus (Lance-headed pit vipers) were responsible for 90.5 % of the accidents while snakes of *Crotalus* genus (South American rattlesnakes) accounted for 7.7 % of total cases, showing however, a much higher lethality index (1.25 %) than that for *Bothrops* snakes (0.35 %) (Ministério da Saúde, 2001). Analysing the different regions of Brazil, the highest proportion of snakebites in relation to the population is localized in the North Region (Amazon Forest) with 53.9 accidents/100,000 inhabitants, probably due to the difficulty of patients to access health services and/or to the delay in the administration of antivenom (Table 1). Among all venomous animals, the scorpion stands out for its high and growing number of accidents in Brazil. Compared to the 2008 year, there was an increase of 7,050 cases in 2009 (45,721 *versus* 38,671 cases in 2008).

According to Chippaux and Goyffon (2008), scorpions are responsible annually for 1.2 million accidents and for about 3,250 deaths in the world. In Brazil, the increased number of scorpion accidents has been attributed to its adaptation to urban and domiciliar areas (Ministério da Saúde, 2001). In this case, the highest incidence was registred in the Northeast Region (Table 1). In contrast, accidents with spiders, caterpillars and bees are a growing problem in states of Southern Brazil. Specifically in the state of Paraná the brown spider (*Loxosceles* genus) is the most important venomous animal responsible for the high incidence of spider envenomation in the whole South Region (da Silva et al., 2004). In contrast, in the states of Rio Grande do Sul and Santa Catarina the caterpillar *Lonomia obliqua*, also called *taturana* (from the American-Indian Tupi-Guarani *tatá*, which means fire, and *raná*, similar to), has been associated with severe cases of hemorrhagic syndrome (Veiga et al., 2009). In this case, although accidents may occur throughout the year, 80 % of cases were reported during summer, when the animal is in the larval stage of its life cycle. Between 1997 and 2005 there were 984 accidents only in the state of Rio Grande do Sul, resulting in a mortality rate of 0.5 % (Abella et al., 2006). Currently, the therapeutic use of specific antivenom (antilonomic serum) has decreased the number of deaths (Table 1). Among bee accidents, the most dangerous are caused by *Apis mellifera* (Africanized bees). In these cases the high number of deaths (30 in 2009) has been associated mainly with the absence of a specific antivenom and the occurence of allergic reactions (Boletim eletrônico epidemiológico, 2010).

ACCIDENTS WITH VENOMOUS ANIMALS IN BRAZIL. REPORTED DATA: YEAR 2009 *.					
	Snakes	**Spiders**	**Scorpions**	**Caterpillars**	**Bees**
Total number of accidents	27,655	23,515	45,721	4,028	6,445
Incidence per 100,000 inhabitants	14.4	12.3	24	2.1	3.4
Number of deaths	125	26	104	5	30
Lethality (%)	0.45	0.11	0.23	0.13	0.05
Brazilian Regions	**Incidence per 100,000 inhabitants**				
North	53.9	3.6	16.2	1.7	1.9
Northeast	14.6	1.3	39.6	0.4	2.5
Midwest	20	2.6	13.3	0.7	2.2
Southeast	7.4	7.1	23.7	1.7	3.2
South	10.1	58.5	3.5	7.3	7

* Data from Brazilian Ministry of Health, 2010 (Boletim Eletrônico Epidemiológico, April 2010).

Table 1. Epidemiological data of accidents with venomous animals in Brazil.

3. Clinical renal manifestations due to snake and arthropod envenomation

A broad clinical spectrum of renal function impairment has been reported in snake and arthropod envenomations (Sitprija, 2006). As the kidneys are highly vascularized organs and have the ability to concentrate substances into the urine they are particularly susceptible

to venom toxins. The most common clinical renal manifestations seen in human patients is acute tubular necrosis, but all renal structures may be involved. Thus, the occurrence of acute tubulointerstitial nephritis, renal cortical necrosis, mesangiolysis, vasculitis, glomerulonephritis, proteinuria, haematuria and myoglobinuria have also been described (Sitprija, 2006).

In this subsection, we reviewed the clinical characteristics of human accidents with snakes and arthropods that cause AKI which are highly prevalent in Brazil and other regions of Latin America. Envenomations by the following animals were analysed: *Bothrops* and *Crotalus* snakes, the brown spider *Loxosceles*, africanized bees, wasps and the caterpillars of genus *Lonomia*. Despite the significant number of accidents with scorpions (Table 1), cases of AKI have not been associated to them. In fact, it is known that the main target of scorpion venom is the nervous and cardiac systems (Cologna et al., 2009).

3.1 Snakebite envenomation

Envenomation by snakebite, indenpendently of the species responsible for the bite, enforces medical emergencies since different organs and tissues can be affected at the same time. In Brazil, most severe cases result from bites by snakes of the family Viperidae (pit vipers and true vipers). Within this family are the *Bothrops* and *Crotalus* snakes. Specifically in the *Bothrops* genus there are more than 30 species distributed from southern Mexico to Argentina, including Brazil. The most important species are *Bothrops asper*, *B. jararaca*, *B. atrox*, *B. moojeni*, *B. jararacussu* and *B. alternatus*. *Bothrops* snakes preferentially inhabit rural areas and moist forest environments. But these snakes also invade cultivated areas and ambients with rodents' proliferation. *Bothrops* snakes have nocturnal habits and an aggressive defensive behavior and its venom present proteolytic, coagulant and hemorrhagic active principles that are directly or indirectly implicated in the local and systemic effects observed upon envenoming acidents (Warrel, 2010). Local effects due to the envenoming by these snakes are characterized by bleeding, swelling, pain and sometimes blisters, and can be frenquently complicated by the development of local abscesses and necrosis. Occasionally, compartmental syndrome may develop, which results in functional or anatomic loss of the bitten limb (Gutiérrez et al., 2006). Signs of systemic envenoming include gingival hemorrhage, microscopic hematuria, ecchymosis and consumption coagulopathy and, more rarely, epistaxis, hemoptosis, menorrhagia and hematemesis (Gutiérrez et al., 2006; Otero et al., 2002). Disturbances of hemostasis also include severe afibrinogenemia, thrombocytopenia and platelet aggregation dysfunction (Santoro and Sano-Martins, 2004). Deaths are usually attributed to renal injury, shock, severe bleeding, and complicating sepsis.

Renal dysfunction can occur early in the human bothropic envenomation which often induces oliguria and is accompanied by an increase in the plasma creatinine concentration. The need for dialysis ranges from 33 % to 75 % of cases (Pinho et al., 2008). AKI is mainly due to acute tubular necrosis and acute cortical necrosis and occasionally glomerulonephritis (Table 2) (Rodrigues-Sgrignolli et al., 2011). These renal pathological alterations have been attributed mainly to hemodynamic changes in response to envenomation, hemoglobinuria, intravascular clot formation and direct venom nephrotoxicity.

By analyzing a series of retrospective studies, Pinho et al. (2008) reported that the prevalence of AKI after *Bothrops* envenomation ranges from 1.6 % to 38.5 %. In most of these reports AKI diagnosis was based on the increase in the plasma creatinine and/or blood nitrogen urea being, the creatinine *clearance* barely estimated. The main reported factors influencing AKI prevalence upon such envenomation are: the patient's age (children under 10 year of age have been shown to be more susceptible to develop AKI); the snake's age (venom composition can vary even within the same species, according to the snake's age); bite site and amount of inoculated venom; and the time elapsed until antivenom treatment. Moreover, pre-existing diseases such as hypertension, diabetes or previous nephropathies may become patients more vulnerable to the effects of venom (Rodrigues-Sgrignolli et al., 2011; Zelanis et al., 2010). Taking in consideration all the above factors, the mortality rate of *Bothrops* venom-induced AKI range from 13 % to 19 %.

Other snakes well known for their nephrotoxicity are the South American rattlesnakes (*Crotalus* snakes). In Brazil, the *Crotalus* genus is represented by a single specie, *Crotalus durissus*, that is composed of six subspecies: *Crotalus durissus terrificus*, *C. d.collilineatus, C.d. cascavella, C.d. ruruima, C.d. marajoensis* and *C.d. trigonicus*. Besides sharing some common characteristics with other venomous snakes, the *Crotalus* genus presents a rattle at the end of its tail, which is a particular characteristic of these snakes making easier their identification. In general the *Crotalus* snakes are found in rocky and drier regions. They are rarely found in humid forests and feed mainly of small rodents. They are robust (may reach 1 meter in length) and are less agressive than *Bothrops* snakes (Ministério da Saúde, 2001).

Among the six different subspecies, *C.d. terrificus* is the most frequently implicated in envenomation cases registered in Brazil. The venom has neurotoxic, myotoxic, and nephrotoxic activities (Table 2). In neuromuscular junctions, the venom leads to a powerful presynaptic inhibition of acetylcholine release, which is responsible for the neuromuscular blockade and progressive flaccid paralysis of variable degrees. Eyelid ptosis, blurred and/or double vision, ophthalmoplegia and facial muscle paralysis are common manifestations of venom neurotoxicity. The myotoxic activity of the venom also produces severe skeletal muscle injury leading to myalgia and rhabdomyolysis with the subsequent release of myoglobin from damaged skeletal muscle into serum and urine (Azevedo-Marques et al., 1987). Indeed, the serum creatine kinase (CK) levels are significantly higher (260-folds that of normal values) in patients who develop AKI after a *Crotalus* bite. Other markers of rhabdomyolysis, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) are also increased in patients with AKI (Pinho et al., 2005). High serum and urine levels of myoglobin are potentially nephrotoxic, leading to acute tubular necrosis, which is the primary and most serious complication of human crotalid envenomation. Tissue damage at the site of the bite has been reported to be minimal or absent, a feature that differentiates the South American rattlesnake from other species of *Crotalus* and from *Bothrops* envenomations. Spontaneous bleeding has only been rarely observed in human patients, despite the presence of blood incoagulability in some cases (Jorge & Ribeiro, 1992). AKI is the main cause of death among patients surviving to the early effects of *Crotalus* snakebites.

In a study of 100 cases of *Crotalus* bites, Pinho et al. (2005) showed that AKI develops within the first 24 to 48 hours after envenomation. Envenomed patients presented a significant

reduction in glomerular filtration rate (estimated by the creatinine *clearance*). AKI patients also presented dark-brown urine and a fractional excretion of sodium significantly higher than the normal (Pinho et al., 2005). The major kidney pathological alteration is acute tubular necrosis, although interstitial nephritis has also been observed (Amaral et al., 1986; Azevedo-Marques et al., 1985) (Table 2). In this type of envenomation the occurrence of severe rhabdomyolysis is one of the more accepted explanations for the acute tubular necrosis. Other factors potentially associated with venom-induced AKI such as shock, hypotension and hemolysis are present in some cases, but have not been confirmed in *Crotalus* envenomation (Azevedo-Marques et al., 1987; Pinho et al., 2008). Despite of *in vitro* hemolytic activity of *Crotalus* venom, it was confirmed that *in vivo C.d. terrificus* envenomation causes myolysis rather than intravascular hemolysis (Azevedo-Marques et al., 1987).

The prevalence of AKI associated with Crotalid envenomation ranges from 10 to 29 % and 68 to 77 % of AKI patients require dialysis treatment. The mortality rate of *Crotalus* venom-induced AKI ranges from 8 to 17 % (Amaral et al., 1986; Pinho et al., 2008; Silveira & Nishioka, 1992). Although most risk factors for AKI are very similar to those described for bothropic envenomation it was reported that early after *Crotalus* snakebite the plasma levels of CK (higher than 2,000 U/L) were associated with a 12-fold increase in the risk of developing AKI (Pinho et al., 2005).

3.2 Brown spider envenomation

Among arthropods, spider and scorpion bites are the most frequent and of medical care importance. Although the number of accidents with scorpions often overcome those with spiders, reports of AKI after human envenomation with scorpion are scarce (Abdulkader et al., 2008). One species of spider that can cause severe renal injury is the brown spider (*Loxosceles* genus). Spiders of the genus *Loxosceles* have a worldwide distribution, since they can live under variable conditions such as temperature ranging from 8 to 43°C and that they can stay long time intervals living without food or water (Hogan et al., 2004; Swanson and Vetter, 2006). In Brazil, seven species have been described, but some of them are the most frequently implicated in bites in humans, namely *Loxosceles intermedia*, *L. gaucho* and *L. laeta*. These spiders are commonly found inside the residences both in rural and urban areas. They are small, measuring between 8 and 15 mm of body length while their legs measure 8-30 mm. Their colour varies from a pale brown (*L. laeta*) to a dark brown (*L. gaucho*). *Loxosceles* spiders are not aggressive and the bites usually occur when they are pressed against the body, mainly while the victim is sleeping or dressing (da Silva et al., 2004).

The venom has proteolytic, dermonecrotic, hemolytic and nephrotoxic activities (Isbister & Fan, 2011) (Table 2). The accident may have local and systemic manifestations that are exhibited in two different clinical forms: cutaneous and viscerocutaneous loxoscelism (da Silva et al., 2004). Most patients have only the local manifestation or cutaneous loxoscelism. In these cases, the accident may cause mild cutaneous inflammatory reaction or a local injury characterized by pain, edema and erithrema, later developing to dermonecrosis with gravitational spreading. In the minority of cases loxoscelism can cause a systemic injury or the viscerocutaneous loxoscelism. This form occurs

predominantly in children, and patients can develop AKI, which is considered the main cause of death after brown spider envenomation. Viscerocutaneous loxoscelism is characterized by fever, malaise, weakness, nausea and vomiting, hemolysis, hematuria, jaundice, thrombocytopenia and disseminated intravascular coagulation. This severe multisystemic clinical picture can occur as early as 24 hours after the bite (Abdulkader et al., 2008; da Silva et al., 2004; Isbister & Fan, 2011).

Analysis of 267 loxoscelism cases reported in Brazil showed that the viscerocutaneous form was diagnosed in 13.1 % of the cases, where *L laeta* was the main specie implicated in the accidents. The investigators reported jaundice in 68.6 %, oliguria in 45.7 %, anuria in 8.6 %, dark urine in 28.6 %, hemorrhage in 25.7 %, and shock in 2.9 % of the patients. AKI occurred in 6.4 % of the patients, and most of them were diagnosed more than 24 hours after the bite. Four patients died (1.5 %), all of them were children under 14 years old (Sezerino et al., 1998). The main factors likely associated with AKI development are hemolysis, hypotension/shock, and direct venom nephrotoxicity (Table 2). Pigment-induced acute tubular necrosis was reported in human necropsies of viscerocutaneous loxoscelism (Zambrano et al., 2005). Thus, it was suggested that the pathological effect of the venom on the kidney may reflect hematological disturbances, such as intravascular hemolysis and disseminated intravascular coagulation (Abdulkader et al., 2008). Although only low myotoxic activity has been reported in *Loxosceles* venom, rhabdomyolysis can also occur after envenomation. In this cases, high levels of serum CK and deposits of myoglobin in tubular cells have been observed (França et al., 2002; Lucato-Junior et al., 2011).

3.3 Bee and wasp envenomation

Stings of insects from the order Hymenoptera, which includes several species of bees, hornets, wasps and yellow jacks, have also been implicated in cases of human envenomation (Vetter et al., 1999).

In general the victims present only local allergic reactions after one or a few stings. However, after a massive attack with hundreds or thousands of stings, a systemic envenomation may occur (Abdulkader et al., 2008). The majority of envenomation cases with medical importance is caused by the so-called Africanized bees (*Apis* genus). These bees are hybrids between bees of European origin (*Apis mellifera mellifera* and *Apis mellifera ligustica*) and African bees (*Apis mellifera scutellata*) which were originated by the introduction of different species in Brazil since 1957. Currently, due to the migratory behavior and a high reproductive rate they are found throughout South America, Central America and parts of North America. Because of their aggressive behavior and the number of accidents associated with them, the Africanized bees are also known as "killer bees" (Abdulkader et al., 2008; França et al., 1994).

The main venom activities are hemolytic, myotoxic, cardiotoxic and nephrotoxic (Table 2). Clinical manifestations can be divided into allergic and systemic reactions. Allergic reactions usually are observed in patients with a history of previous bee stings or asthma or other hypersensitivity disease. These reactions occur immediately after a single sting and can lead to anaphylaxis and death by laryngeal edema. Systemic reactions usually occur after multiple stings and are characterized by pain, erythema, urticaria, release of histamine,

nausea, vomiting, respiratory failure, hypotension and shock (Abdulkader et al., 2008). Rhabdomyolysis and hemolysis can be detected a few hours after the accident (Chao et al., 2004). Fatalities are typically the result of renal damage or from cardiac arrest due to complications of the venom toxicity (Vetter et al., 1999).

AKI has been observed in cases of massive attacks with 150 stings to more than 1,500 stings. Envenomed patients commonly have anuria or oliguria, high levels of serum creatinine (10-30 mg/dL) and CK (>2,000 U/L), hypotension, tachycardia, myocardial damage and anemia (Daher et al., 2003; Gabriel et al., 2004; França et al., 1994; Xuan et al., 2010). Acute tubular necrosis is the main histologic finding in human beings, domestic dogs, and in experimental animals after bee and wasp envenomations. Allergic interstitial nephritis with concurrent pigment tubulopathy resulting from both hemoglobin and myoglobin has also been described after wasp stings (Chao et al., 2004; Zhang et al., 2001) (Table 2). A direct nephrotoxicity of the venom and/or hypotension caused by anaphylactic reaction are also mechanisms implicated in AKI induced by bees of *Apis* genus (Grisotto et al., 2006). By analyzing five cases of severe envenomation by Africanized bees, França et al. (1994) found high venom concentrations in serum and urine which remain for more than 50 h after the stings in two fatal cases; in one of them the total circulating unbound whole venom components was estimated at 27 mg, one hour after the attack. Despite the treatment with dialysis, antihistamines, corticosteroids, bronchodilators, vasodilators, bicarbonate, mannitol and mechanical ventilation, three out four patients died between 22 and 71 h after the attacks. However, in the majority of cases, the renal damage is usually reversible responding well to the dialysis. Complete recovery may require 3-6 weeks (Vetter et al., 1999).

3.4 Caterpillar envenomation

The accidental contact with some lepidopteran caterpillars can also cause human envenomation cases that vary from simple skin irritation and local allergic reactions to a systemic disease characterized by renal damage and hemorrhagic disturbances (Pinto et al., 2010; Veiga et al., 2009). From the medically important Saturniidae family, *Lonomia* genus has been attributed to cause human envenomations since late 1960's in Venezuela (Arocha-Piñango et al., 2000). In Southern Brazil, *Lonomia obliqua* caterpillar is becoming the most important venomous animal responsible for severe injuries, hemorrhagic disorders and often fatal outcome since the 1980's (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 period from 1997 to 2005 (Abella et al., 2006). In fact, based on data from the year 2009, the Brazilian Ministry of Health registered an incidence of 7.3 lepidopteran envenomations per 100,000 inhabitants in Southern Brazil (Boletim eletrônico epidemiológico, 2010) (Table 1). Actually, these numbers are greatly underestimated due to the fact that most accidents are occurring in distant rural areas, where the cases are poorly reported. *Lonomia*'s accidents usually occur when the victim, leaning against tree trunks containing dozens or hundreds of caterpillars, comes into contact with their bristles. These structures are hard and spiny evaginations of the cuticle, underneath which the toxins are stored. Often, the whole animal is smashed in the accident, the insect's chitinous bristles get broken and the venomous secretions, including hemolymph, penetrate the human skin and enter the circulation (Veiga et al., 2001**a**).

The venom presents procoagulant, fibrinogenolytic, proteolytic and hemolytic activities (Table 2). Clinical symptoms of *Lonomia* envenomation include local pain (burning sensation) 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, gross hematuria, hematemesis, melena, pulmonary and intracerebral hemorrhage and AKI (Pinto et al., 2010). Intravascular hemolysis has also been described in human envenomation and experimental studies (Malaque et al., 2006; Seibert et al., 2004). The activation of blood coagulation, fibrinolysis and the systemic inhibition of platelet function are mechanisms that seem to contribute to the hemorrhagic syndrome commonly observed in *Lonomia* envenomation (Berger et al., 2010**a**). In human patients, this hemorrhagic syndrome manifests as a consumptive coagulopathy without thrombocytopenia (Berger et al., 2010**a;** Zannin et al., 2003).

The incidence of AKI varies from 2 to 5 % of envenomation cases reported in the literature (Duarte et al., 1990; Gamborgi et al., 2006). Of the 2,067 patients evaluated in southern Brazil (period from 1989 to 2003), 39 (1.9 %) developed AKI (serum creatinine levels > 1.5 mg/dL). Eleven (32 %) of these patients were treated with dialysis and four (10.3 %) developed chronic renal injury (CRI). All victims with AKI presented concomitantly coagulation disturbances and hematuria and/or hemoglobinuria. Seven deaths (4%) occurred during the period (Gamborgi et al., 2006). The impossibility of conducting early renal biopsies, due the coagulation disturbances inherent to the envenomation, has made it difficult to analyze the acute anatomopathological alterations. The few reports existing in the literature describe thickening of the Bowman's capsule, focal tubular atrophy and acute tubular necrosis (Burdmann et al., 1996; Fan et al., 1998) (Table 2). Similarly, the contribution of other factors possibly associated with AKI, such as hypotension or glomerular fibrin deposition, remains still obscure in *Lonomia* envenomation.

4. Toxins of snake and arthropod venoms and their role in the pathophysiology of acute kidney injury

Animal venoms are mixtures of biologically active proteins and peptides, and also non-protein toxins, carbohydrates, lipids, amines, and other small molecules. The clinical features of envenomation reflect the effects of these different venom components and thus, the contribution of the venom toxins to the pathophysiology of renal injury is complex and multifactorial (Sitprija, 2006).

Based on the current knowledge, the hypothesis for pathogenesis of venom-induced AKI include both a direct cytotoxic action of the venom on different renal structures, and a secondary response of the whole organism resulting from systemic envenomation. The secondary response is usually triggered by inflammation, release of cytokines and vasoactive substances that leads to changes in renal function and hemodynamics (Fig. 1). In fact, there is an increase in plasma concentration of different cytokines and vasoactive substances such as TNF-α, interleukins, nitric oxide, histamine, bradykinin and eicosanoids following several types of envenomations (Petricevich et al., 2000). The elevation of cytokines are mainly due to accumulation of pro-inflammatory cells and immune system response. Together, all these mediators can impair renal function ultimately contributing to

a decrease in renal perfusion pressure, renal blood flow and glomerular filtration rate. As a result in association with the systemic hypotension (Table 2), there will be an inadequate tissue and cellular oxygen delivery which can generate an ischemic process. Since the intermediary metabolism and energy production have an absolute dependence on oxygen, and oxygen cannot be stored intracellularly, the inadequate oxygen availability rapidly leads to cellular dysfunction, injury, and cell death by necrosis (Deitch, 1992). Important contribution to venom-induced renal ischemia is also derived from the process of hemolysis, rhabdomyolysis and/or intravascular deposition of platelets and fibrin in the microcirculation (Table 2). The presence of hemoglobin and myoglobin also have a direct cytotoxic effect on renal tubules (Fig.1) (Khan, 2009; Zager, 1996). Thus, it seems that different, but interrelated processes may contribute to the nephrotoxicity and even to other pathological features observed in envenomed patients.

Venomous animals	Main venom activities	General clinical manifestations	Characteristics of AKI and renal pathology
Bothrops snakes	Hemorrhagic, Procoagulant, Proteolytic and Nephrotoxic	Local abscesses and necrosis, Spontaneous bleeding, DIC, Hypotension	Oliguria/anuria, Hemoglobinuria, Hematuria, ATN, AIN, RCN, GFD
Crotalus snakes	Neurotoxic, Myotoxic and Nephrotoxic	Flaccid paralysis, Myalgia, Rhabdomyolysis	Decrease in GFR, Myoglobinuria, ATN, AIN
Brown spiders (*Loxsosceles*)	Dermonecrotic, Proteolytic, Hemolytic and Nephrotoxic	Local abscesses and necrosis, Hemolysis, Rhabdomyolysis, DIC, Hypotension	Hematuria, Hemoglobinuria, Myoglobinuria, ATN
Africanized Bees (*Apis mellifera*)	Hemolytic, Myotoxic, Cardiotoxic and Nephrotoxic	Allergic reaction (anaphylaxis), Hypotension, Hemolysis, Rhabdomyolysis	Oliguria/anuria,ATN, AIN, Hemoglobinuria, Myoglobinuria
Lonomia caterpillars	Procoagulant, Fibrinogenolytic, Proteolytic and Hemolytic	Ecchymosis, Spontaneous bleeding, DIC, Hemolysis	Hematuria, Hemoglobinuria, ATN

DIC - Disseminated Intravascular Coagulation; ATN - Acute tubular necrosis, AIN - Allergic interstitial nephritis, RCN - Renal Cortical Necrosis, GFD - Glomerular Fibrin Deposition, GFR - Glomerular Filtration Rate.

Table 2. Clinical aspects of venom-induced Acute Kidney Injury (AKI).

Recently, advances in molecular biology, proteomics and transcriptomics, facilitated the isolation of toxins and contributed significantly to the study of their mechanisms of action on renal tissue. In this subsection, we reviewed the renal physiopathological effects of snake and arthropod venoms and their main isolated toxins. Special emphasis was given to

experimental studies with venomous animals highly prevalent in Brazil and other regions of Latin America. As in the previous subsection the following animals were included: *Bothrops* and *Crotalus* snakes, the brown spider *Loxosceles*, africanized bees, wasps and the caterpillars of genus *Lonomia*.
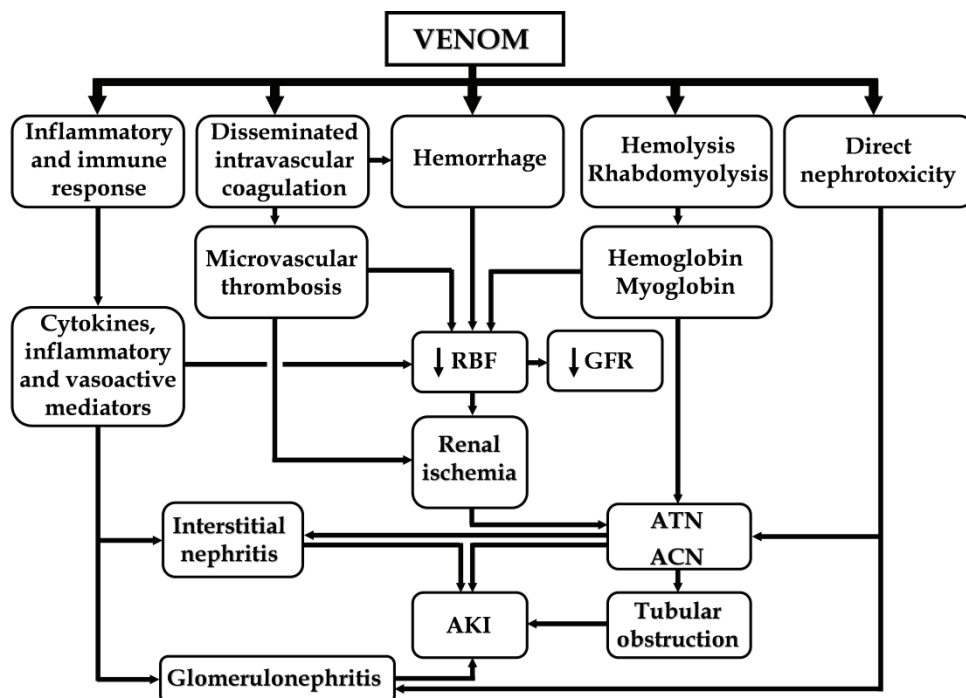
Fig. 1. Schematic summary of pathophysiological phenomena involved in the venom-induced acute kidney injury (AKI). RBF – Renal Blood Flow; GFR – Glomerular Filtratiton Rate; ATN – Acute Tubular Necrosis; ACN – Acute Cortical Necrosis.

4.1 Snake venoms

4.1.1 *Bothrops* venom

The venom of *bothrops* snakes can cause prominent local tissue damage usually characterized by swelling, blistering, hemorrhage and necrosis of skeletal muscle. Such local pathology is mostly due to the venom proteolytic action (Gutiérrez et al., 2006). Snake venom metalloproteinases (SVMPs), phospholipases A_2, , serine proteinases, esterases, L-amino acid oxidases, hyaluronidases, C-type lectins-like and bradykinin-potentiating peptides (BPPs) are the main venom components that acts inducing cellular injury or releasing inflammatory and vasoactive mediators (Warrell, 2010). Transcriptomic and proteomic studies have showed that SVMPs and serine proteinases are the major toxins in the venom, which explained the high local damage and hemorrhage seen in envenomed patients (Table 2) (Cidade et al., 2006; Zelanis et al., 2010). *Bothrops* toxins are also known for their multiple effects on hemostasis. In fact, the venom have thrombin-like enzymes, factor

X and prothrombin activators that are able to directly convert fibrinogen into fibrin (Berger et al., 2008; White, 2005). These actions produce intravascular coagulation and may lead to blood incoagulability by consumption coagulopathy. Systemic inhibition of platelet aggregation and thrombocytopenia are common (Rucavado et al., 2005; Santoro & Sano-Martins, 2004). Moreover, anti-hemostatic principles, such as thrombin and platelet aggregation inhibitors, are also found in *bothrops* venoms (Kamiguti , 2005; Zingali et al., 2005).

Regarding renal function, Boer-Lima et al. (1999) observed that the intravenous injection of *B. moojeni* venom in rats, produced renal tubular disturbances including an increase in proximal and post-proximal fractional excretion of sodium associated with acute tubular necrosis. The glomerular filtration rate decreased significantly, despite the absence of systemic hypotension. Severe morphologic disturbances in the renal glomeruli also occurred. The changes included mesangiolysis, glomerular microaneurysms, and glomerular basement membrane abnormalities. In addition, there was a reduction in the number and width of podocyte pedicels, which caused a reduction in the number of filtration slits. The morphophysiological changes observed in experimental animals also correlated with the levels of proteinuria (Boer-Lima et al., 2002). Similar renal functional alterations were observed after intravenous injection of *B. jararaca* venom into rats. In these animals, differently of human envenomation, *B. jararaca* venom was not able to induce systemic hypotension but significantly reduced the renal plasma flow and increased renal vascular resistance (Burdmann et al., 1993). There was no increase in CK, indicating that rhabdomyolysis is not an important consequence of *B. jararaca* envenomation. However, the venom caused marked fibrinogen consumption and intravascular hemolysis. Indeed, kidney of rats and rabbits envenomed with *B. jararaca* showed an extensive intraglomerular deposition of fibrin and platelets (Burdmann et al., 1993; Santoro & Sano-Martins, 2004). Contrarily to the findings with *B. jararaca* venom, Boer-Lima et al. (1999) did not observed any glomerular fibrin deposition in the *B. moojeni* envenomation. They suggested that the glomerular injury is more likely to be related to structural disorganization of the glomerular capillary tuft, consequent to a direct action of the venom on the mesangial matrix, glomerular basement membrane and podocytes rather than to fibrin deposition in the capillaries.

Studying the kinetic of renal distribution of injected *B. alternatus* venom in rats, Mello et al. (2010) detected the highest venom concentration in renal tissue 30 min post-venom injection. After this time, venom concentration decreased progressively. Venom components were also detected into urine 3, 6 and 24 h post-venom injection. By immunohistochemistry, venom proteins were detected in glomeruli, proximal and distal tubules, and vascular and perivascular tissue, suggesting that toxins bind to kidney structures where they probably exert a direct nephrotoxic action. In accordance to this observation, it was showed that *B. alternatus* venom is cytotoxic to canine renal epithelial cells (MDCK) in culture and causes extensive cytoskeletal alterations inducing impairment of the cell-matrix interaction (Nascimento et al., 2007). Additionally, it was described that *B. jararaca* venom also causes *in vitro* injury of isolated renal proximal tubules and that the *B. moojeni* venom increases cell release of lactate dehydrogenase and decreased cellular uptake of the vital neutral red in MDCK cells (de Castro et al., 2004; Collares-Buzato et al., 2002). Functionally, *B. alternatus* venom induced oliguria, urine acidification, decreased in glomerular filtration rate and

hematuria. Morphologically, the venom caused lobulation of the capillary tufts, dilation of Bowman's capsular space, disruption of renal tubule brush border, and fibrosis around glomeruli and proximal tubules that persisted 7 days after envenomation (Linardi et al., 2011; Mello et al., 2010).

Some purified *Bothrops* toxins are able to reproduce the renal effects obtained with whole venom. Studies on the isolated perfused rat kidney have shown that L-amino acid oxidase (Braga et al., 2008), C-type lectins (Braga et al., 2006), phospholipase A2 myotoxins (Barbosa et al., 2005; Evangelista et al., 2010) and thrombin-like enzyme (Braga et al., 2007) from *Bothrops* venoms can alter renal function. The isolated perfused kidney technique also confirmed the direct acute tubular nephrotoxicity of *Bothrops* venoms and showed that platelet activating factor might play a role in some renal functional disturbances such as the decreased in glomerular filtration rate (Monteiro and Fonteles, 1999). However, the systemic injection of baltergin, a purified metalloproteinase from *B. alternatus* venom, only mildly affected the kidney structure. At high doses, baltergin causes congestion, subcapsular hemorrhage and inflammatory infiltrate (Gay et al., 2009). There was no detection of tubular necrosis indicating that different toxins act synergistically to produce the AKI observed in animals treated with whole venom.

4.1.2 *Crotalus* venom

The venom of *Crotalus* rattlesnakes is a complex combination of different enzymes and toxic peptides that mainly display neurotoxic and myotoxic activities (Boldrini-França et al., 2010). Toxins affecting hemostasis, such as thrombin-like enzymes and platelet activators are also found. The main protein families identified by proteomics included phospholipases A$_2$, serine proteinases, cysteine-rich secretory proteins (CRISP), vascular endothelial growth factor-like molecules (VEGF), L-amino acid oxidases, C-type lectins-like, and snake venom metalloproteinases (SVMP). Crotoxin, a neurotoxic phospholipase A$_2$, represents more than 60 % of the proteins in the whole venom and is the major component responsible for its neurotoxic and myotoxic effects (Boldrini-França et al., 2010). Additionally, crotoxin also exhibits cardiotoxic and direct nephrotoxic activities. Structurally, crotoxin is a heterodimeric β-neurotoxin that consists of a toxic basic phospholipase A$_2$ and a nonenzymatic, non-toxic acidic component (crotapotin). Crotapotin potentiates the activity of crotoxin, since it prevents the basic phospholipase subunit binding to non-specific sites (Sampaio et al., 2010; Soares et al., 2001). Crotoxin targets neuromuscular junctions and inhibits the release of acetylcholine, which leads to neuromuscular blockade and muscular and respiratory paralysis. In the muscle tissue, crotoxin causes selective injury of skeletal muscle groups composed of type I and IIa fibers, which are extremely vascularized and rich in myoglobin (Sampaio et al., 2010). Other important toxins are crotamine, convulxin and gyroxin. Crotamine is a toxic peptide with myonecrotic activity (Martins et al., 2002). Convulxin is a C-type lectin-like glycoprotein with high affinity to specific receptors in rabbit and human platelets. Convulxin binds to the putative collagen receptor glycoprotein VI (GPVI) and mediates platelet adhesion, aggregation and intracellular calcium mobilization (Francischetti et al., 1997). Gyroxin is a serine proteinase that displays several activities including the induction of blood coagulation (thrombin-like activity), vasodilation and neurotoxicity (Alves da Silva et al., 2011).

Intraperitoneal injection of *C.d. terrificus* venom in mice increased plasma creatinine and uric acid and caused urinary hypoosmolality. When compared to control groups injected with saline, the incidence of hypercreatinemia and hyperuricemia (plasma values higher than 1.8 mg/dL) occurred in 60 % and 100 % of the experimental animals, respectively (Yamasaki et al., 2008). *Crotalus* experimental envenomation was also associated with significant renal blood flow and glomerular filtration rate decreases and ischemia with consequent acute tubular necrosis. In isolated perfused rat kidneys treated with crude venom or crotoxin, a large amount of protein material was observed in the glomeruli, probably due to a direct toxic effect of the venom on the glomeruli and tubules and/or to an increase in vascular permeability (Monteiro et al., 2001). Prostaglandins and TNF-α release seems to be important since the treatment with indomethacin and pentoxifylline (inhibitors of cyclooxygenase and TNF-α synthesis, respectively) were able to blockade the renal effects induced by supernatant of macrophages activated with *Crotalus* venom (Martins et al., 2003; Martins et al., 2004). Among the main venom components, crotoxin was able to induce significant changes in glomerular filtration rate and electrolyte transport in isolated kidney. Gyroxin caused only mild alteration in renal parameters and convulxin had no effects (Martins et al., 2002).

Rhabdomyolysis is a well-known cause of AKI and is commonly observed in envenomed patients and envenomed experimental animals. Myoglobin toxicity has been related to renal vasoconstriction, intraluminal cast formation and direct heme-protein cytotoxicity. Myoglobin can contribute to renal vasoconstriction by directly binding to nitric oxide (NO). Thus, acting as NO scavenging molecules, heme-proteins (including myoglobin or hemoglobin) lead to renal hypoperfusion, reductions in the storage of ATP, ischemia and tissue injury (Zager, 1996). Intraluminal casts are formed due to the precipitation of myoglobin inside the renal tubules, forming obstructive casts. Precipitated myoglobin also can be degraded resulting in the release of free iron and heme. Once released, free iron and heme contribute to renal injury by generate reactive oxygen species (ROS) and lipid peroxidation (Khan, 2009; Zager, 1996). Indeed, Yamasaki et al. (2008), showed an increase of oxidized glutathione/reduced glutathione ratio (GSSG/GSH) in renal tissue during *Crotalus* envenomation. This data indicates a rise in the ROS generation by consumption of reduced glutathione (GSH) and production of oxidized glutathione (GSSH) which are the main antioxidant and oxido-reducing agents, respectively. Confirming the participation of ROS in *Crotalus* induced renal injury, envenomed animals treated with lipoic acid (an antioxidant molecule) had their GSSG/GSH ratios normalized when compared to control groups (Alegre et al., 2010). In addition to deleterious effects of obstructive myoglobin casts formation, the high levels of uric acid found in envenomed animals also contribute to tubular obstruction (Yamasaki et al., 2008). Marked hyperuricemia is known to cause AKI by supersaturation, crystallisation and deposition of crystals inside renal tubules (acute urate nephropathy). Moreover, experimental hyperuricemia causes renal vasoconstriction and soluble uric acid has been shown to inhibit endothelial NO bioavailability (Ejaz et al., 2007). Recently, it was observed that systemic inhibition of uric acid synthesis, by allopurinol treatment, significantly reduced lethality rate, normalized GSSG/GSH ratio and ameliorate the renal histopathological changes. Thus, uric acid also seems to have an important role in renal pathophysiology of *Crotalus* envenomation (Frezzatti & Silveira, 2011).

4.2 *Loxosceles* venom

The bites of brown spiders (*Loxosceles* genus) led to several clinical manifestations such as necrotic skin degeneration and gravitational spread at the bite site, renal injury and hematological disturbances. Several studies concerning the structural and biological roles of various venom components have shown the complex nature of these venomous secretions. Likewise, the venom of *Loxosceles* spiders is a complex mixture of protein-based toxins with a molecular mass profile ranging from 5 to 40 kDa. The main components belong to the classes of phospholipases D (or dermonecrotic toxins), serine proteinases, venom allergens, hyaluronidases, astacin-like metalloproteinases and insecticidal peptides (Gremski et al., 2010). Dermonecrotic toxins and astacin-like metalloproteinases are considered the major components responsible for the clinical profile observed in envenomed victims (Table 2) (da Silva et al., 2004). In fact, a transcriptomic study indicated that phospholipases D and astacin-like metalloproteinases represent 20.2 % and 22.6 % of total toxin-encoding transcripts, respectively. Other toxins also important to envenomation, such as serine proteinases, venom allergens and hyaluronidases represent the minority of encoding transcripts (Gremski et al., 2010).

Among all the toxins found in *Loxosceles* spider venom, dermonecrotic toxin is undoubtedly the component most investigated and characterized. This toxin is able to reproduce the major biological effects induced by whole venom. It is involved with the development of dermonecrotic lesions and can trigger neutrophil migration, complement system activation, cytokine and chemokine release, platelet aggregation, lysis of red blood cells, among other effects (Abdulkader et al., 2008; da Silva et al., 2004). Dermonecrotic toxin comprises a family of toxins with different related isoforms that have biological, amino acid and immunological similarities which are found in several *Loxosceles* species. Only in *L. intermedia* venom, many isoforms were described being 9 out of them already expressed as recombinant proteins (Gremski et al., 2010). *Loxosceles* dermonecrotic toxins belong to phospholipases D (30–35 kDa) class of enzymes which was primarily designated as sphingomyelinases D due to their ability to convert sphingomyelin to choline and ceramide 1-phosphate (N-acylsphingosine1-phosphate). As some *Loxosceles* sphingomyelinases D have broad substrate specificity, being able to hydrolyze not only sphingophospholipids but also lysoglycerophospholipids, they are now classified as phospholipases D (Lee and Lynch, 2005). Due to sequence, structural and biochemical differences these toxins are grouped in two classes and their structures and substrate specificities have been recently elucidated (de Giuseppe et al., 2011; Murakami et al., 2005). Other important components of *Loxosceles* venom are the metalloproteinases. The enzymes have molecular weights ranging from 20 to 35 kDa displaying gelatinolytic, fibronectinolytic and fibrinogenolytic activities. They are zinc endopeptidases homologous to the astacin family of metalloproteinases from the crayfish, *Astacus astacus*. The *Loxosceles* astacin-like metalloproteinases possess a digestive function used to initiate the degradation of prey molecules, facilitating the posterior ingestion process (Trevizan-Silva et al., 2010). Furthermore, these enzymes have an important role in the pathogenesis observed in envenomation, particularly inducing hemorrhage into the dermis, injury of blood vessels, imperfect platelet adhesion, and the defective wound healing observed in some cases. Likewise, these metalloproteases can also render tissue structures more permeable, facilitating other noxious toxins to spread throughout the body of victims (Veiga et al., 2000; Veiga et al., 2001**b**).

The nephrotoxic effect of the *L. intermidia* spider venom was demonstrated experimentally in mice exposed to the whole venom (Luciano et al., 2004). Histhopathological analysis showed morphological renal alterations including hyalinization of proximal and distal tubules, erythrocytes in Bowman's space, glomerular collapse, tubule epithelial cell blebs and vacuoles, interstitial edema, and deposition of a protein-rich material inside the Bowman's space and tubule lumen. Morphometric analysis showed that 75–80 % of the kidney area was affected by the venom and no glomerular or tubule leukocyte infiltration was described, suggesting that the involvement of inflammatory process is not important to renal injury in this type of envenomation. Despite the presence of erythrocytes and protein deposits in glomerular and tubular structures, no signs of intravascular hemolysis or hemoglobin were detected in envenomed animals. Supporting the evidence that *L. intermidia* venom has toxins with direct nephrotoxicity, confocal microscopy studies with antibodies against venom proteins were able to show direct binding of toxins to renal structures. Venom proteins were detected in glomerular and tubular epithelial cells and in renal basement membranes. Toxins with molecular weights of 30 kDa were also identified in renal tissue extracts by immunoblotting (Luciano et al., 2004). One of these venom proteins that can bind to the kidney tissue is the dermonecrotic toxin. Chaim et al. (2006), injecting the recombinant dermonecrotic toxin in mice, found glomerular edema and tubular necrosis without signs of inflammatory response. Additionally, the dermonecrotic toxin was detected in kidney tissue and induced changes in renal function such as urine alkalinization, hematuria and elevation of blood urea nitrogen levels. The treatment of renal epithelial cells (MDCK) with recombinant dermonecrotic toxin also caused morphological alterations and reduced the cell viability, confirming its direct citotoxicity (Chaim et al., 2006). Both effects upon renal structures *in vivo* and renal cells *in vitro* were dependent of the phospholipase D catalytic activity, since a mutated toxin without phospholipase activity showed no nephrotoxic effect (Kusma et al., 2008). Another mechanism involved in AKI induced by *Loxosceles* venom is the renal vasoconstriction and rhabdomyolysis. Recently, it was reported that *L. gaucho* caused a sharp and significant drop in glomerular filtration rate, renal blood flow and urinary output and increased renal vascular resistance in rats (Lucato-Júnior et al., 2011). In this model, the authors also found deposits of myoglobin in tubular cells and degenerative lesions indicative of an ischemic process (Lucato-Júnior et al., 2011).

4.3 Bee and wasp venoms

Bee and wasp venoms are composed of a mixture of proteins, peptides, and small molecules, which are related to different mechanisms of envenomation. In the Africanized bee (*Apis mellifera*) venom the most important components are melittin and phospholipase A2. Melittin is a highly toxic peptide and the most abundant component of bee venom comprising about 50 % of its dry weight. This peptide is able to disrupt biological membranes, producing many effects on living cells (Fletcher et al., 1993). Melittin has antibacterial activity, induces voltage-gated channel formation and can also produce micellization of phospholipids bilayers due to its membrane-interacting effect. This peptide is responsible for the direct hemolytic effect of *Apis* venom (Dempsey, 1990; Terra et al., 2007). The enzyme phospholipase A2 represents approximately 11 % of whole venom and acts synergically with melittin. Once melittin has disrupted the membrane, phospholipase

A2 cleaves bonds in the fatty acid portion of the bilipid membrane layer (Vetter et al., 1999; Lee et al., 2001). In association, melittin and phospholipase A2, can act on erythrocytes, myocytes, hepatocytes, fibroblasts, mast cells, and leukocytes (Abdulkader et al., 2008; Fletcher et al., 1993; Habermann, 1972). Additionally, bee venom also has hyaluronidase (an enzyme that disrupts the hyaluronic acid in connective-tissue matrix), apamin (a neurotoxin), mast cell degranulating peptide (a peptide that releases histamine from mast cells) and other small molecules such as histamine, dopamine, and noradrenaline. Among all *Apis mellifera* venom components the main allergens are melittin, phospholipase A2 and hyaluronidase (Vetter et al., 1999). In wasp venoms the components are active amines (serotonin, histamine, tyramine, catecholamines); wasp kinins (similar in composition to bradykinin), which are mostly responsible for pain; and histamine-releasing peptides, which are responsible for the inflammatory response. The major allergens identified in wasp venoms are phospholipase A1, a hyaluronidase and a serine-protease (Pantera et al., 2003; Vetter et al., 1999).

Despite the current knowledge on the composition of wasp venoms, little is known about the participation of its components, and even the whole venom, on the AKI observed in envenomed patients. On the other hand, the mechanisms of bee venom-induced AKI have been more explored in experimental models *in vivo* and *in vitro*. In the case of bee envenomation, the experimental injection of venom in rats caused a significant and early reduction in glomerular filtration rate and diuresis and an increase in plasma creatinine levels (dos Reis et al., 1997; Grisotto et al., 2006). Tubular alterations such as increased fractional sodium and potassium excretions and a reduced water transport through collecting tubules, were also described (dos Reis et al., 1997). The early glomerular filtration rate reduction was concomitant with marked cortical and medullary renal blood flow decrease (Grisotto et al., 2006). Neither hypertension and hypotension nor intravascular hemolysis were detected in experimental models. Despite of the absence of hemolysis, rhabdomyolysis was present with massive myoglobin deposition in the lumen of the tubules as well as into the tubular cells (dos Reis et al., 1997; Grisotto et al., 2006). The injection of purified melittin or phospholipase A2 also induced rhabdomyolysis, due to their capacity to disrupt the membranes of myocytes (Ownby et al., 1997). Additionally, *in vitro* studies have been demonstrated that bee venom is citotoxic to cultured isolated proximal tubule cells (Grisotto et al., 2006). Histological analysis showed acute tubular necrosis mainly in cortex and outer medulla, and cast formation in the distal and collecting tubules (dos Reis et al., 1998). These degenerative lesions observed in AKI induced by the bee venom have been associated with the ischemic process induced by melittin, phospholipase A2 and histamine (Abdulkader et al., 2008; Grisotto et al., 2006). Indeed, melittin and phospholipase A2 may be related to impaired renal blood flow by causing direct vasoconstriction, smooth muscle cell contraction, increased renal renin secretion and release of vasoconstrictor eicosanoids and catecholamines. Histamine and the mast cell degranulating peptides present in the venom also play a role in renal blood flow decrease, since histamine can directly induce vascular changes (Cerne et al., 2010; Churchill et al., 1990).

4.4 *Lonomia* venom

Caterpillars of the species *L. obliqua* are well known in Southern Brazil by causing a severe hemorrhagic syndrome characterized by coagulation disorders, AKI and generalized

hemorrhage. The venom is composed of several active principles, including procoagulant and fibrinolytic activities (Pinto et al., 2010). Even though many studies have been performed with toxic secretions from *L. obliqua* aiming a better elucidation of the hemorrhagic syndrome resulting from this envenomation, few active principles have been purified from the venom and fully characterized so far. Thus, most of the molecules identified in this caterpillar have been characterized as putative enzymes and other proteins based solely on cDNA and amino acid sequences obtained by transcriptomic and proteomic methods (Ricci-Silva et al., 2008; Veiga et al., 2005). Through these techniques, the major protein found in *Lonomia* is a biliverdin-binding protein of the lipocalin family, which is mainly concentrated in the bristles and plays an important role in the caterpillar's camouflage behavior. Along with the lipocalin and other housekeeping proteins, *L. obliqua*'s integument, hemolymph and bristles produce and store a variety of active principles. Among these proteins, the most abundant ones are serine proteases and their inhibitors (serpins) in the integument, and serine proteases, kininogen and lectins in the bristles. Besides these molecules, cysteine proteinases, phospholipase A2, cystatins, Kazal-type inhibitors and other protease inhibitors are also found. Serine proteases 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 factor X and prothrombin (Berger et al., 2010**a**; Veiga et al., 2003), and in the activation of the fibrinolytic system, contributing directly and indirectly to fibrinogen degradation (Pinto et al., 2006) and resulting in the hemorrhagic disorder. In fact, proteases with fibrinogenolytic, prothrombin and factor X activating activities have been purified and characterized in this venom (Alvarez-Flores et al., 2006; Pinto et al., 2004; Reis et al., 2006). The phospholipase A2 enzyme also has a function in envenomation. This enzyme was isolated and characterized as the major component responsible to the *in vitro* and *in vivo* hemolytic activity of *L. obliqua* venom (Seibert et al., 2004; Seibert et al., 2006). Additionally, the phospholipase A2 seems to be involved in platelet aggregation inducing activity present in the venom (Berger et al., 2010**b**). 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 with homology to many snake venom lectins being then another important candidate contributing to the hemorrhagic disorder (Veiga et al., 2005).

Although AKI is the leading cause of death in *L. obliqua* envenomation, the mechanisms involved in kidney disorders are poorly understood. In contrast to hemostatic disturbances, to date, there is no experimental studies describing the renal effects of *L. obliqua* venom. Current knowledge is based only on a few case reports in which hematuria, high levels of serum creatinine and acute tubular necrosis are described as the main features of *L. obliqua* induced AKI. Due to this lack of knowledge, nowadays we are focused on the investigation of the effects of *L. obliqua* venom on renal function in rats. Preliminary results, indicate that subcutaneous injection of *L. obliqua* bristle extract caused severe hematuria with the presence of intact erythrocytes and leukocytes in urinary sediment (Berger et al.,

unpublished data). Envenomed animals also show proteinuria and significant changes in glomerular filtration rate and tubular electrolytic transport (Berger et al., unpublished data). Currently, the contribution of intravascular coagulation, deposits of hemoglobin in renal tubules and hemodynamic changes are under investigation.

5. Diagnosis and management of acute kidney injury in snake and arthropod envenomation

The early intravenous administration of animal-derived antivenoms is the only specific treatment against snake and arthropod envenomations. Antivenoms are concentrated of immunoglobulins (usually pepsin-refined F(ab')2 fragment of whole IgG) purified from the plasma of a horse or sheep that has been immunized with the venoms of one or more species of venomous animal (WHO, 2010**a**). The preparation of antivenoms is expensive and technically demanding. Around the world different manufacturers, which include public and private laboratories of diverse sizes and strengths, are able to produce the antivenoms (Gutiérrez et al., 2010; Williams et al., 2010). Some of them are small facilities, mostly located in public institutions, which manufacture for the needs of specific countries. Others are larger laboratories that manufacture and distribute antivenoms throughout various countries or regions. Although some countries or regions manufacture enough antivenom for their national and regional needs, as in Europe, USA, Brazil, Central America, Mexico, Australia, Thailand and Japan, in other parts of the world, specially in some regions of Africa, there are very few antivenom producers (Gutiérrez et al., 2010). In Brazil, three main Institutions are responsible for production of antivenoms: Instituto Butantan, Fundação Ezequiel Dias and Hospital Vital Brasil. The manufacture is government-subsidized and the antivenom is usually provided free to the patients. However, failures in the distribution of antivenoms to places where they are needed still contribute to the maintenance of high mortality rates (Table 1). In some instances, antivenoms are held in the main cities, where envenomations are rare, instead of being distributed to peripheral health clinics in rural areas where the accidents are frequent. This reflects defective distribution planning which is associated with a lack of coordination between those who understand the epidemiological pattern of the disease and those responsible for the antivenom distribution. Also, inadequate storage and transportation of antivenoms may result in physical destruction of vials and ampoules (e.g. by freezing of liquid antivenom) (WHO, 2010**a**). Besides the inadequate supply, distribution and accessibility to safe and effective antivenoms, another major issue is the lack of trained of health workers on how to use these products and how to conduct appropriate clinical management of medical emergencies. In fact, it is estimated that in Brazil in 2009, 37% of accidents with scorpions and 9% of accidents with spiders received inadequate treatment with antivenom, mainly because the health authorities are uninformed of the treatment protocols (Boletim eletrônico epidemiológico, 2010).

The treatment with antivenom is indicated in moderate and severe cases when systemic signs of envenomation are observed. In general, patients with hemostatic abnormalities, neurotoxic signs, cardiovascular abnormalities, AKI, hemoglobinuria and myoglobinuria should receive antivenom therapy (WHO, 2010**a**). In these cases the time elapsed between the occurrence of the accident and administration of a correct dose of antivenom is decisive for a sucessful therapy. It was demonstrated that the time interval between the accident and

administration of the antivenom of more than 2 hours was associated with the development of AKI, as well as with the risk of death or permanent injuries after *Bothrops* and *Crotalus* envenomations (Otero et al., 2002; Pinho et al., 2005). Although the correct use of antivenom is an effective form of treatment, the sorotherapy is also associated with the occurrence of severe adverse effects. The most serious adverse effect is anaphylactic reactions. Clinical features such as urticaria, itching, fever, tachycardia, vomiting, abdominal colic, headache, bronchospasm, hypotension and angioedema have been described after antivenom treatment (Fan et al., 1999; Ministério da Saúde, 2001). The incidence of adverse effects depends on the quality, dose and speed of intravenous injection or infusion. With antivenoms of good quality profile, there is a low incidence (less than 10%) of generally mild adverse reactions, mostly urticaria and itching. However, for products containing contaminant proteins, the incidence of such reactions may be as high as 85 %, including potentially life-threatening systemic disturbances such as hypotension and bronchospasm (WHO, 2007). Thus the adverse effects are directly associated with lack of good manufacturing practices adopted by laboratories that manufacture antivenoms. Recently, in an attempt to improve the quality of antivenom production the WHO established the guidelines for production, control and regulation of snake antivenom immunoglobulins. These guidelines provide detailed information on the recommended steps for antivenom manufacture and control (WHO, 2010**b**).

A number of additional interventions besides antivenom may be necessary to restore renal function in patients who developed AKI. Special attention should be given to hypotension, shock, electrolyte balance and maintenance of an adequate state of hydration. An urinary flow of 30 to 40 mL/h/kg is recommended for adults and 1 to 3 mL/h/kg for children to prevent AKI after snake envenomations (Ministério da Saúde, 2001; Pinho et al., 2008). Patients presenting oliguria or anuria, despite of fluid administration, are usually treated with intravenous furosemide or mannitol (WHO, 2010**a**). In these cases, a higher urinary flow may decrease the exposure of tubular cells to venom components and myoglobin or hemoglobin, which result in injury attenuation and prevention of tubular lumen obstruction (Zager, 1996). Cases that are unresponsive to fluid intake and diuretics are referred to renal units for dialysis (Pinho et al., 2008). Early alkalinization of urine by sodium bicarbonate in patients with myoglobinuria or hemoglobinuria is also recommended, because in the presence of acidic urine, myoglobin and uric acid precipitate and form obstructive cast (Khan, 2009).

6. Conclusion

Envenomation by different venomous snakes and arthropods is a neglected disease that afflicts the most impoverished inhabitants of rural areas in tropical developing countries. In this chapter, we reviewed some important aspects related to epidemiology, prevalence, clinical manifestations, pathophysiology and treatment of venom-induced AKI, which is one of the most significant and lethal effect of animal venoms. Despite of actual knowledge discussed here, several aspects involving the renal manifestations remain still unclear. Thus, further research are needed to cover the following key points: (i) biochemical composition of different animal venoms and their individual contribution to renal injury; (ii) renal pathological mechanisms induced by some specific venoms that are still unexplored; (iii)

discovery of new and more specific therapeutic alternatives to treat envenomation cases and (iv) improvement in the production, distribution and availability of the antivenoms currently used.

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8. References

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3.4 Capítulo IV

Acute kidney injury induced by experimental *Lonomia obliqua* envenomation.

Neste capítulo, os mecanismos da insuficiência renal aguda causada pelo envenenamento com *L. obliqua* foram investigados em um modelo experimental *in vivo* em ratos.

Os principais resultados obtidos foram:

- O envenenamento causa alterações hemodinâmicas incluindo hipotensão sistêmica, aumento do débito cardíaco, aumento da permeabilidade vascular renal, redução do ritmo de filtração glomerular e alterações tubulares como redução da capacidade de reabsorção de água e eletrólitos;

- As alterações morfológicas são compatíveis com necrose tubular aguda. Cilíndros hialinos, hemáticos e restos de células necróticas podem ser observados nos túbulos. Marcadores de lesão tubular são encontrados nos rins e urina dos animais envenenados;

- Há evidências de deposição de fibrina nos capilares glomerulares. Aumento de atividade de fator tecidual no rim, presença de material PAS positivo nos glomérulos, presença de fibrina e marcadores que indicam ativação da coagulação e complemento no rim;

- A expressão de proteínas associadas com inflamação, dano oxidativo, reparo de DNA, metabolismo e detoxificação de hemoglobina, heme e ferro estão aumentadas no rim de animais envenenados;

- Toxinas do veneno foram detectadas no rim, endotélio, tecido conjuntivo perivascular e urina.

O manuscrito a seguir será submetido para publicação em periódico científico internacional.

Acute kidney injury induced by experimental *Lonomia obliqua* envenomation

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Abstract

Background. *Lonomia obliqua* caterpillar envenomation causes acute kidney injury (AKI), which can be responsible for its deadly actions. This study evaluates the possible mechanisms involved in the pathogenesis of renal dysfunction. **Methods.** To characterize *L. obliqua* venom effects we subcutaneously injected rats and examined renal functional, morphological and biochemical parameters at several time points. We also performed discovery based proteomic analysis to measure protein expression to identify molecular pathways of renal disease. **Results.** *L. obliqua* envenomation causes acute tubular necrosis, which is associated with renal inflammation; formation of hematic casts, resulting from intravascular hemolysis; increase in vascular permeability and fibrosis. The dilation of Bowman's space and glomerular tuft is related to fluid leakage and intra-glomerular fibrin deposition, respectively, since tissue factor procoagulant activity increases in the kidney. Systemic hypotension also contributes to these alterations and to the sudden loss of basic renal functions, including filtration and excretion capacities, urinary concentration and maintenance of fluid homeostasis. In addition, envenomed kidneys increases expression of proteins involved in cell stress, inflammation, tissue injury, heme-induced oxidative stress, coagulation and complement system activation. Finally, the localization of the venom in renal tissue agrees with morphological and functional alterations, suggesting also a direct nephrotoxic activity. **Conclusions.** Mechanisms of *L. obliqua*-induced AKI are complex involving mainly glomerular and tubular functional impairment and vascular alterations. **General significance.** These results are important to understand the mechanisms of renal injury and may suggest more efficient ways to prevent or attenuate the pathology of *Lonomia*'s envenomation.

1. Introduction

Accidents caused by venomous animals (mainly snakes, spiders, scorpions, bees, wasps and caterpillars) are a costly and critically important public health problem. Despite of this, public health authorities, nationally and internationally, have given little attention to this problem worldwide (Warrell, 2010; Williams *et al.*, 2010). Consequentially, the morbidity and mortality associated with envenomation cases produce a great impact on the population and healthcare systems. One of the most important and lethal effects of these animal venoms is nephrotoxicity and a broad clinical spectrum of renal function impairment has been reported in human and experimental models of envenomation (Sitprija, 2006; Berger *et al.*, 2012). As kidneys are highly vascularized organs and have the ability to concentrate substances into urine they are particularly susceptible to venom toxins. The most common clinical renal manifestation seen in human patients is acute tubular necrosis, but all renal structures may be involved. Thus, the occurrences of acute tubulointerstitial nephritis, renal cortical necrosis, mesangiolysis, vasculitis, glomerulonephritis, proteinuria, hematuria, hemoglobinuria and myoglobinuria have also been described (Sitprija, 2006).

Lonomia obliqua caterpillars are well known in southern Brazil where they cause severe hemorrhagic syndrome characterized by perturbed coagulation, ecchymosis, acute kidney injury (AKI) and generalized hemorrhage. Since the 1980's there has been a considerable increase in the number of hemorrhagic incidents in rural areas of the southernmost Brazilian states of Rio Grande do Sul, Santa Catarina and Paraná. The origin of this epidemic is not clear, but can be partially attributed to recent deforestation, as well as to a progressive reduction in the number of natural predators. Usually, accidents occur when the victim, unknowingly leans against a tree trunk containing hundreds of caterpillars and comes into contact with the caterpillar's venomous bristles, which are chitinous evaginations of cuticule. Often, the caterpillar is crushed, the bristles are broken and venomous secretions, including hemolymph, penetrate the human skin (Veiga *et al.*, 2001). The venom is composed of several active constituents with procoagulant, fibrinogenolytic, proteolytic and hemolytic activities (Pinto *et al.*, 2010). Although consumptive coagulopathy secondary to intravascular disseminated coagulation is commonly observed in human and experimental animals, AKI is the leading cause of death from *L. obliqua* envenomation (Zannin *et al.*, 2003; Gamborgi *et al.*, 2006; Berger *et al.*, 2010).

Early in the 1980's, the first registered cases of *L. obliqua*-induced hemorrhagic syndrome indicated that 18 % of envenomed patients had developed AKI. The mortality rate in these patients reached 50 % (Duarte *et al.*, 1990; Duarte *et al.*, 1994). However, a lower incidence (5.2 %) was observed in the Brazilian state of Rio Grande do Sul from 1989 to 1995, when only 15 of 286 envenomed patients developed AKI (Duarte *et al.*, 1997). Another study analyzing a larger group of 2,067 envenomed patients in the Santa Catarina state in Brazil (from 1989 to 2003) reported that 39 victims (1.9 %) developed AKI (with serum creatinine levels $\geq$ 1.5 mg/dL). Eleven (32 %) of these patients were treated with dialysis and four (10.3 %) developed chronic renal injury (CRI). All victims with AKI presented concomitantly coagulation disturbances and hematuria and/or hemoglobinuria. Seven deaths (4 %) occurred during this period (Gamborgi *et al.*, 2006). An important conclusion of this work is that, even after the introduction of antivenom therapy (with anti-lonomic serum) in 1995, there was no reduction in the incidence of AKI, despite the significant decrease in the number of deaths and patients who developed CRI (Gamborgi *et al.*, 2006). In fact, recently we observe that antivenom treatment was able to reduce creatinine and urea levels of rats only if administered 2 h post venom injection. Serotherapy after 6 h of envenomation fails to neutralize the rising in biochemical markers of renal injury (Berger *et al.*, 2013). Since the average time elapsed between the contact of a person with caterpillars and an appropriate medical care can vary from 19 to 37 h (Zannin *et al.*, 2003), it seems imperative to achieve a better understanding of the mechanisms involved in venom-induced AKI. It is clear that having such knowledge available it will then make possible to develop new efficient treatments in order to avoid or at least to reduce the progression of renal disease in *Lonomia*`s and other kind of animal envenoming.

The risk of conducting early renal biopsies in human patients, due to coagulation disturbances inherent to the envenomation, has made it difficult to analyze the acute kidney pathological alterations. There are only two case reports in the literature describing alterations of *Lonomia*-induced AKI. The main findings were oliguria, high levels of serum creatinine, thickening of the Bowman's capsule, focal tubular atrophy and acute tubular necrosis (Burdmann *et al.*, 1996; Fan *et al.*, 1998). Since no experimental studies were available until now, the contribution of several factors possibly associated with AKI, such as hemodynamic changes, vascular permeability alterations, hemolysis, tubular obstruction, glomerular fibrin deposition and even a direct venom nephrotoxicity, remain obscure in *Lonomia*-induced AKI.

In an attempt to better understand the progression of renal disease commonly observed after the contact with *L. obliqua* caterpillars, we have focused on the action of venom in the kidney. Therefore, an experimental rat model was used in order to characterize changes in renal function, tubular hydroelectrolytic transport, histopathology and hemodynamics.

2. Materials and Methods

2.1 Reagents

Evans blue dye, purified coagulation factors (VII, IX and X) and molecular weight standards used in SDS-PAGE and western-blot were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Chromogenic substrate for factor Xa (S2222, Bz-Ile-Glu-Gly-Arg-pNa) was obtained from Chromogenix (Milano, Italy). Ketamine and xylazine were from Syntec, São Paulo, Brazil. *L. obliqua* antivenom (antilonomic serum - ALS), provided by the Butantan Institute (São Paulo, Brazil), was used as primary antibody for the detection of toxins in urine and renal tissue. ALS is a horse-derived concentrate of purified polyclonal antibodies (IgG) that had been raised against *L. obliqua* bristle extract (Rocha-Campos *et al*., 2001). Nonspecific background staining in immunohistochemical reactions was blocked using Ultra V Block reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Venom

L. obliqua caterpillars were kindly provided by the Centro de Informações Toxicológicas (CIT), Porto Alegre, Rio Grande do Sul, Brazil. The specimens used in this study were collected in the cities of Bom Princípio and Progresso, both located in Rio Grande do Sul, Brazil. *L. obliqua* venom was obtained by homogenizing the bristles in cold phosphate-buffered saline (PBS), pH 7.4, as previously described (Berger *et al*., 2010). The venom obtained following this procedure was designated as *Lonomia obliqua* **B**ristle **E**xtract (LOBE). The protein content of the LOBE samples was determined using a BCA assay kit (Pierce, Rockford, USA) and the aliquots were stored at -80 °C prior to use. The total number of caterpillars used for bristle extract preparation was 124 specimens and the protein concentration of the LOBE samples was 4.10 mg/mL. The total amount of venom extracted per caterpillar was 2.4 mg. All of the LOBE samples had similar *in vitro* pro-coagulant activities and the protein pattern for

each sample as monitored by SDS-PAGE and gel filtration chromatography (Pinto *et al*., 2006; Berger *et al*., 2010) were also similar.

2.3 Ethical statements

All procedures involving animals were carried out in accordance with the Guiding Principles for the Use of Animals in Toxicology (International Society of Toxicology, http://www.toxicology.org) and the Brazilian College of Animal Experimentation (COBEA). The experimental protocol was approved by the ethical committee on research animal care of the Federal University of Rio Grande do Sul, Brazil (register number **2008177/2009**) and by the Institute's Animal Ethics Committee of the Federal University of Minas Gerais, Brazil (protocol **177/2008**).

2.4 Experimental protocol

2.4.1 Animals

Adult male Wistar rats, weighing 250-300 g, were supplied by the central animal facility of our institution. They were housed in standard conditions within a temperature controlled room (22-23 ºC, on a 12 h light/dark cycle, with the lights on at 7:00 am) and had free access to water and food.

2.4.2 Selection of the venom dose

The severity of the natural envenoming is related mainly to the number of caterpillars involved as well as to the intensity of the exposure, since the venom is present not only in the caterpillar's bristles but also in their skin and hemolymph (Veiga *et al*., 2001). Considering that accidents with medical importance involves contact with a colony containing at least 40 to 50 caterpillars (Gamborgi *et al*., 2006) and that during venom extraction, after removal of all spicules, each caterpillar produces approximately 2.4 mg of venom, the total amount of venom injected in an individual weighing 70 kg can reach up to 1.4 – 1.7 mg/kg. In fact, these doses were calculated based on an artificial method of venom extraction, in which the caterpillar's bristle was macerated in a solution buffer. Thus, in a real envenomation situation the total amount of venom transferred is probably lower than the amount calculated. In an attempt to reproduce the clinical conditions observed in a real envenomation we selected doses of 1.0 and 1.5 mg/kg injected subcutaneously into rats. These doses were also in accordance with the

amount of venom used in other studies to induce coagulopathy and test the efficacy of antilonomic serum (Berger *et al.*, 2010; Dias da Silva *et al.*, 1996; Rocha-Campos *et al.*, 2001).

2.4.3 Venom administration

To follow the time course of kidney pathophysiological alterations, we used an experimental model of envenomation in rats. For this purpose, animals were divided into three groups (*n* = 6/group): The control animals (CTRL) were injected subcutaneously (s.c.) with 100 µL of sterile PBS solution and the experimental animals received a s.c injection containing 1.0 mg or 1.5 mg of LOBE per kg of body weight in a final volume of 100 µL. Immediately after treatments, the animals were distributed individually into metabolic cages, allowing quantitative urine collections and measurement of water intake. At several time points post-venom injection (2, 6, 12, 24, 48 and 96 h), blood, urine and kidneys were obtained for biochemical, histopathological and immunohistochemical analyses.

2.5 Sample preparation

Blood was collected in conscious rats through the caudal vein in 1:10 (v/v) 3.8 % trisodium citrate. Plasma was obtained by centrifugation at 1500 x g for 10 min and stored at − 80 ºC prior to use. Urine samples were also centrifuged at 2500 x g for 5 min and the supernatants stored at the same conditions. After blood collection, animals from the different groups were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). Then, an intracardiac perfusion was performed through the left ventricle with PBS solution and a circulatory circuit was opened by an incision in the right atrium, to ensure the elimination of intravascular blood. Immediately after perfusion, kidneys were quickly removed. One of them was fixed for histological analysis and the other was frozen in liquid nitrogen and stored at − 80 ºC for the measurement of tissue factor activity and proteomic analysis.

2.6 Biochemical measurements

Urinary and plasma levels of creatinine, urinary γ-glutamyl transferase (γ-GT) activity and proteinuria were determined by spectrophotometry (Turner SP-830 plus Barnstead, Dubuque, Iowa, USA) using commercially available kits (BioClin/Quibasa, Belo Horizonte, Brazil). Plasma and urinary concentrations of Na^+ and K^+ were

measured by flame photometry (CELM 180; Belo Horizonte, Minas Gerais, Brazil). Osmolality was determined in plasma and urine samples by cryoscopic osmometery using a Micro-Osmometer 3320 (Advanced Instruments, Norwood, Massachusetts, USA). Urine proteins were also analyzed by gel electrophoresis which was performed according to Laemmli (1970). Urine samples from animals of different times post-venom injection were diluted (10 X) and aliquots of 10 μL were submitted to SDS-PAGE on 8-20 % gradient gels under reducing conditions. Toxins excreted in urine were detected by Western-blot as previously described (Pinto *et al.*, 2006). Aliquots containing 50 μg of protein were separated by SDS-PAGE and transferred to PVDF membranes. Toxins were recognized using as a primary antibody an equine anti-LOBE IgG (ALS) diluted 1:100 and as a secondary antibody a peroxidase-labeled anti-horse IgG diluted 1:1000.

2.7 Renal function parameters

At the time intervals mentioned above the following renal function parameters were determined: Glomerular filtration rate (GFR), osmolar clearance (C_{osm}), water free clearance (C_{H2O}), fractional water excretion (FE_{H2O}), fractional sodium excretion (FE_{Na}^{+}) and fractional potassium excretion (FE_{K}^{+}). GFR (expressed as mL/min/100 g of body weight) was estimated by the creatinine clearance (C_{cr}), using the standard formula: $C_{cr} = U_{cr}.V/P_{cr}$, where U_{cr} is the urinary creatinine concentration, V is the urinary output and P_{cr} is the plasma creatinine concentration. C_{osm} (expressed as mL/min) was calculated as $C_{osm} = U_{osm}/P_{osm}.V$, whereas U_{osm} and P_{osm} are the urinary and plasma osmolalities, respectively. Values of C_{H2O} (mL/min) and FE_{H2O} (%) were obtained respectively from the equations: $C_{H2O} = V - C_{osm}$ and $FE_{H2O} = V/GFR.100$. FE_{Na}^{+} and FE_{K}^{+} (expressed as %) were calculated according to the equation: $FE = UE/PF.100$. UE represents the urinary excretion of each ion and PF is the amount filtered in plasma (both expressed as nmol/min).

2.8 Hemodynamic parameters

Systemic arterial pressure was measured in conscious rats by an indirect tail-cuff method using an electrosphygmomanometer (LE 5001, Harvard Apparatus, Holliston, Massachusetts, USA) combined with a pneumatic pulse transducer/amplifier, which provides output signals proportional to cuff pressure and amplified Korotkoff sounds.

Three consecutive readings of mean arterial pressure and heart rate were recorded before blood collection for each animal in each time post-venom injection.

2.9 Renal vascular permeability

The extravasation of Evans blue dye into the kidney was used as an index of increased vascular permeability (Pompermayer *et al.*, 2005). Rats received Evans blue dye (30 mg/kg) intravenously (1 mL/kg) via caudal vein 10 min prior to LOBE (1.5 mg/kg, s.c.) or PBS (100 µL, s.c.) injection. After 12 or 24 h, animals were anesthetized and perfused as described above to remove the intravascular Evans Blue. Then, the kidneys were quickly removed, weighed and allowed to dry for 24 h at 40 $^{\circ}$C. The dry weight was determined and Evans blue dye extracted in 2.5 mL of 1 % formamide (48 h at 40 $^{\circ}$C). The absorbance of extracted solution was measured in triplicate using a microplate reader spectrophotometer (SpectraMAX, Molecular Devices Co., Sunnyvale, USA) and the amounts of Evans blue dye were calculated by a standard curve made with known concentrations of Evans Blue. Results are presented as the amount of Evans blue dye extravasated (µg) per 100 mg of kidney tissue.

2.10 Renal tissue factor activity

Renal tissue factor (TF) was measured indirectly based on its ability to form a complex with factor VIIa (TF/FVIIa) to activate factor IX and X (Morrissey, 1995). Briefly, the kidneys were collected as described above, homogenized in cold PBS solution containing 1 % Triton X-100 and centrifuged at 9500 x g for 15 min. Samples of supernatants (with 10 µg of protein) were incubated with a concentrate mixture of FVII+FIX+FX (total of 15 µg) in 20 mM Tris-HCl, pH 7.4 containing 10 mM of $CaCl_2$ for 10 min at 37 $^{\circ}$C. Activated factor Xa (FXa) produced during the reaction was detected by the addition of a specific chromogenic substrate (0.2 mM S2222). The kinetics of *p*-nitroaniline release was monitored at 405 nm for 30 min in a final volume of 100 µL using a microplate reader spectrophotometer (SpectraMAX, Molecular Devices Co., Sunnyvale, USA). Each sample was measured in triplicate and results expressed as µmol of FXa generated per min per mg of kidney tissue.

2.11 Histology and immunohistochemistry

For renal histopathology, kidneys were collected as described above, sectioned sagitally and fixed in 10 % buffered formaldehyde, pH 7.2. After processing in alcohol and xylol, the organs were included in paraffin and 4 µm thick sections were obtained and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or Picro-Sirius reagent.

For immunohistochemical analysis of venom distribution in renal tissue, the kidney sections were deparaffinized and treated with 10 % hydrogen peroxide solution in methanol for 15 min to block endogenous peroxidase activity. Nonspecific binding sites were blocked with Ultra V Block reagent followed by incubation overnight at 4 °C with an equine anti-LOBE IgG (ALS) diluted 1:250 in PBS. After a washing step, the sections were incubated with the secondary antibody (a biotinylated anti-horse IgG produced in goat, diluted 1:100) and streptavidin diluted 1:100. The chromogenic reaction was developed by incubating the sections with 0.05 % diaminobenzidine solution and 0.2 % hydrogen peroxide. The progress of the reaction was monitored by light microscopy and stopped by washing the slides. Finally, the sections were counterstained with diluted Harris's haematoxylin. Control reactions were done by incubating kidney sections from non-envenomed rats with equine anti-LOBE IgG (ALS) under the same conditions as described above. No positive reactions were observed in these sections. Some sections were also used as negative controls. In these cases, the primary antibody was substituted by PBS.

2.12 Morphometric analysis

Glomerular morphological alterations were quantified by computer-assisted morphometric analysis using a method previously described (Caliari, 1997). Briefly, images with 40 X magnification of 30 glomeruli from each animal of different groups were randomly digitalized using a JVC TK1270/RGB microcamera (Tokyo, Japan). The KS300 software coupled to a Carl Zeiss image analyzer (Carl Zeiss, Oberkochen, Germany) was used to measure the total area (μm^2) of each glomerulus, glomerular tuft and bowman's space. The pixels of whole glomerulus and those corresponding to the glomerular tuft were selected and used for the generation of a binary image and subsequent calculation of the corresponding areas. The area of Bowman's space was obtained by the difference between the whole glomerular area and tuft area. The regions

of positive immunohistochemical reaction (expressed as μm^2) were also measured and used as a parameter to quantify venom distribution in the renal tissue.

2.13 Renal tissue proteomics

2.13.1 Sample preparation for mass spectrometry

The kidneys from controls and envenomed animals (at 24 h post-venom injection) were collected as described above, homogenized in cold PBS solution containing 1 % Triton X-100 with protease inhibitor cocktail (Halt protease inhibitor cocktail, Thermo, Rockford, USA) and centrifuged at 9500 x g for 15 min. The resulting supernatants were completely lyophilized and stored at -80 ^{o}C until use. Lyophilized samples were resuspended in water and precipitated using methanol/chloroform protocol. After precipitation samples were dried at room temperature and resuspended in 8 M urea. Each sample containing 100 μg of protein was reduced with 5 mM tris-2-carboxyethyl-phosphine (TCEP) at room temperature for 20 min and alkylated with 10 mM iodoacetamide at room temperature in the dark for 20 min. After reduction and alkylation, proteins were digested with 2 μg of trypsin (Promega, Madison, WI) by incubation at 37 °C during 16 h. Samples were freeze-dried at -80 ^{o}C and, after thaw, formic acid to a final concentration of 5 % was added. Samples were centrifuged at 14,000 rpm for 20 min and the supernatant was collected and stored at -80 °C.

2.13.2 MudPIT

The protein digest was pressure-loaded into a 250-μm i.d capillary packed with 2.5 cm of 5-μm Luna strong cation exchanger (SCX) (Whatman, Clifton, NJ) followed by 2 cm of 3-μm Aqua C18 reversed phase (RP) (Phenomenex, Ventura, CA) with a 1 μm frit. The column was washed with buffer containing 95 % water, 5 % acetonitrile, and 0.1 % formic acid. After washing, a 100-μm i.d. capillary with a 5-μm pulled tip packed with 11 cm of 3-μm Aqua C18 resin (Phenomenex, Ventura, CA) was attached via a union according Klein et al. (2012). The entire split-column was placed in line with an Agilent 1100 quaternary HPLC (Palo Alto, CA) and analyzed using a modified 11-step separation as described previously (Washburn *et al.*, 2001). The buffer solutions used were 5 % acetonitrile, 0.1 % formic acid (Buffer A), 80 % acetonitrile, 0.1 % formic acid (Buffer B), and 500 mM ammonium acetate, 5 % acetonitrile and 0.1 % formic acid (Buffer C). Step 1 consisted of a 70-min gradient from 0–100 % (vol/vol)

buffer B. Steps 2–10 had a similar profile with the following changes: 5-min in 100 % (vol/vol) buffer A, 3-min in X % (vol/vol) buffer C, a 6-min gradient from 0 to 15 % (vol/vol) buffer B, and a 85-min gradient from 15–100 % (vol/vol) buffer B. The 3-min buffer C percentages (X) were 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 % (vol/vol), respectively, for the 10-step analysis.

2.13.3 LTQ-Orbitrap

As peptides eluted from the microcapillary column, they were electrosprayed directly into a LTQ-Orbitrap (Thermo Fisher) with the application of a distal 2.4-kV spray voltage. Full MS spectra were acquired in profile mode, with a mass range of 400 – 1600 in the Orbitrap analyzer with resolution set at 60,000 followed by 10 data-dependent MS/MS spectra at 35 % normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Minimal signal for fragmentation was set to 1,000. Dynamic exclusion was enabled with a repeat count of 1, duration of 30.00 s, list size of 500, exclusion duration of 180.00 s, and exclusion mass with high/low of 1.5 m/z. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system.

2.13.4 Analysis of tandem mass spectra

MS/MS spectra were analyzed using the following software analysis protocol. Protein identification and quantification analysis were done with Integrated Proteomics Pipeline (IP2, Integrated Proteomics Applications, Inc. www.integratedproteomics.com/). Tandem mass spectra were extracted into ms2 files from raw files using RawExtract 1.9.9 (McDonald *et al*., 2004) and were searched using ProLuCID algorithm (Xu *et al*., 2006). MS/MS spectra remaining after filtering were searched with the ProLuCID algorithm against the EBI-IPI_rat_3.30_06-28-2007 concatenated to a decoy data base in which the sequence for each entry in the original data base was reversed (Peng *et al*., 2003). Searches were performed with cysteine carbamidomethylation as a fixed modification. ProLuCID results were assembled and filtered using the DTASelect program (Tabb *et al*., 2002) using two SEQUEST (Eng *et al*. 1994) defined parameters: the cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltaCN) to achieve a false discovery rate of 1 %. The following parameters were used to filter the peptide candidates: -p 1 -y 1 --trypstat --fpf 0.01 --dm. Also, we used 50 ppm as precursor tolerance, fragment mass

tolerance of 600ppm, 3 as number of isotopic peaks and unlimited missed cleavages were allowed.

2.13.5 Assessing differential expression and exclusive proteins / Bioinformatic tools

The software PatternLab (Carvalho *et al.* 2008, 2012) was used to identify exclusive proteins found in the control and treated conditions. PatternLab's Approximately Area Proportional Venn Diagram (AAPVD) module was used for pinpointing proteins uniquely identified in each condition. The following parameter was used: proteins that were not detected in at least 2 out of 3 runs per condition were not considered. G-test spectral counting quantitation was performed in a pair-wise comparison between the two groups, as previously reported (Ambatipudi *et al.* 2009). Proteins are considered differentially expressed with *P* < 0.1. Ingenuity Pathway Analysis tool (Ingenuity Systems; http://www.ingenuity.com) was used to generate functional annotations of identified proteins in known molecular pathways and/or biologic function in disease. The significance of the canonical pathways and biologic function defined by identified proteins was measured in two ways: (*1*) The number of proteins identified from the data set that map to a known pathway or function in disease and (*2*) a *P* value (Fisher exact test) determining the probability that the association between the proteins in the data set and the canonical pathway or function in disease is explained by chance alone.

2.14 Statistical Analyses

Results are expressed as mean ± SE. When appropriate, statistical comparisons were done by using one or two-way analysis of variance followed by the Bonferroni's test. A P value of less than 0.05 was chosen to establish significance. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Renal function

To follow renal alterations in rats several physiological parameters were measured at different times post *Lonomia obliqua* venom injection. The results are presented in **Fig. 1**. Between 2 and 6 h after LOBE administration (doses of 1 mg/kg or 1.5 mg/kg, s.c.), the animals showed signs of acute toxicity, including progressive weakness, lethargy and dyspnea. Compared to PBS-treated animals there was a

reduction of body weight mainly at 6 h in rats that received the higher dose of LOBE (1.5 mg/kg, s.c.) **(Fig. 1A)**. The weight loss was accompanied by a significant reduction in water intake between 6 and 48 h and by an increase in urinary output at 6 and 12 h for both tested doses **(Fig. 1B)**. The polyuria was maximal at 6 h, when urinary output increased from 0.58 ± 0.08 mL/2h in PBS-treated animals to 1.80 ± 0.16 mL/2 h ($p < 0.05$) in rats injected with LOBE (1.5 mg/kg, s.c.). At the same time, animals that received a dose of 1.0 mg/kg had a urinary output significantly lower (0.9 ± 0.2 mL/2h, $p < 0.05$) compared to those treated with 1.5 mg/kg **(Fig. 1C)**.

Important glomerular and tubular functions related to control of fluid filtration, water and electrolyte balance were severely impaired. Despite the polyuria, *L. obliqua*-induced AKI is associated with a marked reduction in the glomerular filtration rate (GFR). In the first 24 h of envenomation, a rapid decrease of GFR was observed in animals treated with both doses of LOBE. At 48 h envenomed rats (injected with 1.5 mg/kg of LOBE) presented values of GFR 16 times lower than controls at the same time. This remarkable effect of the venom on GFR was observed for as long as 96 hours **(Fig. 1D)**. Similarly, the kidney's ability to concentrate urine (a primordial tubular function) was also impaired, since the density and osmolality of urine had a reduction in values between 2 and 48 h **(Fig. 1E an F)**. The decrease in urinary osmolality was accompanied by a significant increase in the plasma osmolality observed mainly in rats injected with the higher dose of LOBE **(Fig. 1G)**. Consistent with these results the osmolar clearance (C_{osm}) was lower in envenomed animals, while the free water clearance (C_{H2O}) increased, which imply that the kidney is producing dilute urine through the excretion of solute-free water **(Fig. 1H and I)**. As expected, with the loss of ability to retain water, the fractional excretion of water (FE_{H2O}) increased from 0.33 ± 0.03 % in PBS-treated animals to 29.1 ± 4.9 % ($p < 0.05$) in rats injected with LOBE (1.5 mg/kg, s.c.) at 48 h **(Fig. 1J)**. Likewise, tubular ability to conserve and maintain the electrolytic balance was lost throughout the envenoming period. The fractional excretion of sodium and potassium (FE_{Na+} and FE_{K+}) was high between 24 and 48 h, indicating a marked impairment in the tubular reabsorption of filtered Na^+ and K^+ **(Fig. 1K and L)**. At 96 h most of the renal function parameters (excepting GFR) tends to return to normal levels (similar from that obtained to non-envenomed rats).

3.2 Proteinuria

Most of envenomed animals produced dark-brown-colored urine in the period of 6 to 12 h, indicating the occurrence of hematuria and/or hemoglobinuria. The presence of intact and fragmented erythrocytes, epithelial cells and leukocytes were observed in the urinary sediment by light microscopy (not shown). Consistent with these observations, *L. obliqua* venom induced massive acute proteinuria, which was maximal at 6 and 12 h post-venom injection **(Fig. 2A)**. As expected, the group treated with 1.5 mg/kg of LOBE had more severe proteinuria compared to animals treated with 1.0 mg/kg, but for both groups, urinary protein excretion decreased progressively between 12 and 48 h, reaching levels similar to controls at 96 h. Analysis of urine by SDS-PAGE revealed the presence of several proteins with molecular weights ranging from 10 to 75 kDa **(Fig. 2B)**. A greater variety of bands were evident mainly at 6 and 12 h after envenomation, confirming this time interval as crucial to the development of glomerular injury. The two most prominent bands (observed around of 70 and 15 kDa) have molecular weights that match to serum albumin (68 kDa) and subunits of hemoglobin (16 kDa). The presence of venom excreted in urine was verified by western-blot. As shown in **Fig. 2C**, we detected at least 5 bands in urine from venom-treated rats at 6 and 12 h, which specifically react with antibodies raised against *L. obliqua* toxic proteins. These bands have molecular weights around of 70-60, 50, 37, 25 and 20 kDa (arrows in **Fig. 2C**) and were not recognized in urine from non-envenomed animals (controls). Several toxins in this range of molecular weights have already been identified through transcriptomic and proteomic analysis of LOBE. Some of them include lectins and c-type lectin-like proteins (70-60 kDa), serine-proteinases (50 kDa), cysteine-proteinases (37 kDa) and lipocalins (25-20 kDa) (Ricci-Silva *et al*., 2008; Veiga *et al*., 2005).

3.3 Renal histopathological alterations

Light microscopy of kidney biopsies from PBS-injected rats revealed a normal renal parenchyma **(Fig. 3A)**. In contrast, envenomed animals showed progressive degenerative lesions compatible with acute tubular necrosis (ATN) **(Fig. 3B–H)**. Increased acidophilia and dilation of renal tubules were observed between 6 and 48 h after venom administration **(Fig. 3C-F)**. Loss of proximal brush border, cytoplasm vacuolation, and in some tubules, degeneration and desquamation of necrotic cells occurred between 12 and 48 h **(Fig. 3D-F)**. The nuclei of the various proximal tubular cells at 24 and 48 h often showed pyknosis with clumping of chromatin material **(Fig.**

3E and F). In several tubules at 48 h, the renal epithelium was completely necrotic whereas the basement membrane was either intact or disrupted by tubular necrosis **(Fig. 3F)**. Consistent with the histological signs of tubular injury, γ-GT urinary activity — which is considered an efficient biomarker for early diagnosis of ATN (Guder and Ross, 1984) — increased in a dose- and time-dependent manner up to 48 h. At 96 h, γ-GT activity decreased, but remained significantly high in animals treated with 1.5 mg/kg of LOBE. Histologically, the signs of lesions were much less marked and the proximal and post-proximal tubular epithelia assumed a normal appearance at 96 h **(Fig. 3G and H)**.

Besides the evidence of degenerative lesions, proximal and distal tubules also had swollen lumens. Hyaline and hematic casts and cellular debris were found within tubules, obstructing their lumens **(Figs. 3C-F and 4)**. Hyaline casts (predominantly formed by a protein-rich material) were more prevalent at 6 and 12 h **(Figs. 3C-D and 4A)**, while hematic casts (predominantly formed by fragmented or intact erythrocytes) and cellular debris have appeared commonly at 12, 24 and 48 h **(Figs. 3D-F and 4A-B)**.

An intense inflammatory response characterized by edema, cellular infiltration and fibrosis was observed mainly at 48 h and 96 h **(Figs. 3F and 5)**. Inflammatory cell infiltrate and edema were detected at 48 h in regions of extensive necrosis **(Fig. 3F)** and within glomeruli **(Fig. 5C)**. At 96 h, despite signs of tubular regeneration, the inflammatory infiltrate increased significantly, probably to help in tissue repair **(Fig. 5B)**. Light microscopy of sections stained with picrosirius revealed extensive peritubular collagen deposition at 96 h after administration of *L. obliqua* venom **(Fig. 5E)** compared to PBS-treated rats **(Fig. 5D)**, indicating fibrosis. Foci of interglomerular collagen deposition were also observed in the renal cortex at the same time **(Fig. 5F)**.

3.4 Glomerular morphometric alterations and renal tissue factor activity

Glomerular alterations in envenomed animals were associated with lobulation of the capillary tufts, dilation of glomeruli, deposition of a hyaline material inside the Bowman's space and distention of Bowman's space **(Figs. 3D, 6A and 7B)**. Nodules that formed a dense, strongly-stained mesangial matrix, evidenced by the presence of a PAS positive stain in the capillary tufts was also observed between 2 and 12 h post-venom **(Fig. 6A)**. Consistent with histological observations, computer-assisted morphometric analysis has indicated a significant increase in glomerular area at all-time intervals examined. There was also an increase in the areas of glomerular tuft and Bowman's space, which confirms that the capillaries and Bowman's space are dilated

(Fig. 6B). The enlargement of Bowman's space observed at 12 h possibly is related to the presence of a hyaline material (rich in plasma proteins) found in several glomeruli at this time (details in **Figs 3D and 7B**).

Since intra-glomerular fibrin deposition can impair renal function and *L. obliqua* venom is able to activate the coagulation system both *in vitro* and *in vivo* (Berger *et al.*, 2010), we decide to measure the levels of renal tissue factor (TF) activity during envenomation. TF is a transmembrane enzyme activator that triggers the coagulation cascade, generating activated factor X and fibrin. As showed in **Fig. 6C**, renal TF activity increased rapidly from the first 2 h, reaching levels 2 times higher than control values at 6 hours. At 12 and 24 h post-venom injection this level remained significantly high, but decreased progressively thereafter **(Fig. 6C)**. Interestingly, the rise in renal TF activity was coincident with the presence of PAS positive deposits (which stain specifically glycoproteins) within the capillary tufts at 2-12 h **(Fig. 6A)**.

3.5 Hemodynamics and renal vascular permeability

Mean arterial blood pressure was lower in animals that received LOBE compared to basal levels in controls **(Table 1)**. Sustained hypotension was detected between 24 and 96 h in rats treated with the both doses of venom. The maximum decrease occurred at 24 h. In contrast, the heart rate increased significantly at 12 and 96 h in animals injected with 1 mg/kg of LOBE and at 48 h in animals injected with 1.5 mg/kg. Hypotension was accompanied by an increase in renal vascular permeability **(Fig. 7)**. Kidney blood vessels were hyperemic and signs of plasma leakage, migration of inflammatory cells and interstitial edema were also evident after 12 h of venom administration **(Fig. 7A)**. As mentioned above, several glomeruli at 12 h have their Bowman's space filled with a protein-rich material that had extravasated from glomerular capillaries **(Fig. 7B)**. Confirming these observations, a marked increase of vascular permeability in the kidney, as measured by the extravasation of Evans blue dye, was detected at 12 and 24 h after LOBE injection (1.5 mg/kg, s.c) **(Fig. 7C)**.

3.6 Immunohistochemical detection of venom in the renal tissue

Venom distribution and its binding to renal structures was investigated by submitting kidney biopsies from venom-treated and control rats to immunohistochemistry using anti-LOBE IgG that reacts specifically with venom toxins **(Fig. 8)**. Positive immunohistochemical reaction was found in cortical and medullar

regions of kidneys from rats injected with 1.5 mg/kg of LOBE **(Fig. 8D and C)**. Venom was detected in glomerular capillaries, Bowman's capsule, proximal and distal tubules and in intra-tubular casts**.** Staining for venom was intense in tubular brush border at 2 and 6 h and was also present in cells of tubules in degeneration at 48 h **(Fig. 8B-D)**. Generally, tubules stained more than glomeruli. There was no immunoreactivity for venom in the renal structures of PBS-treated rats (controls) **(Fig. 8A)**. The highest levels of venom (estimated by the area of positive immunohistochemical reaction) were detected in the renal tissue at 2 h **(Fig. 8F)**. After that, venom immunoreactivity decreased progressively until 96 h when the staining was weak **(Fig. 8E and F)**. *L. obliqua* toxins were also detected in other kidney structures such as in perivascular connective tissue of blood vessels, endothelium and smooth muscle of arteries and veins **(Fig. 9A and B)**.

3.7 Kidney proteins differentially expressed during envenomation

To gain further mechanistic insight which drive venom-induced kidney disease, we applied a semi-quantitative discovery based shotgun proteomic approach to identify the proteins differentially expressed in the kidney of envenomed animals. For this purpose, the proteomic data was acquired by tandem mass spectrometry with subsequent quantification, analysis of differential protein expression, validation, and functional annotations in order to identify the involved molecular pathways. The proteins identified consist of two classes: (1) those that were differentially expressed and met our significance criteria and (2) proteins that were uniquely identified in kidneys of rats injected with PBS or LOBE (1.5 mg/kg) after 24h. **Figure 10A** represents differentially expressed and unique proteins *via* a Venn diagram. Overlaps between PBS- and LOBE-treated animals represent significant differentially expressed proteins and non-overlapping portions of the diagram represent unique protein identifications. A total of 779 proteins were identified. Twenty-four proteins were exclusively identified in envenomed animals; 169 were exclusively identified in control rats and 586 were common proteins in both treatments. Among these 586 proteins, 138 (23.5 %) were identified as being differentially expressed.

Ingenuity Pathway Analysis was used for functional annotations and revealed several key protein categories and pathways significantly enriched in the differentially expressed proteins. Thus, the sets of proteins were assigned to either biologic function in disease or canonical signaling pathways. Through these analyses it was evident that

the set of proteins identified in control kidneys displayed healthy biological functions such amino acid metabolism and small-molecule biochemistry associated with normal renal metabolic pathways **(Fig. 10B)**. On the other hand, protein expression shifts toward "cellular distress" functions in the kidney of envenomed rats **(Fig. 10C)**. In this case, the expression profiles were considerably enriched for proteins that belong to acute phase inflammatory response signaling, LXR/RXR activation (involved in retinoic acid-mediated gene activation triggered by inflammatory stimulus), oxidative stress response pathways such as that mediated by the nuclear erythroid-related factor 2 (NRF2) and coagulation and complement systems (involved in thrombosis, fibrosis, inflammation and vascular alterations) **(Fig. 10C)**.

Accordingly to our functional and histopathological results, several novel proteins related to renal disease were identified in the kidney of LOBE-treated animals. Proteins associated with tubular and glomerular injury, necrosis, inflammation and fibrosis were up-regulated or unique in envenomed rats **(Fig. 10D)**. Some of these molecules, mainly those that were related to renal disease or identified in canonical pathways, are listed in **Table 2**. A complete list with differentially expressed and unique proteins of envenomed and control animals are shown in **supplemental Table 1**. It is worth mentioning, that proteins functionally linked to tissue injury (markers of necrosis and/or apoptosis), osmotic and oxidative stress, electrolytic imbalance, acute phase inflammatory response, fibrosis and thrombosis were expressed in the kidneys of LOBE-treated rats **(Table 2)**. Proteins of the kallikrein-kinin and complement systems which are related to hypotension and control of vascular permeability were also found to be up-regulated in envenomed animals **(Table 2)**. Our proteomic data also confirm that an important mechanism of *Lonomia*-induced AKI is mediated by hemoglobin and the release of its degradation products: free heme and iron. In fact, hemoglobin, hemopexin and ferritin were up-regulated and the expression of heme oxygenase-1 was uniquely induced in the kidneys of envenomed rats **(Table 2)**. As a result of heme and iron release, several antioxidant enzymes were found to be up-regulated, indicating the generation of reactive oxygen species **(Table 2)**.

4. Discussion

AKI is frequently described and is life threatening in several cases of snake and arthropod envenomation (Sitprija, 2006; Berger *et al.*, 2012). Particularly in *Lonomia obliqua* envenomation, AKI is the main cause of death and its mechanisms are

completely unknown until now. In this work we use an *in vivo* experimental model to characterize the *L. obliqua*-induced AKI. According to our results, the pathophysiological mechanism seems to be complex and multifactorial involving four main issues: (1) vascular abnormalities; (2) tubular and glomerular alterations; (3) renal inflammation and (4) a direct venom cytotoxic activity.

4.1 Vascular abnormalities

Rats injected with *L. obliqua* venom presented important hemodynamic alterations characterized by systemic hypotension and increased heart rate. The time of maximal decrease in blood pressure was coincident with the reduction of GFR and the impairment of renal function. In addition, an increase of renal vascular permeability and edema was also observed at the same period of envenomation. These findings are important because systemic vasodilation is associated with the decrease in GFR and can lead to renal hypoperfusion and ischemia in different pathological conditions (Schrier *et al.*, 2004). Specifically in this type of envenomation, one mechanism that could contribute to vasodilation is the activation of kallikrein-kinin system (KKS). It is known that *L. obliqua* venom has toxins (kallikrein activators and kininogenases) able to activate plasma pre-kallikrein and directly release bradykinin (BK) from low molecular weight kininogen (LMWK) (Pinto *et al.*, 2010; Bohrer *et al.*, 2007). Interestingly, our results indicated that the expression of LMWK (the main substrate of tissue kallikrein and venom kininogenases) is up-regulated in the kidneys of envenomed animals, which may favor the generation of BK. The immediate consequence of intravascular activation of KKS is a fall in systemic blood pressure and in peripheral tissues is edema and erythema formation (Bohrer *et al.*, 2007). The participation of BK was already confirmed, since the hypotensive and edematogenic responses elicited by LOBE were inhibited by HOE-140, a B2 receptor antagonist (Bohrer *et al.*, 2007). In agreement with these experimental observations, hypotension and reduced plasma levels of pre-kallikrein are common features observed in patients (Zannin *et al.*, 2003), which support the evidence that KKS is activated during envenomation and is clinically relevant. Moreover, the data presented here also suggest that KKS activation may be involved in venom-induced AKI.

4.2 Tubular and glomerular alterations

Despite the scarce clinical case-reports, the main pathological finding obtained from kidney biopsies is acute tubular necrosis (ATN). Experimental animals also showed histological alterations compatible with ATN such as loss of proximal brush border, cytoplasm vacuolation, pyknotic nuclei, degeneration and desquamation of necrotic cells. These necrotic cells exfoliating into the lumen due to either cell death or defective cell-to-cell or cell-to-basement membrane adhesion, can obstruct the flow of filtrated fluid and give rise to a back pressure limiting glomerular filtration (Trof *et al.*, 2006). Accordingly, in this work, markers of ATN were detected in urine (urinary γ-GT) and several proteins related to tubule injury were found to be up-regulated (Orm-1, YBX1, Alb, CK-B, FABP3 and NHE-RF1) or uniquely expressed (NGAL) in the kidneys of envenomed rats. Proteins linked to apoptosis (CytC, S100-A8 and MFP-1) were also up-regulated. The occurrence of glomerular dysfunction was evident, because envenomed rats presented massive proteinuria, serum Alb had a 2.1-fold increase in the kidneys and a band corresponding to the molecular weight of Alb was detected in urine. In addition, our results confirm that hematuria and hemoglobinuria are predominant characteristics of *L. obliqua*-induced AKI. Indeed, the venom has strong *in vitro* and *in vivo* hemolytic activity and a phospholipase A2 responsible for this effect was already isolated (Seibert *et al.*, 2006; Seibert *et al.*, 2010). Intact erythrocytes were found within the tubules forming intra-tubular casts and, as a result of intravascular hemolysis, different Hb subunits were detected in urine and kidneys. It is known that the formation of Hb deposits may be toxic to renal tubules due to heme cytotoxicity (Zager, 1996). Once reabsorbed by the proximal tubular cells the heme porphyrin ring is rapidly catabolized by Hmox-1 yielding equimolar amounts of free iron, biliverdin and carbon monoxide (Camara and Soares, 2005). Free iron then up-regulates intracellular ferritin expression, a key defense mechanism against iron-induced tissue damage. However, in the presence of large amounts of Hb the levels of released iron also increase, saturating the binding capacity of ferritin. Thus, the iron not removed by ferritin binding is able to readily accept and donate electrons and greatly facilitates free radical production (Zager, 1996; Khan, 2009; Zager *et al.*, 2012). As proteins related to canonical pathways of oxidative stress were identified exclusively in the kidneys of LOBE-treated rats, we believe that heme cytotoxicity plays a significant role in *L. obliqua*-induced AKI. Consistent with this, several anti-oxidant enzymes (Prdx-5, Prdx-2, GST, DJ-1 and SOD) and proteins associated with heme and iron metabolism (Hmox-1, ferritin, Hpx

and Cygb) were up-regulated or uniquely expressed during envenomation, suggesting that heme is effectively metabolized, generating free radicals and inducing oxidative damage to proteins, lipids and DNA. In fact, in previous experiments we demonstrated that LOBE induces kidney DNA damage leading to double-strand breaks and formation of oxidized purines and pyrimidines (Berger *et al.*, 2013). Probably this is associated with the increased expression of Rad50, a double-strand break repair protein, detected in the present work.

As a consequence of ATN, the excretion of Na^+ and K^+ increased significantly during envenomation. Injured proximal tubule cells have alterations in the actin and microtubule cytoskeletal networks that lead to a redistribution of Na^+/K^+ ATPase from the basolateral to the apical membrane, contributing to a decrease in Na^+ transport and reabsorption (Thadhani *et al.*, 1996). It was reported that FE_{Na+} and FE_{K+} also increase in envenomation caused by *Bothrops* snakes (Boer-Lima *et al.*, 1999; Linardi *et al.*, 2011). Despite the redistribution of Na^+/K^+ ATPase to the apical membrane, Linardi *et al.* (2011) reported an increase in expression and activity of Na^+/K^+ ATPase during envenomation by *Bothrops alternatus* and suggest that it is a protective mechanism triggered in response to natriuresis with the aim to preserve renal function during acute damage. In agreement with this observation, we found an increase in expression of type-3 Na^+/H^+ exchanger regulatory cofactor, which is important in Na^+ and HCO_3^- reabsorption in proximal tubule cells (Trof *et al.*, 2006). Likewise Na^+/K^+, water excretion increased significantly and envenomed animals had polyuria. Several parameters measured, such as urinary density and osmolality, C_{osm}, C_{H2O} and FE_{H2O}, indicate that kidneys from envenomed rats are producing dilute urine through the excretion of solute-free water. Possibly, the presence of intratubular Na^+ not reabsorbed by proximal tubule cells may contribute to the increased water excretion and both, Na^+ and water rejection, may also be associated with the fall in blood pressure.

Regarding the glomerular dysfunctions, a valuable hypothesis that should be considered is the deposition of fibrin clots in glomerular capillaries. Indeed, the most potent activity of LOBE *in vitro* is the procoagulant activity (Donato *et al.*, 1998; Veiga *et al.*, 2003). *In vivo*, LOBE also causes activation of coagulation and fibrinolysis leading to a consumptive coagulopathy characteristic of this type of envenomation. Two enzymes responsible for the venom procoagulant activity, activators of prothrombin and factor X, have already been isolated (Alvarez-Flores *et al.*, 2006; Reis *et al.*, 2006). Besides the direct effect on coagulation factors, LOBE is also able to induce a

procoagulant profile in endothelial cells in culture through an up-regulation of TF expression (Pinto *et al*., 2008). Confirming these results obtained in endothelial cells, envenomed kidneys showed an increase in TF procoagulant activity. In addition, the expression of known markers of disseminated intravascular coagulation and thrombosis (serpin 1 alpha-1-antiproteinase, PAI-1 and HC-2) increased in the kidneys of venom-treated rats. The gamma and beta chains of fibrinogen were detected solely in envenomed kidneys, which are probably related to the PAS positive stain observed in glomeruli and is suggestive of fibrin formation.

4.3 Renal Inflammation

Intense inflammatory response is a common feature in *L. obliqua* envenomation. Pain and edema are the most characteristic initial symptoms observed at the local site of contact (Zannin *et al*., 2003). Usually signs of systemic inflammation with neutrophilic leukocytosis, cell infiltrate and edema have also been described in lungs, kidney and heart of experimental animals (Berger *et al*., 2013). The inflammatory response is accompanied by the production of several cytokines (TNF, IL-1β IL-8, IL-6, CCL2 and CXCL1), vasoactive mediators (BK, histamine, prostaglandins and nitric oxide), adhesion molecules (E-selectin, VCAM-1 and ICAM-3) and an increase in leukocyte rolling and adhesion to the endothelium (Alvarez-Flores *et al*., 2006; Bohrer *et al*., 2007; Pinto *et al*., 2008; Berger *et al*., 2010; Nascimento-Silva *et al*., 2012). Specifically in the kidney, it was observed an up-regulation of several proteins related to acute phase inflammatory signaling, nephritis, inflammatory cell infiltration, increase in vascular permeability, glomerular dilation, distention of Bowman's space and interstitial edema. Kidney sections stained with picrosirius revealed extensive collagen deposition in cortical periglomerular and peritubular regions and proteins involved with fibrosis, such as Cygb, PAI-1, complement C3 and complement factor D were identified. Enhanced deposition of extracellular matrix (ECM) proteins in renal tissue has been observed in response to a variety of stimuli, including TGF-β, TNF-α, IL-1, several adhesion molecules and chemoattractants. These stimuli can also increase the levels of tissue inhibitors of matrix metalloproteinases, thereby attenuating ECM turnover, and thus, favoring the deposition of collagen and other matrix proteins (Eddy, 1996; Pawluczyk and Harris, 1998).

Besides fibrosis, activated complement components (C3 and factor D) may also contribute to the alterations in vascular permeability and acts as a chemoattractant for

neutrophils (Turnberg *et al*., 2006). Moreover, complement activation by albumin is a powerful underlying mechanism of tubular and interstitial injury via cytotoxic, proinflammatory, and fibrogenic effects, which often occur in renal diseases where proteinuria is present (Portella *et al*., 2013). In an experiment conducted in proximal tubular cells incubated with serum proteins, *in vitro* complement activation was observed, which could be associated with changes in the cytoskeleton, production of superoxide anion, hydrogen peroxide, and proinflammatory cytokines, such as IL-6 and TNF-α (Abbate *et al*., 2006).

4.4 Direct venom cytotoxic activity

As the kidneys are highly vascularized organs, they are particularly susceptible to direct venom toxicity (Sitprija, 2006). Renal epithelial cells in culture and isolated perfused kidneys have been used to characterize the direct cytotoxic effects of different venoms including bee, snake and spider venoms. The most common isolated toxins, which are nephrotoxic, belong to the classes of metalloproteinases, serine-proteinases, C-type lectins, phospholipases A2, sphingomyelinases D and L-amino acid oxidases (Berger *et al*., 2012). In the case of *L. obliqua* some toxins belonging to these classes have already been isolated (Pinto *et al*., 2010); however, their effects on renal cells are unknown. Our results provide some evidence that the whole venom probably has a direct nephrotoxic effect, since immunohistochemical staining confirmed the presence of venom in renal tissue, with stronger staining in the initial 6 h after venom administration followed by a progressive decrease thereafter. The venom was rapidly excreted in urine, because at least 5 bands which specifically react with antibodies raised against *L. obliqua* toxins were detected in urine. Interestingly, the positive immunohistochemical reaction for venom in different kidney structures agreed with the morphological and histological damage caused by the venom in these anatomical regions and indicated that there was a close correlation between the sites of venom localization and subsequent tissue injury. Previous studies are consistent with our observations. Using immunochemical and radio-labeling methods to analyze venom biodistribution in rats, the highest quantities of LOBE were detected in kidneys, blood and urine (Rocha-Campos *et al*., 2001; Da Silva *et al*., 2004).

5. Conclusion

In this work a rat experimental model was used to study the progression of renal disease during *Lonomia obliqua* envenomation. According to our results, the pathophysiological mechanism involved in *L. obliqua*-induced AKI seems to be multifactorial where events such as systemic hypotension and fibrin deposition contribute to renal hypoperfusion, tubular necrosis and the sudden loss of basic renal functions, including filtration and excretion capacities, urinary concentration and maintenance of body fluid homeostasis. In addition, when compared to control rats, the kidneys from envenomed animals showed to be increasingly enriched for stress-related proteins, which are commonly associated to inflammation, tissue injury, heme-induced oxidative stress, coagulation and complement systems activation. Finally, the localization of the venom in renal tissue agreed with morphological and functional alterations, suggesting a close correlation between venom tissue levels and renal damage.

6. Acknowledgments

7. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1. Functional parameters during *L. obliqua*-induced AKI. Rats were injected subcutaneously with PBS (controls - CTRL) or LOBE (1.5 or 1.0 mg/kg). After different times post administration the following parameters were determined: **(A)** Body weight, **(B)** water intake, **(C)** urine outoput, **(D)** glomerular filtrattion rate (GFR), **(E)** urinary density, **(F)** urine osmolality (urine osm), **(G)** plasma osmolality (plasma osm), **(H)** osmolar clearance (C_{osm}), **(I)** water free clearance (C_{H2O}), **(J)** fractional water excretion (FE_{H2O}), **(K)** fractional sodium excretion (FE_{Na}^+) and **(L)** fractional potassium excretion (FE_K^+). Data are presented as means ± SE (n=6/group). Significant differences: *$p < 0.05$ vs. CTRL and $^\S p < 0.05$ vs. LOBE (1.0 mg/kg, s.c).

Figure 2. Proteinuria. (A) Rats were injected subcutaneously with PBS (controls - CTRL) or LOBE (1.5 or 1.0 mg/kg). After different times post administration the protein levels in urine were measured. Data are presented as means ± SE (n=6/group). Statistical differences of *p < 0.05 vs. CTRL and §p < 0.05 vs. LOBE (1.0 mg/kg, s.c) were considered significant. **(B)** Representative urine samples from CTRL and rats treated with LOBE (1.5 mg/kg, s.c.) were analyzed by SDS-PAGE (8-20 %) under reducing conditions. **(C)** Toxins excreted in urine were detected by western-blot. Samples of urine from CTRL and rats treated with LOBE (1.5 mg/kg, s.c.) at 6 and 12 h post-venom injection were separated by SDS-PAGE and different toxins were detected by immunoreaction with polyclonal antibodies against LOBE. Toxins present in crude bristle extract are also showed (LOBE). The arrows indicate bands detected in urine samples at 6 and 12 h of envenomation. Molecular weight (MW) standards were shown on the left of figure B and C.

Figure 3. *L. obliqua* envenomation induces acute tubular necrosis. Representative kidney sections from control (CTRL) or envenomed animals (injected with 1.5 mg/kg, s.c.) are presented **(A-G)**. Note the normal morphology of kidney from CTRL animal **(A)** in comparison to the progressive degenerative lesions of venom-treated rats **(B-G)**. Increased acidophilia, dilation of renal tubules, loss of proximal brush border, cytoplasm vacuolation, nuclear pyknosis and desquamation of necrotic cells can be observed **(D-F)**. Hyaline (arrowheads in C, D and G) and hematic (arrows in D) casts are also present inside renal tubules. Arrowheads in E indicate necrotic cells. Asterisks in D and F indicate the presence of a hyaline material within the Bowman's space and an inflammatory cell infiltrate and edema, respectively. All sections were stained with H&E. Magnification: 10 X. **(H)** Levels of urinary γ-glutamyl transferase (γ-GT) activity were measured in rats injected with PBS or LOBE (1.5 or 1.0 mg/kg) at different times post-administration. Data are presented as means ± SE (n=6/group). Statistical differences of *p < 0.05 vs. CTRL and §p < 0.05 vs. LOBE (1.0 mg/kg, s.c) were considered significant.

Figure 4. *L. obliqua* envenomation induces renal tubular obstruction. Representative kidney sections from animals injected with LOBE (1.5 mg/kg, s.c.) showing details of tubular obstruction by hyaline and hematic casts and cellular debris at 12 h **(A)** and 24 h **(B)** post-venom injection. Hyaline casts (black arrowheads) are

formed by a protein-rich material (predominantly serum albumin and hemoglobin), while hematic casts (black arrows) are formed by fragmented or intact erythrocytes. Due to tubular necrosis, the basement membrane in some tubules is disrupted, resulting in detachment of necrotic cells into the lumen (white arrowheads). All sections were stained with H&E. Magnification: 4 X (panel A) and 20 X (panel B).

Figure 5. *L. obliqua* **envenomation induces renal inflammation and fibrosis.** Light micrographs showing a marked inflammatory cell infiltrate (arrowheads) in the tubulo-interstitial region at 96 h **(B)** and glomerulus at 48 h **(C)** after LOBE injection (1.5 mg/kg, s.c.). There were no signs of inflammation in control (CTRL) animals **(A)**. It was also observed an extensive peritubular **(E)** and interglomerular **(F)** collagen deposition at 96 h (regions stained in red), indicating fibrosis. Arrowheads in these panels indicate inflammatory infiltrate. There were no signs of fibrosis in CTRL rats **(D)**. Stain: H&E (panels A-C) and picrosirius (panels D-F). Magnification: 10 X (panel A, B, D and E), 20 X (panel F) and 40 X (panel C).

Figure 6. Glomerular alterations. A. Light micrographs showing a time dependent increase in the deposition of a PAS positive material in glomerular capillaries of LOBE-injected animals (1.5 mg/kg, s.c.) in comparison to controls. Also note the increase in glomerular size. All sections were stained with PAS. Magnification: 40 X. **B.** Thirty glomeruli from each animal injected with the dose of 1.5 mg/kg were used to quantify the mean area of glomerulus, glomerular tuft and Bowman's space of animals treated with the dose of 1.5 mg/kg. Data are presented as means ± SE (n=6/group). Statistical differences of $*p < 0.05$ in comparison to the respective control (C) were considered significant. **C.** Renal tissue factor activity was measured in control (C) and envenomed (1.5 mg/kg, s.c.) animals by generation of factor Xa (FXa). Data are presented as means ± SE (n=6/group). Statistical differences of $*p < 0.05$ were considered significant in comparison to the respective control.

Figure 7. Renal vascular permeability. Representative micrographs of a kidney blood vessel **(A)** and glomerulus **(B)** from animals injected with LOBE (1.5 mg/kg, s.c.) after 12 h of envenomation. Note the vascular leakage and edema (asterisks) and migration of inflammatory cells to damaged tissue (arrowheads). Also, the presence of a hyaline material inside the Bowman's space (asterisk in B) was associated with the increase in

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glomerular area observed at this time. All sections were stained with H&E. Magnification: 10 X (panel A) and 40 X (panel B). **C.** Evaluation of changes in renal vascular permeability were assessed by Evans blue dye extravasation. Results are expressed as μg Evans blue dye per 100 mg of renal tissue from control (CTRL) and LOBE-treated (1.5 mg/kg, s.c.) rats at 12 and 24 h post-venom administration. Data are presented as means ± SE (n=6/group). Statistical comparisons are indicated.

Figure 8. Immunohistochemical detection of *L. obliqua* **venom in renal tissue.** Positive immunohistochemical reaction was found in cortical and medullar regions of kidneys from rats injected with 1.5 mg/kg of LOBE **(B-E)**. Venom was detected in glomerular capillaries (arrows in B), Bowman's capsule (arrow in the inset B), tubular brush border (arrows in the inset C), in intra-tubular casts (arrowheads in C) and also was present in cells of tubules in degeneration (arrowheads in the insets C and D). After 96 h the immunoreactivity for venom was weak and mainly localized in tubules (arrows in E). There was no immunoreactivity in the renal structures of control (CTRL) rats (A). Magnification: 10 X (panels A-E) and 40 X (insets in B-D) **F**. The amounts of venom detected in renal tissue was estimated by the area of positive immunohistochemical reaction. Thirty sections per rat were analyzed as described in material and methods. Data are presented as means ± SE (n=6/group).

Figure 9. Immunohistochemical detection of *L. obliqua* **venom in renal vascular tissue.** Positive immunohistochemical reaction was detected in renal arteries **(A)** and veins **(B)** of rats injected with LOBE (1.5 mg/kg, s.c.) at 6 h of envenomation. Note the presence of venom in perivascular connective tissue (arrows) and endothelium and smooth muscle cells (arrowheads). Magnification: 10 X (panel B) and 40 X (panel A).

Figure 10. Kidney proteins differentially expressed during *L. obliqua* **envenomation**. Unique or differentially expressed kidney proteins from control and envenomed (1.5 mg/kg, s.c.) animals were identified at 24 h post-venom injection by proteomic analysis. Exclusive and common proteins in each condition, as well as the total number of proteins identified are showed *via* a Venn diagram **(A)**. Top canonical pathways of differentially expressed and unique proteins identified in control (CTRL) **(B)** and LOBE-treated **(C)** kidneys are shown. Those proteins functionally related to renal disease were also categorized accordingly to their roles in different types of renal

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pathologies **(D)**. Each functional annotation is assigned to a significance score represented as *P* value (Fisher exact test) determining the probability that the association between the proteins in the data set and the canonical pathway or function in disease is explained by chance alone. The number of identified proteins (NIP) that belong to a particular canonical pathway or play a role in renal pathology is shown.

Tables

Table 1. Hemodynamic parameters.
MAP: Mean arterial pressure, HR: heart rate. Data are presented as means ± SE (n=6/group). Statistical differences of $*p < 0.05$ vs. CTRL and $^{\S}p < 0.05$ vs. LOBE (1.0 mg/kg, s.c) were considered significant.

Table 2. Unique and differentially expressed proteins identified in the kidneys of rats envenomed by *L. obliqua***.**
Proteins functionally related to renal disease were selected and their specific role in renal pathology was reviewed based on literature data. Complete proteome analysis was included in supplemental table 1.
[a]Acession number in UniProtKB/Swiss-Prot data base.
[b]Log(2) ratio change.
[c]Spec count G-test p-value (proteins were considered differentially expressed with $p < 0.1.$).

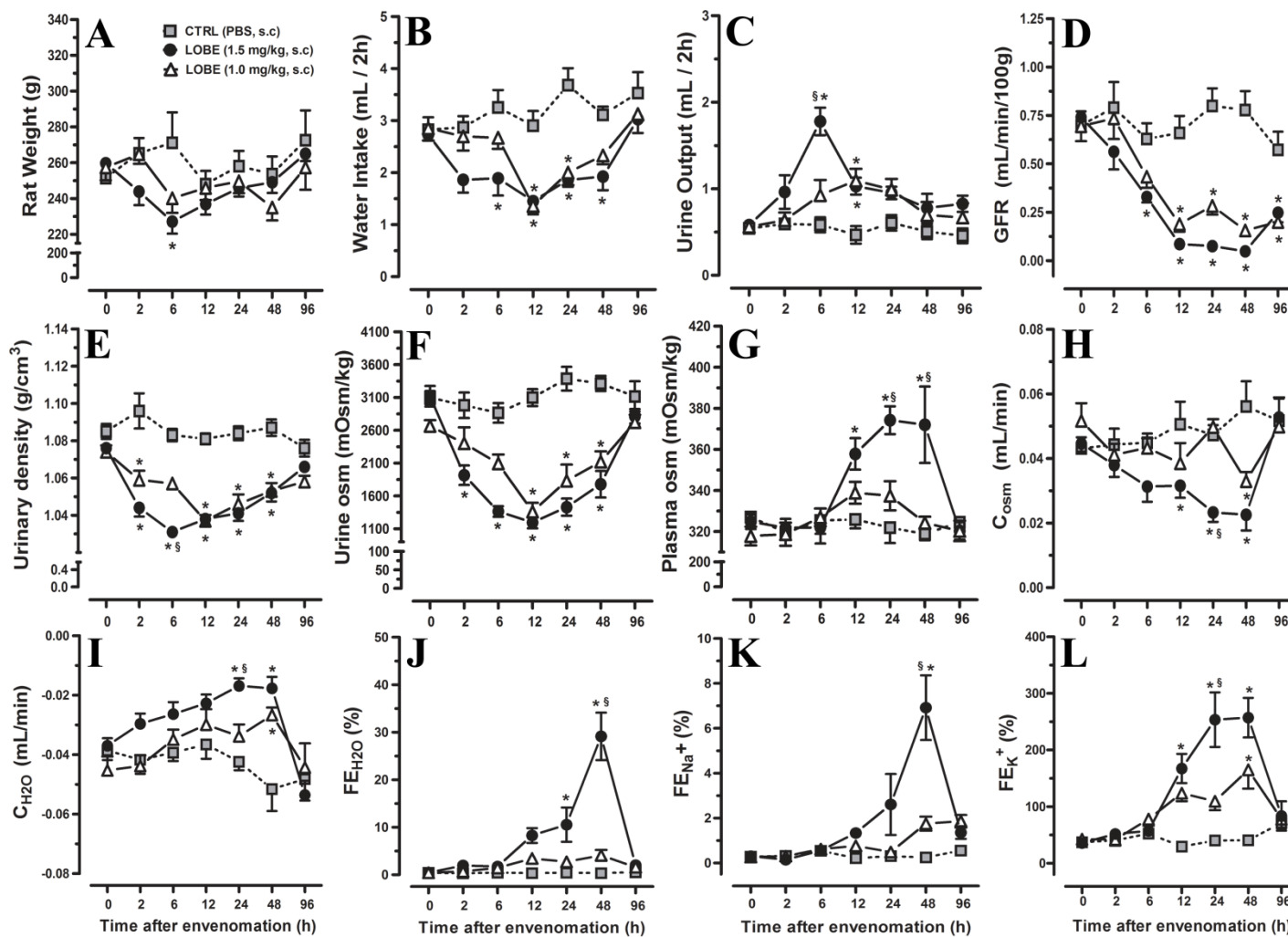**Supplemental Table 1.** Proteins identified in kidney proteome.

Figure 1

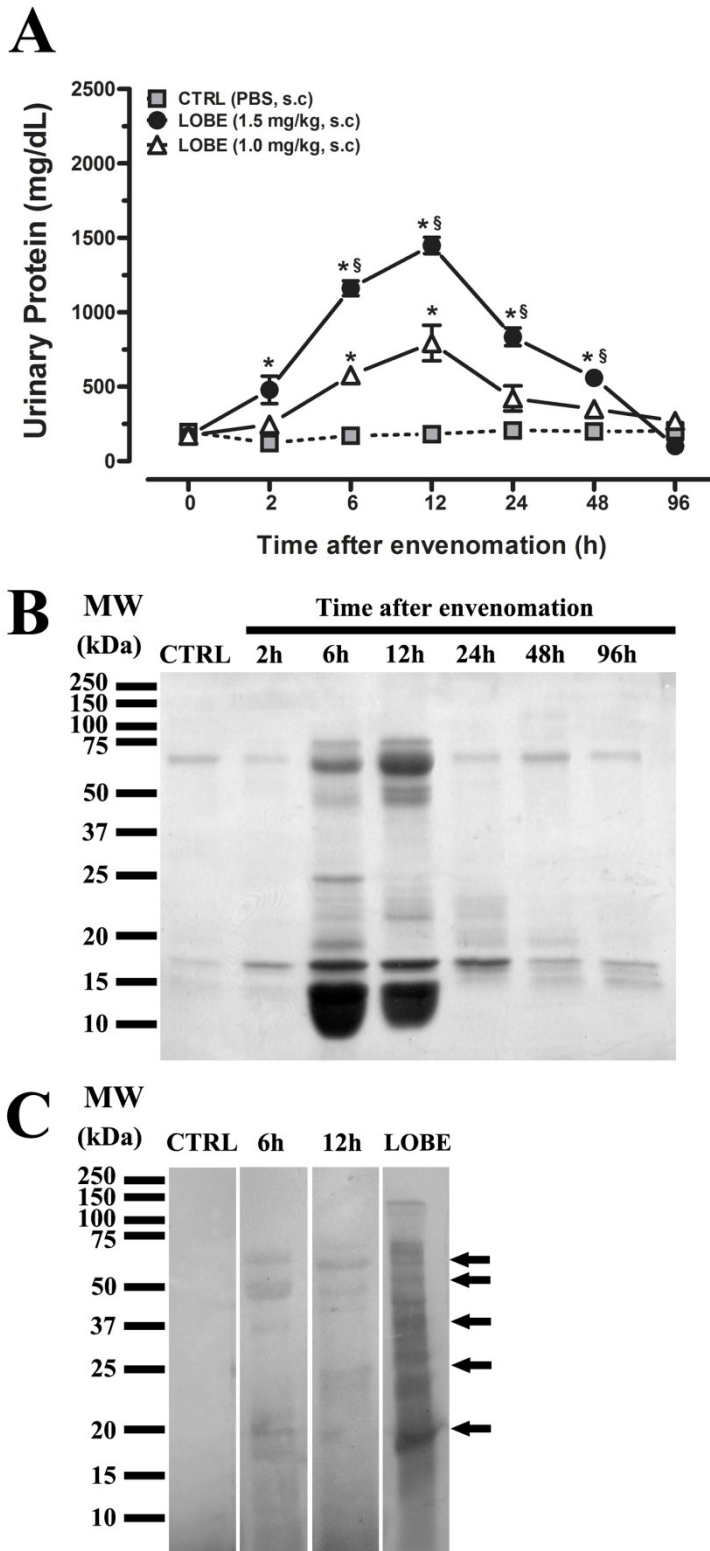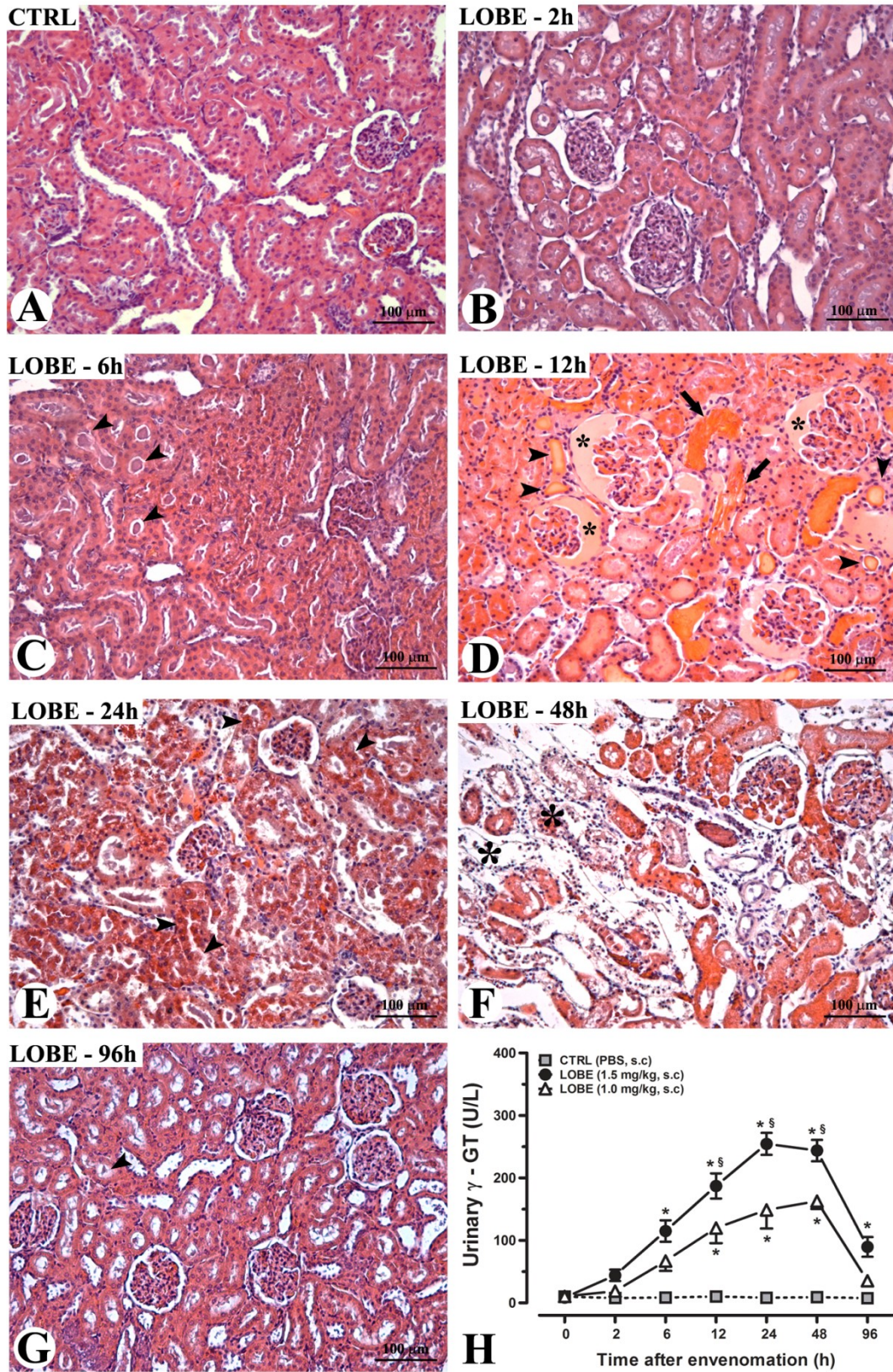**Figure 2**

Figure 3
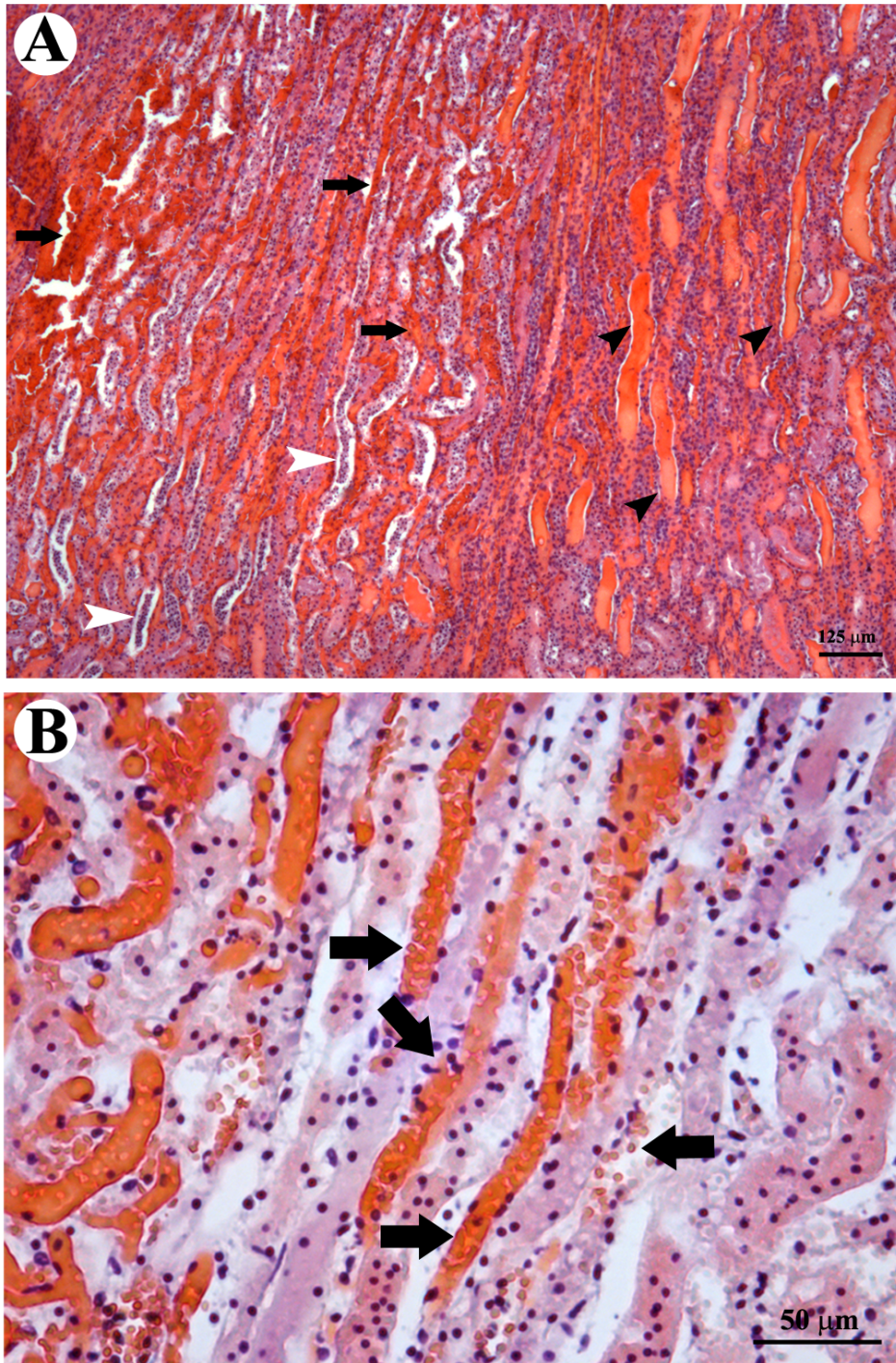
Figure 4

Figure 5
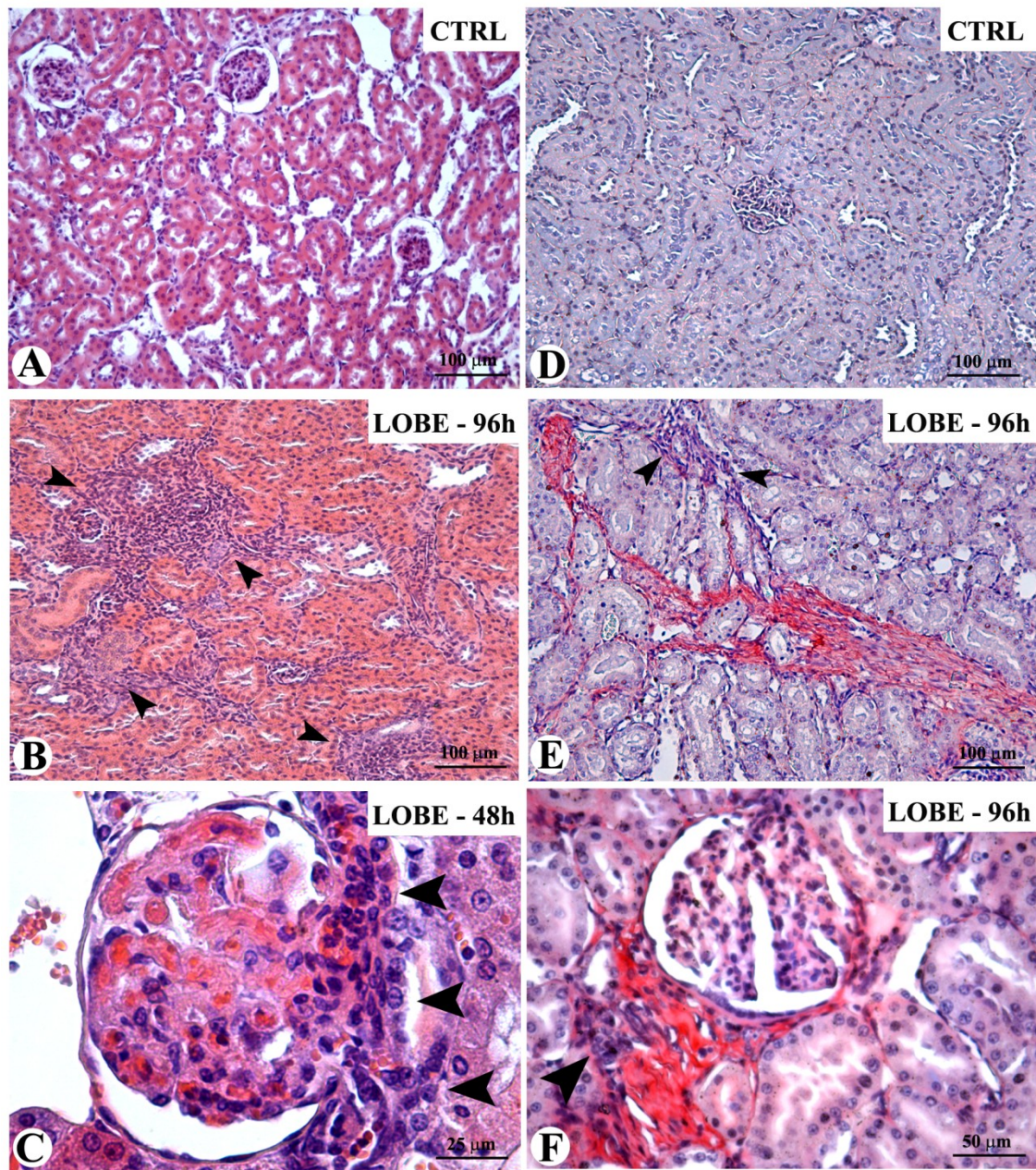
Figure 6
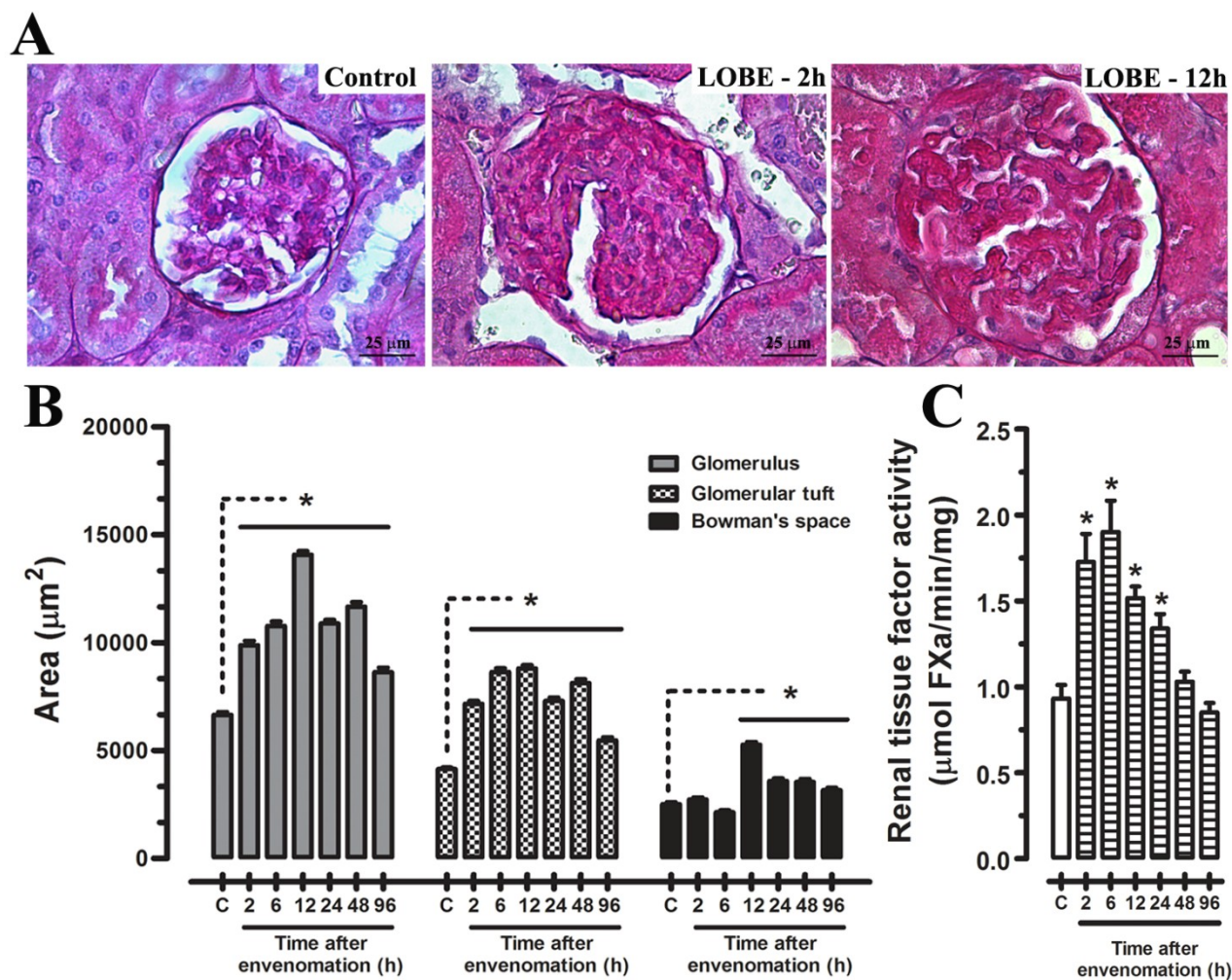
Figure 7
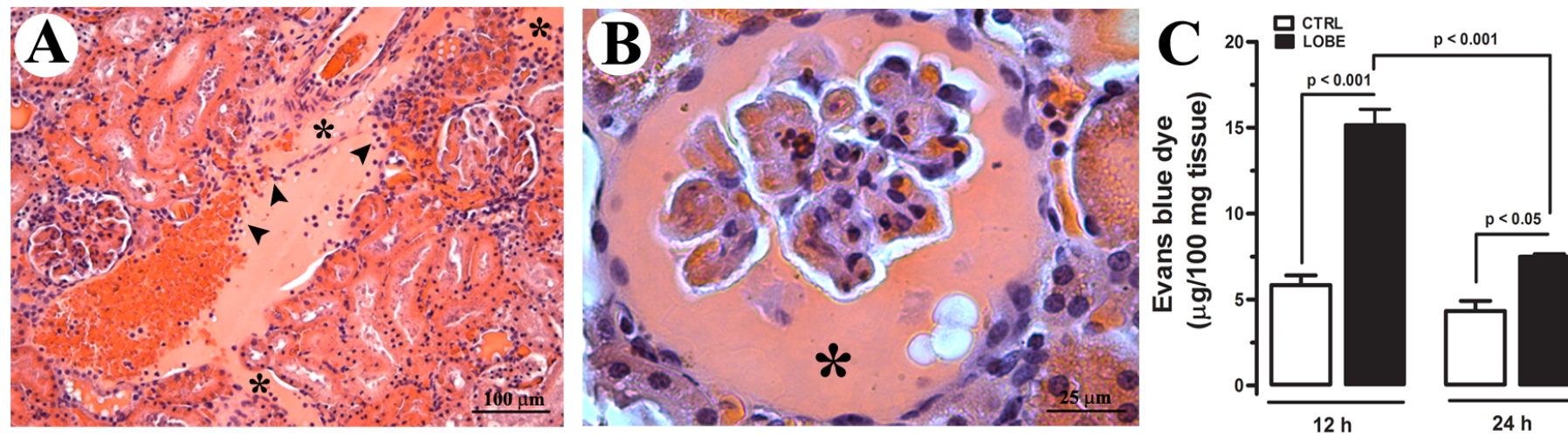
Figure 8

Figure 9

Figure 10

A

CTRL   169   CTRL & LOBE   586   24   LOBE

Total : 779

B **Top Canoninal Pathways - CTRL**

Actin Cytoskeleton Signaling — NIP=7
Glycine Biosynthesis — NIP=2
Folate Transformations — NIP=3
Glycine Betaine Degradation — NIP=4
Aminoacid Metabolism — NIP=19
Small Molecule Biochemistry — NIP=47

-Log(p-value)

C **Top Canoninal Pathways - LOBE**

Complement System — NIP=2
Oxidative Stress — NIP=4
NRF2-mediated Oxidative Stress — NIP=7
Coagulation System — NIP=5
LXR/RXR Activation — NIP=9
Acute Inflammatory Response — NIP=27

-Log(p-value)

D **Proteins Related to Renal Disease**

Renal Fibrosis — NIP=1
Nephrosis — NIP=2
Glomerular Injury — NIP=3
Kidney Failure — NIP=6
Renal Nephritis — NIP=7
Renal Inflammation — NIP=7
Renal Tubule Injury — NIP=9
Renal Damage/Necrosis — NIP=11

-Log(p-value)

Table 1

Parameter	Group	Time after envenomation (h)						
		0	2	6	12	24	48	96
MAP (mmHg)	CTRL	94.6±2.2	107.5±1.5	100.2±3.4	96.3±1.2	115±12.2	111.4±1.1	118±12.6
	LOBE (1.0 mg/kg, s.c.)	103±3.8	92.8±2.6	104.3±2.7	84.5±3.6	88±3.1*	91.6±1.5*	91.7±3.5*
	LOBE (1.5 mg/kg, s.c.)	104±2.3	124.8±8.6§	112.7±4.8	95.1±2.9	74.9±1.5*§	81.5±1.1*	95.1±1.4*
HR (Beats/min)	CTRL	366±5.4	391±1.8	391±5.8	403±2.1	401±4.1	362±11.3	376±8.7
	LOBE (1.0 mg/kg, s.c.)	383±3.7	402±16.1	409±13.1	449±6.9*	398±11.4	381±8.5	443±8.5*
	LOBE (1.5 mg/kg, s.c.)	355±7.2	390±14.3	391±5.4	393±10.2§	427±6.6§	424±5.7*§	369±3.3§

Table 2

Acession number[a]	Protein description	Fold change[b]	p-value[c]	Biological Function and/or Participation in Disease	Reference
Up-regulated					
P24090	Alpha-2-HS-glycoprotein	6.034	0.004	Cysteine-type endopeptidase inhibitor activity/Acute-phase response/ Regulation of inflammatory response/Up-regulation of Ahsg2 is associated with inflammation and tissue edema.	Bowler *et al*., 2004
P02764	Alpha-1-acid glycoprotein (Orm1)	5.093	0.001	Acute-phase inflammatory response to antigenic stimulus/ Up-regulated in response to LPS/ Orm1 is considered an early and accurate biomarker of AKI.	Devarajan *et al*., 2010
P11517	Hemoglobin subunit beta-2 (HbB)	4.395	0.001	Oxygen transport/Up-regulation of Hb is nephrotoxic and associated with kidney injury in several diseases including sepsis, type 2 diabetes and *Crotalus* envenomation.	Zager, 1996; Pinho *et al*., 2005; Larsen *et al*., 2010
Q76MJ6	YBX1	3.37	0.04	DNA-binding/Upregulation of Ybx1 mRNA in kidney is associated with proximal tubular injury in male rat.	Thukral *et al*., 2005
Q6LE95	Low molecular weight kininogen (LMWK)	3.282	0.001	Acute-phase inflammatory response/ Precursor of the active peptide bradykinin (BK)/Related to vasodilation, hypotension and increase of vascular permeability/Participates of the contact-phase reactions of blood coagulation and kallikrein-kinin system/ *L. obliqua* venom is able to release BK from LMWK.	Bohrer *et al*., 2007; Pinto *et al*., 2010
Q921A4	Cytoglobin (Cygb)	3.003	0.015	Heme and iron binding/Up-regulated in conditions of oxidative stress/Plays a role in the development of renal fibrosis/ Overexpression of Cygb improved histological renal injury, preserved renal function, and ameliorated fibrosis.	Mimura *et al*., 2010; Nishi *et al*., 2011
Q9R063	Peroxiredoxin-5, mitochondrial (Prdx-5)	2.796	0.001	Involved in redox regulation of the cell/ Eliminating peroxides generated during metabolism/ Play a major role in the cellular response to oxidative stress/ Up-regulated in conditions of renal ischemia-reperfusion injury and hypoxia.	Godoy *et al*., 2011
P70388	DNA repair protein (Rad50)	2.713	0.061	DNA repair/Plays a central role in double-strand break repair/Up-regulation of Rad50 mRNA is associated with hepatocellular carcinoma.	Stefanska *et al*., 2011;
P02793	Ferritin light chain 1	2.563	0.001	Ferric iron binding/Iron homeostasis and metabolism.	Zager, 1996; Zager *et al*., 2012
P08932	T-kininogen 2 (isoform 2 of LMWK)	2.128	0.001	Acute-phase inflammatory response/ Precursor of the active peptide bradykinin (BK)/Related to vasodilation, hypotension and increase of vascular permeability/Participates of the contact-phase reactions of blood coagulation and kallikrein-kinin system/ *L. obliqua* venom is able to release BK from LMWK.	Bohrer *et al*., 2007; Pinto *et al*., 2010
P02770	Serum Albumin (Alb)	2.096	0.018	The main protein of plasma/ Involved in molecular transport/Regulation of the colloidal osmotic pressure of blood/Up-regulation of serum albumin mRNA and	Thukral *et al*., 2005

				albuminuria is associated with proximal tubular and glomerular injury and interstitial nephritis in male rat.	
P09006	Serine protease inhibitor A3N	1.891	0.001	Acute-phase inflammatory response/ Induced by IL-1, IL-6 and interferon-γ/ Up-regulated during sepsis.	Chinnaiyan *et al*., 2001
P31977	Ezrin	1.706	0.032	Involved in connections of major cytoskeletal structures to the plasma membrane of glomerular epithelium cells (podocytes)/	Thukral *et al*., 2005; Trof *et al*., 2006
P35704	Peroxiredoxin-2 (Prdx-2)	1.691	0.015	Involved in redox regulation of the cell/ Eliminating peroxides generated during metabolism/ Play a major role in the cellular response to oxidative stress/ Up-regulated in conditions of renal ischemia-reperfusion injury and hypoxia.	Godoy *et al*., 2011
Q6P9V2	Ferritin, heavy polypeptide 1	1.543	0.012	Stores iron/ Important for iron homeostasis.	Zager, 1996; Zager *et al*., 2012
P14942	Glutathione S-transferase alpha-4 (GST)	1.516	0.032	Antioxidant enzyme/ Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles/It is a marker of proximal tubular injury.	Thukral *et al*., 2005; Trof *et al*., 2006
P01946	Hemoglobin subunit alpha-1/2 (HbA)	1.495	0.001	Oxygen transport/Up-regulation of Hb is nephrotoxic and associated with kidney injury in several diseases including sepsis, type 2 diabetes and *Crotalus* envenomation.	Larsen *et al*., 2010; Pinho *et al*., 2005; Zager, 1996
B1H216	Hemoglobin alpha, adult chain 2 (HbB)	1.495	0.001	Oxygen transport/Up-regulation of Hb is nephrotoxic and associated with kidney injury in several diseases including sepsis, type 2 diabetes and *Crotalus* envenomation.	Larsen *et al*., 2010; Pinho *et al*., 2005; Zager, 1996
P07335	Creatine kinase B-type (CK-B)	1.452	0.012	Creatine kinase isoenzymes play a central role in energy transduction in tissues. In the kidney localized primarily in the outer medulla in the thick ascending limb and distal convoluted tubule/ Marker of acute tubular injury.	Shashidharamurthy *et al*., 2010
P01026	Complement C3 (Neutrophil chemotactic factor-1)	1.319	0.044	Plays a central role in the activation of the complement system/ Its processing by C3 convertase is the central reaction in both classical and alternative complement pathways/ It is a mediator of local inflammatory process/ It induces the contraction of smooth muscle/ Increases vascular permeability/ Causes histamine release from mast cells and basophilic leukocytes/ Acts as a chemoattractant for neutrophils/ Complement activation increases tubulo-interstitial fibrosis of the kidney.	Turnberg *et al*., 2006
P07483	Fatty Acid Binding Protein 3 (FABP3)	1.286	0.001	FABP3 are thought to play a role in the intracellular transport of long-chain fatty acids and their acyl-CoA esters/ Upregulation of FABP3 mRNA in kidney is associated with proximal tubular injury in male rat.	Thukral *et al*., 2005
Q9JJ19	Na$^+$/H$^+$ exchange regulatory cofactor NHE-RF1	1.196	0.053	Sodium transporter localized in apical membrane of proximal tubular cells/Important in renal sodium transport and phosphate absorption/ It is a marker of tubular injury.	Trof *et al*., 2006

P17475	Serpin 1 Alpha-1-antiproteinase	1.175	0.071	Acute-phase inflammatory response/ Response to hypoxia/ Inhibitor of serine proteases/ Has affinity for plasmin and thrombin/Serpine1 is involved in damage of tubulointerstitium and is up-regulated in cases of disseminated intravascular coagulation (DIC).	Eddy, 2004
P32038	Complement factor D (C3 convertase activator)	1.144	0.017	Plays a central role in the activation of the complement system/ Factor D cleaves factor B when the latter is complexed with factor C3b, activating the C3bbb complex, which then becomes the C3 convertase of the alternate pathway. Its function is homologous to that of C1s in the classical pathway/ Complement activation increases tubulo-interstitial fibrosis in the kidney.	Turnberg *et al.*, 2006
P04276	Vitamin D-binding protein (DBP)	1.088	0.011	It carries the vitamin D sterols and prevents polymerization of actin by binding its monomers. DBP associates with membrane-bound immunoglobulin on the surface of B-lymphocytes and with IgG Fc receptor on the membranes of T-lymphocytes/DBP knockout mice displayed decreased damage in renal cortex.	Safadi *et al.*, 1999
O88767	Protein DJ-1	1.07	0.001	Protects cells against oxidative stress and cell death/ Eliminates hydrogen peroxide/ May act as an atypical peroxiredoxin-like peroxidase that scavenges hydrogen peroxide.	Godoy *et al.*, 2011
P04041	Glutathione peroxidase 1	1.059	0.096	Antioxidant enzyme/ Protects the hemoglobin in erythrocytes from oxidative breakdown	Zager, 1996; Basnakian *et al.*, 2002; Khan, 2009
P62898	Cytochrome c, somatic (CytC)	1.003	0.004	Electron carrier protein/ Plays a role in apoptosis/ Suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome c into the cytosol/ Increases apoptosis in kidney tubule epithelial cells.	Chang *et al.*, 2000
P20059	Hemopexin (Hpx)	0.951	0.065	Participate in heme metabolism/ Binds and neutralizes pro-oxidant free heme/Potentially impacts heme-iron-mediated tubular injury.	Zager *et al.*, 2012
P07632	Superoxide dismutase [cu-zn] (SOD)	0.606	0.001	Antioxidant enzyme/ Destroys radicals which are normally produced within the cells and which are toxic to biological systems	Zager, 1996; Basnakian *et al.*, 2002; Khan, 2009
Down-regulated					
Q63213	Alpha-2u globulin	5.33	0.001	Associated with proximal tubule necrosis and protein droplet accumulation in male rats/ It is down-regulated in chronic hypoxia in the kidney.	Son *et al.*, 2008
P97615	Thioredoxin, mitochondrial	4.301	0.008	Has an anti-apoptotic function/ Plays an important role in the regulation of mitochondrial membrane potential/ Response to hypoxia/ Response to oxidative stress.	Godoy *et al.*, 2011
Q03626	Murinoglobulin-1	3.268	0.045	Peptidase inhibitor activity/ Acute-phase inflammatory response/ It is down-regulated by up to 70% during acute inflammation or tumor development.	Thukral *et al.*, 2005

Q64565	Alanine--glyoxylate aminotransferase 2, mitochondrial	3.112	0.08	Enzyme responsible by the metabolism of dimethylarginine (ADMA)/ ADMA is a potent inhibitor of nitric-oxide (NO) synthase/ This activity provides mechanism through which the kidney regulates blood pressure/ Its inhibition decreases the production of NO.	Caplin *et al*., 2012
D3ZKG1	Methylmalonyl-CoA mutase, mitochondrial (Mut)	2.55	0.05	Involved in the degradation of several amino acids, odd-chain fatty acids and cholesterol via propionyl-CoA to the tricarboxylic acid cycle/ Knockout mouse for Mut gene displayed increased tubulointerstitial nephritis.	Chandler *et al*., 2009
Q62980	Heparin sulfate proteoglycan 2 (HSPG2)	2.391	0.001	Cell adhesion protein/ Increase glomerular and mesangial kidney proliferation/ Positive regulation of endothelial cell proliferation/ Response to hypoxia.	Chen *et al*., 2001
P11232	Thioredoxin	2.191	0.009	Participates in various redox reactions through the reversible oxidation of its active center/ Cell redox homeostasis.	Godoy *et al*., 2011
Q920P6	Adenosine deaminase	2.139	0.019	Catalyzes the hydrolytic deamination of adenosine/ Its inhibition leads to accumulation of adenosine, which has a potent afferent arteriolar vasodilator effect through A_{2B} receptors located in glomerular vessels/ Adenosine also has a platelet aggregation inhibitory activity.	Feng and Navar, 2010/Tofovic *et al*., 1998/
Q64057	Alpha-aminoadipic semialdehyde dehydrogenase (ALDH)	2.083	0.05	Multifunctional enzyme mediating important protective effects/ Protects against hyperosmotic stress/ Protects cells from oxidative stress by metabolizing a number of lipid peroxidation-derived aldehydes.	Brocker *et al*., 2010
P98158	Low-density lipoprotein receptor-related protein 2 (Lrp2)	1.786	0.03	Acts together with cubilin to mediate HDL endocytosis/Downregulation of Lrp2 protein in apical pole from kidney proximal tubule is associated with nephrosis in male rat.	Russo *et al*., 2007
Q5XI73	Rho GDP dissociation inhibitor alpha (ARHGDIA)	0.522	0.005	Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them/Knockout mouse for ARHGDIA displayed increased tubulointerstitial nephritis and degeneration of renal tubular epithelial cells.	Togawa *et al*., 1999
Unique proteins					
P30152	Neutrophil gelatinase-associated lipocalin (NGAL)	-	-	Iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development/ Involved in apoptosis due to interleukin-3 (IL3) deprivation/ It is a marker and predictor of renal injury/ Up-regulation of NGAL is associated with ischemic acute kidney injury.	Trof *et al*., 2006
P06762	Heme oxygenase 1 (HO-1)	-	-	Heme oxygenase cleaves the heme ring to form biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase.	Camara and Soares, 2005; Zager, 1996
P01048	Rat T-kininogen 1	-	-	Acute-phase inflammatory response/ Precursor of the active peptide bradykinin	Bohrer *et al*., 2007;

	(Isoform 1 of LMWK)			(BK)/Related to vasodilation, hypotension and increase of vascular permeability/Participates of the contact-phase reactions of blood coagulation and kallikrein-kinin system/ *L. obliqua* venom is able to release BK from LMWK.	Pinto *et al.*, 2010
P14480-1	Isoform 1 of Fibrinogen beta chain (FbB)	-	-	Precursor of fibrin clots/ Polymerize into fibrin and acting as a cofactor in platelet aggregation/ Conversion of fibrinogen to fibrin is triggered by thrombin/ Up-regulation of Fb mRNA in kidney is associated with proximal tubular injury and intra-glomerular fibrin deposition.	Thukral *et al.*, 2005
P20961	Plasminogen activator inhibitor 1 (PAI-1)	-	-	Serine protease inhibitor. This inhibitor acts as 'bait' for tissue plasminogen activator, urokinase, protein C and matriptase-3/It's the major control point in the regulation of fibrinolysis/Up-regulation of PAI-1 is associated with renal thrombosis and fibrosis in cases of chronic allograft nephropathy.	Revelo *et al.*, 2005
P50115	Protein S100-A8	-	-	Calcium- and zinc-binding protein/ Plays a prominent role in the regulation of inflammatory processes and immune response/ Its proinflammatory activity includes recruitment of leukocytes, promotion of cytokine and chemokine production, and regulation of leukocyte adhesion and migration. Can induce cell death via autophagy and apoptosis and this occurs through the cross-talk of mitochondria and lysosomes via reactive oxygen species (ROS).	Basnakian *et al.*, 2002
P02680-1	Gamma-B of Fibrinogen gamma chain (FbG)	-	-	Precursor of fibrin clots/ Polymerize into fibrin and acting as a cofactor in platelet aggregation/ Conversion of fibrinogen to fibrin is triggered by thrombin/ Up-regulation of Fb mRNA in kidney is associated with proximal tubular injury and intra-glomerular fibrin deposition.	Thukral *et al.*, 2005
Q64268	Heparin cofactor 2 (HC-2)	-	-	Thrombin inhibitor/ It is activated by the glycosaminoglycans, heparin or dermatan sulfate. In the presence of the latter, HC-II becomes the predominant thrombin inhibitor in place of antithrombin III (AT)/ HC-II -levels increase in thrombosis and intravascular disseminated coagulation (DIC).	Takatsuka *et al.*, 2006
P84817	Mitochondrial fission 1 protein (MFP-1)	-	-	Promotes the fragmentation of the mitochondrial network and its perinuclear clustering/ Can induce cytochrome c release from the mitochondrion to the cytosol, ultimately leading to apoptosis. Also mediates peroxisomal fission.	Basnakian *et al.*, 2002

Supplemental Table 1.

OBS: A tabela suplementar deste artigo é apenas complementar à Tabela 2 e está incluída em arquivo (.xls) enviado separadamente via e-mail devido ao grande volume de dados contidos.

3.5 Capítulo V

Pharmacological blockade of the kinin B$_1$ receptor or kallikrein inhibition reduces the renal damage in a model of acute kidney injury induced by *Lonomia obliqua* **venom**

Neste capítulo final, uma abordagem farmacológica foi utilizada para explorar o papel do sistema calicreína-cininas (SCC) na insuficiência renal aguda induzida pelo veneno da taturana em ratos.

Os principais resultados obtidos foram:

- O SCC está ativado no rim durante o envenenamento. Há um aumento na expressão gênica dos receptores B1 e B2 (B1R e B2R) de bradicinina, diminuição na expressão da enzima conversora de angiotensina (ECA), aumento da atividade da calicreína renal e plasmática e aumento na produção de óxido nítrico no rim;
- A ativação de calicreína e o aumento na expressão de B1R indicam que o SCC tem papel fundamental para o desenvolvimento das lesões renais. O tratamento prévio com antagonistas de B1R ou a inibição de calicreína previne as principais alterações de função renal, incluindo a queda acentuada do ritmo de filtração glomerular, aumento de creatinina plasmática e proteinúria. O pré-tratamento com antagonistas de B2R não apresenta qualquer efeito;
- O antagonismo de B1R ou inibição de calicreína protege contra as alterações tubulares degenerativas, inflamação e obstrução tubular. O antagonismo de B2R não apresenta efeito;
- O antagonismo de B1R ou inibição de calicreína previne o desenvolvimento da coagulopatia de consumo, já o antagonismo de B2R não apresenta o mesmo efeito.

O manuscrito a seguir será submetido para publicação em periódico científico internacional.

Pharmacological blockade of the kinin B_1 receptor or kallikrein inhibition reduces renal damage in a model of *Lonomia obliqua* venom-induced acute kidney injury

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Abstract

Lonomia obliqua venom is highly nephrotoxic, and acute kidney injury (AKI) is the main cause of death among envenomed victims. We have previously demonstrated that the pathophysiological mechanism underlying *L. obliqua*-induced AKI seems to be multifactorial and involves renal hypoperfusion, inflammation, tubular necrosis and the sudden loss of glomerular filtration and tubular reabsorption capacities. In the present study, we aimed to investigate the contribution of kallikrein-kinin system (KKS) components to the hemodynamic instability, inflammation and consequent renal functional impairment. To this end, rats were treated with either the bradykinin receptor B_1 (B_1R) antagonists DALBK and R-715, the B_2 receptor (B_2R) antagonist HOE-140, or a kallikrein inhibitor (aprotinin) 30 min prior to the subcutaneous injection of *L. obliqua* venom (1.5 mg/kg). After 24 h of envenomation, plasma, urine and kidney samples were collected to analyze renal function, morphological alterations and the expression of KKS components. The activity of renal tissue kallikrein and the expression of the B_1R and B_2R increased during envenomation, indicating that the KKS was indeed activated. Both B_1R antagonism and kallikrein inhibition prevented glomerular injury and reductions in the rate of glomerular filtration, and restored fluid homeostasis and tubular water retention ability. The main mechanism underlying these beneficial effects was associated with decreased renal inflammation and reduced levels of renal pro-inflammatory cytokines and tubular degeneration. In addition, B_1R antagonists and aprotinin also ameliorated venom-induced blood incoagulability and hemorrhagic syndrome, an outcome that is commonly observed during *L. obliqua* envenomation. In contrast, rats pretreated with the B_2R antagonist, HOE-140, did not display such beneficial effects. Thus, these data indicate that blocking kallikrein or B_1R activity may be therapeutic alternatives that could be used to control the progression of *L. obliqua* venom-induced AKI.

1. Introduction

Envenomation resulting from contact with *Lonomia obliqua* caterpillars has been recognized as a neglected public health issue that mainly occurs in impoverished communities in the rural areas of the southern regions of Brazil. Envenomed victims display systemic hemorrhage secondary to intravascular disseminated coagulation (For review see Veiga *et al*., 2009; Pinto *et al*., 2010). Frequently, the clinical profile evolves to acute kidney injury (AKI), which is the main cause of death following this type of envenomation (Gamborgi *et al*., 2006; Berger *et al*., 2012). In experimental models, *L. obliqua* venom induces the sudden loss of basic renal functions, including filtration and excretion capacities, urinary concentration and the maintenance of body fluid homeostasis. In addition to the direct cytotoxic effects of venom toxins, renal hypoperfusion also appears to be an important underlying mechanism, because signs of glomerular fibrin deposition and hemodynamic instability (systemic hypotension and increased renal vascular permeability) have been detected in rats injected with *L. obliqua* venom (Berger *et al*., unpublished data). In fact, the coagulation, complement and kallikrein-kinin systems are known to be activated during envenomation (Zannin *et al*., 2003; Bohrer *et al*., 2007).

The kallikrein-kinin system (KKS) is composed of potent vasoactive and proinflammatory molecules that are involved in the control of blood pressure, vascular permeability, smooth muscle contraction or relaxation, and pain. Kinins are generated by the proteolytic cleavage of kininogens by tissue or plasma kallikreins. Once released, kinins exert the majority of their biological effects by activating two types of kinin receptors: the bradykinin B_1 receptor (B_1R) and the bradykinin B_2 receptor (B_2R). The B_2R is constitutively expressed in most tissues, and displays a higher affinity for bradykinin (BK) and Lys-BK peptides. In contrast, the B_1R displays high affinities for the kinin metabolites des-Arg^9-BK and Lys-des-Arg^9-BK. The B_1R is not expressed under normal conditions, but is induced following inflammatory, infectious or traumatic stimuli (Regoli and Barabé, 1980, Calixto *et al*., 2004; Campos *et al*., 2006; Kayashima *et al*., 2012). Both the B_1R and B_2R are G protein-coupled, seven transmembrane domain receptors. Stimulation of the latter elevates intracellular Ca^{+2} concentrations and phospholipase A_2 activity, leading to the release of various mediators, including nitric oxide (NO), prostaglandins, arachidonic acids and other inflammatory agents (Kakoki and Smithies, 2009). Notably, the B_1R and B_2R are involved in several inflammation-

related processes, such as atherosclerosis, airway inflammation, diabetic neuropathy, inflammatory bowel diseases, neuropathic pain, and cerebral infarction (Couture *et al*., 2001; Souza *et al*., 2004; Hara *et al*., 2008; Quintão *et al*., 2008; Austinat *et al*., 2009; Duchene and Ahluwalia, 2009; Kakoki *et al*., 2010). In renal diseases specifically, B_1R deletion or antagonism has been associated with renoprotection against ischemia-reperfusion-induced injury, glomerulonephritis and renal inflammation and fibrosis (Wang *et al*., 2008; Klein *et al*., 2009; Klein *et al*., 2010). In contrast, mice lacking the B_2R displayed enhanced diabetic nephropathy (Kakoki *et al*., 2004).

The KKS is involved in the edematogenic and hypotensive responses elicited by *L. obliqua* venom. *L. obliqua* venom induces the release of kinins from low-molecular weight kininogen (LMWK), and both edematogenic and hypotensive responses are reduced following kallikrein inhibition and B_2R antagonism (Bohrer *et al*., 2007). Moreover, envenomed patients presented low levels of plasma prekallikrein, indicating that kallikrein had been activated and released into the blood circulation (Zannin *et al*., 2003). Taken together, these data led us to hypothesize that the generation of kallikrein and BK during envenomation may be involved in *L. obliqua*-induced hemodynamic instability, inflammation and the consequent renal functional impairment.

In the present study, we used different pharmacological treatments to study the roles of kallikrein and the kinin receptors, B_1R and B_2R, in an experimental model of *L. obliqua*-induced AKI. The results indicated that both kallikrein inhibition and B_1R antagonism were able to prevent venom-induced blood incoagulability and restore renal function by reducing tubular necrosis and tissue inflammation. In summary, these data indicate that blocking kallikrein activity or the B_1R may be therapeutic alternatives that could be used to control the progression of the renal pathology that is observed following *L. obliqua* envenomation.

2. Materials and Methods

2.1 Drugs and reagents

[Leu^8]-des-Arg^9-BK (DALBK), R-715 (AcLys[D-βNal^7,Ile^8]des-Arg^9-BK), HOE-140 (D-Arg^0-[Hyp^3,Thi^5, D-Tic^7, Oic^8]-BK, Icatibant), vanadium (III) chloride (VCl$_3$), sulfanilamide (SULF), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) and the purified coagulation factors (VII, IX and X) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Aprotinin (Trasylol$^®$) was obtained from Bayer do

Brasil S.A. (São Paulo, Brazil). The chromogenic substrates for factor Xa (S2222, Bz-Ile-Glu-Gly-Arg-p-nitroanilide), plasma kallikrein (S2302, H-D-Pro-Phe-Arg-p-nitroanilide) and tissue kallikrein (S2366, H-D-Val-Leu-Arg-p-nitroanilide) were obtained from Chromogenix (Milan, Italy). Ketamine and xylazine were obtained from Syntec (São Paulo, Brazil).

2.2 Venom

L. obliqua caterpillars were kindly provided by the Centro de Informações Toxicológicas (CIT), Porto Alegre, Rio Grande do Sul, Brazil. The specimens used in the present study were collected in the cities of Bom Princípio and Progresso, both of which are located at Rio Grande do Sul, Brazil. *L. obliqua* venom was obtained by cutting bristles at the base of each scoli and macerating them in cold phosphate-buffered saline (PBS), pH = 7.4, as previously described (Veiga *et al*., 2005; Pinto *et al*., 2006; Berger *et al*., 2010). The venom obtained following this procedure was designated as *Lonomia obliqua* **B**ristle **E**xtract (LOBE). The protein concentrations of the LOBE samples were determined using a BCA assay kit (Pierce, Rockford, USA), and the aliquots were stored at -80 °C prior to use.

2.3 Animals

Adult male Wistar rats, weighing 250-300 g, were supplied by the central animal facility of our institution. The animals were housed under standard conditions within a temperature controlled room (22-23 ºC, on a 12 h light/dark cycle, with the lights on at 7:00 am), and had free access to water and food. All of the procedures involving animals were carried out in accordance with the Guiding Principles for the Use of Animals in Toxicology (International Society of Toxicology, http://www.toxicology.org) and the Brazilian College of Animal Experimentation (COBEA). The experimental protocol was approved by the ethical committee on research animal care of the Federal University of Rio Grande do Sul, Brazil (register number **2008177/2009**) and by the Institute's Animal Ethics Committee of the Federal University of Minas Gerais, Brazil (protocol **177/2008**).

2.4 Experimental design

2.4.1 Venom induced-acute kidney injury (AKI)

The time course of alterations in KKS components during *L. obliqua* induced-AKI was examined using an *in vivo* experimental model of envenomation. The animals were divided into one of two groups: A control group (CTRL), which contained animals (*n* = 6 per sampling time) that were injected subcutaneously (sc) with 100 µL of a sterile PBS solution, and an experimental group (LOBE), which contained animals (*n* = 6 per sampling time) that were injected sc with a solution containing 1.5 mg of the LOBE per kg of body weight in a final volume of 100 µL. The animals were then distributed individually in metabolic cages, which allowed for quantitative urine collections and measurements of water intake. At several time points post-venom injection (12, 24, 48 and 96 h), blood, urine and kidney samples were obtained for biochemical, histopathological, and renal tissue factor activity and gene expression analyses. The venom dose and times post-envenomation were selected based on the results of previous experiments that were conducted using rats as an animal model (Berger *et al.*, unpublished data) to reproduce the kidney damage and consumption coagulopathy that has been observed in humans (Dias da Silva *et al.*, 1996; Rocha-Campos *et al.*, 2001; Gamborgi *et al.*, 2006; Berger *et al.*, 2010).

2.4.2 Pharmacological treatments

Different pharmacological treatments were tested to determine the roles of the BK receptors and kallikrein. The animals were divided into six groups (*n* = 6/group): (*i*) Control group (CTRL), in which the animals were treated intraperitoneally (ip) with 250 µL of a sterile PBS solution 30 min prior to the sc injection of 100 µL of a sterile PBS solution; (*ii*) LOBE + PBS, in which the animals were treated with 250 µL of PBS (ip) 30 min prior to the injection of the LOBE (1.5 mg/kg, sc); (*iii*) LOBE + HOE-140, in which the animals were treated with the B_2R antagonist, HOE-140 (250 µg/kg, ip, diluted in 250 µL of PBS), 30 min prior to the injection of the LOBE (1.5 mg/kg, sc); (*iv*) LOBE + DALBK, in which the animals were treated with the B_1R antagonist, DALBK (1.0 mg/kg, ip, diluted in 250 µL of PBS), 30 min prior to the injection of the LOBE (1.5 mg/kg, sc); (*v*) LOBE + R-715, in which the animals were treated with the B_1R antagonist, R-715 (1.0 mg/kg, ip, diluted in 250 µL of PBS), 30 min prior to the injection of the LOBE (1.5 mg/kg, sc); and (*vi*) LOBE + APROTININ, in which the

animals were treated with the kallikrein inhibitor, aprotinin (5.6 mg/kg, iv, corresponding to 40,000 kallikrein inhibitory units/kg, diluted in sterile saline), 30 min prior to the injection of the LOBE (1.5 mg/kg, sc). Additional groups were treated only with the drugs (using the same doses) to exclude their own direct effects on renal function parameters. Immediately after the treatments, the animals were distributed individually in metabolic cages. At 24 h post-venom injection, blood, urine and kidney samples were obtained for biochemical, histopathological, renal tissue factor activity and gene expression analyses. All of the drug doses were selected based on previously published data (Day *et al.*, 2006; Bohrer *et al.*, 2007; Austinat *et al.*, 2009; Raslan *et al.*, 2010; Viana *et al.*, 2010)

2.5 Blood, urine and kidney samples

Blood samples were obtained by cardiac puncture in rats that had been anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) and anticoagulated with 1:10 (v/v) 3.8 % trisodium citrate. Plasma was obtained by centrifugation at 1500 x g for 10 min and stored at − 80 ºC prior to the biochemical determinations. Urine samples were collected directly from the metabolic cages and centrifuged at 2500 x g for 5 min. The supernatants were stored under the same conditions used for the plasma samples. After blood collection, the hearts were perfused through the left ventricle with a PBS solution, and a circulatory circuit was opened by an incision in the right atrium to ensure the elimination of intravascular blood. Immediately after perfusion, the kidneys were quickly removed. One of the kidneys was fixed for histological analysis, and the other was frozen in liquid nitrogen and stored at − 80 ºC prior to the measurements of tissue factor and kallikrein activities, nitrate/nitrite levels and the gene expression profile. For the measurements of tissue factor and kallikrein activities and nitrate/nitrite levels, a portion of the kidney was homogenized in a cold PBS solution containing 1 % Triton X-100 and centrifuged at 9500 x g for 15 min. The supernatants were immediately used in the assays.

2.6 Analyses of renal function

The levels of creatinine, urinary γ-glutamyl transferase (γ-GT) activity and urinary proteins were used for renal function assessments. Urine and plasma creatinine, urinary γ-GT activity and proteinuria were determined spectrophotometrically (SP-220 BioSpectro Spectrophotometer, Paraná, Brazil) using commercially available kits

(BioClin/Quibasa, Belo Horizonte, Brazil). The glomerular filtration rate (GFR), expressed as mL/min/100 g of body weight, was estimated by the creatinine clearance (C_{cr}) using the standard formula: $C_{cr} = U_{cr}.V/P_{cr}$, where U_{cr} is the urinary creatinine concentration, V is the urinary output and P_{cr} is the plasma creatinine concentration. Water fractional excretion (FE_{H2O}), expressed as a %, was determined using the equation: $FE_{H2O} = V/GFR.100$. Hydric balance (HB), expressed as a %, represents the relationship between water consumption (WC) and urinary output (V). It was calculated as: $HB = V/WC.100$.

2.7 Gene expression analyses

Renal mRNA levels of angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), angiotensin II receptor (AT_1R), angiotensin (1-7) receptor (MAS), the bradykinin B1 receptor (B_1R), the bradykinin B2 receptor (B_2R) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were estimated using quantitative real-time PCR (qRT-PCR). First, total RNA was extracted from the kidneys using the TRIzol® (Invitrogen, Carlsbad, CA, USA) reagent, according to the manufacturer's recommended protocol. Reverse transcription was performed using 1 μg of total pure RNA, 200 units of reverse transcriptase, 100 mM DTT (dithiothreitol) (1.0 μL), 5× reverse transcription buffer (2.5 μL), 10 mM dNTPs (1.8 μL), 10,000 units of RNAsin (0.2 μL) and 50 ng/ml of oligo(dT) (1.0 μL). The temperature profile for this reaction was: 25 °C for 5 min, 37 °C for 60 min, 70 °C for 5 min and 4 °C for 5 min. The resultant cDNA was used for real-time PCR, as described below. Specific primers were designed using Primer Express software and were synthesized by Applied Biosystems (California, USA). The primers were as follows (forward and reverse, respectively): B_1R, 5'-AACATCGGGAACCGTTTCAAC-3' and 5'-CACCCGGCAGAGGTCAGTT-3'; B_2R, 5'-TTCCGAAAGAAGTCCCGAGA-3' and 5'-GCCTCCCTTCCGGCATATT-3'; AT_1R, 5'-TCTCAGCATCGATCGCTACCT-3' and 5'-AGGCGAGACTTCATTGGGTG-3'; MAS, 5'-TGACCATTGAACAGATTGCCA-3' and 5'-TGTAGTTTGTGACGGCTGGTG-3'; ACE, 5'-CTTCACTGACCAAAAGCTGCG-3' and 5'-CCTAGGGTCTGTACGGATCCG-3'; ACE2, 5'-GTGGAGGTGGATGGTCTTTCA-3' and 5'-TTGGTCCACTGTTCTCTGGGA-3'; and GAPDH, 5'-ATGTTCCAGTATGACTCCACTCACG-3' and 5'-GAAGACACCAGTAGACTCCACGACA-3'. The AT_1 receptor primer set detected

both the AT_1a and AT_1b receptor subtypes. Real-time PCR was carried out using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, California, USA) with SYBR Green PCR Master Mix (Applied Biosystems, California, USA). The relative levels of gene expression were determined using the comparative threshold cycle method, as outlined in the manufacturer's instructions, in which the data for each sample were normalized to GAPDH expression.

2.8 Renal tissue factor activities

Renal tissue factor (TF) was measured indirectly based on its ability to form a complex with factor VIIa (TF/FVIIa) and activate factors IX and X (Morrissey, 1995). Briefly, the kidneys were collected as described above, homogenized in a cold PBS solution containing 1 % Triton X-100 and centrifuged at 9500 x g for 15 min. Supernatant samples (10 μg of protein) were incubated with a concentrated mixture of the FVII+FIX+FX complex (15 μg total) in 20 mM Tris-HCl, pH 7.4, containing 10 mM $CaCl_2$ for 10 min at 37 °C. Activated factor Xa (FXa) that was produced during the reaction was detected by the addition of a specific chromogenic substrate (0.2 mM S2222). The kinetics of p-nitroaniline release were monitored at 405 nm for 30 min in a final volume of 100 μL using a microplate reader spectrophotometer (SpectraMAX, Molecular Devices Co., Sunnyvale, USA). Each sample was measured in triplicate, and the results were expressed as μmol of FXa generated per min per mg of kidney tissue.

2.9 Plasma prekallikrein and renal kallikrein activities

To measure plasma prekallikrein, plasma samples (5 μL) were previously incubated with 20 mM Tris-HCl, pH 7.4, containing 100 μM elagic acid, phospholipids, 0.005 % bovine albumin and 10 mM $CaCl_2$ for 10 min at 37 °C. Activated kallikrein that was produced during the reaction was detected by the addition of 0.2 mM S2302, a specific chromogenic substrate for plasma kallikrein. To measure renal kallikrein, kidney extracts were prepared as described above, diluted (1:2) and incubated (15 μL) in 20 mM Tris-HCl, pH 7.4, for 10 min at 37°C. Kallikrein activity was detected by the addition of 0.2 mM S2366, a specific chromogenic substrate for tissue kallikrein. In both cases, the kinetics of p-nitroaniline release were monitored at 405 nm for 30 min in a final volume of 100 μL using a microplate reader spectrophotometer (SpectraMAX,

Molecular Devices Co., Sunnyvale, USA). Each sample was measured in triplicate, and the results were expressed as μmol of *p*-nitroaniline released per min per mg of protein.

2.10 Nitric oxide determinations

The total plasmatic and kidney levels of nitrate and nitrite were determined as an indication of nitric oxide (NO) production, as described previously (Miranda *et al.*, 2001). Briefly, plasma or kidney samples were deproteinized by the addition of 1:1 (v/v) ethanol and incubated overnight at 4 °C. After centrifugation at 1500 X g for 10 min, the supernatants (100 μL) were mixed with 8 mg/mL of VCl_3 (100 μL) in 96 well microplates. The Griess reagent (100 μL of 2 % SULF + 0.1 % NEED) was immediately added to the wells, and the absorbance was then read at 540 nm after 30 min of incubation at room temperature. The sample values were compared with the absorbance of a standard curve of sodium nitrate (1 – 200 μM).

2.11 Histology

For the renal histopathology analyses, the kidneys were collected, as described previously, sectioned sagitally, and fixed in 10 % buffered formaldehyde, pH 7.2. After processing in alcohol and xylol, the organs were embedded in paraffin, and 4 μm thick sections were obtained and stained with hematoxylin and eosin (H&E). Thirty high-power fields (40 X) of the renal cortex were randomly selected for each section, and the presence of the following alterations was observed: Intratubular casts, tubular degeneration and cellular inflammatory infiltrate. A semi-quantitative score (ranging from 0 to 3) was attributed to each of these alterations as follows: a score of zero was attributed to normal regions, a score of 1 was attributed to changes affecting less than 30 % of the region under examination, a score of 2 was attributed to changes affecting 30 to 60 % of the region, and a score of 3 was attributed to changes affecting greater than 60 % of the region. All scoring was performed in a blinded manner.

2.12 Assessments of renal vascular permeability and pro-inflammatory cytokine levels

The index of vascular permeability was assessed by the extravasation of Evans blue dye into the kidneys, as described previously (Pompermayer *et al.*, 2005). Rats were pretreated with either the B1R antagonist (R-715) or the kallikrein inhibitor (aprotinin) according to the previously outlined protocol. The rats then received Evans

blue dye (30 mg/kg) intravenously (1 mL/kg) via the caudal vein 10 min prior to LOBE (1.5 mg/kg, sc) or PBS (100 µL, sc) injection. After 24 h, the animals were anesthetized and perfused through the left ventricle with a PBS solution to remove the intravascular Evans Blue. Then, the kidneys were quickly removed, weighed and allowed to dry for 24 h at 40 °C. The dry weights were determined and Evans blue dye was extracted in 2.5 mL of 1 % formamide (48 h at 40 °C). The absorbance of the extracted solution was measured in triplicate using a microplate reader spectrophotometer (SpectraMAX, Molecular Devices Co., Sunnyvale, USA), and the amounts of Evans blue dye were calculated based on a standard curve that was generated with known concentrations of Evans Blue. The results are presented as the amount of Evans blue dye extravasated (µg) per 100 mg of kidney tissue. The levels of pro-inflammatory cytokines (interleukin [IL] 1-β and tumor necrosis factor [TNF]-α) were determined in the kidney supernatants (homogenized as described in subsection 2.5) using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's recommendations.

2.13 Statistical analyses

The data are presented as the means ± SE, and significant differences were analyzed using one-way ANOVA followed by an unpaired t-test with a Bonferroni correction for multiple comparisons. P-values of 0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 B_1R and B_2R expression and kallikrein activity are increased in the kidneys during L. obliqua-induced AKI

The results of a previous study indicated that the subcutaneous injection of *L. obliqua* venom (1.5 mg/kg) in rats causes a sudden loss of glomerular filtration capacity, a decrease in tubular hydro-electrolytic transport and tubular necrosis. These alterations peaked between 12 and 48 h. In the present study, we observed that alterations in renal function are also associated with the up-regulation of the expression of the BK receptors, B_1R and B_2R **(Figs. 1A and B)**. B_1R expression was up-regulated in the kidneys throughout the period of envenomation (12-96 h), with a maximal

increase of 2.1-fold observed 12 and 48 h post-venom injection **(Fig. 1A)**. B_2R expression in the kidneys was up-regulated between 24-96 h, with a maximal increase of 2.3-fold observed 48 h post-venom **(Fig. 1B)**. The renal expression of ACE, which is a kininase that is responsible for BK inactivation and the generation of the vasoconstrictor peptide angiotensin II (Ang II), was down-regulated 12 and 24 h after envenomation **(Fig. 1C)**. The decrease in renal ACE expression was accompanied by the simultaneous down-regulation of the AT_1R, the Ang II receptor **(Fig. 1D)**. At 48 and 96 h post-envenomation, ACE expression had significantly increased in the kidneys of envenomed animals, but AT_1R expression remained unchanged **(Figs. 1C and D)**. The mRNA levels of renal MAS, which is the receptor for the vasodilator peptide angiotensin 1-7 (Ang-(1-7)), had increased by 3.2- and 3.4-fold at 48 and 96 h, respectively **(Fig. 1E)**. Despite the up-regulation of MAS expression, the mRNA levels of ACE2, which is the main enzyme responsible for the conversion of Ang II into Ang-(1–7), had only increased slightly, and this increase did not reach statistical significance **(Fig. 1F)**. The ratio of the expression of the MAS/AT_1R receptors increased at all of the time points evaluated, indicating that renal MAS expression predominated during envenomation **(Fig. 1G)**. However, the B_2R/B_1R ratio decreased at 12 h, indicating that B_1R expression predominated at this time point. The B_2R/B_1R ratio increased between 24 and 96 h, indicating that B_2R expression predominated at these time points **(Fig. 1H)**.

Along with the up-regulation of the BK receptors, plasma prekallikrein activity decreased in envenomed animals, suggesting that active kallikrein is generated systemically during envenomation **(Table 1)**. Consistent with this observation, renal kallikrein activity significantly increased between 24 and 96 h post-venom injection. Increases of 3.1-, 2.3- and 2.6-fold in renal tissue kallikrein activities were observed at 24, 48 and 96 h, respectively, indicating that the KKS is activated in the kidneys of envenomed rats **(Table 1)**. Likewise, as a consequence of kallikrein activation and BK formation, nitrate/nitrite levels (which are indicative of nitric oxide production) increased in the plasma and kidneys of envenomed animals, mainly between 12 and 24 h.

3.2 Kinin B₁ receptor blockade or kallikrein inhibition ameliorates renal function during L. obliqua envenomation

Because the KKS appeared to be activated during experimental envenomation, we decided to investigate whether the pharmacological blockade of kinin receptors and kallikrein inhibition could be beneficial during *L. obliqua*-induced AKI. As shown in **Fig. 2**, both B_1R antagonism and systemic kallikrein inhibition prevented venom-induced alterations in renal function. After 24 h of envenomation, significant polyuria was induced **(Fig. 2A)**, which was accompanied by increases in the hydric balance (the ratio of urine output/water consumption) **(Fig. 2B)**, plasma creatinine **(Fig. 2C)**, the fractional excretion of water (FE_{H2O}) **(Fig. 2E)**, urinary protein excretion **(Fig. 2F)**, and a marked reduction in the glomerular filtration rate (GFR) **(Fig. 2D)**. Pretreatment with both B_1R antagonists, DALBK and R-715, completely prevented these alterations. B_1R antagonism restored fluid homeostasis and the tubular ability to retain water, as reflected by the fact that urinary output, hydric balance and FE_{H2O} returned to the levels observed in the PBS-treated animals **(Figs. 2A, B and E)**. DALBK and R-715 pretreatment also restored filtration capacity and protected the animals from venom-induced glomerular injury, as indicated by the levels of GFR, plasma creatinine and urinary protein, which had returned to the levels observed in the control animals **(Figs. 2C, D and F)**. All of these functional parameters also returned to normal (values similar to those observed in the controls) when aprotinin, a kallikrein inhibitor, was administered to the envenomed animals. In contrast, renal protection under B_2R antagonism was not observed, as evidenced by the fact that pretreatment with HOE-140, a B_2R antagonist, failed to prevent renal dysfunction **(Figs. 2A-F)**. It is worth mentioning that isolated treatment with each of the drugs (administered at the doses tested above) did not alter renal function in non-envenomed rats (data not shown).

As expected, aprotinin pretreatment protected envenomed animals from plasma prekallikrein activation and, consequently, the levels of kallikrein in the renal tissue were only slightly increased. These increases were not significant in comparison to PBS-treated rats **(Table 2)**. Nitric oxide production was inhibited by pretreatment with both B_1R (DALBK and R-715) and B_2R (HOE-140) antagonists and aprotinin. Plasma and renal nitrate/nitrite levels were significantly reduced in envenomed animals that were treated with BK antagonists or aprotinin in comparison to those in the LOBE+PBS group **(Table 2)**.

3.3 Kinin B_1 receptor blockade and kallikrein inhibition ameliorate renal histopathological alterations and inflammation during L. obliqua envenomation

We next investigated whether BK antagonists or aprotinin pretreatment would be effective in preventing the development of kidney lesions during *L. obliqua*-induced AKI. Along with the progressive decline in renal function, LOBE injection also induced progressive degenerative lesions that were compatible with acute tubular necrosis (ATN) **(Fig. 3)**. Increased acidophilia, loss of the proximal brush border, inflammatory infiltration, edema, cytoplasmic vacuolation, degeneration and desquamation of necrotic cells forming intratubular casts were common alterations that were observed in envenomed animals (LOBE+PBS group) **(Figs. 3A and B)**. In general, pretreatment with DALBK, R-715 and aprotinin prevented the occurrence of these morphological alterations, despite the presence of intratubular casts in some of the tubules of the rats that had been pretreated with DALBK and aprotinin **(Fig. 3A)**. Nevertheless, the histopathological scores for intratubular casts, tubular degeneration and renal inflammation were ameliorated following B_1R blockade or systemic kallikrein inhibition. Similar effects were not observed following B_2R antagonism **(Fig. 3B)**. Further confirming these histological observations, DALBK, R-715 and aprotinin reduced γ-GT urinary activity, which is considered to be an efficient biomarker for ATN (Guder and Ross, 1984). However, γ-GT activity remained high in envenomed animals that had been pretreated with HOE-140 **(Fig. 3C)**. The main mechanism involved in the beneficial effects of B_1R blockade or kallikrein inhibition appeared to be the suppression of renal inflammation, because pretreatment with R-715 or aprotinin reduced renal vascular extravasation **(Fig. 3D)** and the production of the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) **(Fig. 3E)** and interleukin 1-β (IL-1β) **(Fig. 3F)**.

3.4 Kinin B_1 receptor blockade and kallikrein inhibition ameliorate L. obliqua envenomation-induced hemostatic abnormalities

L. obliqua envenomation is known to induce blood incoagulability and thus, hemorrhagic syndrome, which is characterized by coagulation and fibrinolysis activation with the secondary consumption of coagulation factors, including fibrinogen (Zannin *et al.*, 2003; Berger *et al.*, 2010). In the present study, our results indicated that envenomed animals that had been injected with the B_2R antagonist displayed a prolonged activated partial thromboplastin time (aPTT), while those that had been

pretreated with the B_1R antagonists or aprotinin displayed aPTT values that were similar to non-envenomed animals, indicating that blood clotting remained normal in these rats **(Fig. 4A)**. Despite the normal coagulation time, fibrinogen levels were significantly reduced in envenomed animals that received the B_1R antagonists or aprotinin, although partial recovery occurred in these groups **(Fig. 4B)**. Aprotinin was also the only treatment that was able to protect against the increases in renal tissue factor activity **(Fig. 4C)**.

4. Discussion

Collectively, our results indicate that the renal KKS is activated during *L. obliqua*-induced AKI and that blockade of the B_1R or kallikrein inhibition can reduce the development of tubular injury and renal inflammation and improve not only renal function but also hemostatic abnormalities. Although AKI is a common consequence of accidental contact with *L. obliqua* caterpillars, the present study was the first to demonstrate both the importance of the KKS in venom-induced pathology and that the specific inhibition of the KKS may be a promising therapeutic option. To date, animal-derived antivenoms have provided the only effective treatments for envenomation cases (Bon, 1996). Several laboratories throughout the continents manufacture antivenoms (Gutiérrez *et al*., 2010), which consist of whole IgG molecules or products of their enzymatic digestion, such as bivalent F(ab')$_2$ or monovalent Fab (Lalloo and Theakston, 2003; Theakston *et al*., 2003; Gutiérrez and León, 2009). Despite its efficacy, antivenom therapy has been associated with several problems, including the high cost of production; failures in the distribution of antivenom ampoules to localized, but distant, areas that have a high incidence of accidents and/or the low-income agrarian regions in which they are most needed; inadequate storage and transportation of antivenoms; and the lack of trained health workers who know how to use these products and conduct the appropriate clinical management of medical emergencies (Gutiérrez *et al*., 2010). Another serious problem is the adverse effects caused by antivenom therapy, including potentially life-threatening systemic disturbances, such as anaphylactic reactions, which occur quite frequently (Fan *et al*., 1999). The beneficial effects observed following treatment with the B_1R antagonists in the present study are of particular interest for this reason. Due to the inducible nature of the B_1R at the site of tissue injury, it has been suggested that blocking the proinflammatory B_1R may lack significant adverse effects. This hypothesis has drawn attention to the B_1R as a new therapeutic target for the

treatment of different pathologies, such as airway inflammation, diabetic neuropathy, brain ischemia, arthritis and neuropathic pain (Campos *et al.*, 2006).

Previous data indicated that *L. obliqua* venom possesses kininogenase activity, being able to release BK from low molecular weight kininogen and activate plasma kallikrein *in vitro* (Bohrer *et al.*, 2007). Kallikrein is also believed to be activated *in vivo*, because prekallikrein levels were decreased in the plasma of human victims (Zannin *et al.*, 2003) and in experimental animals, and renal tissue kallikrein activity was increased in envenomed rats (as observed in the present study). In addition, the expression of kininogen increased in the kidneys during envenomation (Berger *et al.*, unpublished data). Taken together, these results are in agreement with the hypothesis that BK is generated during envenomation and are also in accordance with the up-regulation of renal B_1R and B_2R that was observed in the present study. The renal pathophysiological effects produced by locally generated BK can also be enhanced, because ACE expression was down-regulated during the first 24 h, and the vasoconstrictor axis of the renin-angiotensin system (formed by ACE/Ang II/ AT_1R) (Bader *et al.*, 2012) seems to be suppressed during envenomation. In fact, the expression of the AT_1R, which is the receptor for the vasoconstrictor peptide Ang II, decreased during the first 24 h and remained unchanged between 48 and 96 h. In contrast, the vasodilator counter-regulatory axis, which consists of ACE2/Ang-(1-7)/MAS (Passos-Silva *et al.*, 2013), seems to be up-regulated during envenomation. Despite ACE2 expression only being slightly increased, MAS expression and the MAS/AT_1R ratio increased significantly, suggesting that a vasodilatory profile predominated. The B_1R and B_2R also have dual effects on kidney hemodynamics. While BK mediates a vasorelaxant effect through the B_2R, its metabolite, des-Arg^9-BK, induces a renal vasoconstrictor response through the B_1R (Guimarães *et al.*, 1986; Vieira *et al.*, 1994). The imbalance observed in the present study between renal B_2R/B_1R expression may be involved in renal hemodynamic alterations, mainly in the decrease of GFR.

Previous studies have demonstrated that KKS components are modulated in a similar fashion in different models of renal injury. BK receptors are induced during renal ischemia-reperfusion-induced injury, glomerulonephritis and obstructive nephropathy (Wang *et al.*, 2008; Klein *et al.*, 2009; Klein *et al.*, 2010). In general, these models demonstrated that animals treated with B_1R antagonists or B_1R knockout are protected from kidney damage. The protection observed following B_1R deletion or

antagonism was associated with the increased expression of anti-inflammatory molecules and the down-regulation of pro-inflammatory cytokines and pro-fibrotic molecules (Wang *et al.*, 2008; 2009; Klein *et al.*, 2009). Interestingly, neither B_2R deletion nor antagonism have been shown to be beneficial during renal ischemia-reperfusion-induced injury (Wang *et al.*, 2008). Similar results were obtained by Raslan *et al.* (2010), who observed reduced blood-brain barrier leakage and inflammation in mice lacking the B_1R that had been subjected to cerebral ischemia, while animals lacking the B_2R displayed no significant alterations in lesion formation or the development of brain edema. The data obtained in the present study lead us to believe that a similar mechanism occurred in *L. obliqua*-induced AKI, because significant improvements in glomerular function, tubular handling of water and renal hemodynamics were observed only under B_1R, and not B_2R, antagonism. Indeed, both DALBK and R-715 restored the levels of plasma creatinine, GFR, tubular water reabsorption, water imbalance, polyuria and proteinuria in envenomed animals. In addition, treatment with B_1R antagonists also improved histopathological changes, preventing tubular necrosis, the formation of intratubular casts, renal inflammation, vascular leakage and edema. The inhibition of pro-inflammatory signaling mediated by TNF-α and IL-1β seems to be critical for these beneficial effects, because B_1R blockade reduced the renal levels of these cytokines. A similar beneficial effect was observed in envenomed animals treated with the kallikrein inhibitor, aprotinin, confirming that the production of BK through kallikrein activation plays a central role in the renal injury elicited by this venom. The indirect activation of the KKS appears to be an essential mechanism by which *L. obliqua* venom leads to AKI. In other words, despite the presence of several toxins that are potentially cytotoxic (Veiga *et al.*, 2005; Ricci-Silva *et al.*, 2008), a direct cytotoxic effect of the venom on renal cells makes a minor contribution, because blocking BK generation or even B_1R activation is enough to prevent the progression of renal disease.

The mechanisms involved in B_1R up-regulation during venom-induced AKI remain unknown, although evidence provided by other studies has indicated that B_1R induction is controlled by many pro-inflammatory cytokines and growth factors, including IL-1β, TNF-α, and interferon gamma, as well as epidermal growth factor (Calixto *et al.*, 2004; Rocha *et al.*, 2005; Klein *et al.*, 2010). Clear evidence now exists indicating that the induction of the B_1R by many of these factors involves the activation of the transcription factor NF-κB, and, conversely, that B_1R stimulation activates NF-

κB (Medeiros *et al*., 2001; 2004; Calixto *et al*., 2004). An intense inflammatory response is a common feature of *L. obliqua* envenomation. Systemically, the venom induces neutrophilic leukocytosis, while in the kidneys, the occurrence of cellular inflammatory infiltrate, edema, increases in vascular permeability and the up-regulation of several proteins associated with the acute phase of inflammatory signaling and nephritis have been described (Berger *et al*., unpublished data). *In vitro*, when incubated with human fibroblasts or endothelial cells in culture, *L. obliqua* venom induces the production of several cytokines, including TNF-α and IL-1β, and also activates NF-κB and increases the expression of inflammatory inducible enzymes, such as COX-2, iNOS, and HO-1 (Pinto *et al*., 2008; Nascimento-Silva *et al*., 2012). In the present study, we also observed high levels of TNF-α and IL-1β in the kidneys of envenomed animals. Therefore, we propose that pro-inflammatory stimuli are the main factors underlying B_1R induction during envenomation. Moreover, once activated, the B_1R stimulates the release of more TNF-α and IL-1β, amplifying the inflammatory response and leading to leukocyte accumulation and the production of prostaglandins, reactive oxygen species and nitric oxide (NO) (Kayashima *et al*., 2012). As demonstrated previously, the intravascular NO levels increased during experimental envenomation in rats. NO production is associated with venom-induced platelet hypoaggregation, as evidenced by the fact that L-NAME restores platelet aggregation ability (Berger *et al*., 2010). In the present study, plasma NO levels also increased, along with the levels in the kidneys, and both B_1R and B_2R antagonism blocked NO production. However, despite the evident participation of NO in platelet aggregation disturbances, it seems that NO plays a minor role in kidney injury. B_2R antagonism inhibits NO production, but does not prevent renal functional and morphological alterations.

In addition to renal alterations, *L. obliqua* envenomation is also characterized by several hemostatic disturbances. Both human patients and experimental animals displayed signs of consumption coagulopathy with blood incoagulability, prolonged coagulation times, low fibrinogen levels and platelet hypoaggregation (Zannin *et al*., 2003; Berger *et al*., 2010). The venom contains specific toxins that are able to directly activate blood and fibrinolytic cascades, which ultimately contribute to fibrinogen consumption and blood incoagulability (Reis *et al*., 2001; Pinto *et al*., 2004). Surprisingly, in addition to preventing renal alterations, B_1R antagonism was also beneficial to blood incoagulability, because animals pretreated with DALBK or R-715 had similar coagulation times to those in controls that received only PBS. However,

when fibrinogen consumption was analyzed, we observed only partial protection during B_1R antagonism, while the procoagulant activity of renal tissue factor decreased slightly. The mechanism involved in the beneficial effects of B_1R blockade remains unknown and requires further investigation. However, because systemic inflammation leads to coagulation activation (Esmon, 2004), we speculate that inhibiting the pro-inflammatory signaling triggered by B_1R activation may not completely prevent fibrinogen consumption, but it may maintain a basal level of circulating fibrinogen, which may be sufficient to avoid blood incoagulability and the consequent delay in coagulation time. Aprotinin pretreatment was also able to efficiently prevent coagulation disturbances. In this case, the beneficial effect was expected because activated kallikrein is known to trigger the intrinsic coagulation pathway by directly activating factor XII (Shariat-Madar *et al.*, 2002; Renné, 2012). Thus, by inhibiting kallikrein during envenomation, it is possible to counteract the progression of renal injury and consumption coagulopathy.

Taken together, the results of the present study revealed that B_1R blockade or kallikrein inhibition improves renal function during *L. obliqua*-induced AKI by reducing tubular necrosis, renal inflammation, and the production of pro-inflammatory cytokines, and ameliorates the coagulopathy commonly observed during this type of envenomation. Thus, the present study provides consistent evidence linking KKS activation to *L. obliqua*-induced AKI, and indicates that the inhibition of KKS components (mainly kallikrein inhibition or B_1R antagonism) may be a therapeutic alternative that can be used to control the progression of renal injury during such envenomation.

5. Acknowledgments

6. Conflict of interest statement

The authors declare that no conflicts of interest exist.

7. References

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Figure Legends

Figure 1: Expression of components of the renal kallikrein-kinin system during *L. obliqua* **envenomation.** Renal mRNA levels of B_1R **(A)**, B_2R **(B)**, ACE **(C)**, AT_1R **(D)**, MAS **(E)**, ACE2 **(F)**, the MAS/AT_1R ratio **(G)** and the B_2R/B_1R ratio **(H)** were determined using quantitative real-time PCR in control (CTRL) and envenomed animals at different time points post-venom injection (LOBE, 1.5 mg/kg, sc). The data are presented as the means ± SE (n=6/group). Significant differences of *$p < 0.05$ were considered to be significant in comparison to the respective control.

Figure 2: Pharmacological blockade of the B$_1$R or kallikrein inhibition improves renal function in *L. obliqua***-envenomed rats.** Rats were pretreated with antagonists of either the B$_1$R (DALBK and R-715) or the B$_2$R (HOE-140) or the kallikrein inhibitor (aprotinin) 30 min prior to *L. obliqua* venom injection (LOBE, 1.5 mg/kg, sc). After 24 h of envenomation, plasma and urine samples were collected, and the following parameters were determined: **(A)** Urine output, **(B)** hydric balance, **(C)** plasma creatinine, **(D)** glomerular filtration rate (GFR), **(E)** fractional water excretion (FE$_{H2O}$) and **(F)** urinary protein levels. The data are presented as the means ± SE (n=6/group). Significant differences are indicated as follows: *p < 0.05 vs. PBS, §p < 0.05 vs. LOBE + PBS and ‡p < 0.05 vs. LOBE + HOE-140.

Figure 3: Pharmacological blockade of the B$_1$R or kallikrein inhibition ameliorates tubular injury and renal inflammation in *L. obliqua***-envenomed rats. A.** Representative kidney sections from the control animals (pretreated with PBS) and envenomed rats that had been pretreated with antagonists of either the B$_1$R (DALBK and R-715) or the B$_2$R (HOE-140) or the kallikrein inhibitor (aprotinin) 30 min prior to *L. obliqua* venom injection (LOBE, 1.5 mg/kg, sc). Morphological alterations were analyzed 24 h post-venom injection. All of the sections were stained with H&E. Magnification: 10 X. **B.** The kidney sections from the different groups were analyzed, and a semi-quantitative score was attributed according to the presence of intratubular casts, tubular degeneration and renal inflammation. **C.** Levels of urinary γ-glutamyl transferase (γ-GT) activity were also measured in the different groups as biochemical markers of tubular injury. **D.** Changes in renal vascular permeability were assessed using Evans blue dye extravasation in the control animals (pretreated with PBS) and envenomed rats that had been pretreated with either the B$_1$R antagonist (R-715) or the kallikrein inhibitor (aprotinin). The results are expressed as μg of Evans blue dye per 100 mg of renal tissue. **E** and **F.** The renal levels of TNF-α and IL-1β were also measured in these animals using an enzyme-linked immunosorbent assay (ELISA). The results are expressed as pg per 100 mg of renal tissue. All of the data are presented as the means ± SE (n=6/group). In B and C, significant differences of *p < 0.05 vs. PBS, §p < 0.05 vs. LOBE + PBS and ‡p < 0.05 vs. LOBE + HOE-140 were considered to be significant. In D-F, differences of *p < 0.05 vs. PBS, §p < 0.05 vs. LOBE + PBS and ‡p < 0.05 vs. LOBE + R-715 were considered to be significant.

Figure 4: Pharmacological blockade of the B_1R or kallikrein inhibition prevents *L. obliqua* **venom-induced coagulation disturbances.** Rats were pretreated with antagonists of either the B_1R (DALBK and R-715) or the B_2R (HOE-140) or the kallikrein inhibitor (aprotinin) 30 min prior to *L. obliqua* venom injection (LOBE, 1.5 mg/kg, sc). After 24 h of envenomation, the activated partial thromboplastin time **(A)** and fibrinogen levels **(B)** in the plasma were measured, and factor Xa (FXa) generation was measured in kidney extracts to determine renal tissue factor activity **(C)**. The data are presented as the means ± SE (n=6/group). Significant differences of *$p < 0.05$ vs. PBS, §$p < 0.05$ vs. LOBE + PBS, ‡$p < 0.05$ vs. LOBE + HOE-140 and †$p < 0.05$ vs. LOBE + DALBK were considered to be significant.
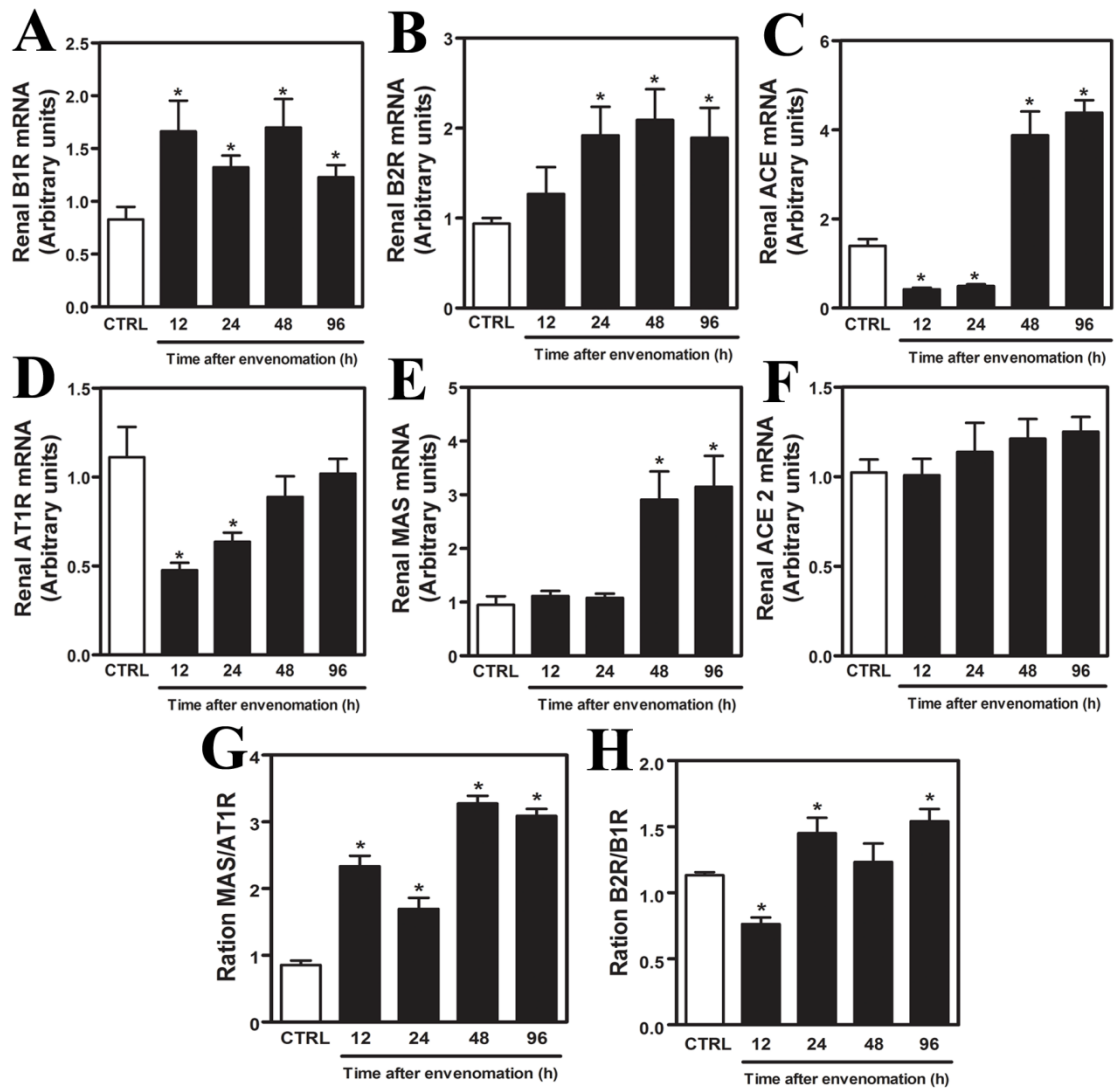
Tables

Table 1: Kallikrein and nitrate/nitrite levels in the plasma and kidneys of control (CTRL) and envenomed rats (LOBE, 1.5 mg/kg, sc) at different time points post-*L. obliqua* **venom injection.**
Significant differences of *$p < 0.05$ vs. CTRL were considered to be significant.

Table 2: Kallikrein and nitrate/nitrite levels in the plasma and kidneys of control and envenomed rats pretreated with bradykinin antagonists or the kallikrein inhibitor.
Rats were pretreated with antagonists of either the B_1R (DALBK and R-715) or the B_2R (HOE-140) or the kallikrein inhibitor (aprotinin) 30 min prior to *L. obliqua* venom injection (LOBE, 1.5 mg/kg, sc). After 24 h of envenomation, the levels of plasma prekallikrein, renal tissue kallikrein and plasma and kidney nitrate/nitrite levels were determined. The data are presented as the means ± SE (n=6/group). Significant differences of *$p < 0.05$ vs. PBS, §$p < 0.05$ vs. LOBE + PBS, ‡$p < 0.05$ vs. LOBE + HOE-140 and †$p < 0.05$ vs. LOBE + DALBK were considered to be significant.
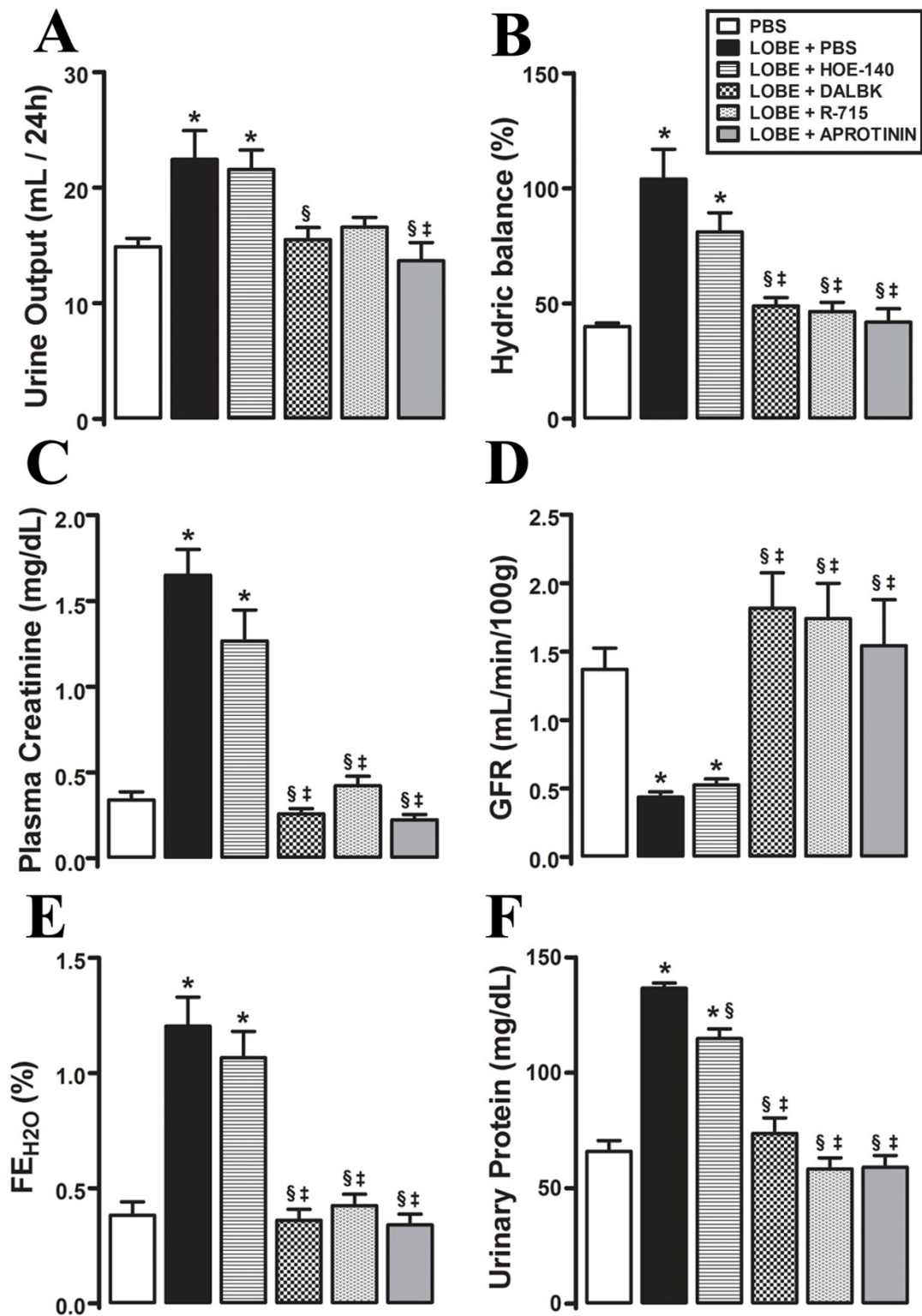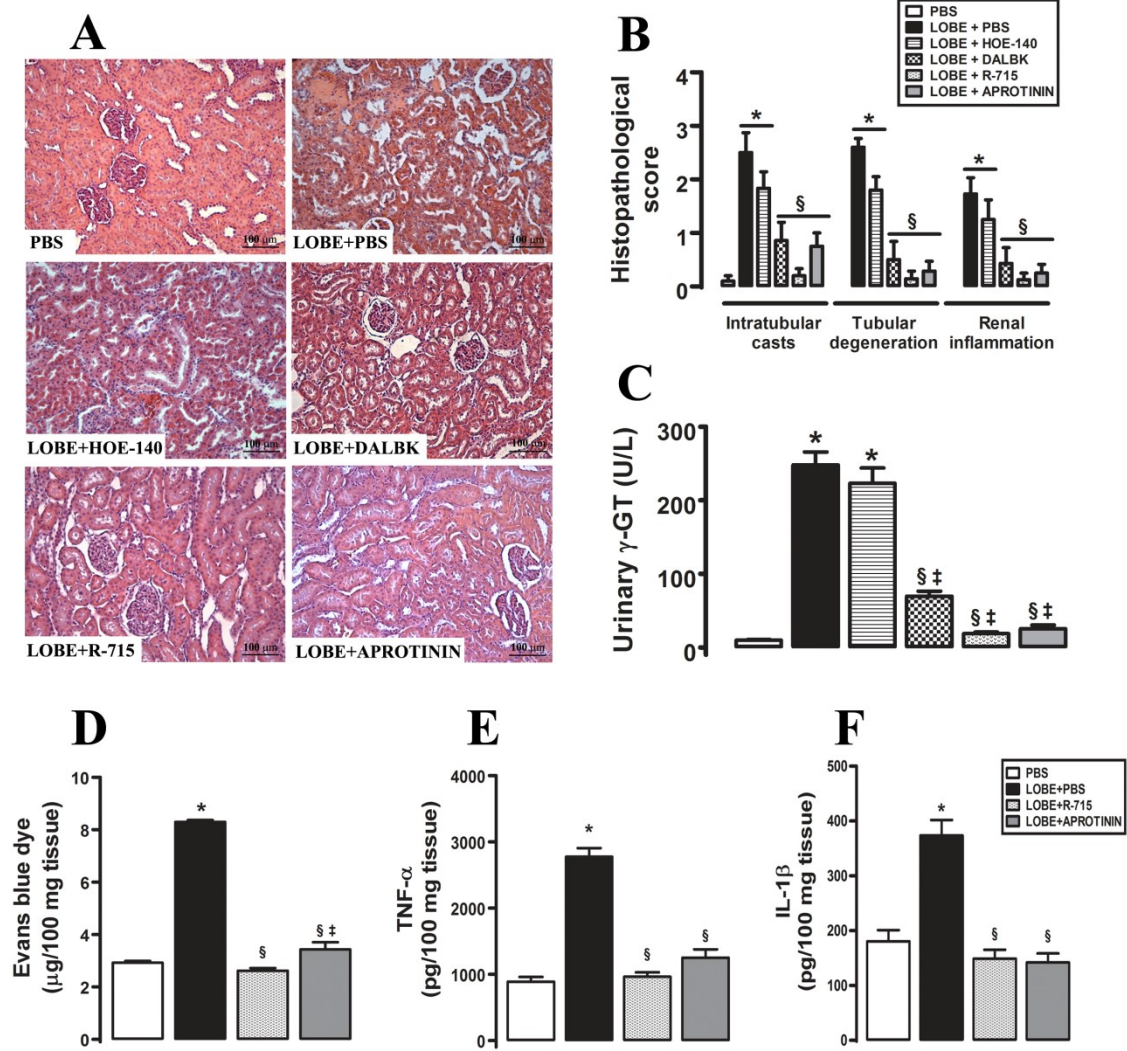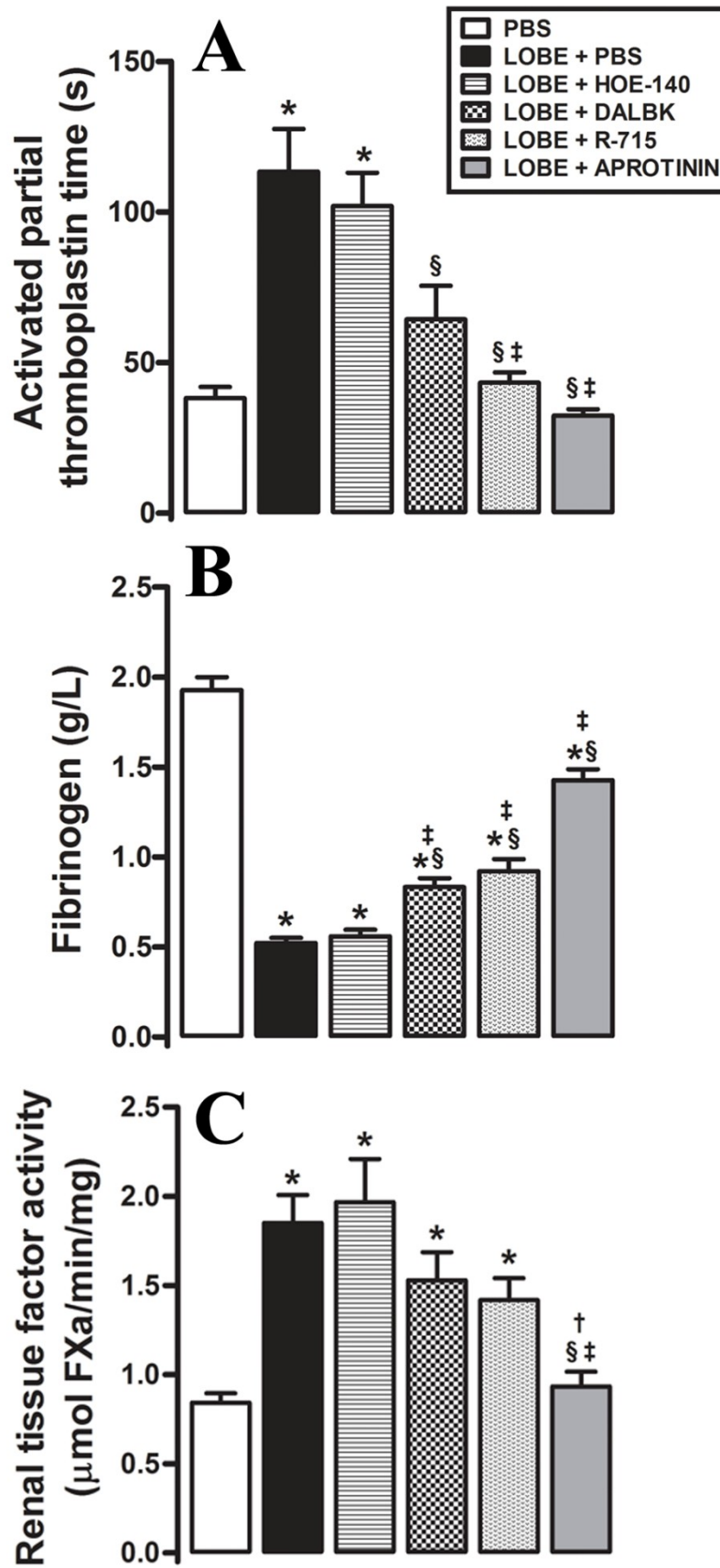
Figure 1

Figure 2

Figure 3

Figure 4

Table 1

	CTRL	Time after envenomation (h)			
		12	**24**	**48**	**96**
Plasma prekallikrein (µmol/min/mg)	2.10±0.2	0.78±0.09*	1.04±0.08*	1.01±0.1*	1.36±0.07
Renal tissue kallikrein (µmol/min/mg)	0.31±0.03	0.41±0.03	0.97±0.1*	0.73±0.06*	0.80±0.05*
Plasma nitrate/nitrite (µM)	22.9±2.5	50.9±5.0*	44.7±5.4*	26.9±7.4	19.1±5.4
Renal nitrate/nitrite (µM)	71.8±6.1	135.9±3.4*	117.5±5.7*	106.4±5.9*	87.3±10

Table 2

	Experimental Groups					
	PBS	**LOBE+PBS**	**LOBE+HOE-140**	**LOBE+DALBK**	**LOBE+R-715**	**LOBE+APROTININ**
Plasma prekallikrein (µmol/min/mg)	2.70±0.1	1.2±0.1*	1.4±0.13*	1.9±0.1§	1.9±0.1§	2.8±0.4§‡†
Renal tissue kallikrein (µmol/min/mg)	0.28±0.03	0.84±0.09*	0.92±0.2*	0.98±0.1*	0.91±0.1*	0.57±0.03
Plasma nitrate/nitrite (µM)	17.5±2.6	33.5±5.4*	21.1±3.3	13.4±0.9§	14.3±0.6§	7.5±0.8§
Renal nitrate/nitrite (µM)	54.6±5.4	85.3±6.9*	34.1±5.7§	55.4±2.6§	68.1±3.1‡	49.4±5.1§

4. DISCUSSÃO

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A patologia desencadeada pelos envenenamentos com animais peçonhentos é complexa e decorrente não só da ação direta das diferentes toxinas presentes nos venenos em tecidos ou sistemas específicos, mas também é resultado da resposta do organismo à lesão provocada por essas toxinas. Neste trabalho, demonstramos que o veneno da taturana *Lonomia obliqua*, reconhecidamente de efeito hemorrágico devido à presença de toxinas pró- e anticoagulantes, é capaz de induzir, além das alterações na hemostasia, uma síndrome de toxicidade sistêmica onde há lesão de múltiplos órgãos. Dentre estes órgãos, o rim é um dos mais afetados, tendo, ademais, importância clínica significativa considerando os casos de vítimas envenenadas. Devido à incidência e aumento da mortalidade associado ao desenvolvimento de lesão renal após o contato com a taturana, empenhamos esforços na investigação da patogênese dessa doença em um modelo experimental *in vivo* em ratos. Os dados obtidos apontam para um mecanismo multifatorial onde a hemólise, a ativação do sistema calicreína-cininas (SCC) e alterações vasculares mostraram ser componentes fundamentais no desenvolvimento da insuficiência renal aguda (IRA) induzida pelo veneno de *L. obliqua*. De acordo com os resultados apresentados nos capítulos anteriores, a **Figura 9** mostra as possíveis vias envolvidas no desenvolvimento da lesão renal. A seguir, uma breve discussão integrativa acerca dos mecanismos da IRA neste tipo de envenenamento é apresentada.

Hemólise. Hemólise intensa ou rabdomiólise podem estar associadas ao desenvolvimento de IRA principalmente devido à nefrotoxicidade do heme liberado a partir da hemoglobina (Hb) ou mioglobina (Mb) (ZAGER, 1996; SITPRIJA, 2006). O heme é uma molécula altamente citotóxica, proinflamatória e prooxidante que está envolvida em uma série de patologias incluindo sepse, malária, lesões isquêmicas, hemorragia pulmonar e cerebral e anemia falciforme (FERREIRA *et al.,* 2008; TRACZ *et al.,* 2007; PAMPLONA *et al.,* 2007; LARA *et al.,* 2009; LARSEN *et al.,* 2010; SIMÕES *et al.,* 2013). Em condições normais, a Hb ou Mb livre na circulação é removida pela ligação à haptoglobina (Hpt) e o heme pela ligação à hemopexina (Hpx). A formação dos complexos Hb-Hpt ou Mb-Hpt e heme-Hpx neutralizam os efeitos prooxidantes e proinflamatórios do heme e, dessa forma, são direcionados para as

células do fígado, ligam-se a receptores específicos e são reutilizados para a síntese de novas moléculas de Hb e Mb para os eritrócitos e células musculares, respectivamente (ZAGER *et al.,* 2012). Entretanto, em condições onde há hemólise ou rabdomiólise intensa, tanto a Hpt quanto a Hpx são consumidas, sua quantidade diminui no plasma e há um acúmulo de Hb ou Mb e heme livres na circulação. Durante o envenenamento por *L. obliqua*, ocorre liberação de Hb a partir dos eritrócitos devido ao efeito hemolítico do veneno *in vitro* e *in vivo* (SEIBERT *et al.,* 2004; 2006). A liberação de Mb também pode ocorrer, pois o veneno tem atividade miotóxica e cardiotóxica, apesar de não induzir rabdomiólise intensa como observado em outros envenenamentos como por *Crotalus* sp., por exemplo, que se caracterizam por fraqueza muscular generalizada. Como mostrado nesta tese, quantidades significativas de Hb acumulam-se no plasma dos ratos envenenados, há redução do número de eritrócitos e Hpt circulantes, presença de eritrócitos fragmentados no esfregaço sanguíneo, aumento dos reticulócitos, eritrofagocitose e deposição de hemosiderina no baço, acúmulo de eritrócitos e Hb nos túbulos renais, hematúria e hemoglobinúria. Especificamente no rim, a expressão da heme-oxigenase 1 (HO-1), Hpx e ferritina aumentam, indicando que o metabolismo do heme está ativado durante o envenenamento. Uma vez reabsorvido pelas células tubulares, o heme é catabolizado pelo sistema da HO-1, gerando quantidades equimolares de biliverdina, monóxido de carbono (CO) e ferro livre (CAMARA & SOARES, 2005; LARSEN *et al.,* 2010). A biliverdina ainda é convertida em bilirrubina pela biliverdina redutase e o ferro livre, por ser um potente agente prooxidante, é neutralizado formando um complexo com a ferritina (KUMAR & BANDYOPADHYAY, 2005). Na presença de pequenas quantidades de heme, esse sistema da HO-1 é efetivo em proteger as células tubulares. No entanto, como é grande o acúmulo de Hb e eritrócitos nos túbulos durante o envenenamento, possivelmente o sistema da HO-1 esteja saturado. Com isso, os níveis de bilirrubina aumentam no plasma e o ferro livre não complexado à ferritina gera espécies reativas de oxigênio (EROs) responsáveis por lesão tissular (ZAGER, 1996). De fato, em nossos experimentos, observamos que a expressão de várias enzimas antioxidantes aumentam no rim juntamente com as atividades de superóxido dismutase, catalase e peroxidase (dados não mostrados) que também aumentam no rim de animais envenenados, indicando a produção de EROs. O aumento na geração de EROs ainda pode estar correlacionado com diferentes eventos observados ao longo do envenenamento, tais como (*i*) as quebras duplas detectadas no DNA de células renais de animais

envenenados que, pelos nossos resultados, parecem ser efetivamente mediadas por EROs; (*ii*) o aumento na expressão de enzimas de reparo de DNA; e (*iii*) a presença de uma série de marcadores de lesão tecidual incluindo marcadores de necrose e apoptose que foram detectados nos rins (**Figura 9**). Além disso, já foi demonstrado que o heme livre, mesmo antes de ser metabolizado pelo sistema da HO-1, também pode mediar a produção de EROs via NADPH-oxidase e induzir a expressão de citocinas proinflamatórias (IL-1β, IL-6 e TNF-α) pela ativação do fator de transcrição NF-κB (GRAÇA-SOUZA *et al.,* 2002; ARRUDA *et al.,* 2004; SIMÕES *et al.,* 2013).

Ativação do sistema calicreína-cininas. O veneno de *L. obliqua* é capaz de ativar a pré-calicreína no plasma e liberar BK diretamente a partir do LMWK. Portanto, a BK pode ser gerada durante o envenenamento por atividade direta de uma cininogenase do veneno sobre o LMWK ou pela ação da calicreína ativada sobre o HMWK (BOHRER *et al.,* 2007) (**Figura 9**). A BK assim gerada e também seus metabólitos ativos, respondem por uma série de efeitos observados no envenenamento, incluindo edema, inflamação, dor e hipotensão (DE CASTRO BASTOS *et al.,* 2004; BOHRER *et al.,* 2007). Os resultados aqui apresentados acrescentam que essa ativação do SCC também é de fundamental importância para o desenvolvimento da IRA durante o envenenamento. A expressão dos genes codificadores de ambos os receptores de BK (B1R e B2R) está aumentada no rim. Verificamos, todavia, que somente o antagonismo de B1R ou a inibição de calicreína são capazes de prevenir as alterações funcionais e morfológicas renais e o desequilíbrio hidroeletrolítico causado pelo envenenamento. O tratamento prévio com antagonistas de B2R não é capaz de proteger contra os efeitos nefrotóxicos do veneno. Ou seja, tanto a ativação de calicreína quanto do B1R são essenciais para os efeitos deletérios do veneno sobre o rim. Resultados semelhantes foram descritos em outros modelos de IRA incluindo nefropatia diabética, lesão renal por isquemia-reperfusão, glomerulonefrite e fibrose renal (KAKOKI *et al.,* 2004; WANG *et al.,* 2008; KLEIN *et al.,* 2009; KLEIN *et al.,* 2010). Especificamente em modelo de isquemia-reperfusão renal, animais nocautes para B1R apresentam menores níveis de creatinina plasmática, melhora da lesão tubular e inflamação, aumento na expressão de citocinas anti-inflamatórias, como IL-4 e IL-10, e redução na expressão de citocinas pró-inflamatórias, como IL-1β e TNF-α (WANG *et al.,* 2006; 2008). Em modelos de glomerulonefrite, fibrose renal e mesmo em biópsias de pacientes com glomerulonefrite o B1R tem sua expressão aumentada e o bloqueio deste receptor

associa-se a uma redução na expressão de citocinas profibróticas, como o TGF-β e CTGF, e quimiocinas responsáveis pelo recrutamento de macrófagos/monócitos, como a MCP-1 e MCP-3 (KLEIN *et al.,* 2009; WANG *et al.,* 2009; KLEIN *et al.,* 2010). Portanto, a ausência ou o bloqueio do B1R parece estar relacionado diretamente a uma diminuição do processo inflamatório no rim. Nos animais envenenados a melhora na função renal provavelmente também esteja relacionada a uma redução da inflamação, já que o bloqueio de B1R reduz o acúmulo de células inflamatórias, edema, extravasamento vascular e a expressão de IL-1β e TNF-α no rim. De fato, estudos relacionados com B1R demonstraram que sua expressão apresenta importante função, principalmente na manutenção da inflamação (CALIXTO *et al.,* 2004; MARCEAU E REGOLI, 2004), e os efeitos proinflamatórios dos B1R incluem dor, edema e aumento do trânsito de leucócitos para as regiões inflamadas (COUTURE *et al.,* 2001). Citocinas proinflamatórias e a ativação de componentes da família MAPK estão envolvidos na regulação e indução de expressão do gene que codifica para o B1R. A região promotora do gene codificador de B1R tem sítios de ligação para o fator transcricional NF-κB, o qual parece ser essencial para o controle da transcrição deste gene após a exposição a agentes inflamatórios como, por exemplo, IL-1β, TNF-α e LPS (MEDEIROS *et al.,* 2004). Dessa forma, uma vez ativado, o B1R induz a translocação de NF-κB para o núcleo, o qual será responsável pelo aumento não só da expressão do próprio gene de B1R, mas também de uma série de citocinas, quimiocinas e prostaglandinas que, em última instância mediarão a migração de células inflamatórias para o tecido lesado, a formação de edema, dor e produção de EROs (PASSOS *et al.,* 2004; KAYASHIMA *et al.,* 2012) (**Figura 9**).

De forma interessante, tanto o bloqueio farmacológico do B1R quanto a inibição sistêmica de calicreína também protegeram os animais das alterações de coagulação sanguínea que são comuns no envenenamento por *L. obliqua*. No caso da inibição de calicreína, este efeito benéfico pode ser explicado pelo fato de que a calicreína ativa na circulação, além de gerar BK, pode também disparar a coagulação pela ativação de fator XII (SHARIAT-MADAR *et al.,* 2002) (**Figura 9**). Entretanto, a ativação do B1R não tem aparentemente qualquer relação direta conhecida com a coagulação. Uma hipótese plausível é que a produção de citocinas proinflamatórias mediadas pelo B1R possam ativar a coagulação e/ou inibir os mecanismos anticoagulantes endógenos que limitam a resposta protrombótica, já que inflamação e coagulação são processos intimamente relacionados (MASSBERG *et al.,* 2010; RENNÉ, 2012). Por exemplo, a produção de

TNF-α estimulada por LPS induz um perfil protrombótico, pois aumenta a expressão de fator tecidual e ao mesmo tempo inibe a via da proteína C e trombomodulina, que são importantes anticoagulantes endógenos (ESMON, 2004). Além disso, o estímulo inflamatório resulta na ativação do sistema complemento e eleva os níveis do inibidor do ativador de plasminogênio (PAI-1), portanto diminuindo a fibrinólise e axacerbando a coagulação e a coagulopatia de consumo (ESMON, 2004; SCHOUTEN *et al.,* 2008). De acordo com esta hipótese, de fato encontramos, além da resposta inflamatória intensa, níveis aumentados de PAI-1 e dos componentes C3 e D do complemento e, também, uma atividade aumentada de fator tissular nos rins de animais envenenados.

Alterações vasculares. O processo de ultrafiltração do plasma nos glomérulos caracteriza-se basicamente pela passagem seletiva de água, eletrólitos e pequenas moléculas para o espaço de Bowman, enquanto os elementos figurados do sangue e as proteínas dissolvidas no plasma ficam retidos. A energia para esse processo provém essencialmente do trabalho cardíaco e depende da pressão arterial sistêmica e da pressão na microvasculatura renal (ZATZ, 2000). Dessa forma, a variação da pressão hidrostática nos capilares glomerulares interfere no ritmo que acontece o processo de filtração. Nos capilares glomerulares, a pressão hidrostática é regulada pela variação do diâmetro das arteríolas aferente e eferente e, portanto, pela resistência oferecida por esses vasos. Quando a resistência da arteríola aferente decresce (por vasodilatação), a pressão hidrostática dentro do capilar glomerular (P_{CG}) aumenta, pois uma maior fração da pressão arterial renal é transmitida ao capilar glomerular. O aumento da P_{CG} eleva o ritmo de filtração glomerular (RFG). Já quando a resistência na arteríola aferente aumenta (por vasoconstrição), a P_{CG} diminui e o RFG também se reduz. Na arteríola eferente, quando a resistência é diminuída, como quando relaxada, acontece diminuição da P_{CG} e do RFG. Por outro lado, quando há constrição na arteríola eferente, observa-se um aumento da P_{CG} e do RFG (ZATZ, 2000). Em situações normais, existe um processo de autorregulação do RFG que o mantém em valores relativamente estáveis mesmo que ocorram flutuações na pressão arterial (CUPPLES and BRAAM, 2007). No entanto, quando há vasodilatação sistêmica ou hemorragia, a pressão arterial pode cair bastante e ficar fora dos limiares da autorregulação. Nesse caso normalmente ocorre uma vasoconstrição na arteríola aferente, como parte da tentativa do organismo de denfender a sua volemia, e, consequentemente, redução da P_{CG} e do RFG (ZATZ, 2000; SCHRIER *et al.,* 2004). A principal evidência de que um mecanismo semelhante tenha

parte na queda acentuada do RFG observada nos animais envenenados, além, obviamente, da hemorragia, é a hipotensão (**Figura 9**). Após a injeção do veneno, há uma queda progressiva da pressão arterial nas primeiras 12 h e os animais permanecem hipotensos entre 24 e 96 h, período em que também é observada a maior queda no RFG. Apesar da diminuição acentuada do RFG, o desbalanço hídrico com o aumento concomitante da fração de excreção de água e eletrólitos também observado nos animais envenenados pode, pelo menos em parte, contribuir para a queda de pressão arterial (**Figura 9**). Estes resultados indicam que os néfrons deixaram de desempenhar adequadamente suas funções: não estão ávidos por sódio, não parecem estar secretando potássio em quantidade significativa, nem estão empenhados em concentrar a urina para reter água. Em outras palavras, não parece estar havendo um processamento da pequena quantidade de filtrado glomerular que ainda se forma. Assim, como a capacidade de reabsorção tubular está diminuída boa parte do filtrado formado é eliminado e ocorre poliúria mesmo com o RFG baixo. Na prática clínica, esse tipo de quadro é conhecido como IRA não-oligúrica (ou IRA poliúrica) e, apesar do evidente comprometimento renal em tal situação, ainda assim é considerada de bom prognóstico se comparada à IRA oligúrica ou anúrica (ZATZ *et al.,* 2000). A diferença básica está no grau de lesão tubular a que cada condição está associada. Na IRA oligúrica a extensão da necrose tubular é grande, de forma que em vários pontos do rim as células tubulares aparecem necrosadas ou ausentes, restando aos túbulos, em certos segmentos, apenas a membrana basal. Isso permite o vazamento puro e simples do filtrado de volta ao interstício. Ou seja, o filtrado não é reabsorvido e nem eliminado corretamente. Já na IRA não-oligúrica, a extensão da necrose tubular é menor e regiões necrosadas contrastam com outras menos comprometidas (geralmente na área medular). O resultado é que, apesar da incapacidade de reabsorção de água e eletrólitos, o filtrado permanece no interior dos túbulos e é eliminado. Assim, o débito urinário se mantém (ou até mesmo aumenta), produzindo uma urina completamente diluída (THADHANI *et al.,* 1996; SCHRIER *et al.,* 2004).

Tanto a ativação da coagulação quanto do SCC também podem interferir na hemodinâmica glomerular e contribuir para a redução do RFG (**Figura 9**). Independente da P_{CG}, se há lesão endotelial e deposição de fibrina no capilar, o fluxo sanguíneo pode sofrer uma redução e, como o processo de filtração também é diretamente proporcional ao fluxo, o RFG cai. Já as cininas geradas pela ativação do SCC alteram significativamente a resistência vascular renal. Em rim isolado de rato perfundido com

BK ou lys-BK observa-se um efeito bifásico de vasodilatação e vasoconstrição. Em uma primeira fase, ocorre vasodilatação mediada pela ligação da BK ou Lys-BK ao B2R e dependente da produção de prostaglandinas. Na segunda fase, ocorre vasoconstrição mediada pela ligação de des-Arg^9-BK ou Lys-des-Arg^9-BK (geradas a partir de BK ou Lys-BK) ao B1R e totalmente independente da produção de prostaglandinas (GUIMARÃES *et al.,* 1986a,b; VIEIRA *et al.,* 1994; REN *et al.,* 2002). *In vivo* durante o envenenamento é difícil prever qual desses efeitos é predominante e, portanto, realmente interfere no RFG. O fato é que a expressão de ambos os receptores de BK aumenta ao longo do envenenamento (**Figura 9**), mas somente o bloqueio do B1R é capaz de prevenir a queda do RFG. Talvez o bloqueio do B1R favoreça o efeito vasodilatador mediado pelo B2R no rim, diminua a resistência vascular e facilite o processo de filtração. É possível que investigações futuras e o uso de outras metodologias como o rim isolado, por exemplo, auxiliem a esclarecer a real influência dos receptores de BK nas alterações hemodinâmicas renais induzidas pelo veneno.
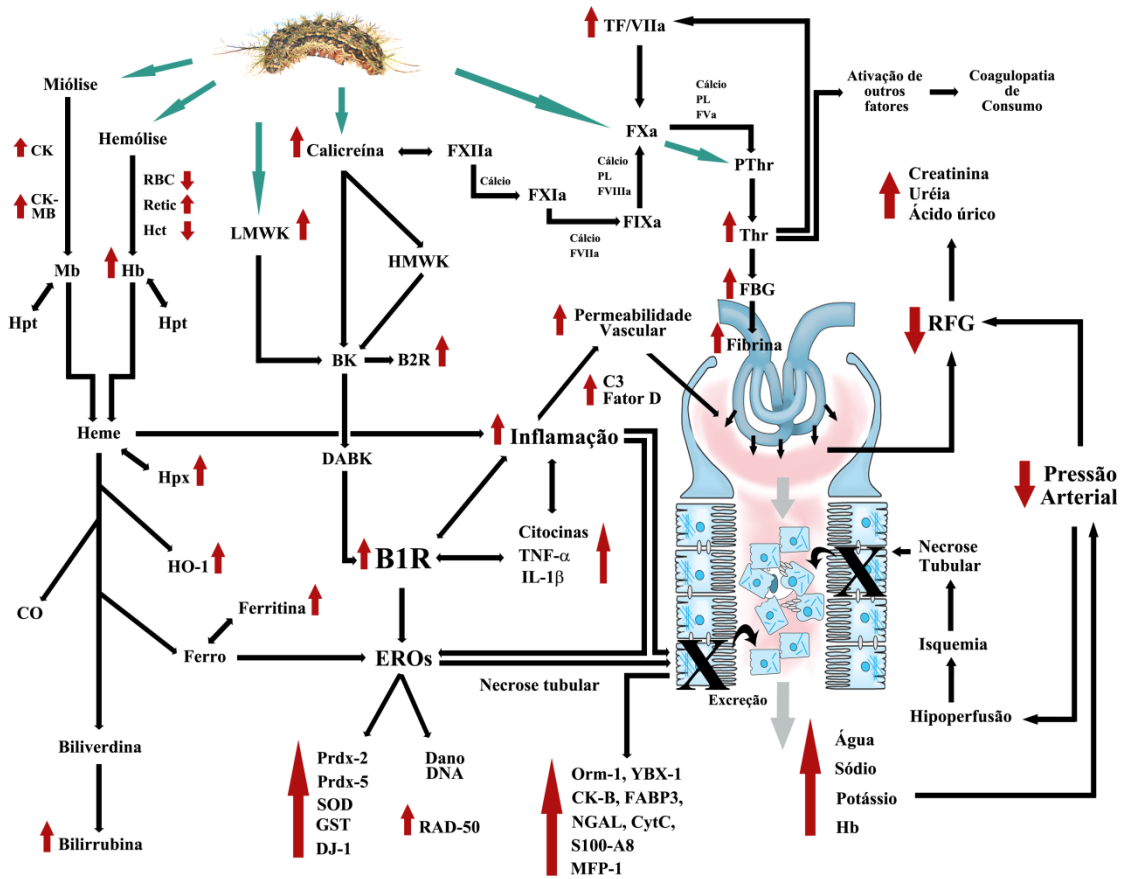
Figura 9. Possíveis mecanismos envolvidos na insuficiência renal aguda (IRA) induzida pelo veneno da taturana *Lonomia obliqua***.** Coletivamente os resultados obtidos indicam que o mecanismo da IRA é complexo, multifatorial e provavelmente envolve vias correlacionadas. São elas: (*i*) Hemólise intravascular e a toxicidade derivada da liberação do heme; (*ii*) Ativação do sistema calicreína-cininas e efeito proinflamatório mediado pelo B1R e (*iii*) Alterações vasculares que contribuem para a diminuição do RFG e hipoperfusão renal, incluindo a ativação da coagulação, deposição de fibrina nos capilares glomerulares, aumento na fração de excreção de eletrólitos e água e hipotensão. Hb: hemoglobina; Mb: mioglobina; Hpt: haptoglobina; Hpx: hemopexina; RBC: contagem de eritrócitos; Hct: hematócrito; RFG: ritmo de filtração glomerular; TF: fator tecidual; Pthr: protrombina; Thr: trombina; FBG: fibrinogênio; TNF-α: fator de necrose tumoral-α; IL-1β: interleucina-1β; HO-1: heme-oxigenase-1; EROs: espécies reativas de oxigênio; LMWK: cininogênio de baixo peso molecular; HMWK: cininogênio de alto peso molecular; BK: bradicinina; DABK: des-Arg[9]-BK; B1R: receptor B1 de bradicinina; B2R: receptor B2 de bradicinina; Prdx: peroxirredoxina; SOD: superóxido dismutase; GST: glutationa s-transferase; DJ-1: proteína anti-oxidante (tipo-peroxirredoxina); RAD-50: proteína de reparo de DNA; CK-B: creatina kinase-B; CK: creatina kinase; CK-MB: creatina kinase-MB; Orm-1: α-1-glicoproteína ácida; YBX-1: marcador de dano tubular; FABP3: proteína ligante de ácidos graxos (marcador de necrose tubular aguda); NGAL: lipocalina associada à gelatinase de neutrófilos (marcador de necrose tubular aguda); CytC: citocromo C; MFP-1: proteína de fissão mitocondrial (marcador de apoptose); S100-A8: proteína envolvida em processos de apoptose. Setas pretas: denotam ativação, liberação ou causa. Setas vermelhas: denotam aumento. Setas verdes: denotam efeitos diretos de toxinas do veneno.

CONCLUSÃO

CONCLUSÃO

A partir dos resultados expostos nesta tese, as seguintes conclusões podem ser formuladas:

❖ O veneno da taturana *Lonomia obliqua* induz, além da síndrome hemorrágica característica, importantes alterações bioquímicas, hematológicas e histopatológicas, indicando que ocorre lesão de múltiplos órgãos ao longo do envenenamento. Essas lesões parecem estar relacionadas principalmente à coagulopatia de consumo e aos efeitos hemolítico, proinflamatório, nefrotóxico, miotóxico, cardiotóxico e genotóxico do veneno. O dano genotóxico em particular parece ser mediado pela produção de espécies reativas de oxigênio;

❖ O soro antilonômico é capaz de neutralizar os efeitos tóxicos sistêmicos do veneno apenas se administrado no início do envenenamento (nas primeiras 2 h). Se administrado após 6 h de envenenamento, o soro é ineficaz em neutralizar o dano renal, cardíaco e o efeito hemolítico, apesar de reverter a coagulopatia de consumo;

❖ Especificamente no rim, as alterações morfológicas são compatíveis com necrose tubular aguda. Há obstrução tubular e o desenvolvimento da lesão renal induzida pelo envenenamento está relacionada com alterações hemodinâmicas incluindo hipotensão sistêmica, aumento do débito cardíaco, aumento da permeabilidade vascular renal, redução do ritmo de filtração glomerular e alterações tubulares como redução da capacidade de reabsorção de água e eletrólitos;

❖ Marcadores de lesão tubular são encontrados nos rins e urina dos animais envenenados. A expressão de proteínas associadas com inflamação, dano oxidativo, reparo de DNA, metabolismo e detoxificação de hemoglobina, heme e ferro estão aumentadas no rim de animais envenenados. Marcadores que indicam ativação da coagulação, complemento e do sistema calicreína-cininas também estão presentes no rim de ratos envenenados;

❖ Toxinas do veneno são detectadas no rim, endotélio, tecido conjuntivo perivascular e urina.

❖ A ativação do sistema calicreína-cininas parece ter um papel fundamental para o desenvolvimento da insuficiência renal aguda no envenenamento por *L. obliqua*;

❖ O bloqueio da atividade da calicreína ou do receptor B1 de bradicinina é capaz de previnir a maioria das alterações morfológicas e funcionais renais e o desenvolvimento da coagulopatia de consumo, constituindo-se, portanto, em uma alternativa terapêutica para o tratamento do envenenamento;

❖ O efeito protetor do antagonismo do receptor B1 de bradicinina ou a inibição sistêmica de calicreína possivelmente está associado a uma diminuição do processo inflamatório no rim e a redução da liberação de citocinas proinflamatórias como TNF-α e IL-1β.

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ANEXOS

Abaixo serão apresentados outros resultados obtidos ao longo do período do doutoramento. Vale lembrar que todos esses resultados estão publicados ou aceitos para publicação em periódicos internacionais indexados e com índice de impacto conhecido e fazem parte de linhas de pesquisas também desenvolvidas no laboratório de Bioquímica Farmacológica do Centro de Biotecnologia da UFRGS.

São eles:

❖ **Susceptibility of** *Loxosceles* sp. **to the arthropod pathogenic fungus** *Metarhizium anisopliae*: **potential biocontrol of the brown spider.** A aranha-marrom (*Loxosceles* spp.) é um animal venenoso capaz de causar lesões dermonecróticas extensas que podem evoluir para um quadro de envenenamento sistêmico potencialmente fatal. No Brasil existem diferentes espécies e algumas áreas (inclusive áreas urbanas e regiões metropolitanas) são infestadas por essas aranhas, o que faz com que o número de acidentes seja muito elevado. Neste trabalho mostramos uma forma alternativa e eficaz de controle biológico desses animais utilizando o fungo entomopatogênico *Metarhizium anisopliae*. **Artigo publicado no Transactions of the Royal Society of Tropical Medicine and Hygiene 2013; 107: 59-61.**

❖ **Antithrombotic effect of chikusetsusaponin IVa isolated from** *Ilex paraguariensis* **(Maté).** Em trabalhos anteriores nosso grupo demonstrou pela primeira vez que a glicirrizina (GL), uma saponina triterpênica conhecida principalmente por suas propriedades anti-inflamatórias, também possui atividade anti-trombótica. A GL é capaz de ligar-se ao exosítio-1 da trombina, inibindo a sua atividade e, portanto, a formação de trombos, tanto *in vitro*, quanto em modelos animais. Neste trabalho, isolamos e identificamos outra saponina triterpênica, a chikusetsusaponin IVa, obtida dos frutos da erva-mate (*Ilex paraguariensis*). A chikusetsusaponin IVa é um inibidor de trombina, liga-se ao exosítio-1, mas também, diferente da GL, é capaz de modular o sítio ativo

❖ da enzima. Também inibe agregação plaquetária e apresenta atividade anti-trombótica *in vitro* e *in vivo*. **Artigo publicado no Journal of Medicinal Food 2012, 15 (12): 1073-1080.**

❖ **Improving the thrombin inhibitory activity of Glycyrrhizin, a triterpenic saponin, through a molecular simplification of the carbohydrate moiety.** Neste trabalho aliamos técnicas de modelagem molecular, dinâmica molecular e síntese orgânica para obter derivados sintéticos da GL com maior atividade inibitória frente à trombina. Entre os diferentes derivados obtidos, um éster-ftálico da GL apresentou atividade inibitória superior ao do composto original e seu arcabouço estrutural será utilizado para o desenho de uma nova classe de compostos anti-trombóticos e para testes em modelos animais de trombose. **Artigo aceito para publicação no Chemical Biology & Drug Design (*in press*). DOI: 10.1111/cbdd.12204.**

Esses trabalhos são apresentados na íntegra nas próximas páginas.

Susceptibility of *Loxosceles* sp. to the arthropod pathogenic fungus *Metarhizium anisopliae*: potential biocontrol of the brown spider

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Background: *Loxosceles* genus (brown spider) is an important pest with great impact on public health. Thus, more effective strategies for spider control are necessary.

Methods: Three isolates of *Metarhizium anisopliae* fungus were tested for the control of *Loxosceles* sp.

Results: *Metarhizium anisopliae* isolate E6 was highly virulent to the *Loxosceles* sp. spider, causing 100% mortality at 10^9 conidia/ml after 12 days and 9 days for juvenile and adult spiders, respectively.

Conclusions: This is the first report of the pathogenicity of *M. anisopliae* against a venomous arthropod. This fungus could offer an interesting alternative to reduce loxoscelism in future biocontrol strategies.

Keywords: *Loxosceles* sp., Loxoscelism, Biological control, *Metarhizium anisopliae*

Introduction

Bites of brown spiders (*Loxosceles* spp.) cause a severe clinical profile associated with dermonecrotic lesions and systemic manifestations that may be intense and occasionally fatal.[1] These spiders have a worldwide distribution, and accidents have been described mainly in developing countries. Some urban regions around the world, such as the metropolitan area of Curitiba city, Brazil, have thousands of cases of loxoscelism (accidents caused by spiders of the genus *Loxosceles*) reported per year, and it is an important health problem with great economic impact on the public health system.[1] Population control strategies for these and other spiders are unspecific and are mostly based on the use of insecticides. In this scenario, re-infestation is a continuous problem and the toxic side effects of indiscriminate use of insecticides for humans have to be considered. Thus, alternative strategies that are more effective to control brown spider populations and the consequent occurrences of loxoscelism have recently gained considerable importance.

The fungus *Metarhizium anisopliae*, a biocontrol agent that is an arthropod pathogen but is not infectious to humans, has been applied safely for several decades in the control of agricultural pests and other arthropods of medical relevance.[2–4] In this work, the susceptibility of the brown spider (*Loxosceles* sp.) to *M. anisopliae* is presented.

Materials and methods

Metarhizium anisopliae isolate E6 (from the hemipteran insect *Deois flavopicta*, Espírito Santo, Brazil, 1981), isolate Nordeste (from the hemipteran insect *Mahanarva posticata*, Pernambuco, Brazil, 1969) and isolate AL (from *M. posticata*, Alagoas state, Brazil, 1995) were maintained in the laboratory (Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil). Conidia production and conidial suspensions were performed as described previously.[5]

Loxosceles sp. spiders were collected on different days (during December 2010–March 2011) from a highly infested and inhabited house in the city of Viamão, RS, Brazil, with the owner's authorisation and only for the bioassays. Spiders were exposed before assay to dead ants (*Atta* sp.) as a food source and were maintained for ≥20 days at 28 °C for observation of their health condition. Spiders were maintained individually in 50 ml plastic tubes covered with screen tissues with free access to sterile water through wet cotton flakes at the bottom of tubes before the bioassay procedure.

For bioassays, 10 adult and 10 juvenile *Loxosceles* sp. individuals were used per treatment. *Metarhizium anisopliae* conidial suspension (2 ml) was applied through sprinkling to cover the total body of the spider (at three concentrations of 10^7, 10^8 or 10^9 conidia/ml). Following fungal inoculation, the spiders were individually placed in tubes covered with screen tissues at 28 °C and >90% relative humidity in a biochemical oxygen demand incubator. Controls consisted of *Loxosceles* sp. spiders treated with sterile distilled water instead of the conidial suspension. Experiments were carried out in three replicates and spiders were observed daily to determine survival. Bioassays were evaluated for 25 days. Statistical analysis, including analysis of Probit for dependent results, was conducted to obtain the lethal concentration (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}) for 50% and 90% of the spiders, respectively, using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1. Cumulative mortality, and lethal times (LT$_{50/90}$) and lethal concentrations (LC$_{50/90}$) in days following immersion of juvenile and adult *Loxosceles* sp. isolates in an aqueous suspension of *Metarhizium anisopliae* conidia

Isolate	Concentration (conidia/ml)	Cumulative mortality (%)[a]		LT$_{50}$ (95% CI) (days)		LT$_{90}$ (95% CI) (days)		LC$_{50}$ (95% CI) (conidia/ml)[a]		LC$_{90}$ (95% CI) (conidia/ml)[a]	
		Juvenile	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult	Juvenile	Adult
E6	10^7	20.3±9.5	80.7±8.1	ND	16.9 (16.3–17.5)	ND	25.0 (23.9–26.3)	4.9×10^7 ($4.3 \times 10^7 - 5.6 \times 10^7$)	8.9×10^6 ($8.2 \times 10^6 - 9.4 \times 10^6$)	1.2×10^8 ($1.1 \times 10^8 - 1.4 \times 10^8$)	2.0×10^7 ($1.5 \times 10^7 - 2.3 \times 10^7$)
	10^8	80.1±7.4	100±1.5	16.4 (15.5–17.5)	9.9 (9.4–10.4)	25.7 (24.9–26.1)	13.3 (12.6–14.2)				
	10^9	100	100	8.3 (8.0–8.6)	6.4 (6.1–6.7)	10.2 (9.8–10.7)	8.1 (7.7–8.6)				
Nordeste	10^7	15.6±7.4	20.6±9.2	ND	ND	ND	ND	6.3×10^7 ($5.6 \times 10^7 - 6.8 \times 10^7$)	2.4×10^7 ($2.1 \times 10^7 - 2.7 \times 10^7$)	1.6×10^8 ($1.3 \times 10^8 - 2.3 \times 10^8$)	4.5×10^7 ($4.1 \times 10^7 - 4.9 \times 10^7$)
	10^8	65.6±10.1	100±2.4	19.8 (19.5–20.1)	17.8 (17.6–18.0)	25.9 (25.3–26.5)	21.7 (21.4–22.0)				
	10^9	100±2.1	100	12.9 (11.7–13.2)	9.7 (8.6–10.8)	16.6 (16.3–17.0)	11.5 (10.3–12.7)				
AL	10^7	15.3±6.6	10.6±1.8	ND	ND	ND	ND	$> 5 \times 10^9$	7.2×10^7 ($7.0 \times 10^7 - 7.6 \times 10^7$)	$> 5 \times 10^9$	2×10^9 ($1.8 \times 10^9 - 2.4 \times 10^9$)
	10^8	35.6±8.8	50.3±12.5	ND	24.5 (24.2–24.9)	ND	25.5 (24.9–26.2)				
	10^9	45.9±11.6	70.6±15.3	ND	19.4 (18.1–20.8)	ND	24.5 (24.2–24.9)				

ND: not determined (mortality <50%).
[a] 25 days after treatment.

Results

Comparing three *M. anisopliae* isolates (E6, Nordeste and AL), it was observed that isolate E6 caused 100% mortality following application of 10^9 conidia/ml after 12 days and 9 days of treatment for juvenile and adult spiders, respectively, being considered highly virulent to *Loxosceles* sp. (Table 1). Isolate Nordeste showed lower virulence (100% mortality in 19 days for juveniles and 13 days for adults at 10^9 conidia/ml), whilst isolate AL was not virulent at all concentrations tested. For all isolates tested, a concentration of 10^7 conidia/ml caused a low mortality rate (Table 1). Interestingly, the most effective isolate (E6) also caused 100% mortality for adult spiders at a concentration of 10^8 conidia/ml (15 days). In all experiments, the survival rate in the brown spider control group was 100%. All dead spiders infected with *M. anisopliae* developed intense surface mycosis (Supplementary Figure 1). No fungal growth was observed in the control spiders.

Adults were more susceptible to fungal infection compared with juvenile spiders, as evidenced by the LT$_{50}$ and LT$_{90}$ values. In some cases, especially for isolate AL, mortality values were <50% at the end of experiment; thus, LT$_{50}$ and LT$_{90}$ values could not be calculated. LC$_{50}$ values on day 25 post treatment for the most infective *M. anisopliae* isolate (E6) were 4.9×10^7 and 8.9×10^6 conidia/ml for juvenile and adult spiders, respectively (Table 1).

Discussion

To our knowledge, this is the first study evaluating the effect of a fungus to control a venomous spider of medical relevance. We have shown in vitro that the fungus *M. anisopliae* can be used to control juvenile and adult *Loxosceles* sp. spiders, especially fungal isolate E6. Biocontrol strategies with *M. anisopliae* are considered of low impact in nature because this fungus is specific to its arthropod target host.[4] Moreover, use of biocontrol agents decreases the application of chemical pesticides, which greatly impact on the environment, humans and animals.

Metarhizium anisopliae appears to be a promising alternative to unspecific chemical insecticides in use owing to its verified efficacy in *Loxosceles* sp. infection and control. The potential use of this fungus might be its application inside homes in specific sites associated with the brown spider's lifestyle, such as basements, garages, behind and under furniture, and baseboards. It could also be a good alternative for highly infested areas in abandoned constructions, wastelands, warehouses, deposits and sheds. *Metarhizium anisopliae* can be applied directly in the field or at specific sites through pulverisation. Another way to apply the fungus in biocontrol in domestic areas might be the use of dry conidia associated with any kind of spider attractant, allowing contact of conidia onto the spider's body, like a spider trap.

Development of specific strategies based on the use of *M. anisopliae* conidia and treatment of highly infested sites by *Loxosceles* sp. with a conidial suspension could be an alternative for spider population control, consequently reducing loxoscelism in Brazil and other countries.

Supplementary data

Supplementary data are available at *Transactions* Online (http://trstmh.oxfordjournals.org/).

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Antithrombotic Effect of Chikusetsusaponin IVa Isolated from *Ilex paraguariensis* (Maté)

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ABSTRACT   The triterpene chikusetsusaponin IVa was isolated from the fruit of *Ilex paraguariensis*. Using biochemical and pharmacological methods, we demonstrated that chikusetsusaponin IVa (1) prolongs the recalcification time, prothrombin time, activated partial thromboplastin time, and thrombin time of normal human plasma in a dose-dependent manner, (2) inhibits the amidolytic activity of thrombin and factor Xa upon synthetic substrates S2238 and S2222, (3) inhibits thrombin-induced fibrinogen clotting (50% inhibition concentration, $199.4 \pm 9.1\,\mu M$), and (4) inhibits thrombin- and collagen-induced platelet aggregation. The results also indicate that chikusetsusaponin IVa preferentially inhibits thrombin in a competitive manner ($K_i = 219.6\,\mu M$). Furthermore, when administered intravenously to rats, chikusetsusaponin IVa inhibited thrombus formation in a stasis model of venous thrombosis, although it did not induce a significant bleeding effect. Chikusetsusaponin IVa also prolonged the *ex vivo* activated partial thromboplastin time. Altogether, these data suggest that chikusetsusaponin IVa exerts antithrombotic effects, including minor hemorrhagic events. This appears to be important for the development of new therapeutic agents.

KEY WORDS: ● *chikusetsusaponin* ● *hemostasis* ● Ilex paraguariensis ● *platelet aggregation* ● *thrombosis* ● *thrombin*

INTRODUCTION

THROMBOSIS AND CARDIOVASCULAR diseases are the major causes of morbidity and mortality in the Western world and frequently involve a prothrombotic state such as myocardial infarction, stroke, and atherosclerosis.[1] Anticoagulant and antithrombotic agents, such as heparin, vitamin K antagonists, and acetylsalicylic acid, are currently used to treat and prevent these thromboembolic events.[2] Considering the high incidence of side effects (*e.g.*, gastrointestinal bleeding and thrombocytopenia), important efforts have been made to find new antithrombotic substances.[2–5]

In the context of hemostasis, glycyrrhizin [3-*O*-(2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl)-18β-glycyrrhetinic acid (GL)] (Fig. 1), a triterpenoid saponin obtained from the roots of *Glycyrrhiza glabra*, presented anticoagulant and antithrombotic effects. GL prolonged the *in vitro* coagulation time and inhibited the thrombin-induced

clotting of plasma and fibrinogen.[6] In addition, the GL antithrombotic effect was also observed in animal models of thrombosis.[7]

Ilex paraguariensis, a South American tree of the Aquifoliaceae family, is rich in saponins (approximately 10%).[8] Dried leaves and twigs from this tree are used to prepare a tea known as *maté* that is commonly consumed in Brazil, Uruguay, Paraguay, and Argentina. The saponins isolated from its leaves are glycosides derived mainly from ursolic and oleanolic acids.[8] Other triterpenoid glycosides were isolated from the fruits of *I. paraguariensis*.[9] Pentacyclic triterpenoids such as ursolic and oleanolic acids and their derivatives possess pharmacological properties, such as anti-human immunodeficiency virus, hepatoprotective, anti-inflammatory, antimalarial, antiglycation, and cytotoxic effects,[10–12] suggesting their potential use for the design of new bioactive compounds. In this sense, the triterpenoid chikusetsusaponins (identified by numbers I–IV) possess several pharmacological activities such as anti-inflammatory,[13] antiviral,[14] fibrinolytic,[15] and anticancer[16–18] properties. In particular, chikusetsusaponin IVa has also been isolated from other species of *Ilex*, such as *Ilex dumosa*,[19] *Ilex pubescens*,[13] and *Ilex rotunda*.[20]

In the present study, we describe the *in vitro* and *in vivo* anticoagulant and antiplatelet activity of chikusetsusaponin IVa (Fig. 1).

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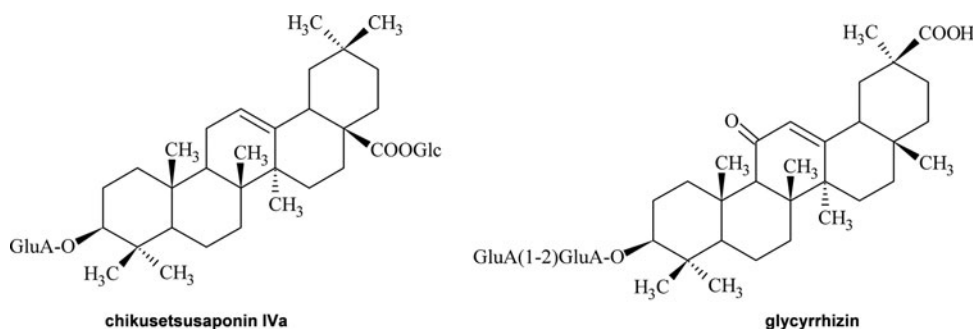
FIG. 1. Chemical structures of chikusetsusaponin IVa and glycyrrhizin. Glc, glucose; GluA, glucuronic acid.

MATERIALS AND METHODS

Drugs and reagents

Bovine fibrinogen, human factor Xa, bovine type I collagen, ADP, and GL were purchased from Sigma-Aldrich (St. Louis, MO, USA). Arachidonic acid was purchased from Chrono-Log Co. (Havertown, PA, USA). Human α-thrombin was purified from plasma of healthy volunteer donors according to the precedure of Ngai and Chang.[21] Synthetic substrates for thrombin (S2238 [H-D-Phe-Pip-Arg-p-nitroanilide]) and factor Xa (S2222 [Benzyl-Ile-Glu-Gly-Arg-p-nitroanilide]) were purchased from Chromogenix (Milan, Italy). Anasedan® (xylazine) and Dopalen® (ketamine) were purchased from Cristália Produtos Químicos Farmacêuticos (São Paulo, Brazil). Calcium thromboplastin was purchased from Wiener Lab (Rosario, Argentina).

Apparatus

Mass spectrometry experiments were carried out using a Micromass/Waters (Milford, MA, USA) quadrupole-time of flight mass spectrometer microequipped with a nano-electrospray ionization source. Data were analyzed with the Waters MassLynx software. [1]H and ^{13}C nuclear magnetic resonance were recorded on a Varian (Palo Alto, CA, USA) Inova spectrometer (300 MHz) in CD_3OD. The spectrophotometer used was a SpectraMax (Molecular Devices Co., Sunnyvale, CA, USA), equipped with temperature control and shaking systems.

Isolation and structural elucidation of saponin

Fruits of *I. paraguariensis* A. St. Hil. were harvested in a cultivated area in Rio Grande do Sul, Brazil. A voucher specimen was deposited at the Herbarium of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, as voucher number ICN/UFRGS (163413). The hydro-ethanolic extract was prepared using 10 g of dried and powdered fruits in 40% ethanol by maceration (1:10, plant:solvent). After evaporation of the solvent, the extract was subjected to chromatography on a silica gel column (Merck, Darmstadt, Germany) using gradient mixtures of chloroform:ethanol:water. Fractions were collected and pooled according to thin-layer chromatography (silica gel GF$_{254}$; chloroform:ethanol:water, 40:40:5 by volume). Then

pooled fractions were subjected to further purification, separately, using Lichroprep® (Merck; particle size, 40–63 μm) and gradient mixtures of water:ethanol as eluate. Pure saponin was obtained (90 mg), corresponding to 2% of the dried ethanolic extract.

Chikusetsusaponin IVa

The identification of saponin in its native form was mostly deduced from a combination of mass spectrometry and nuclear magnetic resonance spectroscopy. This combination allowed the identification of an already known saponin named chikusetsusaponin IVa, previously isolated by us from leaves of *I. dumosa*.[19] It was obtained as an amorphous powder; its main peak in the mass spectrum was *m/z* 817.4354 Da, corresponding to the chemical species $[(C_{42}H_{66}O_{14})\,Na]^+$ (expected *m/z*, 817.4350 Da), together with the peak *m/z* 833.4175 Da $[(C_{42}H_{66}O_{14})\,K]^+$ (Fig. 2). [1]H and ^{13}C nuclear magnetic resonance (300 MHz, CD_3OD) data were similar to those previously described.[19]

Effects of chikusetsusaponin IVa on hemostatic system: in vitro *studies*

Coagulation assays. The anticoagulant activity of chikusetsusaponin IVa (0–2000 μM) was verified by the determination of the following coagulation parameters in plasma: recalcification time (RT), activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT). Anticoagulant experiments were performed using commercial kits following the manufacturer's instructions (Wiener Lab). Human venous blood was collected from healthy volunteer donors in 1:10 (vol/vol) 3.8% trisodium citrate and centrifuged at 1500 *g* for 10 min to obtain plasma. Assays were conducted using a 96-well microplate SpectraMax as described.[22] Results are expressed as the coagulation time (in seconds) and represent the mean ± SEM values of three independent experiments performed in quadruplicates.

Amidolytic assay. Saponin (0–2650 μM) was incubated with thrombin (2 μg/mL) or factor Xa (3.4 μg/mL) in 20 mM Tris-HCl (pH 7.5) for 20 min at 37°C. Enzymatic reactions were started by addition of the chromogenic substrate S2238 (0.2 mM) or S2222 (0.2 mM) in a volume of 100 μL for the determination of thrombin and factor Xa activities,
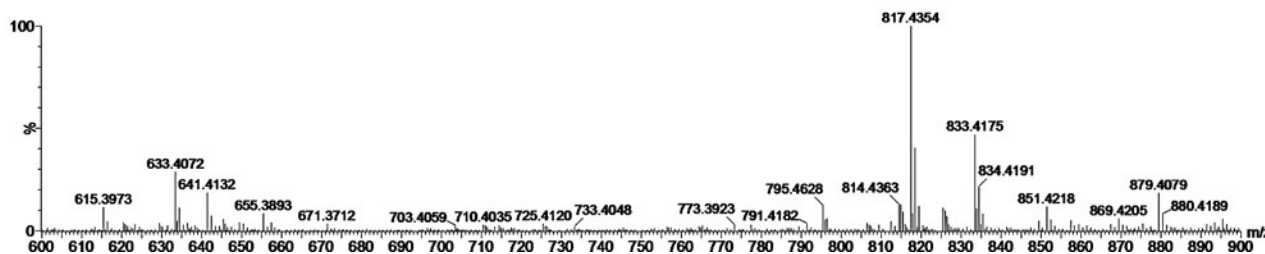
FIG. 2. Mass spectrum of chikusetsusaponin IVa using a Micromass/Waters quadrupole-time of flight (TOF) mass spectrometer (MS) equipped with a nano-electrospray (ES) ionization source.

respectively. The amount of *p*-nitroaniline produced was monitored at 405 nm in intervals of 14 s for 30 min using a SpectraMax spectrophotometer. The initial rate of hydrolysis (expressed as milli-optical density units per minute) was used to calculate the percentage of thrombin inhibition. These data were plotted versus the concentration of inhibitor, and a nonlinear regression analysis was performed to calculate the values for concentration causing 50% inhibition (IC_{50}) values. For kinetic analysis, saponin (0–450 μM) was incubated with thrombin prior to the addition of S2238 (50 and 100 μM). Data from the initial rate, expressed as (μM *p*-nitroaniline generated/min)$^{-1}$, was plotted versus the inhibitor concentration to construct a Dixon's plot. The curves were analyzed by linear regression to calculate the inhibition constant (K_i) value and to determine the type of inhibition.[23] Results are presented as mean±SEM values ($n=3$).

Thrombin-induced fibrinogen clotting. Chikusetsusaponin IVa (0–900 μM) and GL (0–1100 μM) were solubilized in 20 mM Tris-HCl (pH 7.5) and incubated with thrombin (2 μg/mL) for 10 min at 37°C. Reactions were initiated by the addition of fibrinogen (2 mg/mL) in a volume of 100 μL. The kinetics of fibrin formation were measured at 14-s intervals for 30 min at 650 nm using a SpectraMax spectrophotometer. The initial rate of clotting formation (expressed as milli-optical density per minute) was used to calculate the percentage of the thrombin inhibition. These data were plotted versus the concentration of inhibitor, and a nonlinear regression analysis was performed to calculate the IC_{50} (mean±SEM, $n=3$).

Platelet aggregation. The platelet function was measured by the *in vitro* photometric method,[24] using a SpectraMax spectrophotometer. Whole human blood was collected from healthy volunteer donors. Washed human platelets were then prepared by gel filtration in a Sepharose 2B column.[25] Platelet concentration was adjusted to 350,000 cells/μL in a Neubauer chamber. Platelet aggregation was measured by the decreased rate in washed human platelet optical density in a final volume of 150 μL. The agonists used were as follows: ADP (10 μM), collagen (2.5 μg/mL), arachidonic acid (10 μM), or human thrombin (3 μg/mL). Saponin (0–1000 μM) was preincubated with washed human

platelets for 20 min before the addition of agonist. The results are expressed as the percentage area under curves (mean±SEM, $n=4$).

Effects of chikusetsusaponin IVa saponin on hemostatic system: In vivo *studies*

Animals. Male Wistar rats (weighing 300–350 g) were housed in a temperature-controlled room (21–25°C, in a 12-h light/dark cycle) with free access to water and food. All procedures involving animals were carried out in accordance with the guiding principles of the International Society of Toxicology and the Brazilian College of Animal Experimentation. The experimental protocol was approved by the Ethical Committee on Research Animal Care of the Federal University of Rio Grande do Sul, Brazil (protocol number 2008177/2009).

In vivo *model of deep venous thrombosis.* A rat thrombosis model that is a combination of stasis and hypercoagulability induced by a tissue factor-rich component was used according to the literature[26] with minor modifications.[27] In brief, rats were anesthetized with xylazine (16 mg/kg, i.p.) followed by ketamine (100 mg/kg, i.p.). The abdomen was surgically opened, and the vena cava was exposed and dissected free from surrounding tissues. Subsequently, rats received the following treatments via the left femoral vein (final volume of 0.7 mL): (1) saline, $n=4$; (2) chikusetsusaponin IVa at 15 and 50 mg/kg, $n=4$ per dose; or (3) GL at 15 and 50 mg/kg, $n=4$ per dose. After 3 min, calcium thromboplastin (3 mg/kg) was injected into the vena cava, and stasis was immediately established by the ligation of caudal vena cava (above the insertion point of the right renal vein). The distal ligations of the vena cava, left renal vein, and other major tributaries were conducted 20 min after thromboplastin administration. The isolated segment of caudal vena cava was removed, and the thrombus was separated, rinsed with saline (at 37°C), dried on a filter paper at 60°C (1 h), and weighed. The ratio value of thrombus/rat weight was used for data comparison.

Bleeding effect. Rats were anesthetized as described above, and the following treatments were administered via the left femoral vein (final volume of 0.7 mL): (1) saline,

$n=4$; (2) chikusetsusaponin IVa at 15 and 50 mg/kg, $n=4$ per dose; or (3) GL at 15 and 50 mg/kg, $n=4$ per dose. After 5 min, the rat's tail was cut off 3 mm from the tip and carefully immersed in 40 mL of distilled water at room temperature. Blood loss was evaluated 60 min later as a function of hemoglobin concentration in aqueous solution.[7] Hemoglobin concentration was determined at 540 nm using a standard curve made with hemoglobin. The hemoglobin content in the water of saline-treated animals was used as a control.

Ex vivo *determination of aPTT and hemoglobin.* Animals were anesthetized as described above, and the following treatments were administered via the left femoral vein (final volume of 0.7 mL): (1) saline, $n=4$; (2) chikusetsusaponin IVa at 15 and 50 mg/kg, $n=4$ per dose; or (3) GL at 15 and 50 mg/kg, $n=4$ per dose. Blood samples were collected from the right femoral vein (0.3 mL in 3.8% trisodium citrate, 1:10 [vol/vol]) at different times post-treatment. Each blood sample was centrifuged, and plasma was used to measure the aPTT as described above. To estimate the hemolytic potential of saponins, hemoglobin content was measured in plasma samples of animals treated with 50 mg/kg chikusetsusaponin IVa or GL. The concentration of free plasma hemoglobin was measured with a kit commercially available following the manufacturer's instructions (Labtest SA, Lagoa Santa, MG, Brazil).

Statistical analysis

Results are expressed as mean ± SEM values, and significance was determined by Student's *t* test. When more than two groups were compared, an analysis of variance was used followed by a Bonferroni's test to compare pairs of means. The data were considered significant with a probability of < .05.

RESULTS

Effects of chikusetsusaponin IVa on hemostatic system: in vitro *studies*

Chikusetsusaponin IVa showed anticoagulation effects by elongating the time for RT, aPTT, PT, and TT in a dose-dependent manner (Table 1). At 500 μM, a significant inhibition was observed in PT and TT. At 1000 μM, the saponin caused an increase of 1.3-fold in RT, 2.3-fold in aPTT, 1.8-fold in PT, and 1.9-fold in TT compared with respective control values (Table 1). In relation to the hydrolysis of synthetic substrates, chikusetsusaponin IVa inhibited, in a dose-dependent manner, the enzymes tested (S2238 and S2222) with IC$_{50}$ values of 384.2 ± 44.2 μM for thrombin-catalyzed hydrolysis (Fig. 3A) and 1585.7 ± 96.9 μM for factor Xa-catalyzed hydrolysis (Fig. 3B). Enzyme kinetic studies indicated that chikusetsusaponin IVa inhibited the thrombin catalytic hydrolysis of S2238 in a competitive manner with a K_i value of 219.6 ± 32 μM (Fig. 3C).

TABLE 1. EFFECTS OF CHIKUSETSUSAPONIN IVa ON BLOOD COAGULATION PARAMETERS *IN VITRO*

Saponin (μM)	Coagulation time (s)			
	RT	aPTT	PT	TT
0	385.6 ± 9.6	55.7 ± 2.7	17.3 ± 1.6	167.5 ± 7.3
500	374.6 ± 3.1	81.6 ± 2.3	25.5 ± 0.9*	240.7 ± 4.5*
1000	485.7 ± 6.6***	126.5 ± 11.4**	31.7 ± 2.4***	330.1 ± 6.3***
1600	555.5 ± 4.8***	139.6 ± 3.6**	40.0 ± 1.5***	412.2 ± 27.8***
2000	840.6 ± 25.0***	158.3 ± 18.6***	53.3 ± 1.2***	428.9 ± 1.0***

After incubation of chikusetsusaponin IVa with human plasma, the following coagulation parameters were determined: recalcification time (RT), activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT). Data are mean ± SEM values (in s).

*$P<.05$, **$P<.01$, ***$P<.001$, statistically significant difference compared with coagulation time measured in the absence of saponin (control).

As shown in Figure 4, chikusetsusaponin IVa inhibited the thrombin-induced fibrinogen clotting in a dose-dependent manner with an IC$_{50}$ value of 199.4 ± 9.1 μM. This inhibitory activity was similar to that obtained for GL (235.7 ± 1.4 μM; Fig. 4). In relation to the platelet aggregation, the saponin had no effect on ADP or on arachidonic acid-induced aggregation (Fig. 5). However, it significantly inhibited collagen- and thrombin-induced platelet aggregation in a dose-dependent manner. The calculated IC$_{50}$ values for collagen- and thrombin-induced platelet aggregation were 482 and 190 μM, respectively.

Effects of chikusetsusaponin IVa on hemostatic system: in vivo *studies*

As shown in Figure 6A, chikusetsusaponin IVa reduced the size of the thrombus by 56.2% and 91.2% at doses of 15 and 50 mg/kg, respectively. In comparison, GL was able to reduce the thrombus only 21.2% and 53.1% at doses of 15 and 50 mg/kg, respectively (Fig. 6A). In addition, chikusetsusaponin IVa did not induce a significant bleeding effect after intravascular administration (Fig. 6B). However, GL caused an increase in blood loss of approximately 1.8-fold compared with the control at the dose of 50 mg/kg (Fig. 6B).

The anticoagulant effect of chikusetsusaponin IVa observed *in vitro* was confirmed by the measurement of aPTT *ex vivo*. Compared with the control, chikusetsusaponin IVa at 50 mg/kg was responsible for a significant prolongation of aPTT at different times following intravascular administration (Table 2). A similar result was observed after the injection of GL at 50 mg/kg (Table 2). To verify the hemolytic potential of chikusetsusaponin IVa and GL, we measured the hemoglobin content in the plasma of animals previously treated with saponins. No signs of hemolysis were observed in the plasma of animals treated with 50 mg/kg GL (control, 43.21 ± 8.2 mg/dL; treated, 43.35 ± 4.5 mg/dL) or chikusetsusaponin IVa (control, 42.11 ± 5.6 mg/dL; treated, 40.75 ± 4.7 mg/dL).
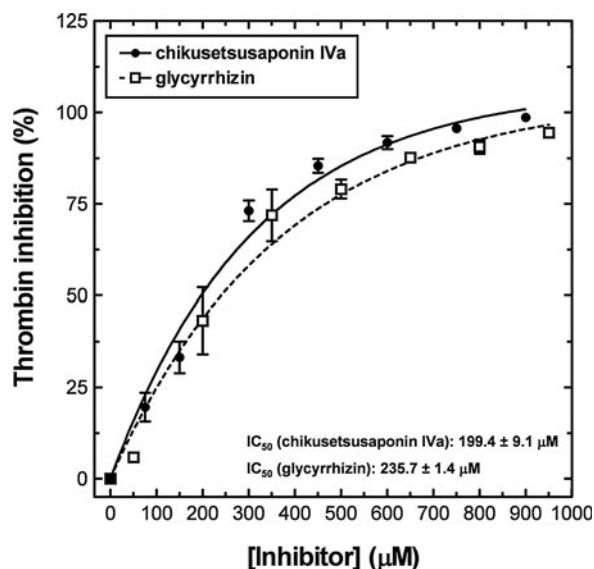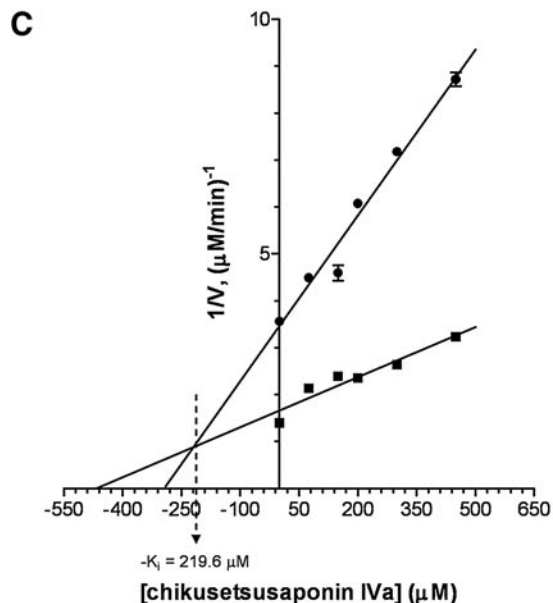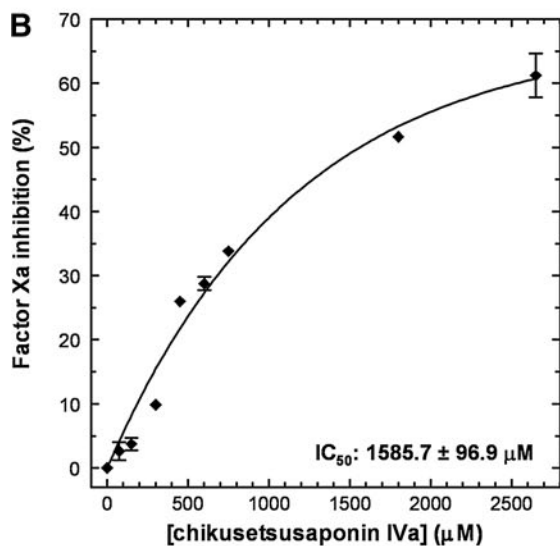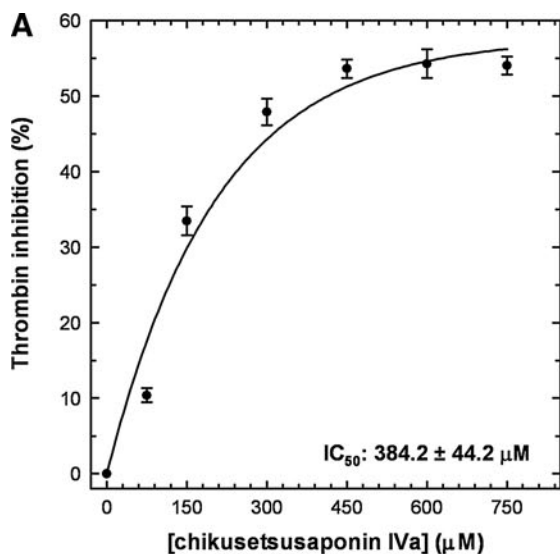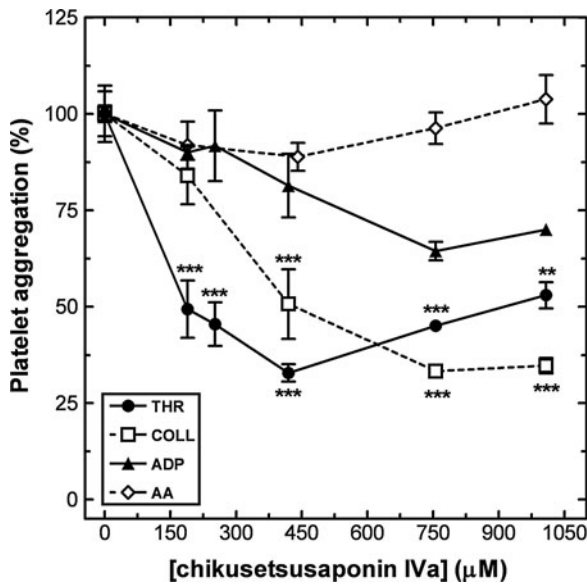
FIG. 4. Effects of chikusetsusaponin IVa and glycyrrhizin on thrombin-induced fibrinogen clotting. Increasing concentrations of chikusetsusaponin IVa or glycyrrhizin were incubated with human thrombin, followed by the addition of fibrinogen. The kinetics of fibrin formation were monitored at 650 nm as described in Materials and Methods. Data are mean ± SEM values of three independent determinations.

DISCUSSION

Herein, the anticoagulant, antiplatelet, and *in vivo* antithrombotic activities of chikusetsusaponin IVa were described for the first time. When tested *in vitro*, chikusetsusaponin IVa caused a dose-dependent prolongation of RT, aPTT, and PT, suggesting that the major targets for this compound were factors Xa, Va, and thrombin. Additionally, chikusetsusaponin IVa was able to inhibit the TT, indicating thrombin as a potential target for this saponin in plasma. A dose-dependent inhibition of thrombin- and factor Xa-catalyzed hydrolysis of specific synthetic substrates (S2238 and S2222, respectively) was observed after incubation with chikusetsusaponin IVa. This observation confirms that the main targets of this saponin are common coagulation factors. Moreover, the IC_{50} value for factor Xa inhibition was four times higher than that for thrombin inhibition, indicating a preference for thrombin over factor Xa. We demonstrated that chikusetsusaponin IVa is a competitive inhibitor of thrombin, similar to other thrombin

FIG. 3. Effects of chikusetsusaponin IVa on thrombin and factor Xa amidolytic activities. Data are mean ± SEM values of three independent determinations. IC_{50}, concentration causing 50% inhibition. **(A, B)** Effects of increasing concentrations of chikusetsusaponin IVa on the activity of **(A)** human thrombin upon synthetic substrate S2238 and **(B)** human factor Xa upon synthetic substrate S2222. **(C)** Dixon's plot for the inhibitory pattern of chikusetsusaponin IVa on thrombin upon S2238 hydrolysis. Saponin at different concentrations was incubated with thrombin prior to the addition of 50 μM (●) and 100 μM (■) S2238. The K_i value obtained from the graph is indicated.

FIG. 5. Effects of chikusetsusaponin IVa on platelet aggregation. Increasing concentrations of chikusetsusaponin IVa were incubated with washed human platelets. Platelet aggregation was triggered by the addition of the following agonists: thrombin (THR), collagen (COLL), ADP, or arachidonic acid (AA). Data are mean ± SEM values of four independent determinations. $**P < .01$, $***P < .001$, statistically significant difference compared with platelet aggregation in the absence of inhibitor.

TABLE 2. *Ex Vivo* ANTICOAGULATION EFFECT OF CHIKUSETSUSAPONIN IVa

Treatment, dose (mg/kg)	aPTT (s)			
	5 min	15 min	30 min	60 min
Saline	17.2 ± 0.2	16.8 ± 0.2	16.8 ± 0.6	17.4 ± 0.6
Glycyrrhizin				
15	17 ± 0.2	17.7 ± 0.4	17.2 ± 0.3	16.8 ± 0.5
50	16.6 ± 0.2	19.6 ± 1.0	20.4 ± 0.7*	25 ± 0.3***
Chikusetsusaponin IVa				
15	18.6 ± 0.9	17.6 ± 0.6	19.1 ± 0.8	14.8 ± 1.1
50	20.5 ± 0.3***	24 ± 0.6***	21.3 ± 1.4*	20.4 ± 0.2*

Ex vivo aPTT was measured in plasma of rats 5, 15, 30 and 60 min after intravenous injection of saline (control), glycyrrhizin, or chikusetsusaponin IVa. Data are mean ± SEM values (in s).

$*P < .05$, $***P < .001$, statistically significant difference compared with coagulation time of the control group.

inhibitors such as the thrombin receptor peptide or glycoprotein GPIb that are known to act in the micromolar range.[28,29]

Chikusetsusaponin IVa also inhibited thrombin-induced fibrinogen clotting activity with an IC_{50} that was two times

lower than that for the thrombin-catalyzed hydrolysis of S2238. These values indicate that chikusetsusaponin IVa most likely interacts with sites in the thrombin molecule other than the catalytic site because a more potent inhibition was observed when the macromolecular substrate fibrinogen was used. Chikusetsusaponin IVa has an *in vitro* antiplatelet effect when induced by thrombin and collagen as well as an *in vivo* antithrombotic property. In comparison with GL, chikusetsusaponin IVa had a significantly more potent antithrombotic effect at the doses of 15 and 50 mg/kg. In addition, chikusetsusaponin IVa had no bleeding effect, which may be considered an advantage compared with known antithrombotic agents such as hirudin, argatroban, heparin, or acetylsalicylic acid.[30] Furthermore, intravenous injection of chikusetsusaponin IVa
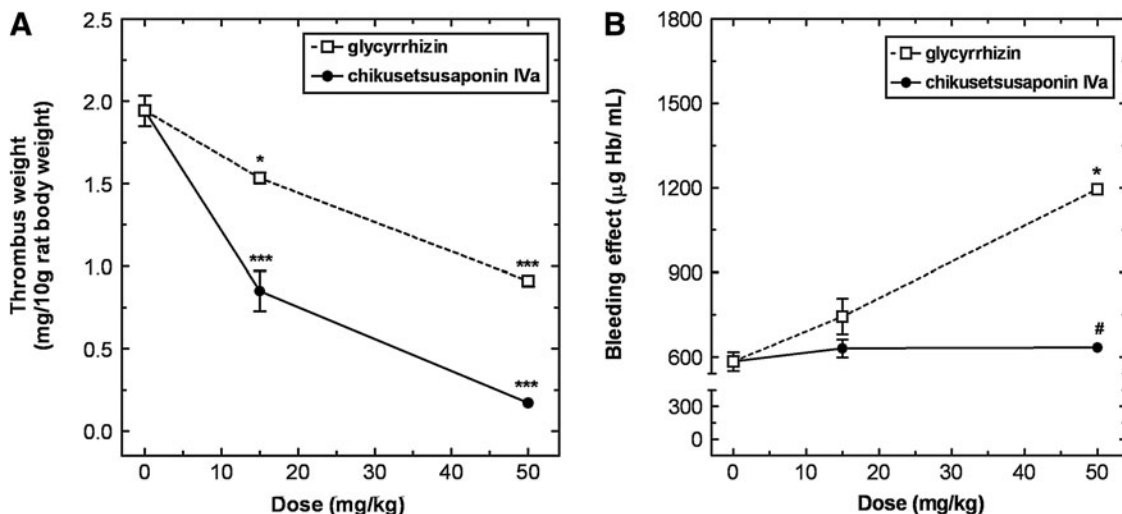
FIG. 6. *In vivo* antithrombotic and bleeding effects of chikusetsusaponin IVa and glycyrrhizin. **(A)** The venous thrombosis model. Saponins at the indicated doses were administered intravenously to rats 3 min before the induction of thrombosis by thromboplastin combined with stasis as described in Materials and Methods. Data are mean ± SEM values of four animals. $*P < .05$, $***P < .001$, statistically significant difference compared with animals that received saline instead of saponins. **(B)** The bleeding effect. Rats were injected intravenously with different doses of chikusetsusaponin IVa or glycyrrhizin. Five minutes after injection, the rat's tail was cut off and carefully immersed in distilled water. Blood loss was evaluated 60 min later by measuring the hemoglobin (Hb) concentration in aqueous solution. Data are mean ± SEM values of four animals. Statistically significant differences ($P < .001$) are indicated for comparison with *animals that received only saline solution and #animals that received 50 mg/kg glycyrrhizin.

or GL had no toxic hemolytic effect in treated animals and caused only a slight increase in *ex vivo* aPTT. Moreover, this saponin had a significant antithrombotic activity at a dose of 15 mg/kg without affecting the aPTT. Recently, it was reported that potent orally active factor Xa inhibitors such as darexaban and darexaban glucuronide are able to inhibit thrombus formation and prolong the coagulation time *in vivo* without inducing any bleeding effect.[31] It was reported[15] that chikusetsusaponins III, IV, and V increased the *in vitro* activity of urokinase, an important plasminogen activator during fibrinolysis. Possibly, the increase in fibrinolysis induced by chikusetsusaponins contributed to the antithrombotic and anticoagulant effects described in this research.

In conclusion, chikusetsusaponin IVa is a new plant-derived antithrombotic compound that increases the coagulation time and promotes antiplatelet and antithrombotic activity.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Improving the Thrombin Inhibitory Activity of Glycyrrhizin, a Triterpenic Saponin, Through a Molecular Simplification of the Carbohydrate Moiety

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Glycyrrhizin, a saponin, and its aglycone glycyrrhetinic acid are natural products found in the Liquorice (*Glycyrrhiza glabra* L.) root extract. This saponin is known for its *in vitro* and *in vivo* thrombin inhibitory activity. The design and synthesis of five glycyrrhizin derivatives were carried out to improve the natural product activity. Compound 3b, a phthalic ester derivative of glycyrrhizin, presented a more pronounced thrombin inhibition ($IC_{50} = 114.4 \pm 1.3~\mu M$) than the saponin ($IC_{50} = 235.7 \pm 1.4~\mu M$). Molecular docking simulations performed to investigate the molecular interaction between compound 3b and the enzyme indicate that this product is, as previously determined for glycyrrhizin, an allosteric thrombin inhibitor.

Pathologies associated with hypercoagulable states, including myocardial infarction, deep vein thrombosis and pulmonary embolism, are one of the most important causes of morbidity and mortality worldwide (1). In spite of the arise of several new synthetic agents in the recent years for treating such diseases, mainly direct thrombin and fXa inhibitors, concerns still exist for side-effects, specially bleeding (2,3).

In the recent years, the saponin glycyrrhizin (**1**) was found to be an *in vitro* and *in vivo* thrombin inhibitor, being the first plant-derived natural product with this activity reported in the literature (4,5). Additionally, the *in vitro* assays indicate that its observed thrombin inhibitory effect is due to the selective inhibition of the enzyme's anion-binding exosite 1 (3).

Glycyrrhizin (**1**) and its aglycone glycyrrhetinic acid (**2**) are natural products that are abundant in the Liquorice (*Glycyrrhiza glabra* L.) root extract. This saponin is used worldwide as a natural sweetener and employed in many Chinese, Indian and Tibetan traditional preparations. Both compounds are known in the literature for its wide range of biological activities, and they have been employed as scaffolds for the semisynthesis of several derivatives with interesting pharmacological proprieties (6,7). 'Stronger Neo-Minophagen C', a glycyrrhizin-containing i.v. formulation, has been used for more than 30 years in Japan for the treatment of chronic hepatitis diseases (8), being also marketed in China, Korea, Taiwan, Indonesia, India and Mongolia (9).

While synthetic derivatives of heparin analogues as fondaparinux are already in therapeutic use (1), such compounds are difficult and expensive to obtain, which has supported the simplification of the carbohydrate scaffold by other, non-saccharidic structures (10). In this context, the current work is focused on the structural simplification of the carbohydrate moiety of **1**, searching for potential new non-saccharidic scaffolds with improved antithrombotic activity.

Methods and Materials

Assuming that the carboxylic acid groups present at the carbohydrate units of **1** were crucial to the interaction with

the enzyme's basic exosite, we devised a simple and straightforward molecular simplification via the synthesis of hemi-esters employing the triterpene **2** as starting material (Scheme 1) and cyclic and acyclic anhydrides, leading to the esters **3a** (also known as carbenoxolone), **3b** and the 3-O-acetyl derivative **3c** (6,7). We also employed the classical Jones oxidation protocol (6,7) to obtain the ketone derivative (**4**). The reaction of **4** with hydroxylamine hydrochloride gave its respective oxime **5** in moderate-to-good yields (Scheme 1). Chemical purity and properties of all synthesized compounds (melting point, TLC profiles when available and ^{1}H- and ^{13}C-NMR) were compared with previous reports in the literature [**3a** and **3b** (10), **3c** (11), **4** (12) and **5** (13)].

Results and Discussion

Because no previous structure–activity relationship was available to guide compound **1** structural modifications, our first step was to evaluate the importance of the carbohydrate moiety on thrombin modulation. Accordingly, the saponin **1** and its aglycone **2** were tested in the thrombin-induced fibrinogen clotting assay. While **1** presented an $IC_{50} = 235.7 \pm 1.4$ μM, compound **2** was found to be inactive on thrombin-induced fibrinogen clotting assay (Figure 1), pointing to the importance of the carbohydrate moiety interactions with thrombin.

As previous works from our group described the binding of **1** to thrombin exosite 1, known for its basic properties, the carbohydrate moiety was substituted by acidic, hydrogen-bonding acceptor groups (Scheme 1) to evaluate (i) the influence of the substituent configurational entropy and flexibility (**3a** and **3b**), (ii) the role of the chemical function nature at C-3 upon removal of the carbohydrate residues (**4** and **5**) and (iii) the importance of the ester group at C-3 (**3a–c**).

Compounds **4** and **5** presented IC_{50} values >1000 μM in the thrombin-induced fibrinogen clotting assay, pointing to an absence of influence of the hydrogen bond characteristics
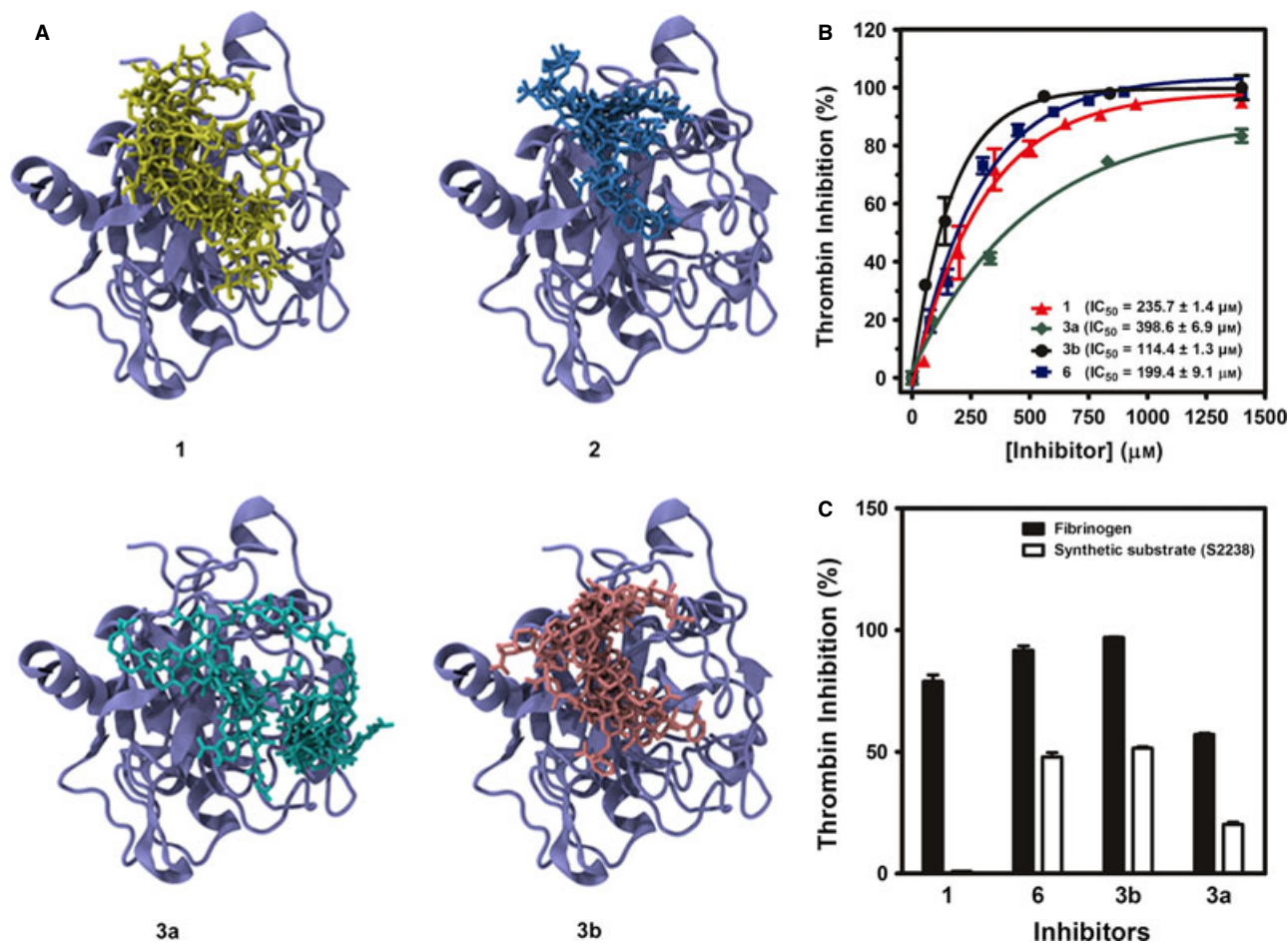
Figure 1: Structural and functional features of thrombin inhibition. (A) Conformational ensemble of the inhibitors at the anionic exosite 1, superimposed at every 10 ns of MD. (B) Inhibitory activity of compounds on thrombin-induced fibrinogen clotting. (C) Comparison of thrombin inhibitory activity using fibrinogen and the synthetic substrate S2238 (H-D-Phe-Pip-Arg-*p*-nitroanilide) as substrates. All inhibitors were tested at 500 μM.
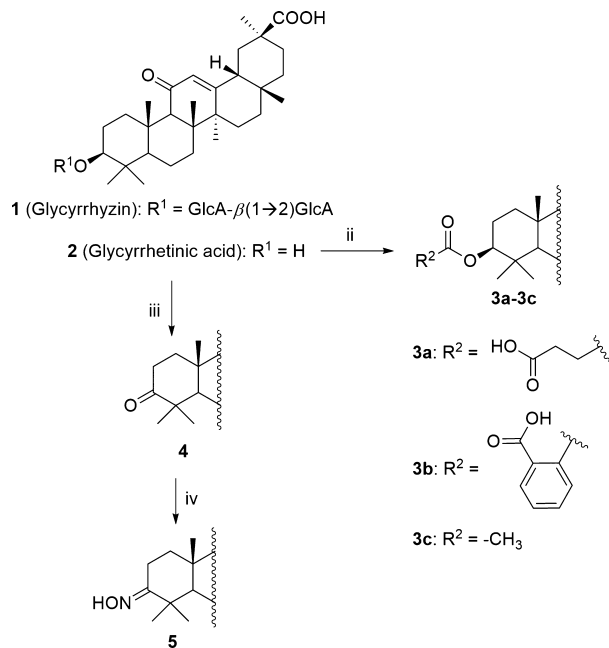
Scheme 1: Reagents and conditions: (i) anhydride (10 equiv.), pyridine, 24–48 h, reflux, 30–90%; (ii) Jones oxidation; (iii) hydroxylamine hydrochloride, pyridine, reflux, 24 h, 38%.

Table 1: Pharmacological data on thrombin inhibition by compounds **1**–**6**

Compound	Thrombin time (s)[a]	Thrombin IC$_{50}$ (μM)
1	86.3 $\pm$ 3[b]	235.7 $\pm$ 1.4
	191.4 $\pm$ 40.0[c]	
2	NT[d]	NA[e]
3a	62.6 $\pm$ 2.1[b]	392.6 $\pm$ 6.9
	107.0 $\pm$ 15.4[c]	
3b	118.3 $\pm$ 8.0[b]	114.4 $\pm$ 1.3
	282.6 $\pm$ 62.2[c]	
3c	NT	927.1 $\pm$ 1.5
4	NT	2111.5 $\pm$ 11.2
5	NT	1319.2 $\pm$ 3.6
6	92.4 $\pm$ 2.7[b]	199.4 $\pm$ 9.1
	240.7 $\pm$ 4.5[c]	
Control[a]	45.8 $\pm$ 4.8	–

[a]Thrombin time (TT) was measured by the incubation of human plasma in the absence (control) or presence of inhibitors.
[b]Measured at 100 μM.
[c]Measured at 500 μM.
[d]NT = not tested.
[e]NA = not active.

of the group at C-3, that is, donor or acceptor. Similar results were obtained for **3c**, indicating that the ester group at C-3 has minor or no participation on interactions with thrombin, being unable to recover the interactions lost from glycan removal.

Compounds **3a** and **3b**, however, indicate that such carbohydrate moiety is not an absolute requirement for thrombin binding and inhibition, with the phthalic ester **3b** showing an almost twofold increase in its activity (IC$_{50}$ = 114.4 $\pm$ 1.3 μM) accompanied with a significant simplification of the molecule structure (Figure 1A,B). In fact, substitution of carbohydrates by aromatic rings has been described previously regarding antithrombin activation by heparin mimetics, based on sulphated flavonoids (14). In spite of a great reduction in the number of hydrogen bond-capable groups from **1** to **3b**, there is also a great reduction in configurational entropy lost upon complexation, which may explain such increase in activity. This possibility correlates with the low activity of compound **3a** (IC$_{50}$ = 398.6 $\pm$ 6.9 μM) in comparison with **3b** (Figure 1B). Also, compound **3b** showed a more pronounced anticoagulant activity when tested in human plasma. In fact, compound **3b** caused a significant prolongation of thrombin time compared with normal plasma values (Table 1).

With the purpose of obtaining further insights into the reported activities, compounds **1**, **2**, **3a** and **3b** were docked on thrombin exosite I, previously observed as the binding site for glycyrrhizin (4). Additionally, as the inhibitory effect upon thrombin is expected to modulate the conformation of its catalytic site, each docking com-

plex was further refined through molecular docking (MD) simulations to search for potential allosteric influences of the compounds, in comparison with non-inhibited thrombin, as reported for the modulation of thrombin by sulphated arabinans (15). A simulation of uncomplexed thrombin was employed as a negative control, and in addition to the use of glycyrrhizin as the positive control, another anticoagulant saponin, through thrombin inhibition, chikusetsusaponin IVa (β-D-glucopyranosiduronic acid, (3β)-28-(β-D-glucopyranosyloxy)-28-oxoolean-12-en-3-yl) (**6**) (16) was also simulated in complex to its target protein. The so-obtained data from the combination of docking and MD are presented in Figure 1 and Table 2.

Table 2: Structural data on thrombin inhibition by compounds **1**–**6**

| Compound	Distance between catalytic triad residues (nm)[a]			Interaction energy (kJ/mol)[a]
	His57–Asp102[b]	His57–Ser195[c]	Sum	
1	0.30 $\pm$ 0.03	0.55 $\pm$ 0.10	0.85	−136 $\pm$ 46
2	0.32 $\pm$ 0.04	0.65 $\pm$ 0.11	0.97	−44 $\pm$ 21
3a	0.31 $\pm$ 0.03	0.61 $\pm$ 0.11	0.92	−120 $\pm$ 42
3b	0.46 $\pm$ 0.15	0.83 $\pm$ 0.34	1.29	−147 $\pm$ 61
3c	NT[d]	NT	NT	NT
4	NT	NT	NT	NT
5	NT	NT	NT	NT
6	0.33 $\pm$ 0.10	0.76 $\pm$ 0.14	1.09	−178 $\pm$ 43
Control[e]	0.29 $\pm$ 0.02	0.57 $\pm$ 0.12	0.86	–

[a]Average values measured after the first 20-ns MD simulations.
[b]Between atoms ND1 from His and OD2 from Asp.
[c]Between atoms NE2 from His and OG2 from Ser.
[d]NT = not tested.
[e]Control: simulation of free, uncomplexed thrombin.
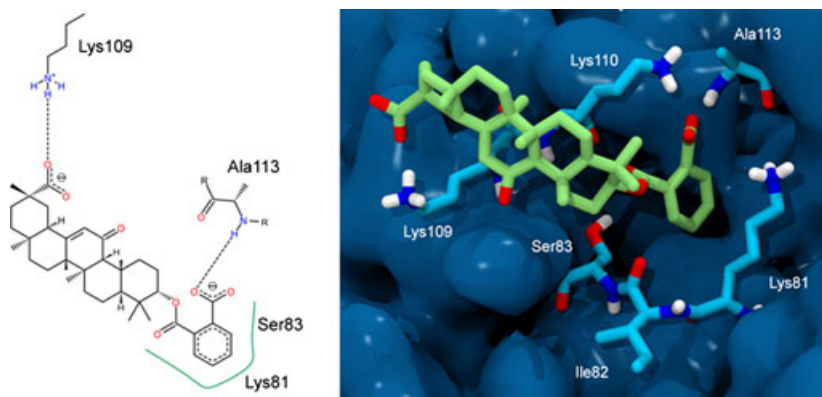
Figure 2: Schematics of thrombin interaction with compound **3b** based on the combination of docking and MD simulation. In the left, a 2D representation based on results obtained from PoseView Server, where a green line indicates hydrophobic interactions, while a dashed line indicates hydrogen bonds. In the right, thrombin is represented in a blue surface, while the highlighted residues (cyan) and compound **3b** (green backbone) are represented as sticks.

The MD simulations indicated a progressive reorientation of the docking-derived conformations, further exploring the exosite surface and refining the interactions with the protein along the simulations (Figure 1). Regarding compounds **1** and **3b**, the dynamics of these products indicate that the anionic exosite 1 is properly occupied, and there are similarities between the binding regions of both molecules. The less active compounds **2** and **3a** appear to populate the site in a more diffuse manner on the simulations, moving either to the northwest region of the site (**2**) or to the southern region (**3a**). The binding of **1** and **3b** to the anionic exosite 1 is important for inhibition because this site is essential for fibrinogen recognition by thrombin. Thus, MD simulations corroborate our previous results, which showed the ability of compound **1** to displace hirudin (a specific ligand of thrombin exosite 1) from its binding site (4).

Additionally, MD simulations also indicated that the compounds could modulate the conformation of thrombin catalytic site. As showed in Table 2, the distance between catalytic triad residues had an increase after binding of compounds **3b** and **6** in comparison with uncomplexed thrombin. Interestingly, these compounds also had the lowest IC_{50} values in the thrombin-induced fibrinogen clotting assay and the most potent anticoagulant activity in plasma. Our hypothesis is that the binding to exosite 1 could induce these changes in catalytic triad residues via allosteric modulation and thus contribute to the inhibitory activity. In fact, we recently showed that compound **6** is also able to inhibit the thrombin-induced hydrolysis of chromogenic substrate S2238 (H-D-Phe-Pip-Arg-p-nitro-anilide), a synthetic peptide designed specifically to the thrombin catalytic site (16). Unlike fibrinogen, the cleavage of synthetic peptide is not dependent on interaction with exosite 1, indicating that compound **6** can modulate the active site. Similar to compound **6**, compound **3b** was also able to inhibit hydrolysis of the synthetic peptide, while **1** was not (Figure 1C).

Based on the final frame of thrombin-**3b** complex, the main interactions are depicted in Figure 2. The carboxyl group at the aromatic ring contributes to the majority of the interactions, through hydrogen bonding with Ala113 backbone and Lys110 side chain. Furthermore, the phenyl ring participates in hydrophobic interactions involving mostly Lys81, Ile82, Lys109 and Ser83 (Figure 2). These findings suggest that the dual nature of the substituent, comprehending polar and apolar groups, provides multiple possibilities for the compound binding to the anionic exosite 1 and, most important, future insights for further compound optimizations.

Conclusions

Although the synthesized compounds are already known in the literature, this is the first report regarding the synthesis of thrombin inhibitory glycyrrhizin (**1**) derivatives. Compound **3b**, with a simpler molecular scaffold than **1**, presented a greater thrombin inhibitory activity and prolonged thrombin time and, unlike **1**, was able to inhibit hydrolysis of the synthetic peptide S2238, indicating that this ester can modulate the active site. These results, along with the docking and MD simulations performed, can be used for the design and synthesis of new glycyrrhizin derivatives towards new thrombin inhibitory compounds.

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APRESENTAÇÕES DE TRABALHOS

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