UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Distúrbios de agregação plaquetária e coagulação sangüínea no envenenamento pela taturana *Lonomia obliqua*

Markus Berger

Porto Alegre

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Orientador: Dr. Jorge Almeida Guimarães

Dissertação 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 Mestre.

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“As conquistas da indústria que enriqueceram tantos homens práticos nunca teriam ocorrido se apenas esses homens práticos tivessem existido, e se não tivessem sido antecedidos por loucos desprendidos que morreram pobres, que nunca pensavam no útil e que, no entanto, tinham outro guia que não suas fantasias”.

Jules Henri Poincaré (1854-1912).

ÍNDICE

LISTA DE ABREVIATURAS

ADP	*Adenosine 5'- diphosphate* (5'- difosfato de adenosina)
AMPc	*Adenosine 3',5'- cyclic monophosphate* (3',5'-monofosfato de adenosina cíclico)
α_2AP	α_2-Antiplasmina
ATP	*Adenosine 5'- triphosphate* (5'- trifosfato de adenosina)
CIT/RS	Centro de Informações Toxicológicas do Rio Grande do Sul
CIT/SC	Centro de Informações Toxicológicas de Santa Catarina
Da	Dáltons
D-D	D-Dímeros
F_{1+2}	Fragmento 1+2 da ativação de protrombina
Fg	Fibrinogênio
5-HT	5-hidróxitriptamina (serotonina)
LOPAP	*Lonomia obliqua Prothrombin Activator Protease* (ativador de protrombina de *Lonomia obliqua*)
LOSAC	*Lonomia obliqua Stuart* (ativador de fator X de *Lonomia obliqua*)
PA	*plasminogen activator* (ativador de plasminogênio)
p-BPB	*p-bromophenacyl-bromide* (brometo de *p*-bromofenacila)
PC	Proteína C
PDF	Produto de degradação de fibrina
PK	*Pre-Kallikrein* (pré-calicreína)
Plg	Plasminogênio
TAT	complexo Trombina-Antitrombina
TF	*Tissue factor* (fator tecidual)
TFPI	*Tissue factor pathway inhibitor* (inibidor da via do fator tecidual)

TP	Tempo de Protrombina
tPA	*Tissue type plasminogen activator* (ativador de plasminogênio tipo tecidual)
TTPa	Tempo de Tromboplastina Parcialmente ativada
TT	Tempo de Trombina
TXA_2	Tromboxana A_2
uPA	*Urokinase type plasminogen activator* (ativador de plasminogênio tipo uroquinase)
vWF	*Von Willebrand factor* (fator de Von Willebrand)

LISTA DE FIGURAS

LISTA DE TABELAS

RESUMO

O envenenamento causado pela lagarta *Lonomia obliqua* é um problema de saúde pública nas regiões do Sul do Brasil. As vítimas envenenadas apresentam uma síndrome hemorrágica grave que pode evoluir para insuficiência renal aguda, hemorragia intracraniana e óbito. Com o objetivo de compreender os mecanismos que levam à hemorragia, neste trabalho foram avaliados a função plaquetária e os parâmetros de coagulação sangüínea em um modelo experimental de envenenamento em ratos e também foram investigados os efeitos diretos do veneno de *L. obliqua* sobre plaquetas isoladas *in vitro*. Os ratos envenenados apresentaram hipofibrinogenemia e hipoagregação plaquetária. A produção intravascular de óxido nítrico e a geração dos produtos de degradação de fibrinogênio/fibrina parecem estar envolvidos na hipoagregação plaquetária. Os animais envenenados também apresentaram incoagulabilidade sangüínea e um aumento significativo na atividade de trombina, plasmina e uroquinase no plasma. Apesar desta geração intravascular de trombina, somente uma pequena redução na contagem de plaquetas foi detectada. Quando testado *in vitro*, o veneno de *L. obliqua* foi capaz de induzir diretamente agregação e adesão de plaquetas isoladas. A agregação plaquetária induzida pelo veneno foi significativamente inibida por brometo de *p*-bromofenacila (*p*-BPB), um inibidor específico de fosfolipases A2. Experimentos com diferentes antagonistas farmacológicos indicaram que a agregação plaquetária disparada pelo veneno ocorre por um mecanismo dependente de cálcio envolvendo a via das ciclooxigenases e a ativação da fosfodiesterase 3A, uma enzima que leva ao consumo dos níveis intracelulares de AMPc. Se analisados em conjunto, os resultados aqui apresentados são importantes para a compreensão da síndrome hemorrágica resultante do contato acidental com a lagarta *L. obliqua* e podem auxiliar na descoberta de novas formas de tratamento para o quadro clínico.

ABSTRACT

The envenomation caused by the caterpillar *Lonomia obliqua* is a public health hazard in Southern Brazil regions. Envenomed victims present a severe hemorrhagic syndrome that can progress to acute renal failure, intracranial hemorrhage and death. To understand the mechanisms that lead to hemorrhage, we evaluated the platelet function and blood coagulation parameters in an experimental model of envenomation in rats and investigated the direct effects of *L. obliqua* venom on isolated platelets *in vitro*. Envenomed rats presented hypofibrinogenemia and platelet hypoaggregation. The intravascular production of nitric oxide and generation of fibrinogen/fibrin degradation products seems to be involved in platelet hypoaggregation. Animals also showed intense blood incoagulability and a significant increase in thrombin, plasmin and urokinase plasmatic activities. Despite this intravascular thrombin generation, only a slight decrease in platelet numbers was detected. When tested *in vitro*, *L. obliqua* venom was able to directly induce aggregation and adhesion of isolated platelets. The venom-induced platelet aggregation was significativly inhibited by *p*-bromophenacyl bromide (*p*-BPB), a specific inhibitor of phospholipases A2. Experiments with different pharmacological antagonists indicate that the aggregation response triggered by venom occurs through a calcium-dependent mechanism involving the cyclooxygenase pathway and activation of phosphodiesterase 3A, an enzyme that leads to the consumption of intracellular cAMP content. Altogether, these findings may be important to the understanding of the complex hemorrhagic syndrome resulting from accidental contact with *L. obliqua* caterpillars and may give new insights in the management of the clinical profile.

1. INTRODUÇÃO

1.1 O estudo dos venenos animais e sua relevância

O envenenamento por animais peçonhentos constitui um importante problema de saúde pública em muitas regiões, particularmente em áreas tropicais e subtropicais (GUTIÉRREZ *et al*, 2006). Nesses acidentes, os agricultores 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 esta população desempenha muitas vezes um trabalho exclusivamente 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 farmacologia dos venenos animais, o que acabou gerando o conhecimento científico necessário ao desenvolvimento dos soros antivenenos para o tratamento das vítimas de acidentes ofídicos, muitos deles fatais (BARRAVIERA, 1999).

Atualmente, a utilização dos antivenenos produzidos nos Institutos Butantan (São Paulo, SP), Vital Brasil (Niterói, RJ) e outros, vem reduzindo significativamente o número de óbitos ocasionados por acidentes com animais

peçonhentos. Todavia, nem sempre tais produtos estão disponíveis em localidades distantes, onde mais freqüentemente os acidentes ocorrem, sendo também, ainda muito elevados os índices de seqüelas decorrentes das lesões geradas por tais envenenamentos (FAN & CARDOSO, 1995).

Apesar de difícil a estimativa da incidência dos acidentes com animais peçonhentos, um estudo recente realizado pelo Centro de Informações Toxicológicas do estado do Rio Grande do Sul (CIT-RS) apontou a ocorrência de 61.172 notificações de acidentes no período de 1980 – 2005 só neste estado. O estudo indica que a partir de 1984 ocorreu um aumento excepcional no número dos acidentes com animais peçonhentos chegando, em alguns anos da década de 90, a superar o registro de acidentes com medicamentos (ABELLA *et al*, 2006).

No Brasil, os principais acidentes que resultam em quadros de envenenamento são causados por serpentes e aranhas (MINISTÉRIO DA SAÚDE, 1998). No entanto, a partir de 1989 acidentes hemorrágicos com lagartas da espécie *Lonomia obliqua* (Lepidoptera, Saturniidae) assumiram proporções epidêmicas nas regiões do sul do Brasil (DUARTE *et al*, 1990). Somente no ano de 2005 foram registrados 127 acidentes com essas lagartas no Rio Grande do Sul, sendo os níveis de letalidade considerados de 3 – 6 vezes maiores do que os observados em envenenamentos por serpentes (DIAZ, 2005; ABELLA *et al*, 2006). O quadro clínico resultante do contato com *Lonomia 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 que podem causar a morte (VEIGA *et al*, 2009).

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 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 sangüínea, hemorragias e insuficiência renal aguda (DIAZ, 2005). No Brasil, poucas espécies 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 foi

realizado pelo médico mineiro Zoroastro de Alvarenga em 1912, que descreveu o caso de um agricultor que teve contato com várias lagartas (ZANNIN, 2002).

No período de 1978 a 1982 um estudo prospectivo 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 *Lonomia achelous* (FRAIHA *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, Paraguai, Peru e 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. Em Santa Catarina e no Rio Grande do Sul foram identificadas como responsáveis pelos acidentes, lagartas da espécie *Lonomia obliqua*. No período de 1997 a 2005 foram registrados pelo CIT/RS, um total de 1009 acidentes com a taturana *Lonomia obliqua*, sendo que 984 ocorreram no Rio Grande do Sul (Figura 1). Deste total, cinco pacientes evoluíram para o óbito, resultando numa taxa de letalidade de 0,5% (ABELLA *et al*, 2006).

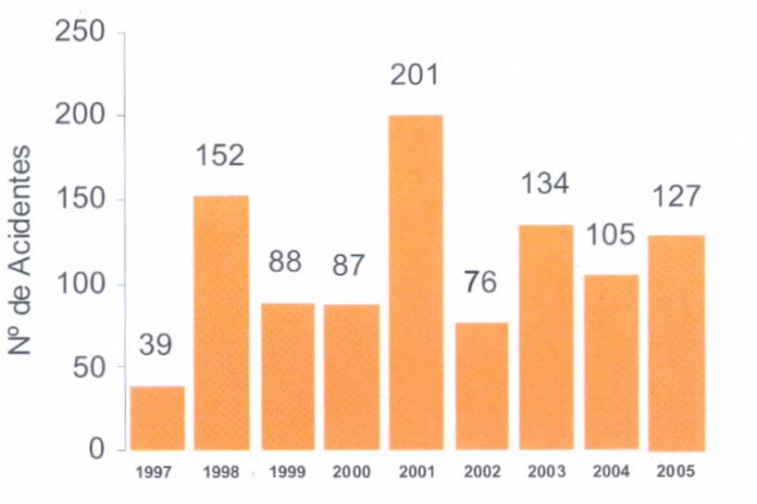

Figura 1. Números de acidentes causados por *Lonomia obliqua* **registrados pelo CIT/RS no período de 1997 a 2005**. Fonte: ABELLA *et al*, 2006.

No Rio Grande do Sul, várias cidades (Figura 2A) têm registrado a ocorrência de acidentes ou identificação da presença da taturana. Entretanto, os maiores percentuais de notificações foram registrados em Passo Fundo com 9,7 % e Bento Gonçalves com 5,4 % dos casos (Figura 2B) (ABELLA *et al*, 2006).

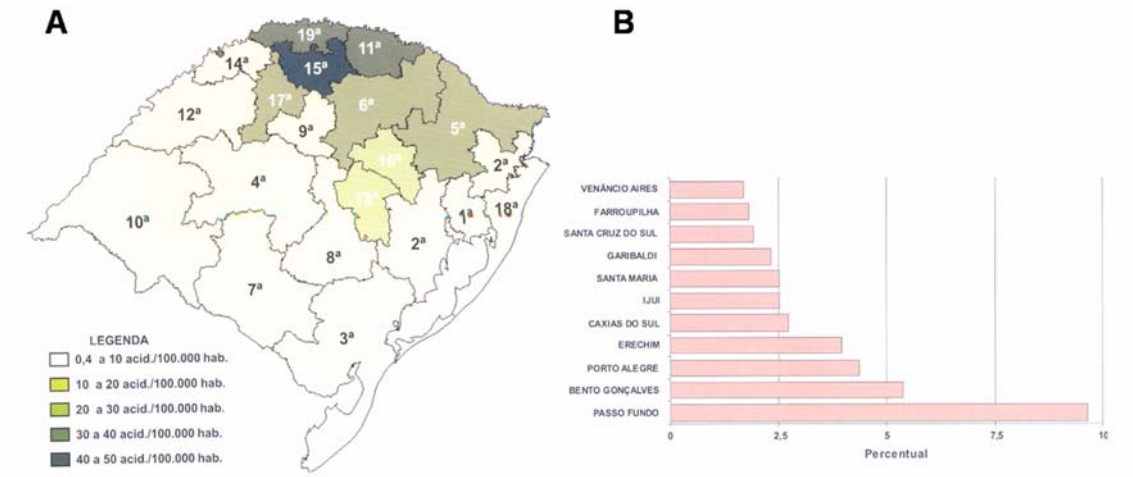

Figura 2. Localidade dos acidentes com *Lonomia obliqua* **no Rio Grande do Sul. A.** Incidência de acidentes com *L. obliqua* por 100.000 habitantes, nas respectivas Coordenadorias Regionais de Saúde. **B.** Percentual do número de acidentes causados por *L.obliqua* nas principais cidades do RS (1997-2005). 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 *Lonomia 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).

1.2.2 O agente

A taturana *Lonomia 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, 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 3B). 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).

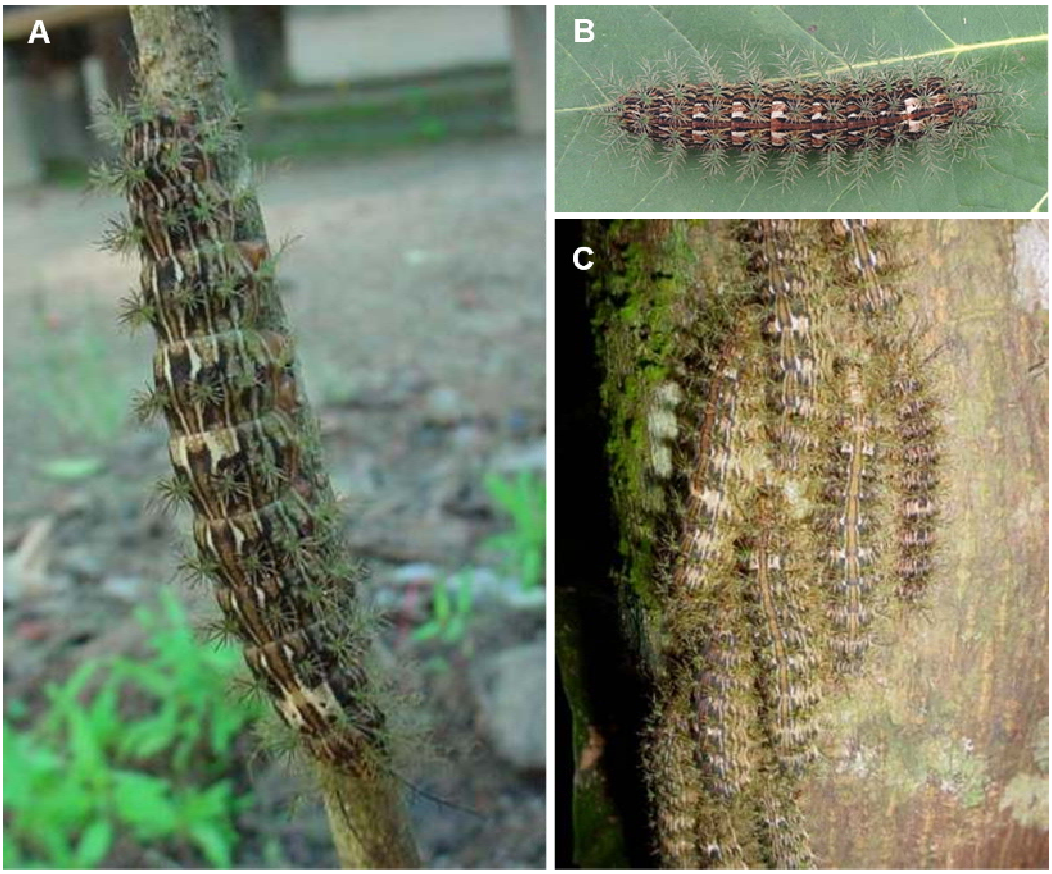

Figura 3. A lagarta *Lonomia obliqua* **(taturana). A** e **B.** A lagarta *Lonomia obliqua* evidenciando aspectos da morfologia externa. **C.** Colônia de lagartas sobre o tronco de uma árvore. Fotos: CIT/SC.

A lagarta *Lonomia 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. A fase de lagarta tem duração média de 85 dias, sendo que cada ínstar perdura por aproximadamente duas semanas (LORINI, 1997).

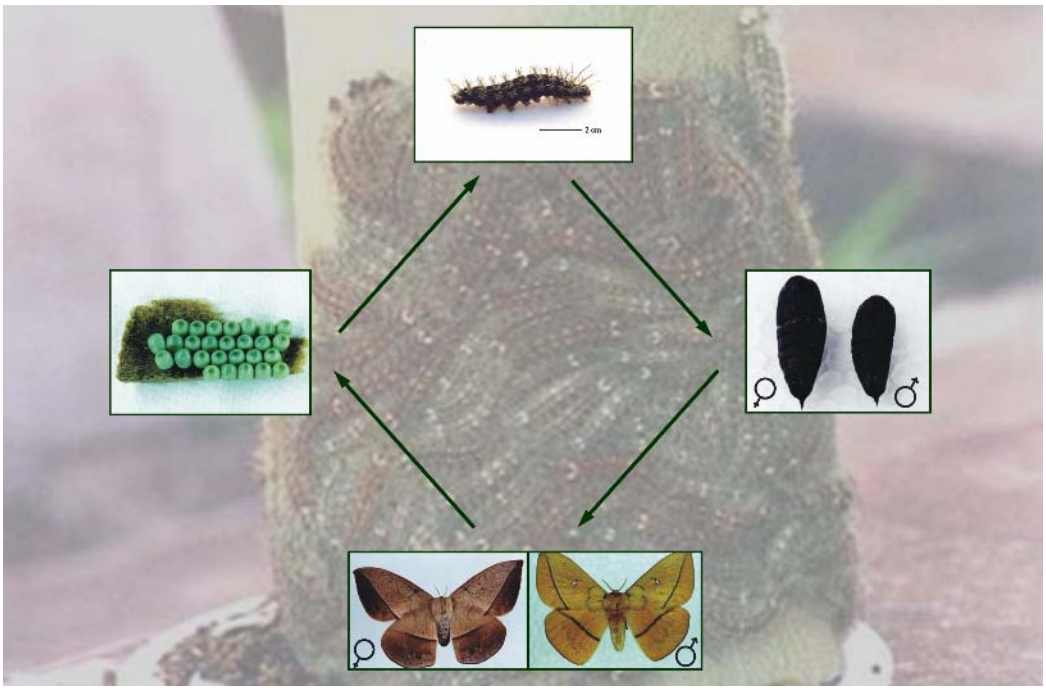

Figura 4. Ciclo de vida do lepidóptero *Lonomia obliqua*. Fotos: CIT/RS.

Em um estudo detalhado da morfologia da lagarta *Lonomia 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). Estas espículas, por serem constituídas de quitina, são de fácil ruptura (Figura 5D), o que acaba facilitando o contato da secreção venenosa da taturana com a pele e mesmo com o tecido subcutâneo da vítima, causando o envenenamento (VEIGA, 2001).

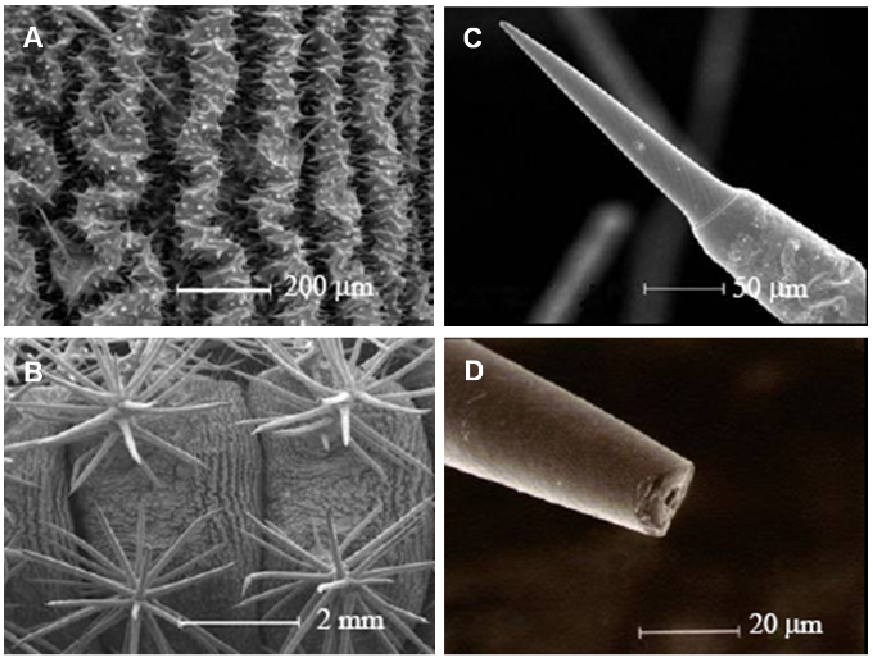

Figura 5. Ultraestrutura do tegumento de *Lonomia obliqua*. **A.** Estruturas quitinosas que compõem a base do tegumento. **B.** Região dorsal do tegumento com vários scoli contendo as espículas. **C.** Espícula íntegra. **D.** Espícula quebrada na extremidade com canal interno exposto. Fonte: VEIGA *et al*, 2001.

1.2.3 A síndrome hemorrágica

O contato com as secreções venenosas de *Lonomia obliqua* provoca, no local, uma reação imediata caracterizada por um ardor intenso (dor caracterizada por queimação), hiperemia, prurido, edema e bolhas sanguinolentas (Figura 6A), 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 freqüência 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 6D), equimose (Figura 6B 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, insuficiência renal, podendo evoluir para o óbito. As mortes relatadas foram provocadas por complicações do tipo insuficiência renal aguda e hemorragia intracraniana (DUARTE *et al*, 1990, 1996; KOWACS *et al*, 2006).

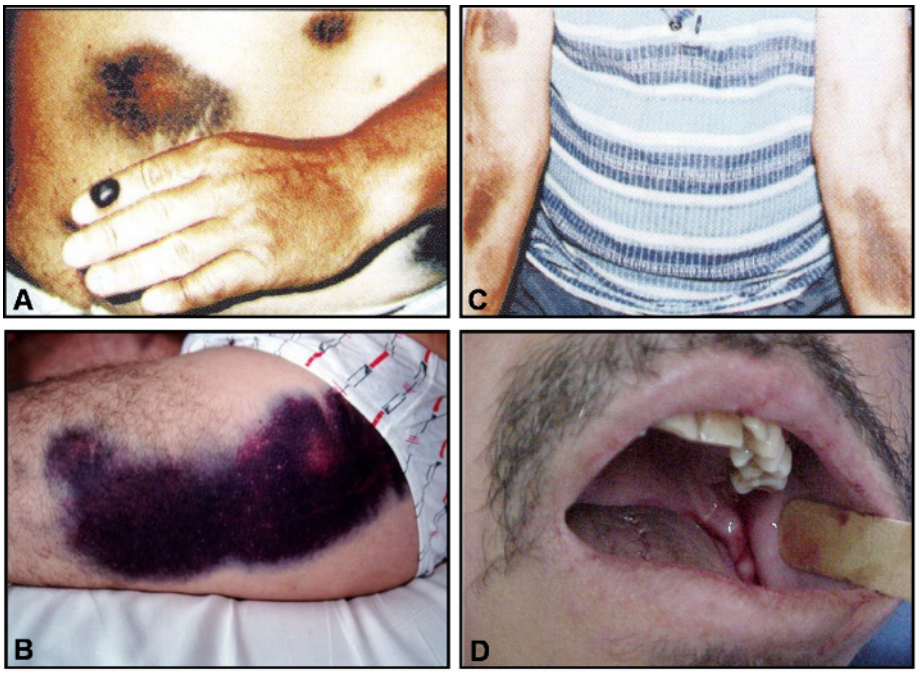

Figura 6. Sinais clínicos do envenenamento por *Lonomia obliqua*. **A.** Hematoma no dedo da mão de um paciente. **B** e **C.** Equimoses em diferentes regiões do corpo. **D.** Gengivorragia. Fotos: CIT/RS e ZANIN *et al*, 2002.

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, pré-calicreína (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 (F1+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 (CID). De fato, os elevados níveis de TAT e F1+2 indicam que há geração de trombina intravascular através 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 sangüínea, 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 a presença de intensa fibrinólise. Provavelmente, componentes do veneno são capazes de ativar direta ou indiretamente o Plg gerando quantidades grandes 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.

Apesar da ativação da coagulação, o número de plaquetas encontra-se normal na maioria dos pacientes. Somente foi observada trombocitopenia importante em casos graves de envenenamento (ZANNIN *et al*, 2003; GAMBORGI *et al*, 2006), o que não significa necessariamente que a atividade

ou função plaquetária esteja normal na maioria das vítimas. De fato, até a realização do presente trabalho não havia sido realizado nenhum relato sobre as alterações de função plaquetária durante o envenenamento por *L. obliqua*, apesar dessas alterações serem comuns em envenenamentos por serpentes (SANO-MARTINS *et al*, 1997; RUCAVADO *et al*, 2005).

Com relação aos distúrbios renais, Gamborgi e colaboradores (2006) relataram que 18% das vítimas acidentadas entre 1989 e 2003 desenvolveram insuficiência renal aguda e, entre esses pacientes, a taxa de mortalidade foi de 50%. Mesmo sendo uma conseqüência grave, pouco se sabe sobre a patogênese da falência renal aguda induzida pelo envenenamento por *L. obliqua*. Um dos fatores que contribuem para essa falta de conhecimento é a impossibilidade de realização de biópsia renal nos pacientes envenenados, devido ao fato de esses pacientes apresentarem concomitantemente os distúrbios hemorrágicos graves (DUARTE *et al*, 1990).

A gravidade dos acidentes com *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 (GONÇALVES *et al*, 2007). Uma vez que a produção do soro requer o uso das espículas da taturana em quantidade suficiente para a imunização dos cavalos, permanecem como desafios a produção do soro suficiente para atender à crescente demanda devido ao aumento dos acidentes

e a necessidade de disponibilizar o produto em locais distantes onde os acidentes vêm ocorrendo com mais freqüência.

1.3 Hemostasia

A hemostasia representa um complexo, redundante e eficiente mecanismo fisiológico de defesa contra a perda não controlada de sangue. O estado normal de fluidez do sangue circulante é mantido pelas propriedades não-trombogênicas das paredes intactas das células dos vasos. O dano a esses vasos provoca uma pronta resposta hemostática que previne a hemorragia.

O sistema é um conjunto de processos finamente regulados e com máxima eficiência incluindo a parede vascular, estruturas e agentes vasoativos envolvidos na vasoconstrição e vasodilatação, fatores que levam à adesão e agregação das plaquetas circulantes que formam um tampão hemostático e a ativação dos fatores da cascata de coagulação que levam à formação de coágulos de fibrina. Na regeneração total do tecido danificado, os coágulos são subseqüentemente degradados pelo sistema fibrinolítico. Em situações em que qualquer componente desses mecanismos esteja alterado, a hemostasia é comprometida e o resultado pode ser tanto trombose como hemorragia (DAVIE *et al*, 1991; DAHLBÄCK, 2000).

O rompimento da monocamada de células endoteliais que recobre a parede vascular seja fisiologicamente ou em conseqüência a uma lesão tissular, causa a exposição da matriz subendotelial, ocorrendo, inicialmente, a atração para o local das plaquetas circulantes. Como conseqüência desse contato, as plaquetas sofrem profunda mudança de sua forma discóide (processo de *shape change*), caracterizada pela transformação desse formato nativo em uma forma mais esférica, com a emissão de inúmeros pseudópodes

(ALLEN *et al*, 1979). Tal transformação induz à adesão progressiva de outras plaquetas que se espraiam sobre a matriz subendotelial através da interação com proteínas adesivas como fator de Von Willebrand e colágeno. Os receptores glicoprotéicos GPIb-IX-V e GPVI e a integrina $\alpha 2\beta 1$ são importantes nesta interação e são responsáveis por manterem as plaquetas no sítio da lesão. Em seguida ao processo de adesão, ocorre a ativação e a agregação das plaquetas pela interação de agonistas como colágeno, trombina e adrenalina, liberados na lesão tissular, com receptores de superfície. Outros receptores tornam-se funcionais quando as plaquetas são ativadas. Assim, na formação de um agregado compacto, são mobilizados alguns receptores de membrana, particularmente as glicoproteinas IIb e IIIa (GPIIb-IIIa, complexo conhecido como integrina αIIbβ3), em resposta aos agonistas extracelulares. A ativação e a agregação inicial das plaquetas próximas ao local da lesão tissular induz uma efetiva reatividade plaquetária que produz a secreção de organelas plaquetárias de estoque contendo outros agonistas como ADP, tromboxana A_2 (TXA_2) e serotonina (5-HT), potentes agentes pró-agregantes. O complexo processo de adesão, reatividade e ativação plaquetária acaba provocando o recrutamento e agregação de outras plaquetas circulantes à primeira camada, levando à formação de um tampão celular (Figura 7). Juntamente com a mudança de forma e adesão, um rearranjo nas fosfolipoproteínas de membrana forma uma apropriada superfície catalítica pró-coagulante (BLOCKMANS *et al*, 1995; ANDREWS & BERNDT, 2004), potenciando sobremaneira o processo hemostático como um todo.

O estado de ativação plaquetária é dinamicamente modulado pelo balanço de ações estimulatórias e inibitórias da função plaquetária. O processo

de ativação é, em geral, iniciado pela exposição a um agonista plaquetário que se liga a receptores de superfície e desencadeia uma cascata de eventos bioquímicos. Como já mencionado anteriormente, a trombina, o colágeno, o ADP, a epinefrina e a tromboxana A2 são estímulos fisiológicos para a ativação plaquetária. Os eventos posteriores têm elementos comuns e outros que se diferenciam, especialmente como resultado da estimulação de receptores específicos (BLOCKMANS *et al*, 1995). De uma maneira geral, após a ligação do agonista ao receptor, os segundos mensageiros inositol-1,4,5-trifosfato (IP3) e diacilglicerol (DAG) são formados e modulam vias de ativação independentes, podendo atuar sozinhos ou sinergisticamente (BLOCKMANS *et al*, 1995). O IP3 se liga a receptores do sistema tubular denso, principal local de estoque de cálcio, fazendo com que as concentrações citoplasmáticas do cátion aumentem. Esse aumento nos níveis intraplaquetários de cálcio acaba por promover o rearranjo do citoesqueleto, sendo responsável pela mudança de forma característica do processo de ativação das plaquetas. Além disso, a ligação do IP3 ao sistema tubular denso e o aumento do cálcio, medeiam a oxidação citosólica do ácido araquidônico a tromboxana A2, que posteriormente é liberado pela plaqueta. O DAG causa a ativação da proteína quinase C (PKC), o que contribui para a liberação do conteúdo dos grânulos de secreção contendo outros agonistas (Figura 7) (JACKSON & SCHOENWAELDER, 2003; ROBERTS *et al*, 2004).

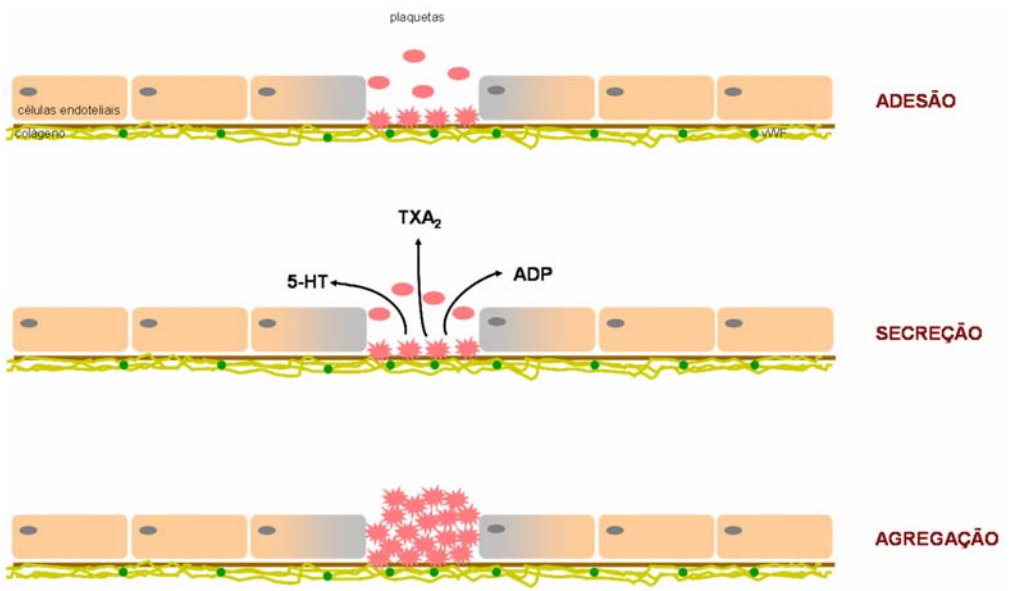

Figura 7. Adesão, secreção e agregação plaquetária. As plaquetas aderem ao local da lesão e são ativadas pela presença de colágeno e outros agonistas. Após a adesão, plaquetas já ativadas secretam agonistas como tromboxana A_2 (TXA_2), adenosina difosfato (ADP) e serotonina (5-HT), que são responsáveis pela ativação e recrutamento de outras plaquetas circulantes. vWF Fator de von Willebrand. Modificado de KROLL & SULLIVAN, 1998.

Paralelamente à agregação plaquetária, a reação de coagulação é iniciada culminando na formação de fibrina. Em 2001, um modelo de hemostasia baseada em células foi proposto, enfatizando a interação de fatores plasmáticos da coagulação com superfícies celulares específicas (HOFFMAN, 2003a; HOFFMAN, 2003b; HOFFMAN & MONROE, 2001; ROBERTS *et al*, 2006). O modelo de coagulação baseado em células é uma evolução conceitual do processo da cascata da coagulação formulado em 1964 por Davie e Ratnoff e MacFarlane (DAVIE & RATNOFF, 1964; MACFARLANE, 1964), que propunham os conhecidos modelos das vias intrínseca e extrínseca para a cascata de coagulação sanguínea. Este novo modelo mostra a importância da interação entre as proteínas plasmáticas e superfícies celulares para o início da coagulação e confirma que a manutenção do processo depende das reações bioquímicas de ativação dos fatores da coagulação.

O modelo celular da hemostasia baseia-se em uma série de três etapas que ocorrem no plasma e em superfícies de diferentes tipos celulares. Como mostra a Figura 8, a primeira fase, ou iniciação, ocorre em células extravasculares carreadoras de fator tecidual (*tissue factor*, TF). Na fase de amplificação, plaquetas e cofatores são ativados propiciando a geração de trombina. Finalmente, a propagação ocorre na superfície das plaquetas ativadas, aderidas ao local da lesão, resultando na produção de grandes quantidades de trombina e na subseqüente formação do coágulo de fibrina (HOFFMAN & MONROE, 2001; MONROE *et al*, 2002; MONROE & HOFFMAN, 2006).

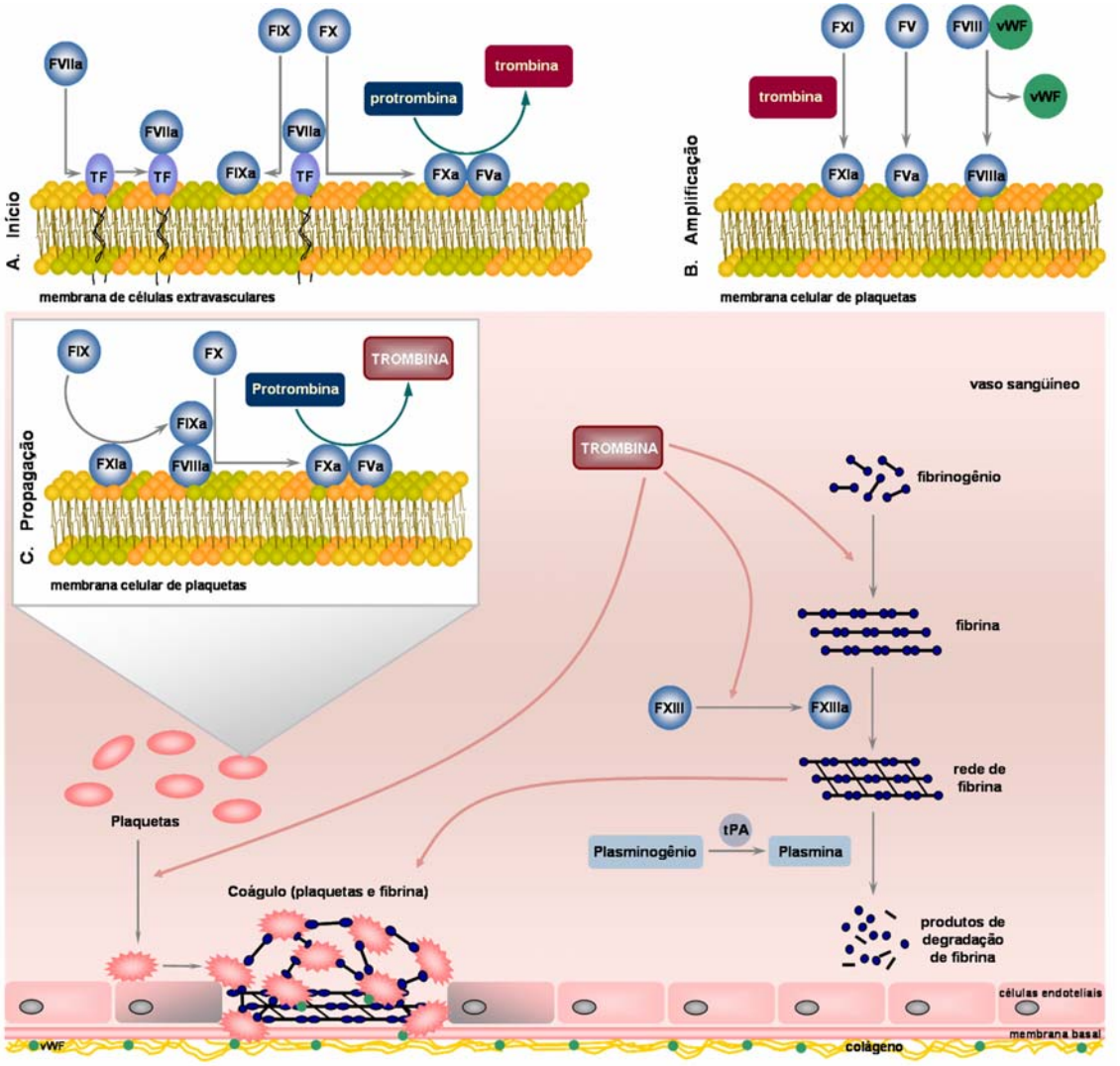

Figura 8. Modelo da cascata de coagulação baseado em células. A. Fase de iniciação; **B.** Fase de amplificação e **C.** Fase de propagação. Fatores de coagulação estão representados em azul e a trombina ativa em vermelho. TF fator tecidual; vWF fator de von Willebrand; tPA ativador de plasminogênio tecidual. Figura: M. BERGER, 2006.

A fase de iniciação da coagulação (Figura 8A) ocorre nas superfícies de células contendo TF (fibroblastos do estroma, células mononucleares, macrófagos e células endoteliais que não expõem TF ao sangue até que ocorra dano vascular ou inflamação) que estão normalmente localizadas no espaço extravascular. O fator VII liga-se ao TF celular e é rapidamente ativado por proteases do sistema de coagulação. O complexo FVII/TF ativa pequenas quantidades dos fatores IX e X. Uma vez ativado, o FXa juntamente com o FVa

formam o complexo protrombinase que será responsável pela geração de pequenas quantidades de trombina na superfície das células carreadoras de TF. O fator V pode ser ativado por fator Xa ou por outras proteases não-coagulantes. A presença de inibidores específicos (*tissue factor pathway inhibitor*, TFPI e anti-trombina III) localiza o fator Xa somente na superfície em que ele é formado (HOFFMAN & MONROE, 2001).

Além disso, baixos níveis de atividade da via do TF ocorrem no espaço extravascular todo o tempo. Algumas proteínas da coagulação atravessam a camada endotelial dos vasos, sendo encontradas na linfa em quantidades que variam de acordo com suas massas moleculares (proteínas menores são encontradas em maior quantidade). Assim, fator VII pode ser encontrado ligado a TF mesmo na ausência de lesão vascular e fatores IX e X podem ser ativados quando passam pelos tecidos. Esse fenômeno, chamado coagulação basal, não leva à formação de coágulos em circunstâncias normais devido a ausência de componentes de alta massa molecular do processo de coagulação, como plaquetas e complexo fator VIII/vWF. O processo de coagulação prossegue para a fase de amplificação apenas quando algum dano vascular permite que plaquetas e fator VIII/vWF entrem em contato com tecido extravascular (HOFFMAN, 2003a).

A fase de amplificação (Figura 8B) é preparatória para a subseqüente ativação e produção de trombina em grandes quantidades na fase de propagação. A pequena quantidade de trombina gerada nas células carreadoras de TF (fase de iniciação) possui várias funções, entre elas a ativação de plaquetas, expondo receptores e sítios de ligação para fatores da coagulação ativos e liberando formas de fator V parcialmente ativadas em suas

superfícies. A trombina formada na fase inicial também ativa os fatores V e VIII na superfície de plaquetas ativadas. Nesse processo, o complexo fator VIII/vWF se dissocia, permitindo que vWF plasmático atue como mediador adicional na adesão e agregação plaquetária. Também nessa fase, o fator XI na superfície de plaquetas é ativado a fator XIa (HOFFMAN & MONROE, 2001).

A fase de propagação (Figura 8C) ocorre na superfície das plaquetas ativadas, aderidas e agregadas no local da lesão. O fator IXa, tanto o ativado na fase de iniciação quanto o ativado por fator XIa, se liga ao fator VIIIa nas plaquetas. Já que o fator Xa não pode se mover das células carreadores de TF até as plaquetas ativadas, este deve ser suprido diretamente na superfície plaquetária pelo complexo fator IXa/VIIIa (complexo Xase). O fator Xa rapidamente se associa ao fator Va ligado às plaquetas na fase de amplificação. A formação do complexo protrombinase leva a ativação de protrombina em grandes quantidades, levando à clivagem de fibrinogênio e formação de fibrina. A trombina formada também ativa fator XIII a fator XIIIa, uma transglutaminase plasmática. Fator XIIIa catalisa a modificação covalente entre monômeros de fibrina, formando a rede estável de fibrina.

O processo de coagulação é regulado pela serpina antitrombina III e a via anticoagulante da proteína C/trombomodulina, que inativa os fatores Va e VIIIa. Além disso, a via regulatória composta pelo sistema do plasminogênio também tem um papel importante na degradação da rede de fibrina gerada pela ativação do processo hemostático. A via é ativada por dois ativadores fisiológicos de plasminogênio (PA): ativador de plasminogênio do tipo tissular (t-PA) e ativador de plasminogênio tipo uroquinase (u-PA). A via de ativação

mediada por t-PA está primariamente envolvida na homeostase da fibrina. Por outro lado, a via mediada por u-PA está envolvida em fenômenos como migração celular e remodelagem de tecidos. O plasminogênio ativado gera plasmina, que atua na degradação de fibrina e fibrinogênio. A inibição do sistema de regulação do plasminogênio ocorre na etapa de ativação de plasminogênio, pela ação específica de inibidores de ativadores de plasminogênio (PAI) e diretamente na plasmina ativa, através da serpina α2-antiplasmina (VAUGHAN & DECLERCK, 1998; COLLEN, 1999).

1.4 Atividades tóxico-farmacológicas do veneno de *Lonomia obliqua*

Com o objetivo de entender as alterações fisiopatológicas observadas nos pacientes envenenados, algumas toxinas têm sido isoladas e caracterizadas. Muitas dessas toxinas são capazes de interferir em processos essenciais da hemostasia, como os ativadores de fator X e protrombina, envolvidos com o processo de coagulação intravascular (DONATO *et al*, 1998), e as enzimas fibrinolíticas identificadas em mais de uma secreção da lagarta e envolvidas com a degradação de fibrinogênio e fibrina (FRITZEN *et al*, 2005; PINTO *et al*, 2004; PINTO *et al*, 2006; VEIGA *et al*, 2003). Também já foi identificada uma fosfolipase A2, envolvida no processo de hemólise intravascular (SEIBERT *et al*, 2004), e outras toxinas envolvidas nos processos de edema, nocicepção e ativação do sistema calicreínas-cininas (DE CASTRO BASTOS *et al*, 2004; BOHRER *et al*, 2007). Além disso, o veneno é capaz de induzir aumento na expressão de proteínas relacionadas à coagulação e inflamação, como fator tecidual (FT), interleucina 8 (IL-8) e ciclooxigenase-2 (COX-2) (PINTO *et al*, 2008).

A presença de outras toxinas e das já isoladas foi demonstrada por um estudo de transcriptoma de tecidos da lagarta, o que gerou um catálogo de várias proteínas presentes no veneno. Essas proteínas são inoculadas na vítima envenenada e, provavelmente constituem princípios ativos envolvidos direta ou indiretamente nas manifestações clínicas (VEIGA *et al*, 2005, http://www.ncbi.nlm.nih.gov/ projects/omes/). A tabela 1 apresenta um resumo das toxinas já isoladas do veneno e suas atividades.

Tabela 1. Toxinas isoladas do veneno de *Lonomia obliqua* (Modificado de Veiga *et al*, 2009).

Toxina	Nome	Atividades			Mw (Da)	Molécula identificada[3]	Gen Bank[4]	Referências
		Amidolítica[1]	Coagulação[2]	Outras				
Fibrinogenase (enzima fibrin(ogen)olítica)	Lonofibrase	DL-BAPNA	Anti	ND	35.000	LOqua-SP6	AY829843	Pinto *et al*, 2004; Veiga *et al*, 2005
Ativador de protrombina, Ca^{+2} -dependente	Lopap	S-2222	Pro	Induz produção de NO, PGI_2 e IL-8 pelas células endoteliais	69.000	NI	NI	Reis *et al*, 2001; Fritzen *et al*, 2005; Reis *et al*, 2006
Ativador de fator X, Ca^{+2} -independente	Losac	ND	Pro	Induz produção de NO, tPA e aumenta proliferação celular	45.000	LOqua-PPOAF	AY829844	Alvarez-Flores *et al*, 2006
Hialuronidases	Lonogliases	ND	ND	Atividade hidrolítica sobre matriz extracelular	53.000 49.000	NI NI	NI NI	Gouveia *et al*, 2005
Fosfolipase A2	-	ND	ND	Atividade hemolítica indireta	15.000	LOqua-PLA	AY829845	Seibert *et al*, 2006
Proteína anti-apoptótica	-	ND	ND	Inibe apoptose de células de inseto em cultura	51.000	NI	NI	Souza *et al*, 2005

ND: não determinado; NI: não identificado

[1]Atividade amidolítica sobre substratos cromogênicos sintéticos

[2]Atividade pro ou anti-coagulante sobre plasma humano

[3]Molécula identificada no estudo de transcriptoma (Veiga *et al*, 2005)

[4]Acesso no Gen Bank

2. OBJETIVOS

Apesar do conhecimento adquirido sobre as toxinas e as alterações na coagulação sangüínea, pouco se sabe sobre o papel das plaquetas na patogenia do envenenamento e, principalmente, sobre a relação das plaquetas com os distúrbios hemorrágicos. Portanto, o presente trabalho tem como objetivo geral investigar as alterações de agregação plaquetária e correlacioná-las com a coagulopatia observada no envenenamento pela taturana *Lonomia obliqua*.

Os objetivos específicos são:

- Estabelecer um modelo experimental *in vivo* em ratos para o estudo das alterações fisiopatológicas do envenenamento;
- Avaliar *ex vivo* as alterações de função plaquetária durante o envenenamento e investigar os mecanismos envolvidos;
- Avaliar *ex vivo* as alterações de coagulação sangüínea durante o envenenamento e investigar os mecanismos envolvidos;
- Avaliar a capacidade pró-agregante do veneno de *L. obliqua* sobre plaquetas humanas *in vitro*;
- Identificar o(s) componente(s) do veneno responsável pelo efeito pró-agregante;
- Investigar mecanismos de sinalização envolvidos na agregação plaquetária induzida pelo veneno.

3. RESULTADOS

A seção de resultados apresentada a seguir está dividida em duas subseções que correspondem a um artigo já aceito para publicação e a um manuscrito submetido. Cada subseção inicia-se com um breve texto introdutório em português seguido de uma cópia do artigo ou do manuscrito em inglês.

3.1 Hipoagregação plaquetária e incoagulabilidade sangüínea durante o envenenamento pela taturana *Lonomia obliqua* em ratos – Artigo I

Neste primeiro trabalho, foram avaliados a função plaquetária e os parâmetros de coagulação sangüínea em um modelo experimental de envenenamento em ratos. Os animais apresentaram uma diminuição drástica na capacidade de agregação plaquetária durante o envenenamento. Essa diminuição foi correlacionada com a produção intravascular de óxido nítrico e com o aumento na concentração plasmática dos produtos de degradação de fibrina. A contagem de plaquetas foi significativamente diminuída em relação aos animais não envenenados, no entanto não houve trombocitopenia importante como ocorre nos envenenamentos por serpentes do gênero *Bothrops*. Os animais apresentaram hipofibrinogenemia e incoagulabilidade sangüínea. Indícios de produção de trombina e plasmina intravascular também foram encontrados.

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Lonomia obliqua caterpillar envenomation causes platelet hypoaggregation and blood incoagulability in rats

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ABSTRACT

Envenomation caused by *Lonomia obliqua* is a public health hazard in Southern Brazil. Envenomed victims present severe hemorrhagic syndrome that can progress to intracranial hemorrhage and death. To understand the mechanisms that lead to hemorrhage, we investigated the platelet dysfunction and blood coagulation disturbances following experimental envenomation in rats. *L. obliqua* bristle extract was injected (s.c.) and blood collected at different times post-venom administration for determination of platelet response and analysis of blood coagulation. Rats presented hypofibrinogenemia and platelet hypoaggregation in platelet rich plasma (PRP). After addition of exogenous fibrinogen to PRP, platelet hypoaggregation was not corrected. Interestingly, normoaggregation was observed when platelets were separated from plasma. In addition, incubation of plasma from envenomed rats inhibits aggregation response of normal washed platelets. These results indicate that an aggregation inhibitor is generated in plasma during envenomation. Moreover, rats presented an increase in nitric oxide plasmatic levels which coincided with maximum inhibition in platelet aggregation. Animals also showed blood incoagulability and a significant increase in thrombin, plasmin and urokinase plasmatic activities. Despite this intravascular thrombin generation, only a slight decrease in platelet numbers was detected. Certainly, the platelet hypoaggregation and blood incoagulability described herein contribute to systemic bleeding observed in patients.

1. Introduction

Incidents involving venomous animals such as *Lonomia obliqua* (Lepidoptera, Saturniidae) caterpillars are an important public health hazard in some Southern Brazil regions (Duarte et al., 1990; Donato et al., 1998). This importance does not lie solely in its high incidence rate, but also in morbidity and mortality rates associated with the hemorrhagic disorders it causes (Caovilla and Barros, 2004). Epidemiological studies in the state of Rio Grande do Sul conducted between 1997 and 2005 indicate the occurrence of 1009 accidents, which resulted in seven deaths (Abella et al., 2006). According to the study of Abella et al. (2006), envenomed patients present a severe hemorrhagic syndrome and in the most serious cases the clinical profile can evolve into acute renal failure and intracranial hemorrhage. The accidents usually occur when the victim, leaning against tree trunks hosting 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 crushed in the accident, the insect's chitinous bristles are broken and the venomous secretions, including hemolymph, penetrate the human skin and enter the blood circulation (Veiga et al., 2001).

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Clinical symptoms of *L. obliqua* envenomation include a local inflammatory reaction, which starts immediately after contact; systemic reactions such as headache, fever, vomiting and asthenia, which appear few hours after exposure; and bleeding diathesis characterized by hematomes and ecchymosis, hematuria, pulmonary hemorrhage, intracerebral hemorrhage and acute renal failure (Burdmann et al., 1996; Kowacs et al., 2006; Garcia and Danni-Oliveira, 2007). Poisoned patients present low levels of fibrinogen, plasminogen, α2-antiplasmin and high levels of fibrinogen/fibrin degradation products and D-dimers. Moreover, high concentrations of thrombin–anti-thrombin complex and prothrombin fragments 1 + 2 may be detected in blood (Kelen et al., 1995; Zannin et al., 2003). These findings indicate that the fibrinolytic system is activated in *L. obliqua* envenomation, and that a significant amount of intravascular thrombin is generated.

Several biochemical studies have been conducted to isolate and characterize the toxins responsible for the pathological effects observed in *L. obliqua* envenomation. Some of the purified active principles so far include: factors II and X activators, α-fibrinogenases, hyaluronidases and a phospholipase A2 (Reis et al., 1999; Pinto et al., 2004; Gouveia et al., 2005; Alvarez-Flores et al., 2006; Seibert et al., 2006). The presence of these different toxins has been confirmed in a transcriptome study, which also revealed several other molecules that may be directly or indirectly involved in the clinical profile manifestations (Veiga et al., 2005). Besides, some aspects of the *L. obliqua* envenomation were previously studied in animal models, such as edema formation, hypotension, nociception and hemolysis (De Castro Bastos et al., 2004; Seibert et al., 2004; Bohrer et al., 2007).

To date, in comparison with blood coagulation and fibrinolysis disturbances, platelet dysfunctions have been poorly investigated in *L. obliqua* envenomation. In spite of significant amounts of thrombin generated in circulation by the factors II and X activators, thrombocytopenia is detected in some of the patients envenomed, mainly in the most serious cases, but is usually normal in mild ones (Zannin et al., 2003; Gamborgi et al., 2006; Malaque et al., 2006). The mechanism that leads to bleeding disorders without causing significant alterations in platelet numbers is unknown. Moreover, the platelet aggregation capacity during envenomation by *L. obliqua* 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 (Hoffman and Monroe, 2001), these elements probably make a decisive contribution to the appearance of hemorrhagic syndrome during *L. obliqua* envenomation. Thus, the investigation of pathophysiological mechanisms that induce platelet disturbances is indispensable to understand the causes of systemic bleedings, which may be very severe and cause death by intracranial hemorrhage in envenomed individuals (Duarte et al., 1996; Kowacs et al., 2006).

In this work we described, for the first time, the platelet hypoaggregation and blood coagulation disturbances following *L. obliqua* experimental envenomation in rats. Our data present evidences that the *in vivo* platelet aggregation inhibition and the blood incoagulability are decisive for the bleeding disorders observed after contact with *L. obliqua* caterpillars.

2. Materials and methods

2.1. Experimental 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 in the Use of Animals in Toxicology (International Society of Toxicology, http://www.toxicology.org) and the Brazilian College of Animal Experimentation (COBEA). Experiments also following the recommendations of Ethical Committee for the Use of Animals of Federal University of Rio Grande do Sul, Brazil. All efforts were made to minimize the number of animals used and their suffering.

2.2. Drugs and reagents

Rat fibrinogen, bovine type I collagen, adenosine diphosphate (ADP), vanadium (III) chloride (VCl_3), sulfanilamide (SULF), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) and N^G-nitro-L-arginine-methyl-ester (L-NAME) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). GPRP (H-Gly-Pro-Arg-Pro-NH_2) was kindly provided by Dr. Robson Queiroz Monteiro (Institute of Medical Biochemistry, Federal University of Rio de Janeiro, Brazil). Human thrombin was purified in our laboratory from plasma of healthy donors according to Ngai and Chang (1991). Chromogenic substrates for thrombin (S2238, H-D-Phe-Pip-Arg-pNa), factor Xa (S2222, Bz-Ile-Glu-Gly-Arg-pNa), plasmin (S2251, H-D-Val-Leu-Lys-pNa) and urokinase (S2444, pyroGlu-Gly-Arg-pNa) were obtained from Chromogenix (Milano, Italy). Sodium thiopental was from Cristália Produtos Químicos Farmacêuticos (São Paulo, Brazil) and anti-lonomic serum was provided by Instituto Butantan (São Paulo, Brazil). All other chemicals were of the highest commercially available purity.

2.3. L. obliqua *caterpillars and bristle extract*

L. obliqua caterpillars were kindly provided by Centro de Informações Toxicológicas (CIT), Porto Alegre, Rio Grande do Sul, Brazil. *L. obliqua* bristle extract was obtained by cutting bristles at the caterpillar's tegument insertion and the excised material was kept at 4 °C until preparation of the extract, which occurred immediately after dissection. Bristles were macerated in cold saline solution (150 mM NaCl) and centrifuged at 9600 × *g* for 20 min. The protein content of the supernatant, designed as crude bristle extract, was determined by the BCA assay kit (Pierce, Rockford, USA) and aliquots were stored at −20 °C until use. For the experiments, four different preparations of bristle extract were used and the total number of caterpillars varied between 50 and 80 specimens per preparation. The mean protein concentration of bristle extracts was 4.41 ± 0.93 mg/mL (mean ± S.E.M). Considering these different preparations the total amount of venom extracted per caterpillar was 2.56 ± 0.52 mg (mean ± S.E.M).

2.4. Time course of experimental envenomation

In order to follow the evolution of platelet and blood coagulation disturbances we developed an experimental

model of envenomation in rats. For this purpose, animals were divided into two groups: an experimental group, which was injected subcutaneously (s.c.) with 100 μL of a solution containing 1 mg protein of crude *L. obliqua* bristle extract per kg of body weight, and a control group that received subcutaneously 150 mM NaCl in a volume of 100 μL, under the same conditions. Two, 6, 12 and 24 h after injection, rats were anesthetized and blood was collected carefully to avoid platelet activation. A total of 8 animals were utilized in each group, for each time interval.

2.5. Blood collection and platelet preparation

After treatment, animals of experimental and control groups were anesthetized with sodium thiopental (i.p. 83 mg/kg) and blood was collected by cardiac puncture. Blood samples were anticoagulated with 1:5 (v/v) ACD solution (2.5% trisodium citrate, 1.37% citric acid, 2% D-glucose) or 1:10 (v/v) 3.8% trisodium citrate containing 2% anti-lonomic serum. Platelet rich plasma (PRP) was prepared from blood anticoagulated with trisodium citrate, centrifuged at $200 \times g$ thrice for 5 min. Washed platelets (WP) were obtained from blood collected on ACD, centrifuged as above to obtain PRP suspension and then re-centrifuged at $620 \times g$ for 10 min. The platelet pellet was re-suspended in 0.3 mL ACD and washed by gel filtration in a Sepharose 2B column equilibrated and eluted with Tyrode–albumin buffer, pH 7.4, according to Timmons and Hawiger (1989). Washed platelets suspensions were supplemented with 2 mM $CaCl_2$ and 0.5 mg/mL fibrinogen and their density adjusted to $3–4 \times 10^5$ cells/μl. In all experiments platelet blood counts were performed manually in a Neubauer chamber (hemocytometer) using optical microscopy.

2.6. Studies on platelet aggregation ex vivo

Platelet aggregation and shape change (in PRP and washed platelets of envenomed animals) were monitored turbidimetrically, using a Lumi-aggregometer (Chrono-Log Co., Havertown, USA), and light transmission across the platelet suspension was registered on a chart recorder for 5 min (Reck Jr. et al., 2009). Platelet aggregation assays were also performed on a SpectraMax microplate reader (Molecular Devices Co., Sunnyvale, USA) as described (Fuly et al., 2004). Briefly, the platelet aggregation agonists ADP (10 μM), collagen (2.5 μg/mL) and thrombin (3 μg/mL) were incubated for 2 min at 37 °C in 96-well flat-bottomed plates with Tyrode–albumin buffer, pH 7.4. Aggregation was triggered by the addition of PRP or WP suspensions ($3–4 \times 10^5$ cells/μl) and monitored at 650 nm in intervals of 11 s for 30 min. Change in turbidity was measured in absorbance units and results are expressed as area under the aggregation curves. To assay thrombin-induced aggregation directly in PRP, platelets were incubated for 5 min at 37 °C with 8 mM GPRP (H-Gly-Pro-Arg-Pro-NH_2) prior to the addition of agonist. This peptide inhibits fibrin polymerization and avoids plasma coagulation during the aggregation assay (Oenick, 2004).

2.7. Experiments of fibrinogen replacement in platelet rich plasma

Six hours after envenomation, animals presented an accentuated reduction in fibrinogen levels. To verify whether this reduction was the cause of platelet dysfunctions we designed an experiment of fibrinogen replacement in PRP of envenomed animals. For this purpose, rats were separated in two groups: a control group ($n = 8$) that was injected with saline solution and an experimental group ($n = 16$) that was injected with *L. obliqua* bristle extract (1 mg/kg, s.c.). Six hours after envenomation animals were anesthetized, blood was collected and PRP prepared as described above. At this point, the experimental group was subdivided into two subgroups ($n = 8$/subgroup). The fibrinogen levels of one subgroup were replaced by addition of exogenous fibrinogen (2.5 mg/mL, final concentration) directly to the PRP suspension. PRP of other subgroup received equal volume of Tyrode's buffer and platelet response was tested in an aggregation assay.

2.8. Inhibition of platelet aggregation induced by plasma of envenomed animals

The effect of cofactors present in envenomed plasmas on washed platelet aggregation was also verified. For this purpose, rats ($n = 8$/group) were separated into two groups: a control group that was injected with saline solution and an experimental group that was injected with *L. obliqua* bristle extract (1 mg/kg, s.c.). After 6 h, platelets of control rats were washed through a gel filtration column and directly incubated with either the plasmas of envenomed or non-envenomed animals (controls). The cell density was adjusted to $3–4 \times 10^5$ cells/μL and the suspensions used for aggregation assays.

2.9. Studies on blood coagulation parameters

Rats ($n = 8$/group) were envenomed with *L. obliqua* bristle extract as described above. At different times of envenomation (2, 6, 12 and 24 h) blood was collected with 1:10 (v/v) 3.8% trisodium citrate containing 2% anti-lonomic serum. Platelet poor plasma (PPP) was obtained by blood centrifugation at $1500 \times g$ for 10 min. The following coagulation parameters were determined: plasma fibrinogen levels, activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT). Fibrinogen concentration was measured according to Claus (1957). Evaluation of aPTT, PT and TT were performed using commercial kits following manufacturer's instructions (Wiener Lab, Rosario, Argentina). All assays were conducted using a 96-well microplate spectrophotometer (SpectraMax, Molecular Devices Co., Sunnyvale, USA) equipped with temperature control and shaking systems.

2.10. Activity of blood coagulation and fibrinolytic enzymes

To assess the activity of blood coagulation enzymes we used synthetic chromogenic substrates specific for enzymes of the blood coagulation and fibrinolysis. Briefly, 5 μL plasma of envenomed rats (at different times of

envenomation) was incubated in 20 mM Tris–HCl buffer, pH 7.4 at 37 °C. The enzymatic activity generated during *L. obliqua* envenomation in plasmas was measured by adding substrates for thrombin (S2238), factor Xa (S2222), plasmin (S2251) or urokinase (S2444) at 0.2 mM (final concentration) in a volume of 100 μL. The amount of p-nitroaniline produced was monitored at 405 nm in intervals of 14 s for 30 min using a SpectraMAX microplate reader and compared with basal values obtained from plasmas of non-envenomed rats (control group).

2.11. Nitric oxide determination

The total plasmatic levels of nitrate and nitrite (NO_x^-) as an indication of nitric oxide (NO) production were determined as described previously (Miranda et al., 2001). Briefly, plasmas from control and experimental groups were deproteinized by addition of 1:1 (v/v) ethanol and incubated overnight at 4 °C. After centrifugation at 1500 × *g* for 10 min, supernatants (100 μL) were mixed with 8 mg/mL VCl_3 (100 μL) in 96-well microplates. The Griess reagent (100 μL of 2% SULF + 0.1% NEED) was rapidly added to wells and then absorbance was 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).

2.12. Experiments to verify the role of nitric oxide in platelet hypoaggregation

The role of nitric oxide in platelet hypoaggregation was verified in rats by a pretreatment with L-NAME, an inhibitor of NO synthesis. Animals ($n = 8$/group) were injected intraperitoneally with L-NAME at a dose of 50 mg/kg, 24 h and 30 min before *L. obliqua* venom injection (Chaves et al., 2006). After 6 h of envenomation, the platelet aggregation response to ADP and collagen were compared with another groups of animals that received: saline solution (control group), L-NAME without *L. obliqua* venom and *L. obliqua* venom without L-NAME. In these different groups, the total plasmatic levels of nitric oxide were determined as described above.

2.13. Statistical analyses

Results are expressed as mean ± SEM. The significance of differences between mean values of two experimental groups was determined using 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. 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. Clinical symptoms during L. obliqua experimental envenomation

Within the first 6 h after envenomation rats present general signs of toxicity, including lethargy, dyspnea, prostration and high sensitivity on the venom injection site. Several animals presented dark reddish–brown urine at 6–12 h of envenomation, indicating hematuria. No macroscopic signs of hemorrhage, such as petechiae, suffusions or nasal and eye bleeding were observed. After 24 h all rats recovered from the clinical symptoms and no deaths were observed during experiments. Animals of the control group (injected with 150 mM NaCl) showed none of the effects seen in envenomed rats.

3.2. Platelet hypoaggregation in platelet rich plasma (PRP) of rats experimentally envenomed with L. obliqua venom

To evaluate platelet dysfunction, rats were subcutaneously injected with bristle extract (1 mg/kg) and *ex vivo* platelet aggregation was assayed at different post-envenomation times. The PRP of envenomed animals presented a significant platelet hypoaggregation (Fig. 1). The ADP-induced platelet aggregation showed an evident decrease 2 h after venom administration, reaching 98.6% of inhibition in the aggregation response at 6 h (Fig. 1A). Collagen-induced platelet aggregation was also reduced at 2 h and presented a decrease of 98.4% 6 h post-envenomation (Fig. 1B). Both ADP and collagen-induced aggregation were partially recovered after 12 h and no significant difference was observed after 24 h, as compared to control values. To test thrombin-induced aggregation directly on PRP, platelets of envenomed animals were previously incubated with 8 mM GPRP (H-Gly-Pro-Arg-Pro-NH_2), a peptide that inhibits fibrin polymerization (Oenick, 2004). As shown in Fig. 1C, thrombin-induced platelet aggregation was reduced by 90.9% after 6 h venom injection.

3.3. Platelet blood counts and fibrinogen levels during L. obliqua experimental envenomation

There was a significant decrease in platelet counts of envenomed rats after 6 h (429,500 ± 27/μL) and 12 h (442,885 ± 67/μL) in comparison to the control group (577,383 ± 18/μL) (Fig. 2A). Yet, this decrease represents a reduction of only 26% (at 6 h) and 24% (at 12 h) in platelet blood numbers when compared to non-envenomed animals.

Plasma fibrinogen levels presented an abrupt fall after bristle extract administration (Fig. 2B). After 2 h fibrinogen have already been consumed in comparison to control values (1.16 ± 0.10 g/L vs. 2.63 ± 0.14 g/L) and reached the lowest levels (0.32 ± 0.02 g/L) after 6 h. In 12 h a slight increase in fibrinogen was observed (0.66 ± 0.05 g/L), becoming normal in 24 h (3.00 ± 0.28 g/L) (Fig. 2B).

3.4. Effects of fibrinogen replacement on platelet hypoaggregation induced by L. obliqua experimental envenomation

The possibility of platelet hypoaggregation as a consequence of the low levels of plasma fibrinogen was investigated. For this purpose, PRP of envenomed animals was prepared as described in the Materials and methods section and, immediately before the aggregation assay, fibrinogen levels were replaced by addition of exogenous fibrinogen (2.5 mg/mL, final concentration) directly to the PRP suspension. As shown in Fig. 3, the addition of fibrinogen to

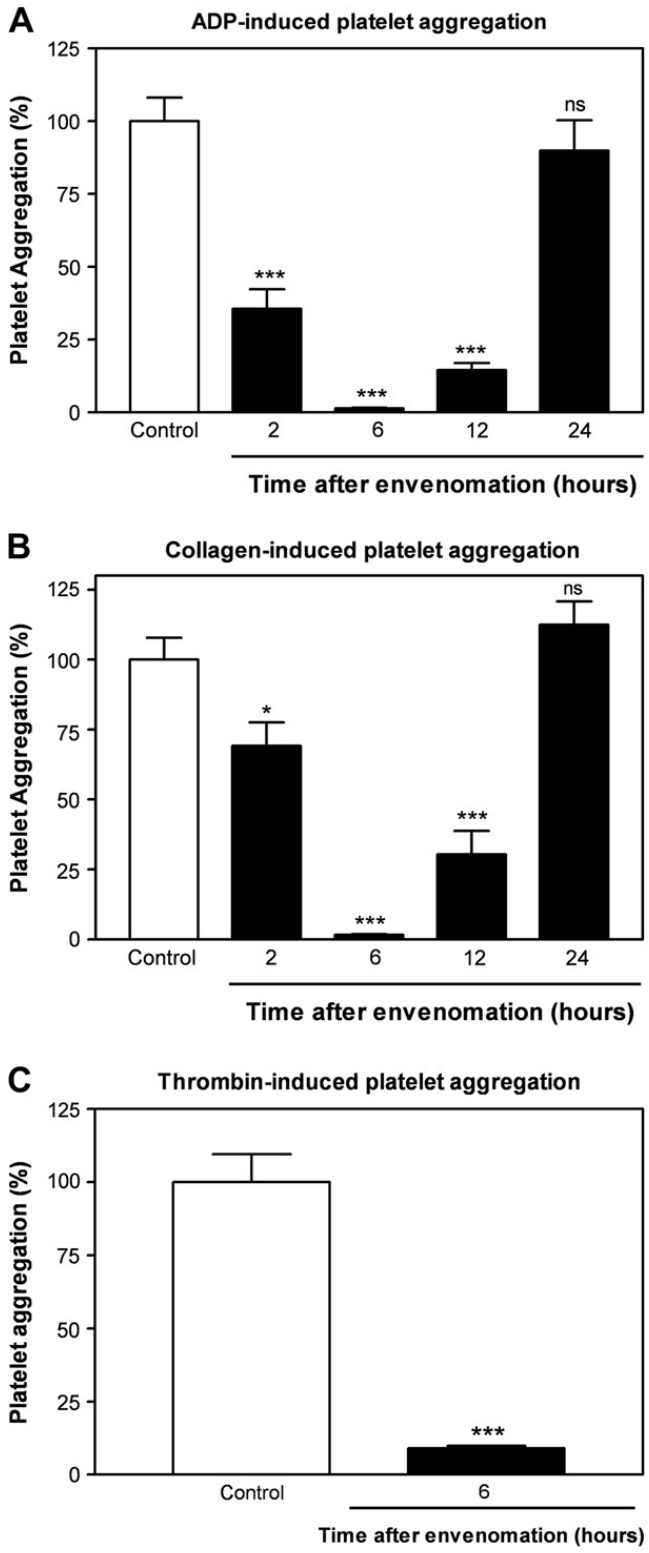

Fig. 1. Time course of platelet aggregation in platelet rich plasma (PRP) of rats envenomed with *L. obliqua* venom. The aggregation response induced by (A) 10 μM ADP and (B) 2.5 μg/mL collagen was determined directly in PRP of control groups (non-envenomed animals) and experimental groups 2, 6, 12 and 24 h post-envenomation. Thrombin-induced aggregation (C) was also tested in PRP after 6 h post-venom injection by addition of 3 μg/mL thrombin. Results were expressed as mean ± S.E.M. of 8 animals in each group, for each time interval. Values of $^{*}p < 0.05$ and $^{***}p < 0.001$ were considered statistically significant in comparison to the control group. (ns) Non-statistically different from the control.

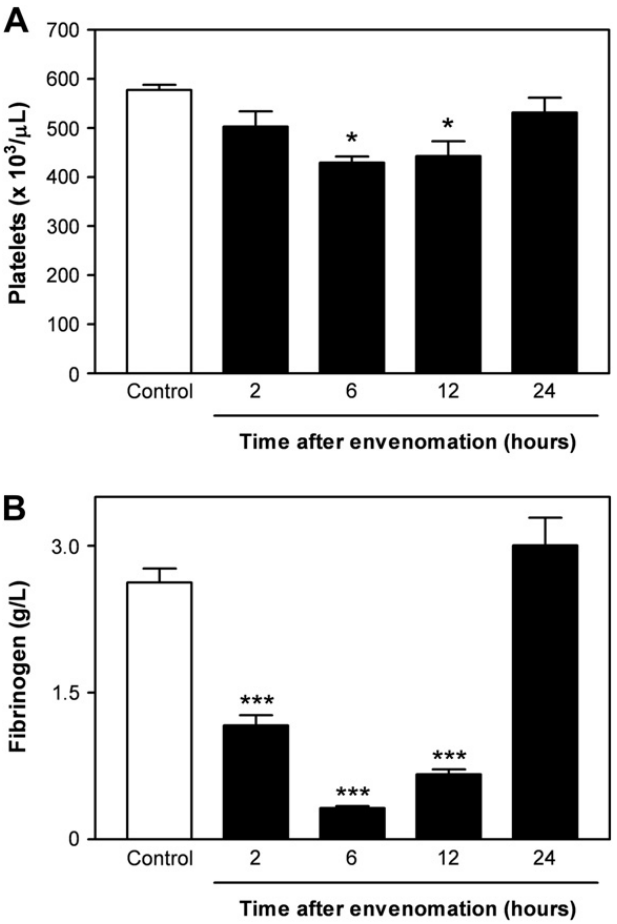

Fig. 2. Platelet blood counts and fibrinogen levels during *L. obliqua* experimental envenomation in rats. Platelet blood counts (A) and fibrinogen levels (B) were determined in control groups (non-envenomed animals) and experimental groups 2, 6, 12 and 24 h post-envenomation. Results were expressed as mean ± S.E.M. of 8 animals in each group, for each time interval. Values of $^{***}p < 0.001$ and $^{*}p < 0.05$ were considered statistically significant in comparison to the control group.

PRP did not improve the aggregation response to ADP (Fig. 3A) and collagen (Fig. 3B) at 6 h of *L. obliqua* envenomation.

3.5. Platelet aggregation in washed platelet suspensions of rats envenomed with L. obliqua venom

To rule out the possible influence of a plasma cofactor on platelet aggregation inhibition observed in PRP (Figs. 1 and 4A), a suspension of washed platelets (WP) was prepared from envenomed rats 6 h after venom injection. Interestingly, washed platelets did not present any dysfunction in aggregation capacity (Fig. 4B) when compared with an evident hypoaggregation observed in PRP (Fig. 4A). No difference was observed between control and experimental groups for aggregation response induced by ADP, collagen or thrombin in WP suspensions (Fig. 4B).

3.6. Platelet aggregation inhibition induced by the plasma of rats envenomed with L. obliqua

In order to confirm the existence of a putative inhibitory cofactor of platelet aggregation in plasma of *L. obliqua*

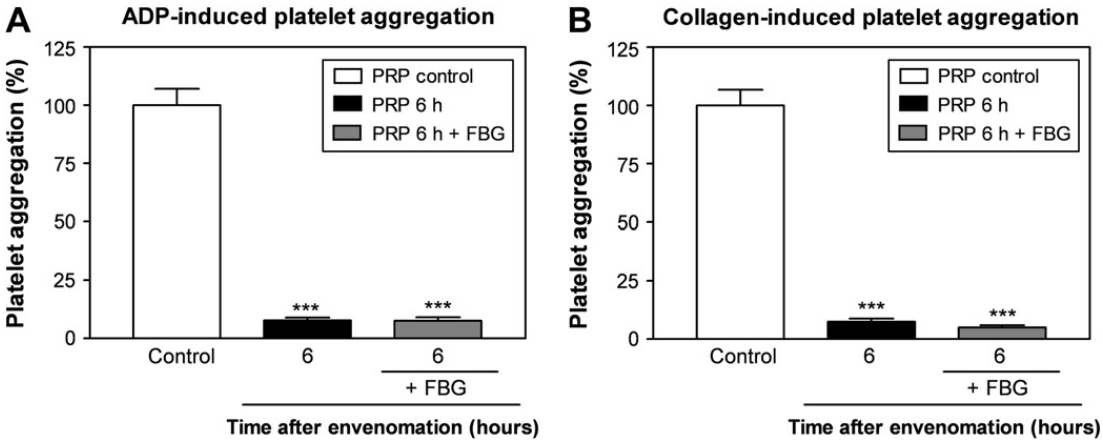

Fig. 3. Effects of fibrinogen replacement on platelet hypoaggregation induced by *L. obliqua* envenomation in rats. Rats were divided into control ($n = 8$) and experimental ($n = 16$) groups and treated as described in Materials and methods. After 6 h, PRP was obtained and the experimental group was subdivided into two subgroups ($n = 8$/subgroup). The fibrinogen (FBG) levels of one subgroup were replaced and other subgroup received Tyrode's buffer. Then the platelet aggregation response was tested by addition of ADP (A) and collagen (B). (6 h + FBG) Represent PRP of subgroup that received fibrinogen addition and (6 h) PRP of subgroup that not received fibrinogen. Results were expressed as mean ± S.E.M. and values of $^{***}p < 0.001$ were considered statistically significant in comparison to the control group.

envenomed rats, a suspension of washed platelets from non-envenomed rats (control groups) was incubated with either the plasma from envenomed animals, or with a plasma pool obtained from controls. As shown in Fig. 5, normoaggregation was observed when normal washed platelets were incubated with plasma of non-envenomed

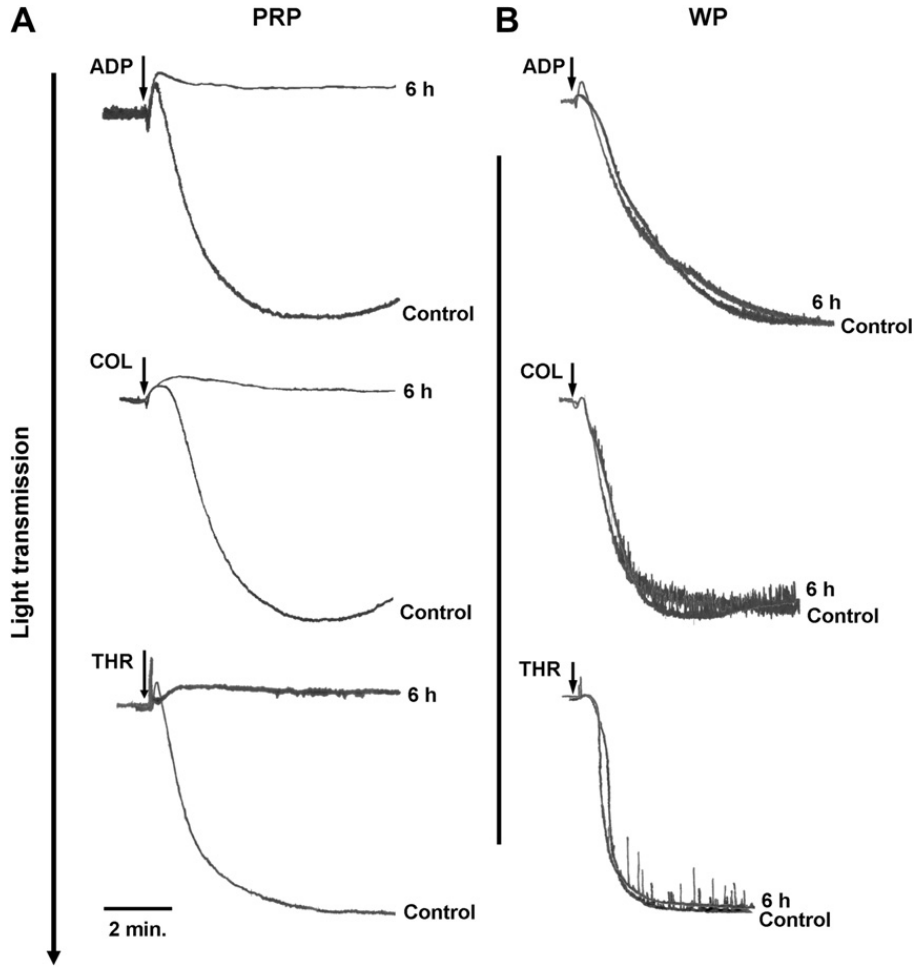

Fig. 4. Comparison between platelet aggregation in platelet rich plasma (PRP) and washed platelet (WP) suspensions of rats envenomed with *L. obliqua* venom. PRP and WP suspensions from non-envenomed (control, $n = 8$) and envenomed ($n = 8$) animals were obtained after 6 h of venom injection. The aggregation response was registered on a Lumi-aggregometer by addition of 10 μM ADP, 2.5 μg/mL collagen and 3 μg/mL thrombin directly to PRP (A) and WP (B) suspensions. Tracing shows representative results of quadruplicate experiments.

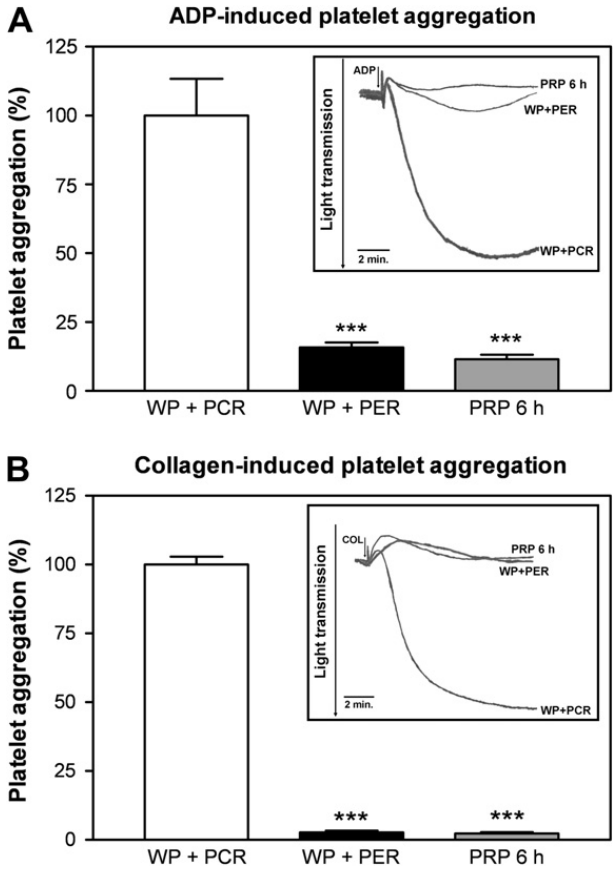

Fig. 5. Platelet aggregation inhibition induced by the plasma of rats envenomed with *L. obliqua* venom. Rats ($n = 8$/group) were divided into control and experimental groups and treated as described in Materials and methods. After 6 h, platelets of control rats were washed and directly incubated with the plasmas of either envenomed or non-envenomed animals. Then, the aggregation ability was tested by addition of ADP (A) and collagen (B). (□) WP + PCR, washed platelets of control rats + plasma of control rats; (■) WP + PER, washed platelets of control rats + plasma of envenomed rats; (▭) PRP 6 h, platelet rich plasma of envenomed rats at 6 h. Results were expressed as mean ± S.E.M. and values of $^{***}p < 0.001$ were considered statistically significant in comparison to the WP + PCR group. The insets show a typical platelet aggregation tracings registered on a Lumi-aggregometer after addition of ADP and collagen.

rats. However, when normal washed platelets were incubated with plasma obtained from envenomed rats (6 h of envenomation) an accentuated inhibition in the platelet aggregation response induced by ADP (Fig. 5A) and collagen (Fig. 5B) was noticed. This reduction in platelet aggregation was similar to that observed in PRP of animals 6 h post-envenomation (Fig. 5A and B).

3.7. The role of nitric oxide in platelet hypoaggregation induced by L. obliqua experimental envenomation

The production of nitric oxide (NO) during the time course of envenomation was assessed by the determination of total nitrite and nitrate (NO_x^-) levels in plasma. A slight increase in total NO production was observed early in the course of envenomation (2 h) and a significant elevation of 1.7-fold after 6 h (Fig. 6A). After this time, total NO levels decreased (12 h) and reached similar concentrations to those of control rats in 24 h (Fig. 6A). In the crude bristle extract, the level of nitrite/nitrate was 0.25 ± 0.02 μM (mean ± S.E.M.). Considering the total dilution of venom in blood circulation the concentration reaches approximately 0.0062 μM. This concentration was much lower than obtained in plasma of control (6.3 ± 0.2 μM) and envenomed (11.0 ± 0.9 μM) animals, indicating that the influence of nitrate/nitrite levels present in bristle extract preparation can be discarded.

The participation of NO in platelet hypoaggregation was verified by systemic inhibition of NO synthesis. For this purpose, envenomed rats were pretreated with L-NAME, a nonselective inhibitor of NO synthase activity. As show in Fig. 6B, animals pretreated with L-NAME that received bristle extract injection presented an increase of 25.01 ± 2.22% and 30.77 ± 2.41% in the aggregation response to ADP and collagen, respectively, when compared with animals that received only bristle extract injection. Increase in aggregation response was coincident with a reduction of NO levels in envenomed rats pretreated with L-NAME (Fig. 6B). In addition, no significant alterations in platelet count and clinical symptoms were observed between envenomed animals treated and non-treated with L-NAME (results not show).

3.8. Blood coagulation and fibrinolytic parameters during L. obliqua experimental envenomation

Blood coagulation disturbances were evaluated by coagulation tests of PT (extrinsic pathway), aPTT (intrinsic pathway) and TT (common pathway) as well as by the measuring of enzymatic activity of blood clotting and fibrinolytic enzymes. Severe coagulopathy was verified during *L. obliqua* envenomation (Table 1). An intense prolongation of PT, aPTT and TT tests occurred in envenomed animals at 6 h. Twelve hours after venom administration PT and TT tests were still prolonged and 24 h after the coagulation time it was back to normal, in comparison with values of non-envenomed rats (Table 1).

Thrombin activity presented a 2.6-fold increase at the beginning of envenomation (2 h). Afterwards, a decrease in thrombin activity was observed, reaching basal values at 24 h (Table 2). Factor Xa activity was not statistically different from control values, despite a mild increase during the first 12 h of envenomation. At all time intervals envenomed rats had higher levels of plasmin activity as compared to the control group. Nevertheless, maximal increases in plasmin activity were observed at 2 h (2.5-fold) and 24 h (2.9-fold) (Table 2). Urokinase activity showed a slight increase during the first 12 h, reaching maximal activity 24 h post-envenomation (Table 2).

4. Discussion

Venomous animals, such as snakes, spiders, scorpions and the caterpillars of *Lonomia* genus are rich sources of molecules that destabilize hemostasis (Arocha-Piñango et al., 2000; Escalante et al., 2003; Chaim et al., 2006). In the experimental model presented herein, rats subcutaneously injected with *L. obliqua* venom showed severe platelet and blood coagulation disturbances, reproducing the clinical

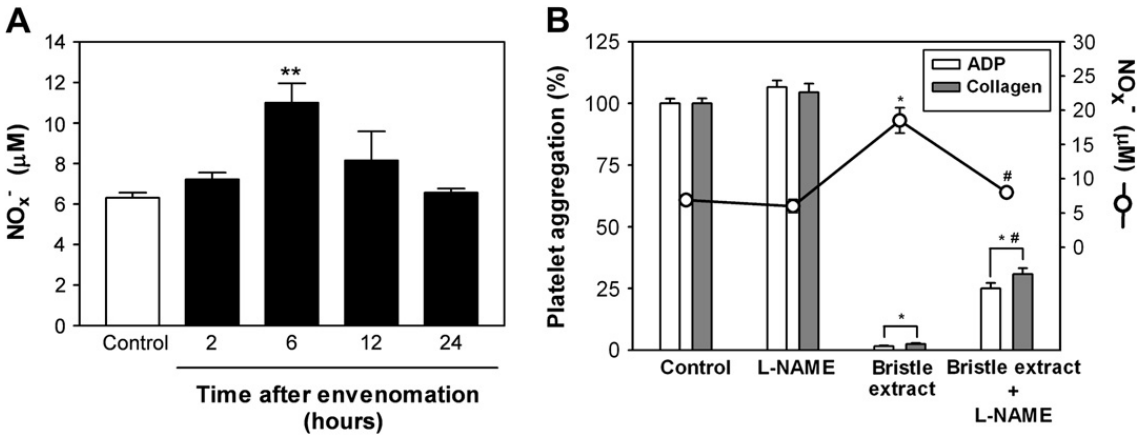

Fig. 6. The role of nitric oxide in platelet hypoaggregation induced by *L. obliqua* experimental envenomation. (A) Total levels of nitrate and nitrite (NO_x^-) were determined in plasmas of control and experimental groups 2, 6, 12 and 24 h post-envenomation. Results were expressed as mean ± S.E.M. of 8 animals in each group, for each time interval. Values of $^{**}p < 0.01$ were considered statistically significant in comparison to control group. (B) Rats ($n = 8$/group) were pretreated with L-NAME, and were then injected with *L. obliqua* bristle extract. Aggregation response to ADP and collagen were tested in PRP and compared with groups that received only saline solution (control), L-NAME or bristle extract. Plasmatic NO levels of different groups were also determined. Results were expressed as mean ± S.E.M. (*) Statistical difference compared with control group ($p < 0.001$) and (#) statistical difference compared with group injected with bristle extract ($p < 0.001$).

symptoms and biochemical effects of envenomation caused by these caterpillars in humans.

The major pathological alterations observed in envenomed patients are subcutaneous hemorrhages and systemic reactions associated with severe coagulopathy. In some cases the clinical profile may evolve into acute renal failure and intracerebral bleeding. The severity of the 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 spines but also in their skin, which consists of a complex tegument with several cuticular specializations such as spicules or scoli (Veiga et al., 2001). Considering that accidents with medical importance in general involve the contact with a colony containing at least 40–50 caterpillars (Gamborgi et al., 2006) and that during venom extraction, after removal of all spicules, each caterpillar produces approximately 2.56 mg of venom, the total amount of venom injected in an individual weighing 70 kg can reach up to 1.46–1.82 mg/kg. In fact, these doses were calculated based on an artificial method of venom extraction, in which the caterpillar's spicules were macerated in a solution buffer. Thus, in a real envenomation situation the total amount of venom inoculated is probably lower than the amount calculated. In an attempt to reproduce the clinical conditions observed in a real envenomation we selected a dose of 1 mg/kg injected via subcutaneous route into rats. This dose was also in accordance with the amount of venom used in other studies to induce coagulopathy and test the efficacy of anti-lonomic serum (Dias da Silva et al., 1996; Rocha-Campos et al., 2001).

Table 1
Blood coagulation parameters during the time course of *L. obliqua* envenomation in rats.

Time after envenomation (h)	Blood coagulation parameters (s)		
	PT	aPTT	TT
Control	18.6 ± 0.93	18.1 ± 0.6	23.9 ± 2.5
2	36.2 ± 2.7***	58.6 ± 5.2**	32.9 ± 4.3
6	92.0 ± 1.5***	187.9 ± 16.0***	183.1 ± 11.0***
12	39.7 ± 2.1***	24.5 ± 1.6	50.1 ± 6.6*
24	18.9 ± 0.7	19.8 ± 1.6	21.0 ± 1.9

Plasma of animals ($n = 8$/group) was collected at different times post-envenomation and the following coagulation parameters were determined: Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT). Results are expressed in seconds as mean ± S.E.M. Values of $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ were considered statistically significant when compared to coagulation time of non-envenomed rats (control group).

The data obtained in the present work demonstrate that the platelet aggregation response, induced by either ADP (Fig. 1A) or collagen (Fig. 1B), is decreased in rat's 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 PRP of envenomed rats (Fig. 1C). Probably, the hemostatic function of platelets is not maintained following envenomation, since other reports demonstrated that rats previously injected with crude bristle extract presented prolongation in their bleeding times (Prezoto et al., 2002). In human patients bleeding manifestations such as gengivorrhagia or bleeding from recent wounds are also common (Kelen et al., 1995). Taken together, these data indicate that platelets are not able to aggregate and perform their normal hemostatic function to avoid blood loss.

During the course of these experiments, envenomed animals presented a slight reduction of approximately 26% in the platelet count at 6 h, being normal after 24 h of envenomation (Fig. 2A). These results are in accordance with those obtained 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). In contrast, moderate and severe thrombocytopenia are common findings in several snake

Table 2
Activity of blood coagulation and fibrinolytic enzymes during the time course of *L. obliqua* envenomation in rats.

Time after envenomation (h)	Enzyme activity (mOD/min)			
	Thrombin	Factor Xa	Plasmin	Urokinase
Control	2.16 ± 0.18	0.84 ± 0.07	1.00 ± 0.17	1.80 ± 0.17
2	5.67 ± 0.47***	0.94 ± 0.05	2.53 ± 0.37**	1.93 ± 0.13
6	3.73 ± 0.16**	1.09 ± 0.10	2.01 ± 0.15*	2.20 ± 0.15
12	3.59 ± 0.24*	1.05 ± 0.07	2.21 ± 0.13*	2.76 ± 0.26
24	2.79 ± 0.13	0.77 ± 0.02	2.99 ± 0.42***	3.12 ± 0.46*

Plasma of rats (n = 8/group) was obtained and enzyme activity determined by addition of specific synthetic substrates. Kinetic of the reactions were monitored at 405 nm and results (mean ± S.E.M) expressed as mOD/min. Values of ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ were considered statistically different when compared to the basal activity in plasma of non-envenomed rats (control group).

envenomations (Sano-Martins et al., 1997; Rucavado et al., 2005). Rabbits experimentally envenomed with *Bothrops jararaca* venom present a decrease of 50% in platelet counts, which persists for 24 h (Santoro and Sano-Martins, 2004). In this type of envenomation, the thrombin generated intravascularly (Berger et al., 2008) seems to play a crucial role in inducing platelet consumption, since previous administration of heparin to dogs injected intravenously with *B. jararaca* venom inhibited the development of thrombocytopenia (Nahas et al., 1975). Furthermore, intravenous administration of thrombin causes thrombocytopenia, hypofibrinogenemia and accumulation of platelet aggregates and fibrin in lungs and kidneys (Bergentz et al., 1972; Shimamura et al., 1983; van Oost et al., 1983). In the *L. obliqua* envenomation, animals also presented an accentuated decrease in fibrinogen levels between 2 and 12 h (Fig. 2B) and an expressive increase in thrombin activity was detected in their plasmas (Table 2). In fact, data obtained from human patients showed intense consumption of fibrinogen plasmatic levels and an increase in concentrations of prothrombin fragments 1 + 2, which also indicates that significant amounts of thrombin are generated in the circulation of envenomed patients (Zannin et al., 2003). Nonetheless, our data suggest that aggregation or consumption of platelets in blood circulation does not seem to occur, since only a slight reduction in platelet numbers was observed. Indeed, histopathological analysis in biopsies from envenomed patients showed acute renal tubular necrosis, but no deposition of platelets nor of fibrin was found in kidneys and lungs (Burdmann et al., 1996; Gamborgi et al., 2006). Probably, the platelet aggregation inhibition can prevent platelet consumption and deposition of thrombi in the vascular beds of these organs. Thus, platelet hypoaggregation can decisively contribute to the hemorrhagic clinical profile observed in *L. obliqua* envenomation.

Fibrinogen is considered an essential molecule for platelet aggregation. Since fibrinogen is a symmetrical molecule, platelet aggregation occurs through the formation of fibrinogen (and/or von Willebrand factor) bridges between GPIIb–IIIa molecules of adjacent platelets (Blockmans et al., 1995). As envenomed animals presented significant hypofibrinogenemia (Fig. 2B), we tested whether these low levels of fibrinogen could contribute to the dysfunction observed in platelet aggregation during *L. obliqua* envenomation. Despite an apparent correlation between low levels of fibrinogen and inhibition in platelet aggregation, the addition of exogenous fibrinogen to PRP of envenomed rats does not correct the deficiency in ADP and collagen-induced aggregation 6 h after venom injection (Fig. 3).

To verify a possible direct action of *L. obliqua* venom on the function of platelet surface receptors, platelets of envenomed animals were separated from other plasmatic components and then tested for their aggregation ability. For this purpose, platelets were washed through a gel filtration column. Interestingly, gel filtrated-platelets from envenomed rats presented normal aggregation intensity when stimulated either with ADP, collagen or thrombin (Fig. 4B). These results indicate that, despite the high proteolytic activity in the bristle extract (Pinto et al., 2006), none of the venom enzymes seems to have a direct action on platelet surface receptors, since normoaggregation was observed after the washing procedure. Moreover, the data suggest that an inhibitor of platelet aggregation is generated in plasma of envenomed rats. Confirming this hypothesis, when normal washed platelets were incubated with plasma of envenomed animals, a reduction of platelet aggregation intensity was observed in comparison with aggregation of platelets incubated with plasma of control rats (Fig. 5). Similar results were obtained by Sano-Martins et al. (1997) incubating washed human platelets with plasma of patients bitten by *B. jararaca*. In addition, rabbits experimentally envenomed with *B. jararaca* venom presented platelet hypoaggregation and it was also suggested that a plasma inhibitor, generated during envenomation, might contribute to platelet dysfunction (Santoro and Sano-Martins, 2004).

Until now, the identity of this plasmatic aggregation inhibitor is unknown. Fibrinogen consumption and the rise of fibrinogen/fibrin degradation products (FfDP) and D-dimer levels are important factors, 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). On the other hand, FfDP does not seem to be the only factor responsible for diminishing platelet aggregation, because patients bitten by *B. jararaca* snakes showed hypoaggregation in response to ADP even after normalization in FfDP levels and increase in platelet numbers (Sano-Martins et al., 1997). In this work, the activation of the fibrinolytic system was assessed by increase in plasmin and urokinase activities (Table 2). So, it is possible to think that FfDP and D-dimer could also be elevated in the present model of envenomation.

A significant increment in plasmatic nitric oxide (NO) levels has been described in *Bothrops atrox*, *Bothrops asper* and *B. jararaca* envenomations (Barros et al., 1998; Petricevich et al., 2000). During *L. obliqua* envenomation, rats also presented an increase in NO, which reached the maximum concentration after 6 h (Fig. 6). Endothelium-derived NO is an important endogenous mediator in the control of vascular tone due to its powerful vasodilating activity (Umans and Levi, 1995). Besides its physiological role, NO has been implicated in the genesis of a variety of pathological conditions (Olsen, 1972; Büsse and Mülsch, 1990; Libby and Hansson, 1991). In the pathogenesis of envenomation, NO might participate by several mechanisms: (a) it may induce tissue damage due to its ability to generate peroxynitrites and hydroxyl radicals after interacting with the anion superoxide (Radi et al., 1991; Hogg et al., 1992), (b) it might contribute to the hypotension characteristic of envenomations due to its strong vasodilating action (Joseph et al., 2004; Bohrer et al., 2007), and (c) it might increase the hemorrhagic effect of venoms by inhibiting platelet aggregation (Fritzen et al., 2005). In fact, by the activation of guanylyl cyclase, inhibition of the phosphoinositide 3-kinase (PI3K) pathway and suppression of intracellular calcium flux, NO can suppress both P-selectin expression and the conformational change in GPIIb–IIIa required for fibrinogen binding and normal platelet aggregation (Michelson et al., 1996; Ekmekçi et al., 2006; Kleinbongard et al., 2007). In this model of *L. obliqua* envenomation, platelet hypoaggregation coincided with elevation in total NO levels, mostly after 6 h, when the maximum inhibition in platelet response was observed. In addition, the *in vivo* blockade of NO synthase activity by L-NAME pretreatment partially revert the platelet hypoaggregation response induced by envenomation. Thus, we suggest that NO can be one of the mediators that participate in platelet dysfunctions. It is possible that the generation of NO, production of FfDP and probably other unknown factors can act synergistically to impair platelet function. The mechanisms that lead to *in vivo* platelet aggregation inhibition are currently under investigation.

The injection of *L. obliqua* bristle extract in rats induces blood incoagulability as demonstrated by the significant prolongation of hemostatic parameters, such as prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) (Table 1). Blood coagulation disturbances have been associated with the consumption of coagulation factors, mainly to fibrinogen consumption (Zannin et al., 2003; Gonçalves et al., 2007). In fact, envenomed rats presented normal hemostatic parameters (PT, aPTT and TT) only after the fibrinogen returned to normal levels (at 24 h of envenomation). *L. obliqua* venom possesses procoagulant and fibrin(ogen)olytic enzymes that are able to activate clotting and fibrinolytic cascades, leading to consumption of fibrinogen and other coagulation factors (Veiga et al., 2003). Envenomed patients showed high concentrations of thrombin–anti-thrombin complex and prothrombin fragments $1+2$ in blood, which indicates that significant amount of intravascular thrombin was generated (Zannin et al., 2003). Our results confirm these data, since an elevation in thrombin activity (Table 2) was found in plasma of envenomed rats, which was likely due to a strong prothrombin activating activity present in bristle extract. Nonetheless, factor Xa activity was not altered, despite the presence of a factor X activator in the venom (Alvarez-Flores et al., 2006). To date, no direct plasminogen activator activity was reported in *L. obliqua* venom. Only a mild alteration in plasmatic urokinase plasminogen activator (uPA) has been reported in envenomed patients. However, plasminogen and α2-antiplasmin levels are reduced in these patients (Zannin et al., 2003) and, in envenomed animals, an increase in plasmin and urokinase activity was observed (Table 2). Besides, a recent gene expression study of envenomed cells shows an up-regulation of the urokinase plasminogen activator receptor (uPAR), a glycosylphosphatidylinositol-anchored protein that binds uPA with high affinity and facilitates plasminogen activation and plasmin generation (Pinto et al., 2008). Taken together, these results indicate that *L. obliqua* venom is able to activate the fibrinolytic system *in vivo* and generate active plasmin, despite the absence of a direct plasminogen activator in the venom.

On the other hand, the increased activity of fibrinolytic enzymes detected in plasma of envenomed rats, mainly plasmin activity, could be correlated to the absence of thrombocytopenia observed in animals and patients. In studies performed with chimpanzees that received an infusion of a combination of factor Xa and phosphatidylcholine/phosphatidylserine vesicles an abrupt fall of circulating platelets in the first few minutes following the procoagulant stimulus was observed (Toh et al., 1993). The loss of circulating platelets resulted from the formation of intravascular aggregates which were then filtered and sequestered into the microcirculation of lungs (Bergentz et al., 1972). After 60 min, sequestered platelets returned to the circulation and this return coincided with the fibrinolytic response observed in these animals. Thus, it was suggested that the dispersion of platelet aggregates in the microcirculation results from a proteolytic event mediated by plasmin (Giles et al., 1990; Toh et al., 1993). Significant amounts of plasmin was generated in the early phase of *L. obliqua* envenomation and remained active in plasma even 24 h after venom injection (Table 2). Moreover, *L. obliqua* venom present fibrin(ogen)olytic enzymes that are able to cleave both fibrinogen and fibrin (Pinto et al., 2004). It is possible that the generated plasmin, fibrin(ogen)lytic enzymes and the plasma-derived platelet aggregation inhibitor can prevent the formation of platelet aggregates and the deposition of thrombi on microcirculation. Thus, the hemorrhagic effect is maximized, despite the high *in vitro* procoagulant activity of the venom (Veiga et al., 2003).

In the present work, we describe platelet hypoaggregation and blood coagulation disturbances following *L. obliqua* experimental envenomation. The data obtained support the hypothesis that the activation of coagulation and fibrinolytic enzymes play an important role, leading to consumption of clotting factors and consequently to blood incoagulability. Moreover, during envenomation a platelet aggregation inhibitor seems to be generated in plasma, impairing platelet function. Certainly this *in vivo* inhibition of platelet aggregation contributes decisively to systemic bleeding observed in *L. obliqua* envenomation. The

mechanisms involved in platelet aggregation inhibition are the aims of a current investigation.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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3.2 Atividade do veneno de *Lonomia obliqua* sobre agregação e adesão de plaquetas humanas *in vitro* – Artigo II

No trabalho anterior foram investigadas as alterações de agregação plaquetária causadas pelo envenenamento em um modelo experimental *in vivo*. Neste segundo trabalho apresentamos os resultados relativos aos efeitos diretos do veneno sobre a agregação e adesão de plaquetas *in vitro*. Os dados obtidos indicam que o veneno de *Lonomia obliqua* é capaz de induzir diretamente agregação e adesão de plaquetas humanas. O veneno também foi capaz de induzir secreção de ATP de maneira dose-dependente. O mecanismo de ativação plaquetária induzido pelo veneno envolve a mobilização de cálcio do meio externo para o interior da plaqueta e a produção de metabólitos da via das ciclooxigenases. A ativação da fosfodiesterase 3A e a conseqüente redução do conteúdo intracelular de AMPc também parecem estar envolvidos. No entanto, a secreção de grânulos de ADP não foi essencial para o processo de agregação induzido pelo veneno. Os principais componentes do veneno responsáveis pela atividade pró-agregante foram enzimas da classe das fosfolipases A2.

Os resultados apresentados a seguir estão na forma de um manuscrito que está submetido para publicação no periódico **Journal of Thrombosis and Thrombolysis**.

Lonomia obliqua venomous secretion induces human platelet adhesion and aggregation

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Abstract

The caterpillar *Lonomia obliqua* is a venomous animal that causes numerous accidents, especially in southern Brazil, where it is considered a public health problem. The clinical manifestations include several haemostatic disturbances that lead to a hemorrhagic syndrome. Considering that platelets play a central role in hemostasis, in this work we investigate the effects of *L. obliqua* venomous secretion upon blood platelets responses *in vitro*. Results obtained shows that *L. obliqua* venom directly induces aggregation and ATP secretion in human washed platelets in a dose-dependent manner. Electron microscopy studies clearly showed that the venomous bristle extract was also able to produce direct platelets shape change and adhesion as well as activation and formation of platelet aggregates. Differently from other enzyme inhibitors, the venom-induced platelet aggregation was significativly inhibited by *p*-bromophenacyl bromide, a specific inhibitor of phospholipases A2. Additional experiments with different pharmacological antagonists indicate that the aggregation response triggered by the venom active components occurs through a calcium-dependent mechanism involving arachidonic acid metabolite(s) of the cyclooxygenase pathway and activation of phosphodiesterase 3A, an enzyme that leads to the consumption of intracellular cAMP content. It was additionally found that *L. obliqua*-induced platelet aggregation was independent of ADP release. Altogether, these findings are in line with the need for a better understanding of the complex hemorrhagic syndrome resulting from the envenomation caused by *L. obliqua* caterpillars, and can also give new insights into the management of its clinical profile.

Keywords: Lonomia, platelets, hemorrhagic syndrome, coagulation, phospholipase A2.

1. Introduction

Lonomia obliqua (Lepidoptera, Saturniidae) is a medically important animal particularly venomous in its larval stages, which occur during spring and summer in Southern Brazil (mainly in states of Paraná, Santa Catarina and Rio Grande do Sul) [1]. At these stages the caterpillar can cause severe and even fatal accidents upon contact with the bristles that cover the animal's body. These structures are hard and spiny evaginations of the cuticle, underneath which the venom is stored. Since the whole animal is crushed in this type of accident, the insect's chitinous bristles are broken and the venomous secretions penetrate the human skin, reaching the blood circulation [2]. Victims of the envenomation present a typical profile of an acquired hemorrhagic syndrome [3]. Initial symptoms include pain and burning sensation at the site of contact, generally followed by more severe clinical manifestations, such as bleeding from skin and mucous membranes, epistaxis, hematuria, acute renal failure and melena. If the victim is not quickly treated, intracerebral bleeding may occur, leading to death [4]. Due to their gregarious habits the accidents with *L. obliqua* frequently involves contact with whole colonies containing dozens or hundreds caterpillars, which increases clinical consequences and complicate the prognosis of the envenomation [5].

Some of the biochemical components responsible for blood coagulation and fibrinolysis disturbances were identified and their contributions to venom-induced pathology have been determined [1, 6-9]. Although thrombocytopenia has been detected in some envenomed patients, mainly in the most serious cases, no reports exist about platelets involvement and their possible function in the clinical profile produced by such envenomation [5, 10, 11]. Thus, the role of

platelets in the physiopathology of *L. obliqua* envenomation has not been fully investigated so far. Recently, we demonstrated that rats experimentally envenomed with *L. obliqua* venom presented a severe impairment of the platelet function *in vivo* [12]. Despite of this *in vivo* characterization, the direct effect of *L. obliqua* venom on isolated platelets remains unknown. Considering that the platelets are important not only to prevent blood loss by platelet plug formation, but also by playing an essential role in the regulation of the coagulation system and thrombin generation [13], we thought that these elements would probably make a decisive contribution to the appearance and development of the hemorrhagic syndrome. Thus, characterization of venom-induced effects on platelets seems to be essential to help a better understanding of the physiopathology of *L. obliqua* envenomation.

In this work we describe, for the first time, the ability of *Lonomia obliqua* venom to directly induce activation, aggregation and adhesion of human platelets. Here, we performed experiments to elucidate the molecular pathway mechanisms by which venom-induced platelet aggregation occurs and, in addition, we were able to identify venom components that may contribute for this activity.

2. Materials and methods

2.1 Drugs and reagents

Luciferin-luciferase (Chrono-lume) was obtained from Chrono-log (Havertown, PA, USA). Verapamil (Sandoz pharmaceuticals, Brazil) and cilostazol (Libbs pharmaceuticals, Brazil) were obtained as commercial drugs. Indomethacin, esculetin, apyrase grade VII, PMSF (phenylmethanesulfonyl fluoride), E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane), *p*-BPB (*p*-bromophenacyl bromide), EGTA (ethylene glycol-bis(aminoethyl ether)-N, N, N', N'-tetraacetic acid), ADP (adenosine diphosphate), bovine collagen type I, bovine fibrinogen, BCA assay kit and TOX-7 *in vitro* toxicology assay kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). D-Phe-Pro-Arg-chloromethylketone (PPACK) was from Gibco Life Technologies (Gaithersburg, USA). Human thrombin was purified in our laboratory from plasma of healthy donors according to Ngai and Chang (1991) [14]. All other chemicals were of the highest purity commercially available.

2.2 Lonomia obliqua caterpillars and bristle extract

L. obliqua caterpillars were kindly provided by Centro de Informações Toxicológicas (CIT), Porto Alegre, Rio Grande do Sul, Brazil. *L. obliqua* bristle extracts were obtained as previously described [7], by cutting bristles at the caterpillar's tegument insertion, macerating in cold saline solution (150 mM NaCl) and centrifuging at 9,600 x g for 20 minutes. The protein content of the supernatant, designed as *L. obliqua* bristle extract (Lobe), was determined by the BCA assay kit (Pierce, Rockford, USA) and aliquots were stored at -20 °C until use. For the experiments, four different preparations of bristle extract were used and the total number of caterpillars varied between 50 to 80 specimens

per preparation. The average protein concentration of bristle extracts was 4.41 ± 0.93 mg/mL (mean ± S.E.M).

2.3 Blood collection and preparation of human washed platelets

Whole human blood was collected from healthy volunteers, into 1:5 (v/v) of ACD solution (2.5 % trisodium citrate, 1.37 % citric acid and 2 % dextrose). The procedure described by Reck Jr *et al*. (2009) [15] was employed to obtain washed human platelets (WHP). Briefly, blood was centrifuged for three times at 200 g for 5 min to obtain platelet rich plasma (PRP). PRP was centrifuged at 650 g for 10 min. The supernatant was discarded and the platelet pellet was suspended in 0.3 ml of ACD solution. Then, platelets were washed through a gel filtration column (sepharose-2B) equilibrated and eluted with Tyrode-albumin buffer, pH 7.4. Washed platelet suspensions were supplemented with 2 mM $CaCl_2$ and 0.5 mg/ml of fibrinogen. The platelet count was performed manually in a Neubauer chamber (haemocytometer) and the platelet concentration was adjusted to $3 – 4 \times 10^5$ cells/µL. To test the influence of calcium ions and fibrinogen on Lobe-induced platelet aggregation, some experiments were performed in the absence of calcium and fibrinogen.

2.4 Platelet aggregation and ATP secretion

To determine the direct effect of *L. obliqua* venom and its components upon platelets, the tests for measurement of platelet function were selected based on classical methodologies well established [16, 17]. Briefly, washed platelet aggregation was measured turbidimetrically using a dual-channel whole blood Lumi-aggregometer (Chrono-log Corporation, Havertown, USA) and/or a SpectraMax microplate reader (Molecular Devices, USA), according to Fuly *et al.* (2004) [18]. The following agonists were used: ADP (10 µM, final

concentration), collagen (2.5 μg/mL, final concentration), thrombin (3 μg/mL, final concentration) or different doses of *L. obliqua* bristle extract (Lobe). The final volume of assay performed on Lumi-aggregometer and SpectraMax microplate reader was 300 μL and 150 μL, respectively. Changes in turbidity were measured in absorbance units and results are expressed as area under the aggregation curves. In some experiments, inhibitors of different pathways of platelet aggregation were incubated with WHP for 20 min, previously to the addition of agonists.

The amount of adenosine triphosphate (ATP) released from platelets was measured by addition of luciferin-luciferase reagent (15 μl) to the platelet reaction mixture. ATP secretion was monitored through the generation of luminescence on Lumi-aggregometer, as described [18].

2.5 Platelet adhesion assay

A modified platelet adhesion procedure described by Bellavite et al. (1994) [19] was used. Microtiter plates of 96 wells (Flacon, USA) were coated overnight at 4 °C and then for 1 hour at 37 °C by adding 100 μL/well of a solution containing bovine albumin (2 mg), Lobe (7.5 μg) or type I collagen (2.0 μg). Immediately before the assay, plates were manually washed twice with 150 mM NaCl solution. Aliquots (100 μL each) of washed platelets preparation (2.5 X 10^6 cells) were added to the wells and incubated at 37 °C for 30 minutes. At the end of incubation the wells were washed twice with 150 mM NaCl and immediately supplemented with 100 μL of 0.1 M citrated buffer, pH 5.4, containing 5 mM ρ-nitrophenyl phosphate and 0.1 % Triton X-100 in order to measure platelet acid phosphatase activity. The plate was incubated for one hour at room temperature and the reaction was stopped with 50 μL of 2 M

NaOH. The amount of ρ-nitrophenol produced by the reaction was measured at 405 nm against a platelet-free blank by using a SpectraMax microplate reader. The percentage of adherent platelets was calculated based of a standard curve obtained with known number of lysed platelets.

2.6 Chemical treatments for inhibition of specific enzymatic activities of L. obliqua venom

Samples of Lobe (0.3 mg/mL) or thrombin (0.2 mg/mL) were added to well plates containing: (a) phenylmethanesulfonyl fluoride (PMSF) and (b) D-Phe-Pro-Arg-chloromethylketone (PPACK) for serine proteases inhibition [20]; (c) *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) for cysteine proteases inhibition [21]; (d) *p*-bromophenacyl bromide (*p*-BPB) for phospholipases A_2 inhibition [22] or (e) ethylene glycol-bis(aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) for inhibition of metal-dependent proteases [21]. Samples were incubated for 2 h at 37 ^{0}C, followed by overnight dialysis against 20 mM Tris.HCl, pH 7.4. Control samples of Lobe and thrombin experiments were submitted to incubation and dialysis, without an inhibitor treatment. The aggregation response induced by the controls was considered as 100 %.

2.7 Measurement of lactate dehydrogenase (LDH) release

The integrity of human platelets after incubation with Lobe was verified using a kit for measurement of LDH release (TOX-7 in vitro toxicology assay kit, Sigma- Aldrich, St Louis, MO, USA), according to manufacturer's instructions. As controls, platelets were lysed with 0.1 % Triton X-100 to estimate their total LDH content.

2.8 Scanning electron microscopy (SEM)

SEM sample preparation procedure was modified from Gear (1984) [23]. Briefly, aliquots of washed human platelets (300 μL) were pre-warmed at 37 ^{0}C and exposed to ADP (10 μM, final concentration), saline or *L. obliqua* bristle extract (15 μg) under low stirring. Platelets were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodilyc buffer, pH 7.2. The samples were washed twice for 30 minutes in 0.1 M cacodilyc buffer and filtered in a 0.4 μm polycarbonate membranes (Millipore, USA). The fixed cells were sequentially dehydrated for 5 minutes in 30, 50, 70 and 90% (v/v) acetone and finally treated twice for 10 minutes in 100% acetone. Critical-point drying and gold coating treatments were performed at the University's Center of Electron Microscopy (CEM-UFRGS, Brazil). Specimens were visualized in a JEOL-JSM 6060 scanning electron microscope with automated image digitization and archiving.

2.9 Statistical analysis

Data are expressed as mean ± S.E.M. of five independent experiments. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test correction. A p value of less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) software.

3. Results

3.1 Effects of L. obliqua venom on platelet activation, aggregation and adhesion

The addition of *L. obliqua* bristle extract (Lobe) to a suspension of human washed platelets triggered a potent platelet aggregation and ATP-secretion (Fig. 1). In a range of 0.2 to 56 μg, Lobe produced platelet aggregation and ATP-secretion in a dose-dependent manner (Fig. 1A). Half-maximal response (EC_{50}) of platelet aggregation was obtained at a dose of 10.7 μg of protein. Lobe-induced platelet aggregation occurs after a lag phase that varied from 2 to 8 min depending on the concentration used (Fig. 1A, insert). Other agonists, such as ADP (10 μM), collagen (2.5 μg/mL) or thrombin (3 μg/mL) induced aggregation to a similar magnitude at a considerably faster rate. At the same manner, Lobe was also able to trigger platelet aggregation in platelet rich plasma (data not show). For these experiments, platelet rich plasma was previously incubated with GPRP (H-Gly-Pro-Arg-Pro-NH_2), a peptide that inhibits fibrin polymerization induced by venom procoagulant enzymes [24].

In order to evaluate the interaction between Lobe and platelets a direct adhesion experiment was conducted. Bovine serum albumin (control), venom or collagen were immobilized in a 96-well plate and then incubated with washed platelets. Adhered platelets were lysed and acid phosphatase activity measured in a colorimetric assay. Figure 1B shows that Lobe was able to induce direct adhesion of human platelets. In experiments containing 2.5 X 10^6 cells, 41% of the platelets adhered to the venom proteins. This result was similar to that obtained for collagen (42 % of platelet adhesion), a well known adhesive protein for platelets (Fig. 1B). In addition, platelet adhesion was specific, since little cell attachment was obtained when plates were coated with albumin (control), while

a consistent adhesion could be observed when Lobe or collagen were used as coating agents (Fig. 1B).

Electron microscopy techniques showed (Fig. 1C) that the platelet aggregates produced by 15 μg of Lobe presents a morphological pattern similar to the one induced by 10 μM ADP. It also confirms that, at this dose, Lobe does not lyses human platelets. Accordingly, very little lactate dehydrogenase (LDH) activity were released by platelets incubated with Lobe at doses of 10.7, 36 and 56 μg: 5.2 ± 0.3%; 6.6 ± 0.4% and 7.3 ± 0.2%, respectively, when compared with LDH activity of Triton X-100 lysed platelets (considered as 100 %). Lobe LDH values were similar to that obtained for supernatant of platelets incubated with thrombin (5.1 ± 0.2 %) and collagen (7.6 ± 0.2 %).

3.2 Influence of different chemical treatments on L. obliqua-induced platelet aggregation

In an attempt to verify the effect of enzyme inhibitors on the platelets pro-aggregating activity of Lobe, several known inhibitors of serine proteases (PMSF, PPACK), cysteine proteases (E-64), phospholipases A2 (*p*-BPB) or metal-dependent proteases (EGTA), were investigated. These inhibitors have been used in several previous works to identify and characterize the different classes of enzymes present in *L. obliqua* and some snake venoms [18, 21, 22, 25]. Here, we used a similar approach in order to verify the possible contribution of each of these enzymes to the venom platelet pro-aggregating activity. Results are summarized in Table 1. Differently from what happens with thrombin, were the serine protease inhibitors (PMSF and PPACK) caused an expressive inhibitory effect of thrombin-induced platelet aggregation, the treatment of Lobe with both inhibitors leads to a slight reduction of platelets

response (Table 1). Similarly E-64 (cysteine protease inhibitor) and EGTA (inhibitor of metal-dependent proteases) produced partial or no inhibition on Lobe-induced platelet aggregation (Table 1). On the other hand, *p*-BPB, a specific inhibitor of phospholipases A2, was the most effective inhibitor among all compounds tested. This compound reduced Lobe's pro-aggregating properties to approximately 70 % at 2 mM and to 80 % at 5 mM, suggesting that phospholipases A2 are the major venom components responsible for this activity (Table 1).

3.3 Effects of fibrinogen and calcium ions on L. obliqua-induced platelet aggregation

As fibrinogen and calcium ions play important roles in platelet aggregation, the effects of these elements on Lobe-induced aggregation were tested. As shown in Fig. 2A, the presence of fibrinogen (0.5 mg/mL, final concentration) in WHP suspension slightly increased the aggregation response induced by Lobe. On the other hand, the addition of calcium ions (2 mM, final concentration) to WHP preparation was essential for Lobe-induced platelet aggregation, since no aggregation was observed in the absence of calcium (Fig. 2B). To confirm the participation of calcium on Lobe-induced aggregation, platelets were pretreated with different concentrations of verapamil, a voltage-dependent calcium-gated channels blocker. As depicted in Fig. 2C, the aggregation response was significantly inhibited by verapamil at 25 and 50 μM concentrations and completely blocked at 100 μM. These results clearly indicate that Lobe-induced platelet aggregation depends on the influx of external calcium into the platelets.

3.4 Effects of pharmacological treatments on L. obliqua-induced platelet aggregation

In order to characterize the mechanisms involved in Lobe-induced platelet activation we investigated the effect of a number of anti-platelet drugs. When the cyclooxygenase (COX) pathway of the arachidonic acid metabolism was manipulated, we found that the COX-inhibitors aspirin and indomethacin were both able to produce a significant dose-dependent reduction in platelet response induced by Lobe (Fig 3A and 3B). The same COX-inhibitors were also able to reduce the response elicited by collagen, although this reduction was more pronounced that those observed with Lobe (Fig 3D and 3E). Contrarily, esculetin, the 12-lipoxygenase (12-LOX) pathway inhibitor, at different concentrations had no effect on platelet aggregation triggered by Lobe or collagen (Fig. 3C and F).

The possible role of adenosine diphosphate (ADP)-release from stimulated platelets was also investigated. Different concentrations of apyrase (an ADP scavenger enzyme) were then incubated with platelets, previously to the addition of Lobe or collagen. As seen in Fig. 4A, apyrase did not show any inhibitory effect on Lobe-induced aggregation (Fig. 4A), while at the same concentrations, this enzyme strongly reduced collagen-induced aggregation (Fig. 4B).

It is known that several platelet activators are effectively antagonized by cyclic adenosine monophosphate (cAMP)-elevating agents. Thus, cilostazol, a specific inhibitor of phosphodiesterase 3A (PDE3A), an enzyme that reduces intraplatelet cAMP content was also tested. As depicted in Fig. 5A, pretreatment of platelets with cilostazol cause significant inhibition of Lobe-induced platelet

aggregation, an effect even more expressive than that observed when collagen was used as a control agonist (Fig. 5B).

4. Discussion

Severe envenoming by *L. obliqua* caterpillars can lead to a hemorrhagic syndrome in humans, followed by cerebral hemorrhage and death. The clinical profile results from activation of coagulant and fibrinolytic systems and subsequent consumption of coagulation factors [10]. More recently, we have demonstrated that platelet dysfunctions also play an important role in the clinical manifestations of *L. obliqua* envenomation. Rats experimentally envenomed presented *in vivo* platelet hypoaggregation caused by intravascular generation of nitric oxide and fibrin/fibrinogen degradation products, though the platelet counts were only partially reduced (26 %) [12]. Quite similarly, platelet hypoaggregation was also observed in *Bothrops jararaca* envenomation, however with more accentuated thrombocytopenia, where platelet counts reduced approximately to 50 % [26, 27].

In the present study, we showed that *L. obliqua* venom is also able to induce aggregation and adhesion of human platelets *in vitro* (Fig. 1). It is possible that this pro-aggregating activity contributes to the reduction in platelet counts observed in rats and in envenomed patients [11, 12, 28]. Actually, a similar effect has been shown with *B. jararaca* and *B. asper* venoms that also induce *in vitro* platelet aggregation [29-31]. Moreover, the pro-aggregating activity of either the snakes or Lonomia's venoms can increase intravascular thrombin generation by supplying the activated platelet surface necessary for positive feedback prothrombin activation [13]. Thus, coagulation disturbances triggered by *L. obliqua* venom procoagulant enzymes are further maximized.

L. obliqua pro-aggregating activity is produced in a dose-dependent manner and occurred in parallel with the ATP-release reaction. The maximal

magnitude of the platelet aggregatory response induced by Lobe was comparable with that observed for collagen, ADP or thrombin. However, different from these agonists, *L. obliqua*-induced platelet aggregation was preceded by an extended lag-phase (2 – 8 min.), probably related with the low concentrations of active principle(s) present in crude venom. Interestingly, it was also found that the crude bristle extract, similarly to collagen and thrombin did not lyses blood platelets, as indicated by the poor release of LDH activity measured in the supernatant of platelets incubation. Instead, as demonstrated by electron microscopy studies, the venom was able to induce platelet adhesion and a shape change reaction similar to that observed with ADP.

Pretreatment of Lobe with specific inhibitors of serine and cysteine-proteases did not significantly block the aggregation effects elicited by the venom (Table 1). These findings suggest that serine and cysteine-proteases play a minor role in *L. obliqua*-induced platelet aggregation, despite the presence of these classes of proteases in venom [32] and the high proteolytic activity found in bristle extract [21]. In a similar way, pretreatment with EGTA, a chelating agent, presented no effect on the pro-aggregating effect of Lobe, which discards the participation of metal-dependent enzymes, such as metallo-proteases. On the other hand *p*-BPB, a specific inhibitor of phospholipases A2 (PLA2), significantly reduced the aggregation response thus indicating an active participation of these enzymes in the pro-aggregating activity of the venom. In fact, PLA2 from animal venoms are well known for their ability to induce a wide variety of pharmacological actions, including neurotoxic, miotoxic and hemolytic effects [33]. Some types of PLA2 also interfere with coagulation and platelet functions, displaying anti- and/or pro-aggregating properties [18, 34, 35]. In *L.*

obliqua venom, a 15 kDa PLA2 has been isolated and described [9]. In addition, its N-terminal sequence showed high homology to a sequence of a putative PLA2 obtained from a cDNA library of *L. obliqua* bristles [32]. The isolated PLA2 presented high indirect hemolytic activity and the intravascular hemolysis observed in animal models after injection of bristle extract has been attributed to the presence of this enzyme [36]. Our data presented here confirms the presence of a PLA2 enzyme in *L. obliqua's* venom which displays strong platelets pro-aggregating activity devoided, however, of lytic effect upon these cells.

Fibrinogen is considered an essential molecule for platelet aggregation, since it serves as a necessary bridging molecule that binds to the integrin receptor GPIIbIIIa and, thus, promotes the linkage of adjacent platelets [37]. Jennings *et al*. (2005) [38] described the isolation of Ba25, a C-type lectin from the venom of *Bitis arietans* that specifically binds to GPIIbIIIa and enhances the affinity of fibrinogen to its receptor, leading to agglutination of platelets. This protein induced agglutination of WHP only in the presence of exogenous fibrinogen. Differently, *L. obliqua* bristle extract was able to triggered platelet aggregation even in the absence of fibrinogen (Fig. 2A), indicating that the effects observed for *L. obliqua* venom involve more than a simple interaction between fibrinogen and GPIIbIIIa. Moreover, Ba25 induces agglutination without platelet activation, since the release of platelet granules was not detected [38]. In contrast, Lobe leads to the release of dense granules containing ATP, suggesting that *L. obliqua* venom is able to induce activation and aggregation of WHP and not an agglutination process.

It is well established that collagen induces platelet activation and aggregation by a transmembrane signaling process mediated by the activation of phospholipase Cγ2 and a subsequent cleavage of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Inositol 1,4,5-trisphosphate induces the release of calcium from dense tubular system, whereas 1,2-diacylglycerol activates protein kinase C [37, 39]. The collagen-induced inositol 1,4,5-trisphosphate-mediated increase in intracellular calcium concentration is accompanied by an influx of calcium from the extracellular milieu [40, 41]. For Lobe- induced platelet activation the influx of calcium from the extracellular milieu shows to be equally important, since the aggregation response was completely abolished in the absence of exogenous calcium (Fig. 2B). Furthermore, the blockade of calcium channels by verapamil inhibited Lobe-induced platelet aggregation in a dose-dependent manner (Fig. 2C), indicating that calcium is essential to the intracellular signaling triggered by the venom.

In addition to phospholipase C activation and increase in intracellular calcium concentration, the release of thromboxane A2 (TXA2) has a pivotal role for platelet activation triggered by various agonists, including collagen, thrombin or ADP [41, 42]. In fact, it is known that calcium concentration rising together with protein kinase C activation may lead to the activation of an endogenous membrane PLA2 enzyme, which can in turn hydrolyses arachidonic acid forming TXA2 [43].. As collagen-induced activation requires the release of TXA2, inhibitors of cyclooxygenase (aspirin and indomethacin) antagonized its aggregation response (Fig. 3D and 3E). The same cyclooxygenase inhibitors also showed an antagonizing effect on platelet aggregation elicited by Lobe

(Fig. 3A and 3B). However, when the 12-lipoxygenase pathway of arachidonic acid metabolism was blockade by esculetin, no inhibitory influence on platelet activation induced either by collagen (Fig. 3F) or Lobe (Fig. 3C) was observed. Thus, our results suggest that Lobe-induced platelet activation involves the release of arachidonic acid and production of TXA2 through the cyclooxygenase pathway, without the participation of 12-lipoxygenase products.

Such as TXA2, the secretion of dense granules containing ADP also participates in a positive feedback loop that leads to irreversible aggregation after platelet stimulation with collagen [43]. In accordance, the results found here confirm that the pretreatment of platelets with apyrase (an enzyme that hydrolyses ADP) inhibited the collagen-induced aggregation in a dose-dependent fashion (Fig. 4B). Distinctly to this, Lobe-induced aggregation was insensitive to the pretreatment with apyrase (Fig. 4A), suggesting that the positive feedback pro-aggregating mechanism resulting from ADP release from platelets do not play a significant role to further strengthen the aggregation response triggered by Lobe as happens with collagen. Similar observations have been made for Bthtx-II, a phospholipase A2 from *Bothrops jararacussu* snake venom [18].

The levels of intracellular cAMP are a control mechanism in platelets that interrupts multiple signaling pathways and plays a significant role in down-regulating platelet activation [44]. On the other hand, the levels of cAMP can be regulated by the degradation of cAMP via the cyclic nucleotide phosphodiesterases, a group of enzymes that catalyze the hydrolysis of 3', 5'–cyclic nucleotides to inactive 5'–nucleotides by cleaving a phosphodiester bond. One possible mechanism by which the aggregation response can be enhanced

involves the activation of phosphodiesterase 3A (PDE3A), an isoform of phosphodiesterase present in platelets [45, 46]. In this work, we used the selective PDE3A inhibitor cilostazol to verify the participation of this enzyme in Lobe- and collagen-induced platelet activation. Both agonists were inhibited by cilostazol, presenting evidence that the cAMP-dependent phosphodiesterase (PDE3A) is a component of the collagen and Lobe signaling pathways in platelets.

In conclusion, this study shows that *L. obliqua* venom is able to induce human platelet aggregation and adhesion *in vitro*. The aggregation response triggered by venom occurs through a calcium-dependent mechanism involving arachidonic acid metabolite(s) of the cyclooxygenase pathway and activation of PDE3A, which probably leads to the consumption of intracellular cAMP content. Moreover, *L. obliqua*-induced platelet aggregation was independent of ADP secretion and it seems that the major venom component responsible for this activity is a phospholipase A2. The purification and characterization of this phospholipase A2 are the aims of a current investigation in order to confirm its pro-aggregating ability and to determine the *in vivo* effects of isolated enzyme. Certainly the results presented here add new important elements to help better understand the contribution of platelet impairment to the hemorrhagic syndrome resulting from patients envenomed upon accidental contact with *L. obliqua* caterpillars.

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Tables

Table I

Influence of different enzyme inhibitors on platelet aggregation induced by *L. obliqua* venom.

Figure legends

Figure 1. *L. obliqua* **venom induced-platelet aggregation and adhesion. (A)** Dose-response curves of platelet aggregation and ATP-secretion were obtained on Lumi-aggregometer after incubation of WHP with different doses of Lobe (0.2 – 56 μg) in a final volume of 300 μL. Results (mean ± S.E.M) are expressed as percentage of maximal aggregation for five independent experiments. The inset shows typical platelets aggregation tracings after addition of 0.2 (a), 1 (b), 14 (c), 33.9 (d), 44 (e) and 56 μg (f) of Lobe. **(B)** Direct adhesion was measured as number of platelets adhered to Lobe (7.5 μg/well) or collagen (2.0 μg/well). Control represent platelets adhered to bovine albumin (200 μg/well). Results (mean ± S.E.M) are expressed as percentage of adherent platelets and values of ***$p<0.001$ were considered statistically significant in comparison to control. **(C)** Scanning electron microscopy. (Control) WHP + saline solution; (ADP) WHP + 10 μM ADP; (Lobe 5 min.) WHP + 15 μg Lobe incubated for 5 min.; (Lobe 10 min.) WHP + 15 μg Lobe incubated for 10 min.

Figure 2. Effects of fibrinogen and calcium ions on *L. obliqua***-induced platelet aggregation.** Platelet aggregation triggered by 15 μg of *L. obliqua* bristle extract (Lobe) was tested in the presence and absence of 0.5 mg/mL fibrinogen **(A)** or 2 mM calcium (final concentrations) **(B)**. The influence of different concentrations of verapamil (a calcium channel blocker) on Lobe-

induced aggregation was also tested **(C)**. These experiments were performed on a Lumi-aggregometer with a final volume of 300 μL. Results are expressed as typical aggregation tracings representative of five independent experiments.

Figure 3. Effects of cyclooxygenase and 12-lipoxygenase inhibitors on *L. obliqua***-induced platelet aggregation.** Washed human platelets were previously incubated with different concentrations of cyclooxygenase (aspirin and indomethacin) and 12-lipoxygenase (esculetin) inhibitors in a final volume of 150 μL. Then, the aggregation response was measured in a SpectraMax microplate reader by addition of 7.5 μg Lobe **(A, B and C)** or 0.4 μg collagen **(D, E and F)**. Results (mean ± S.E.M) are expressed as percentage of maximal aggregation for five independent experiments. Values of *p<0.05 and ***p<0.001 were considered statistically significant in comparison to control.

Figure 4. Effects of apyrase on *L. obliqua***-induced platelet aggregation.** Washed human platelets were previously incubated with different concentrations of apyrase (an enzyme that hydrolyses ADP) in a final volume of 150 μL. Then, the aggregation response was measured in a SpectraMax microplate reader by addition of 7.5 μg Lobe **(A)** or 0.4 μg collagen **(B)**. Results (mean ± S.E.M) are expressed as percentage of maximal aggregation for five independent experiments. Values of ***p<0.001 were considered statistically significant in comparison to control.

Figure 5. Effects of cilostazol on *L. obliqua***-induced platelet aggregation.** Washed human platelets were previously incubated with different concentrations of cilostazol (a phosphodiesterase 3A inhibitor) in a final volume of 150 μL. Then, the aggregation response was measured in a SpectraMax microplate reader by addition of 7.5 μg Lobe **(A)** or 0.4 μg collagen **(B)**. Results

(mean ± S.E.M) are expressed as percentage of maximal aggregation for five independent experiments. Values of *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant in comparison to control.

Figure 1

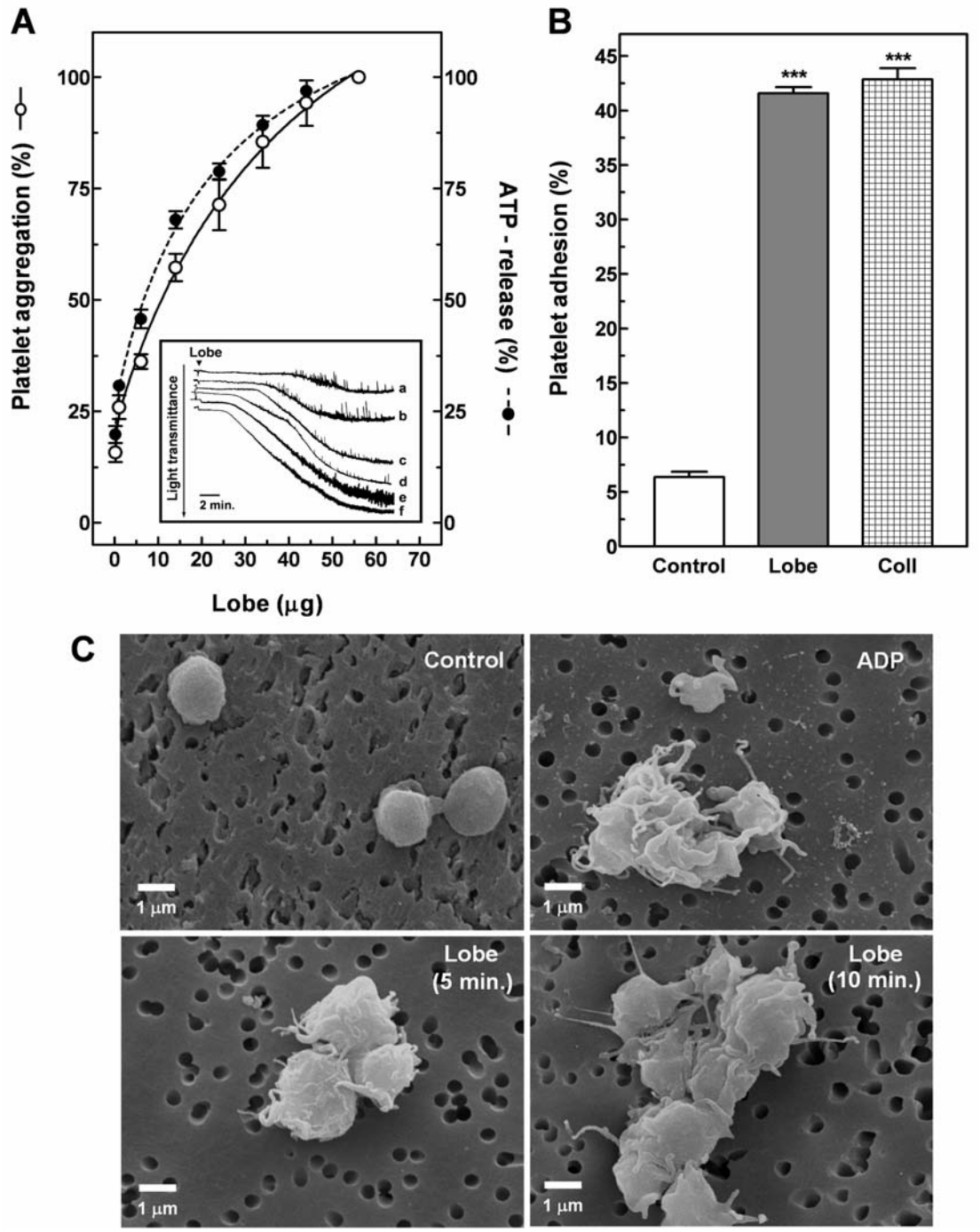
A
Platelet aggregation (%)
ATP - release (%)
Lobe (μg)
Lobe
Light transmittance
2 min.
a
b
c
d
e
f
B
Platelet adhesion (%)
***
***
Control
Lobe
Coll
C
Control
ADP
Lobe (5 min.)
Lobe (10 min.)
1 μm

Figure 2

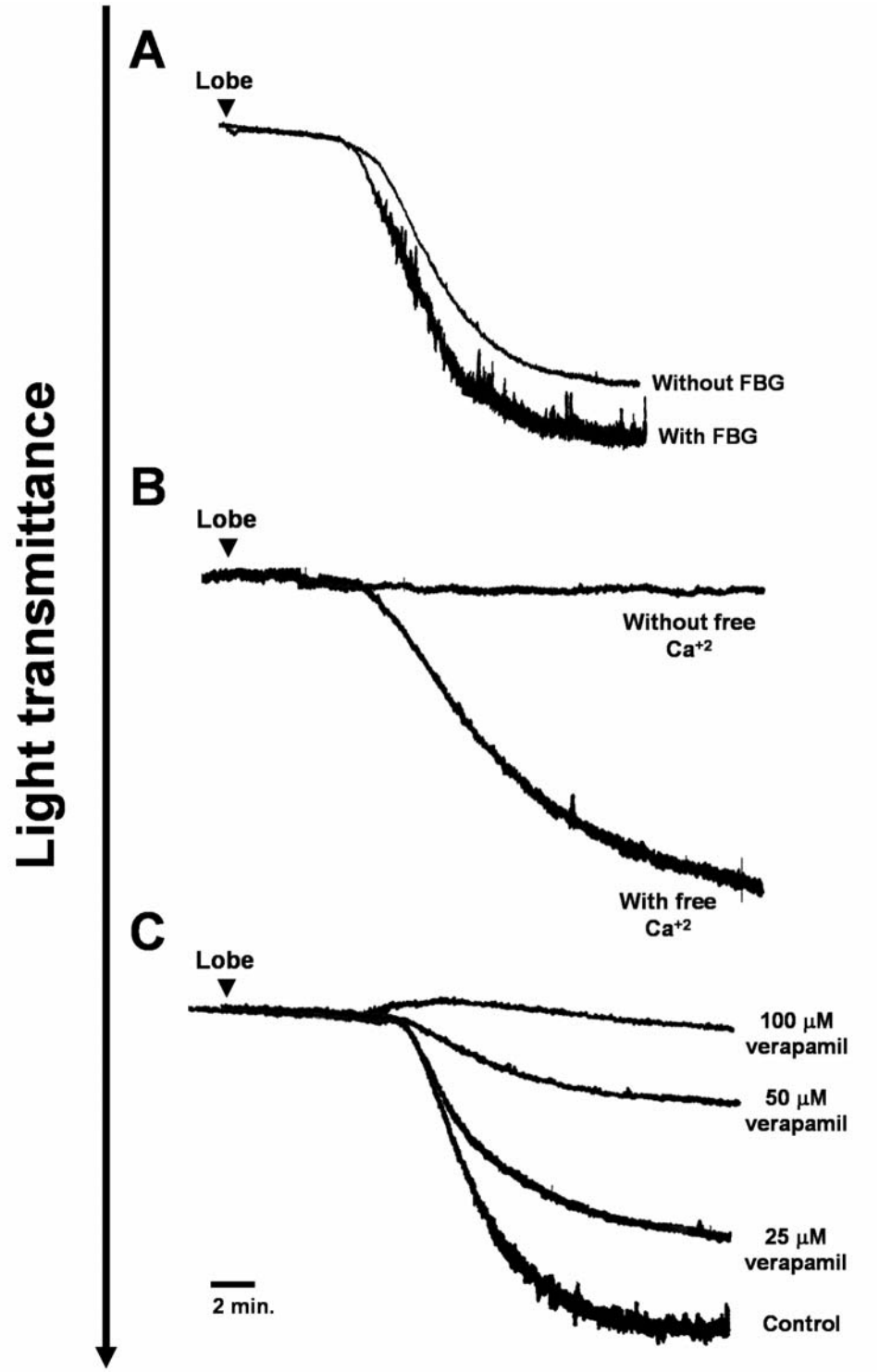
A
Lobe
Without FBG
With FBG
B
Lobe
Without free Ca+2
With free Ca+2
C
Lobe
100 μM verapamil
50 μM verapamil
25 μM verapamil
Control
2 min.
Light transmittance

Figure 3

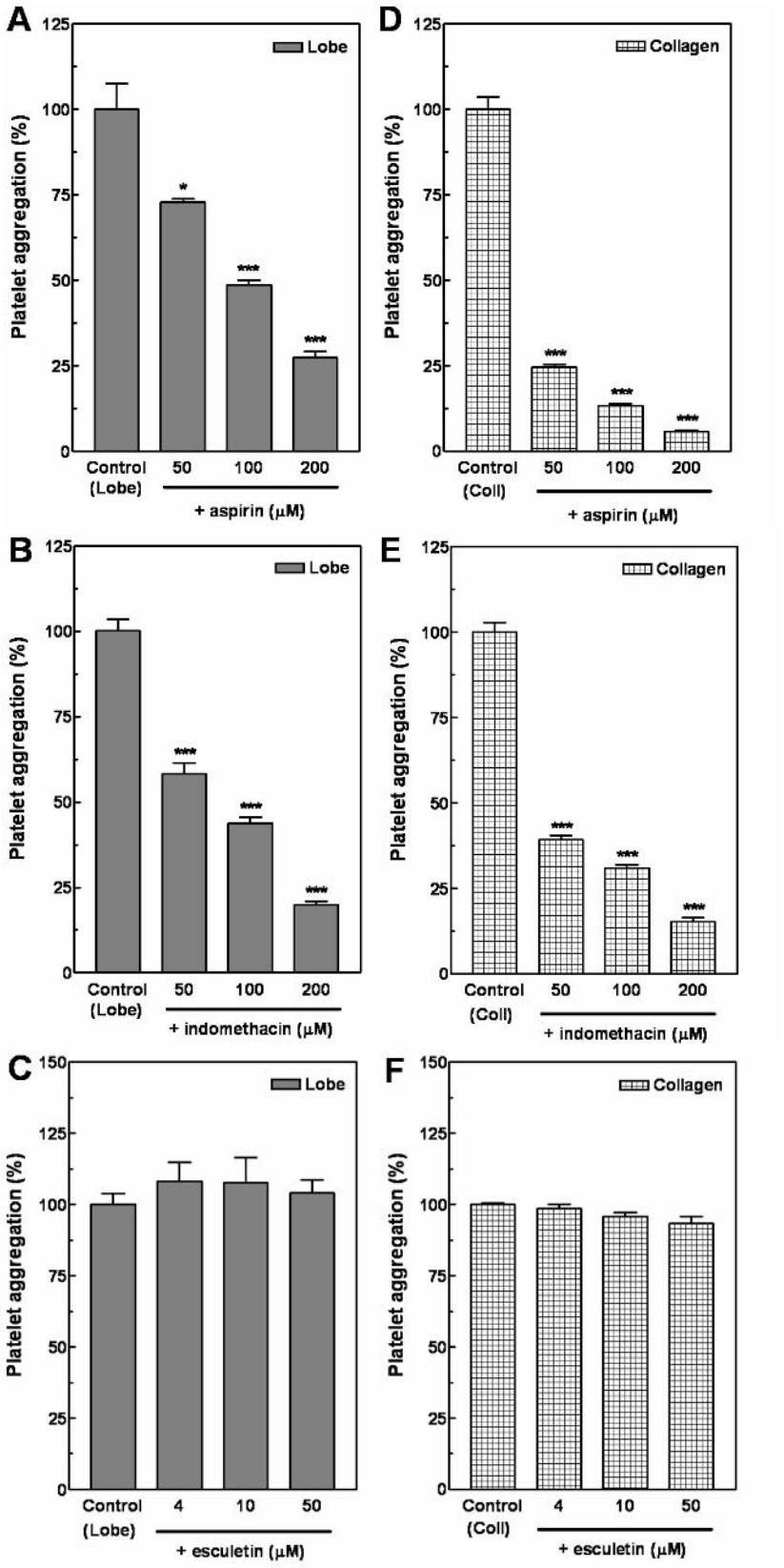
A
Lobe
Platelet aggregation (%)
125
100
75
50
25
0
*
***
***
Control (Lobe)
50
100
200
+ aspirin (μM)
D
Collagen
Platelet aggregation (%)
***
***
***
Control (Coll)
50
100
200
+ aspirin (μM)
B
Lobe
Platelet aggregation (%)
***
***
***
Control (Lobe)
50
100
200
+ indomethacin (μM)
E
Collagen
Platelet aggregation (%)
***
***
***
Control (Coll)
50
100
200
+ indomethacin (μM)
C
Lobe
Platelet aggregation (%)
150
125
100
75
50
25
0
Control (Lobe)
4
10
50
+ esculetin (μM)
F
Collagen
Platelet aggregation (%)
Control (Coll)
4
10
50
+ esculetin (μM)

Figure 4

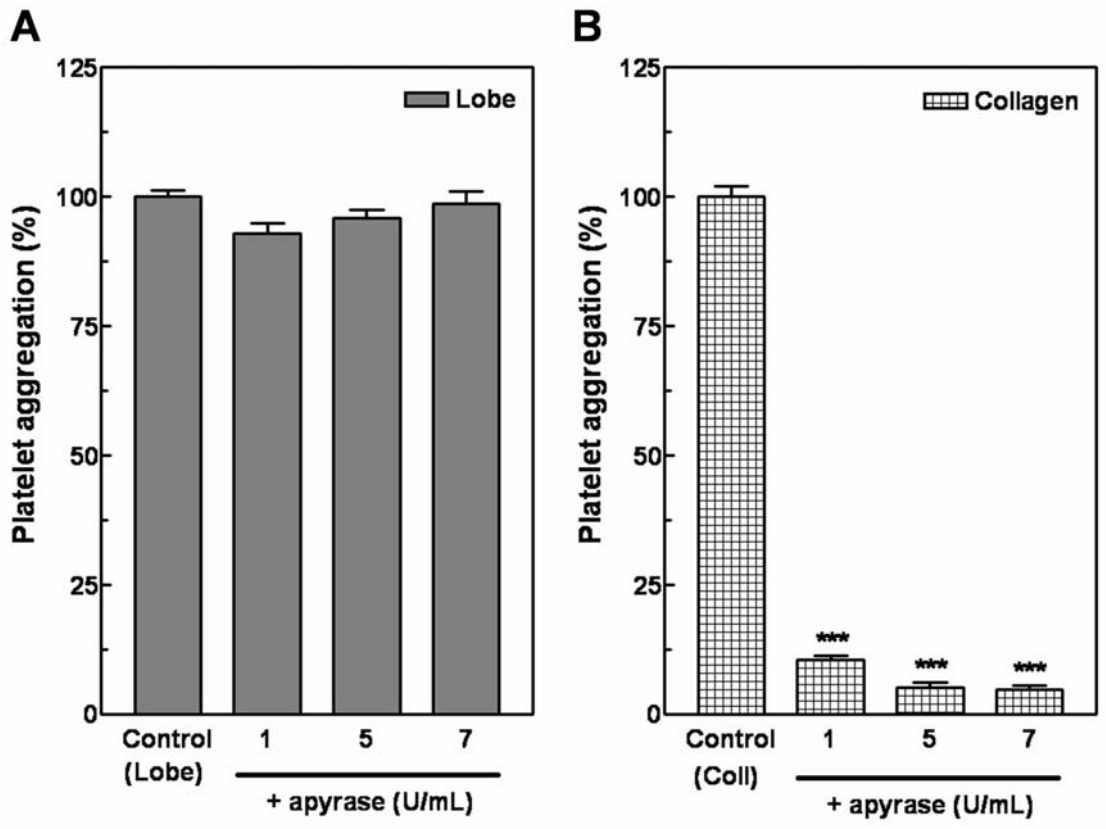
A
125
100
75
50
25
0
Platelet aggregation (%)
Lobe
Control
(Lobe)
1
5
7
+ apyrase (U/mL)
B
125
100
75
50
25
0
Platelet aggregation (%)
Collagen
***
***
***
Control
(Coll)
1
5
7
+ apyrase (U/mL)

Figure 5

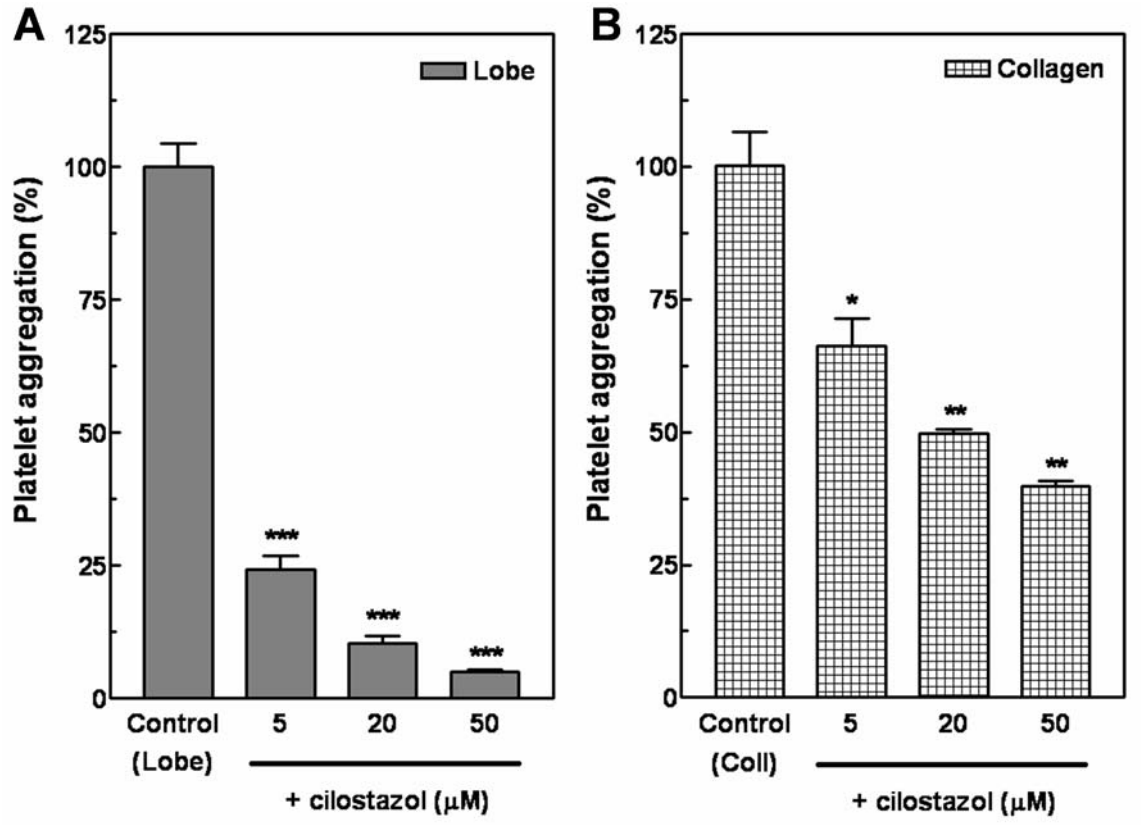
A
Platelet aggregation (%)
125
100
75
50
25
0
Lobe
***
***
***
Control
(Lobe)
5
20
50
+ cilostazol (μM)
B
Platelet aggregation (%)
125
100
75
50
25
0
Collagen
*
**
**
Control
(Coll)
5
20
50
+ cilostazol (μM)

Table 1

Agonist	Dose	Inhibitor	Concentration	Platelet aggregation (%)
Lobe	7.5 μg	---	---	100 ± 4.1
Lobe	7.5 μg	PMSF	1 mM	85.8 ± 2.6
Lobe	7.5 μg	PMSF	5 mM	84.6 ± 3.4
Lobe	7.5 μg	PPACK	0.2 mM	97.3 ± 4.8
Lobe	7.5 μg	PPACK	1 mM	89.5 ± 4.9
Lobe	7.5 μg	E-64	5 μM	104.2 ± 3.6
Lobe	7.5 μg	E-64	10 μM	89.4 ± 1.7
Lobe	7.5 μg	*p*-BPB	2 mM	29.6 ± 2.1[a]
Lobe	7.5 μg	*p*-BPB	5 mM	20.9 ± 2.0[a]
Lobe	7.5 μg	EGTA	1 mM	95.4 ± 0.4
Lobe	7.5 μg	EGTA	5 mM	91.2 ± 0.4
Thrombin	0.5 μg	---	---	100 ± 1.4
Thrombin	0.5 μg	PMSF	1 mM	15.8 ± 1.4[b]
Thrombin	0.5 μg	PPACK	0.2 mM	16.2 ± 0.3[b]

Different classes of enzymes were inactivated by incubation of Lobe with specific inhibitors for 2 h at 37 ^{0}C. The excess of inhibitors were removed and ability of venom to induce platelet aggregation was measured in a SpectraMax microplate reader (final volume 150 μL) as described in materials and methods.

[a] Statistically different ($p<0.001$) in comparison with platelet aggregation induced by Lobe in the absence of inhibitors.

[b] Statistically different ($p<0.001$) in comparison with platelet aggregation induced by thrombin in the absence of inhibitors.

4. DISCUSSÃO

O contato acidental com a taturana *Lonomia obliqua* pode levar a uma grave síndrome hemorrágica bastante comum nos estados da região sul do Brasil. Nesta dissertação apresentamos resultados que demonstram, pela primeira vez, o envolvimento de distúrbios de função plaquetária na patologia desencadeada pelo envenenamento.

Assim como demonstrado para alguns venenos de serpentes (WHITE, 2005), o veneno de *L. obliqua* parece exercer uma ação paradoxal sobre a função plaquetária, uma vez que é capaz de inibir a agregação plaquetária *in vivo* e possuir uma atividade pró-agregante *in vitro*. No caso dos venenos de serpentes essa ação paradoxal é resultado da presença de toxinas diferentes que possuem ação inibitória ou indutora de agregação (FULY *et al*, 1997; FULY *et al*, 2004; CLEMETSON *et al*, 2005; WHITE, 2005). Para o veneno de *L. obliqua*, a inibição da agregação plaquetária *in vivo* não parece ser resultado da ação de uma toxina específica sobre as plaquetas e sim da produção de substâncias endógenas que são geradas pelo organismo em resposta ao envenenamento e têm a capacidade de inibir a função plaquetária. De fato, os animais envenenados apresentaram um aumento nos níveis plasmáticos de óxido nítrico (NO) e uma ativação do sistema fibrinolítico, o que indica também uma possível elevação na concentração plasmática dos produtos de degradação de fibrina (PDF). Tanto NO quanto PDF são potentes inibidores de agregação plaquetária e certamente contribuem para a hipoagregação plaquetária observada.

Diferente da ação inibitória *in vivo*, a ação pró-agregante observada nos experimentos *in vitro* deve-se a presença de uma toxina específica presente no veneno. Os resultados indicaram a participação de enzimas da classe das fosfolipases A2 que foram capazes de induzir agregação, secreção de grânulos de ATP e adesão de plaquetas. Uma possível função para esta atividade no envenenamento pode estar relacionada com a diminuição na contagem de plaquetas observada nos animais experimentais e nas vítimas de envenenamento grave. Já foi demonstrado, por exemplo, que a aspercetina, uma toxina com atividade pró-agregante do veneno de *Bothrops asper*, é a principal responsável pela ocorrência de trombocitopenia no envenenamento por esta serpente (RUCAVADO *et al*, 2001). Camundongos injetados com veneno de *B. asper* apresentaram uma redução drástica de mais de 50 % na contagem de plaquetas circulantes (RUCAVADO *et al*, 2005); entretanto, os ratos injetados com veneno de *L. obliqua* apresentaram uma redução de apenas 26 % no número de plaquetas, sugerindo que a atividade pró-agregante não tenha uma ação *in vivo* tão pronunciada quanto aquela relatada no envenenamento por *B. asper*. Além disso, a atividade indutora de agregação plaquetária pode ser importante por ativar as plaquetas circulantes fornecendo uma superfície adequada para a geração de trombina e, conseqüentemente contribuindo para o processo de coagulação intravascular disseminada.

Os dados aqui apresentados juntamente com aqueles já existentes na literatura permitem melhor compreender o quadro hemorrágico decorrente do envenenamento por *L. obliqua*. Resumidamente, o quadro clínico parece ser resultado da ativação dos sistemas de coagulação sangüínea e fibrinólise, dos distúrbios de agregação plaquetária e da resposta do sistema vascular ao

envenenamento (Figura 9). A ativação da coagulação e das plaquetas aumenta a produção intravascular de trombina, o que leva ao consumo dos fatores de coagulação como protrombina, fator V e fibrinogênio. Paralelamente, a ativação do sistema fibrinolítico leva à produção de plasmina que causa o aumento nos níveis dos produtos de degradação de fibrina. Como resposta ao envenenamento, há um aumento nos níveis de óxido nítrico, muito provavelmente gerado pelas células endoteliais dos vasos sangüíneos. Em conjunto, estas alterações levam à perda da função plaquetária e a incoagulabilidade sangüínea. Dessa maneira, a capacidade do organismo de conter a perda excessiva de sangue fica extremamente prejudicada durante o envenenamento (Figura 9).

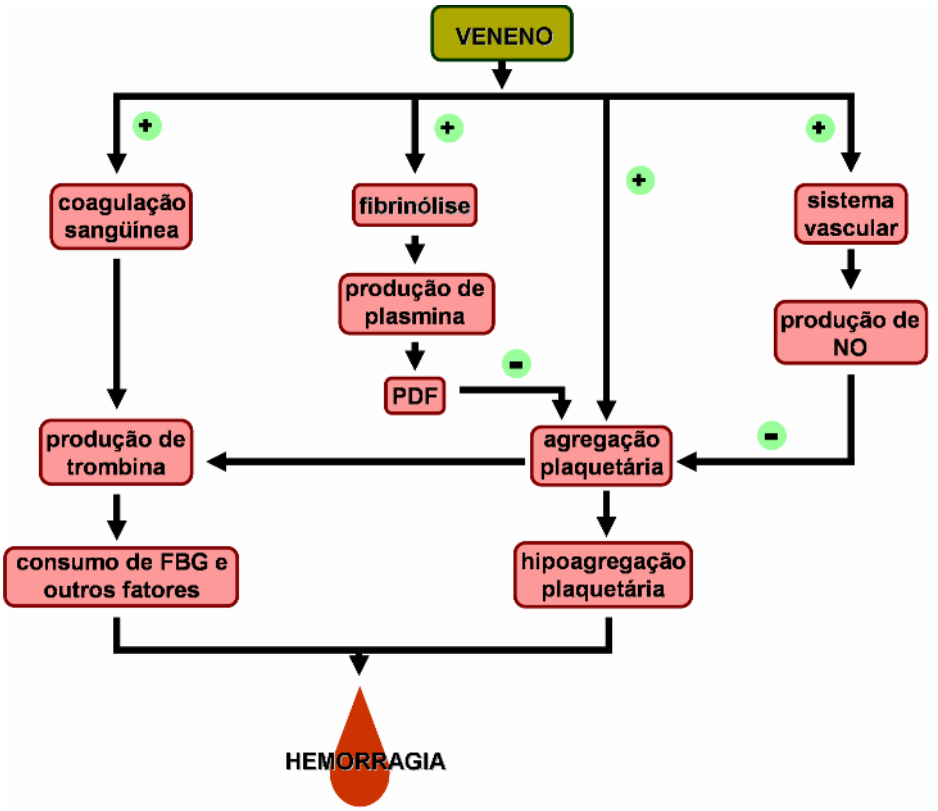

Figura 9. Resumo das alterações hemostáticas no envenenamento pela taturana *L. obliqua***.** O veneno é capaz de ativar diretamente o sistema de coagulação, fibrinólise, plaquetas e estimula a produção de óxido nítrico (NO) pelo sistema vascular. A ativação da fibrinólise gera os produtos de degradação de fibrina que, juntamente com o NO, inibem a agregação plaquetária levando à diminuição da capacidade de agregação. A ativação da coagulação leva ao consumo de fibrinogênio (FBG) pela formação de trombina. Parte das plaquetas ativadas diretamente pelo veneno também podem aumentar a concentração da trombina formada. A perda da capacidade de agregação plaquetária e a incoagulabilidade sanguínea gerada pelo consumo dos fatores de coagulação acabam contribuindo de forma decisiva para a hemorragia. (+) indica ativação; (-) indica inibição.

CONCLUSÃO

Os resultados obtidos no desenvolvimento deste trabalho e aqui apresentados evidenciam que:

1. Foi possível estabelecer um modelo experimental em ratos que reproduziu o quadro clínico de envenenamento. Através deste modelo, as alterações de coagulação sangüínea e função plaquetária foram descritas;

2. O veneno de *L. obliqua* injetado em ratos pela via subcutânea induz uma diminuição acentuada da capacidade de agregação plaquetária no plasma rico em plaquetas. No entanto, foi observada uma redução de apenas 26 % na contagem de plaquetas circulantes;

3. A redução na resposta de agregação plaquetária é causada por um inibidor gerado no plasma dos animais durante o envenenamento;

4. Um possível inibidor é o óxido nítrico (NO) gerado em resposta ao envenenamento, uma vez que o bloqueio sistêmico da produção de NO reverte parcialmente o efeito inibitório sobre a agregação plaquetária;

5. Possivelmente os produtos de degradação de fibrina também tenham um papel importante na redução da capacidade de agregação, uma vez

que o sistema fibrinolítico está ativado ao longo de todo o período de envenenamento;

6. O veneno de *L. obliqua* também induz hipofibrinogenemia e prolongamento dos parâmetros de coagulação sangüínea;

7. Durante o envenenamento, há geração de trombina e plasmina intravascular, indicando que os sistemas de coagulação e fibrinólise são ativados;

8. Quando testado *in vitro*, o veneno de *L. obliqua* possui efeito pró-agregante e induz adesão plaquetária;

9. O principal componente do veneno responsável pela atividade pró-agregante parece ser uma enzima da classe das fosfolipases A2;

10. O mecanismo de indução de agregação plaquetária envolve a mobilização de cálcio do meio externo para o interior da plaqueta e a produção de metabólitos da via das ciclooxigenases, principalmente de tromboxana A2.

11. A ativação da fosfodiesterase 3A e a conseqüente redução do conteúdo intracelular de AMPc também parecem estar envolvidos. No entanto, a secreção de grânulos de ADP não foi essencial para o processo de agregação induzido pelo veneno.

Em conjunto, os resultados demonstram a contribuição das alterações de função plaquetária e coagulação sangüínea para o quadro hemorrágico decorrente do envenenamento pela taturana *L. obliqua*. No entanto, novas investigações sobre a fisiopatologia do envenenamento ainda são necessárias, principalmente para elucidar alguns aspectos relacionados ao processo de falência renal que não são bem compreendidos.

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PRÊMIOS E TÍTULOS

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