

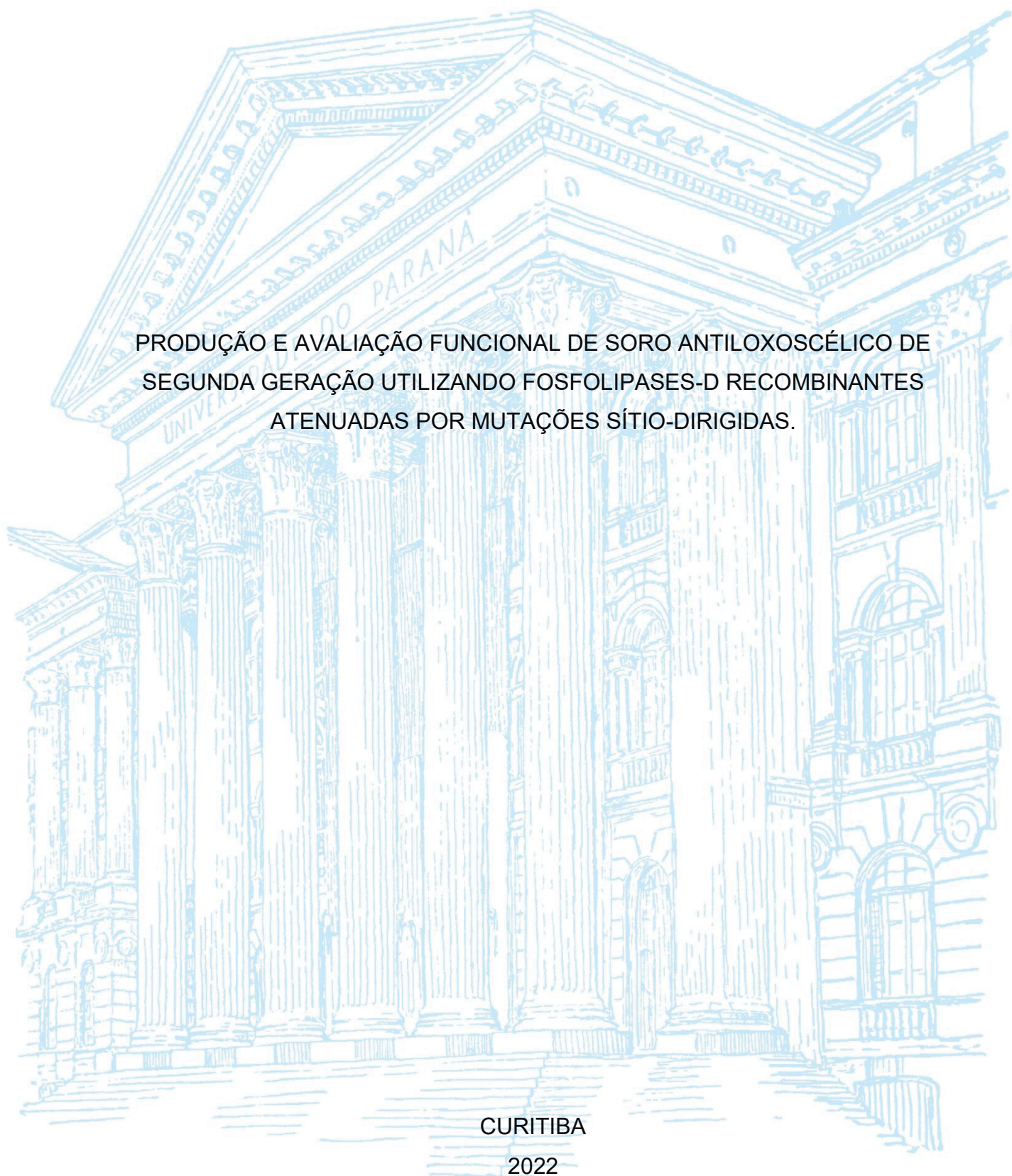
UNIVERSIDADE FEDERAL DO PARANÁ

BRUNO CESAR ANTUNES

PRODUÇÃO E AVALIAÇÃO FUNCIONAL DE SORO ANTILOXOSCÉLICO DE
SEGUNDA GERAÇÃO UTILIZANDO FOSFOLIPASES-D RECOMBINANTES
ATENUADAS POR MUTAÇÕES SÍTIO-DIRIGIDAS.

CURITIBA

2022



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

Orientador: Prof. Dr. Silvio Sanches Veigas
Coorientadora: Prof. Dra. Luíza Helena Gremski

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação BIOLOGIA CELULAR E MOLECULAR da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **BRUNO CESAR ANTUNES** intitulada: **Produção e avaliação funcional de soro antiloxoscélico de segunda geração utilizando fosfolipases-D recombinantes atenuadas por mutações sítio-dirigidas**, sob orientação do Prof. Dr. SILVIO SANCHES VEIGA, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa. A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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“A vida sem luta é um mar morto no centro do organismo universal.”

Machado de Assis

(1839 – 1908)

RESUMO

Os acidentes loxoscélicos são caracterizados principalmente por uma lesão dermonecrótica no local da picada, acompanhado de espalhamento gravitacional e intensa resposta inflamatória. Manifestações sistêmicas podem, menos frequentemente, estar presentes e são representadas por distúrbios renais e hematológicos podendo levar a óbito. As espécies mais comumente envolvidas nesses acidentes no Brasil são *L. intermedia*, *L. gauch*o e *L. laeta*. O único tratamento específico é o soro antiloxoscélico, que possui a capacidade de reduzir os efeitos do envenenamento. O soro é produzido a partir da imunização de equinos com os venenos das três espécies de *Loxosceles* citadas. Sabe-se que as fosfolipases-D são as responsáveis pela maioria dos efeitos biológicos observados no loxoscelismo. Esta tese apresenta o desenvolvimento de um soro de segunda geração e avalia sua capacidade de neutralizar os efeitos tóxicos do veneno. Para isso, foi proposto o enriquecimento dos venenos brutos das aranhas-marrons utilizados como antígenos em imunizações com toxinas dermonecróticas (fosfolipases-D) recombinantes com mutações pontuais e biologicamente inativas, mas ativas como estimulantes antigênicos, ou a substituição dos venenos por estas toxinas mutadas para a produção do soro. Sendo assim, foram produzidas três isoformas de toxinas dermonecróticas com mutações sítio-dirigidas comprovadamente sem atividade biológica: D32A-E34A de *L. gauch*o, W230A de *L. intermedia* e H12A-H47A de *L. laeta* para imunizações de coelhos em associação ou não com o veneno bruto das três espécies de *Loxosceles*. Ensaio de ELISA revelaram que as imunizações começaram a estimular o sistema imune dos coelhos de maneira significativa a partir da segunda imunização. Além disso, observaram-se maiores absorvâncias no grupo Proteínas Recombinantes. Também foram realizados Western Blottings e todos os soros produzidos, exceto o Controle Negativo, foram capazes de reconhecer os venenos de *L. intermedia*, *L. gauch*o e *L. laeta* entre as faixas de 25 kDa e 35 kDa. Nos ensaios *in vivo*, os soros recombinantes (Mix e Proteínas Recombinantes) foram capazes de reconhecer os venenos loxoscélicos e diminuir edema e dermonecrose causados pela ação do veneno de *L. intermedia*. No teste de incubação, frente a 1 DMN de veneno de *L. intermedia* observou-se redução de até 63% no diâmetro médio da lesão necrótica e 82% no diâmetro médio do edema, quando soro Proteínas Recombinantes foi utilizado. Na soroneutralização em orelha de coelhos, similar à etapa de controle de qualidade realizado no Soro Antiloxoscélico observou-se redução de até 56% no diâmetro médio da lesão dermonecrótica, quando o Soro Proteínas Recombinantes foi aplicado. Exames hematimétricos realizados tanto nas imunizações quanto no ensaio de soroneutralização em orelha, demonstraram que as proteínas recombinantes mutadas e os soros produzidos são seguros, não gerando danos aos animais. Os resultados apresentados demonstram o potencial de utilização das proteínas recombinantes mutadas na substituição total ou parcial dos venenos loxoscélicos nas imunizações de animais visando a produção de soro, uma vez que sua capacidade de soroneutralização e diminuição dos sinais clínicos equiparou-se ao do Soro Antiveneno Total produzido em coelhos. Tal substituição possui impacto direto no bem-estar animal, havendo uma dependência menor de aranhas mantidas em laboratório para a extração de veneno.

Palavras-chave: veneno, loxoscelismo, soro, Fosfolipases-D.

ABSTRACT

Loxoscelic accidents are characterized mainly by a dermonecrotic lesion at the bite site with gravitational spread and intense inflammatory response. Systemic manifestations such as hematological and renal disturbances may be present and can lead to death. The most common species involved on accidents in Brazil are *L. intermedia*, *L. gaucho* and *L. laeta*. The Antiloxoscelic Serum is the only specific treatment capable to reduce the effects of the envenoming. Horses are immunized with venoms from the three cited spiders in order to produce the serum. It is known that phospholipase-D are responsible for the majority of the biological effects seen on loxoscelism. This thesis presents the development of a second-generation serum and evaluates its neutralizing capability against the venom's toxic effects. The enrichment of brown spider's venoms used as antigens on immunizations with recombinant dermonecrotic toxins (phospholipase-D) containing site-directed mutations that result on biological inactivity was proposed. Total substitution of the venoms for the mutated toxins was also assessed on the serum's production. Three dermonecrotic toxins isoforms with site-directed mutations and no biological activities were obtained: D32A-E34A from *L. gaucho*, W230A from *L. intermedia* and H12A-H47A from *L. laeta*. These toxins were used on rabbits' immunizations in association with *Loxosceles* venoms or without the venoms. ELISA assays revealed that antibodies concentration started to rise after the second immunization. Besides that, higher absorbances were seen on the Recombinant Proteins groups. Western Blotting tests also showed that all the sera produced were capable to recognize venoms from *L. intermedia*, *L. gaucho* and *L. laeta* between 25 kDa e 35 kDa. Recombinant sera (groups Mix and Recombinant Proteins) were effective decreasing edema and dermonecrosis on rabbits when tested against *L. intermedia* venom. Incubation assays against 1 NMD from *L. intermedia* venom showed that Recombinant Proteins serum reduced up to 63% on the medium diameter of the lesions and up to 82% on the medium diameter of the edema. Reduction on the medium diameter of the dermonecrotic lesion (up to 56% when Recombinant Proteins serum was used) was observed when the neutralization was tested on rabbits' ears, which is the gold standard for the quality control step on the Antiloxoscelic Serum production. Blood tests were run on the immunizations and on the neutralization assay on rabbits' ears. They demonstrated that the mutated recombinant proteins and sera are safe and didn't cause any damage to the animals. The results presented reveal the potential for the utilization of the mutated recombinant proteins on the total or partial substitution of the loxoscelic venoms, since the serum obtained has a similar capability of neutralization to the Antiloxoscelic Serum produced in rabbits. There were no significant differences on the comparison between the sera produced. This substitution would be significant to animal welfare, once the need of maintaining spider to extract their venom would be much smaller.

Keywords: venom, loxoscelism, serum, phospholipase D.

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1. INTRODUÇÃO

Uma vez que acidentes com animais peçonhentos são considerados um problema de Saúde Pública no Brasil (MINISTÉRIO DA SAÚDE, 2001), é natural que sistemas de vigilância epidemiológica, vigilância em saúde e abordagem do paciente acidentado com a utilização de novos medicamentos sejam estudados ao longo dos anos e alternativas aos métodos atualmente empregados sejam apresentadas.

Neste contexto, acidentes causados por aranhas do gênero *Loxosceles*, popularmente conhecidas por aranhas-marrons, merecem destaque.

Atualmente, o soro antiloxoscélico administrado em pacientes que o necessitam é produzido a partir da imunização de equinos com soluções do veneno total de três espécies de aranhas-marrons consideradas de maior importância médica no país. O sangue dos equinos, contendo os anticorpos específicos contra os venenos, é colhido e processado industrialmente para posterior envio ao Ministério da Saúde e distribuição a diversos polos de saúde.

Para a obtenção de veneno em quantidade suficiente visando a produção do soro, é necessária a captura *in situ* e manutenção *ex situ* de milhares de espécimes de aranhas, o que caracteriza um trabalho oneroso e de risco.

Contudo, atualmente, através de estudos de caracterização dos venenos loxoscélicos e da capacidade de produção em laboratório das principais enzimas causadoras dos sinais clínicos dos acidentes, as fosfolipases-D, novas propostas de produção de soro e imunizações vêm sendo apresentadas à comunidade científica nos últimos anos.

Sendo assim, levando-se em consideração a importância das fosfolipases-D no contexto do acidente com aranhas-marrons, o desenvolvimento de um soro antiveneno produzido a partir de imunizações com fosfolipases-D recombinantes biologicamente inativas, porém com capacidade de serem reconhecidas pelo sistema imune, torna-se uma alternativa considerável para ser estudada e eventualmente sugerida como uma nova estratégia de produção de soros.

2. REVISÃO BIBLIOGRÁFICA

As aranhas do gênero *Loxosceles*, ou aranhas-marrons, como são popularmente conhecidas, podem ser encontradas em regiões de diferentes latitudes, e são adaptadas a distintas condições ambientais das zonas de clima tropical e temperado do globo terrestre (TAVARES *et al.*, 2011). O gênero possui mais de 150 espécies descritas, podendo ser encontradas nos cinco continentes (CHAVES-MOREIRA *et al.*, 2019). Na América do Sul, apenas, encontram-se 53 espécies (WORLD SPIDER CATALOG, 2021).

Há relatos de acidentes com seres humanos envolvendo o gênero *Loxosceles* em diferentes regiões do planeta (LOPES *et al.*, 2019), como por exemplo, na Europa (MARTÍNEZ-DOMENÉCH *et al.*, 2019; JERUSALEM *et al.*, 2018; PERNET *et al.*, 2010), no Oriente Médio (DEHGHANI *et al.*, 2017), na África (BUSER, 2020), na Oceania (VETTER, ISBISTER, 2008), nas Américas do Norte (LOPES *et al.*, 2020; SAUPE *et al.*, 2011) e do Sul (BUCARETCHI *et al.*, 2010).

No Brasil, das 19 espécies descritas e distribuídas no território nacional (WORLD SPIDER CATALOG, 2021), considera-se que *L. intermedia*, *L. gaucho* e *L. laeta*, encontradas especialmente nas regiões Sul e Sudeste, tenham maior importância do ponto de vista médico e epidemiológico (OLIVEIRA *et al.*, 2016).

De acordo com dados de 2019 do Sistema Nacional de Notificações de Agravos do Ministério da Saúde, no Estado do Paraná foram registrados 3810 casos de envenenamento loxoscélico (SISTEMA NACIONAL DE NOTIFICAÇÕES DE AGRAVOS – MINISTÉRIO DA SAÚDE – BRASIL, 2021). Em vista disso, os acidentes são considerados um importante problema de saúde pública no Brasil (GREMSKI *et al.*, 2014), assim como em outros países da América do Sul, como Chile e Peru (GREMSKI *et al.*, 2020).

O quadro clínico desenvolvido por indivíduos picados por aranhas-marrons é conhecido como loxoscelismo (MORGON *et al.*, 2016) e o envenenamento pode se manifestar de duas formas: o loxoscelismo cutâneo ou dermonecrótico (84-97% dos casos) e o quadro cutâneo visceral ou sistêmico (3-16% dos casos) (GRASHOF *et al.*, 2020; BARBARO *et al.*, 1992).

O quadro cutâneo é caracterizado pelo aparecimento de lesão dermonecrótica na região da picada. Geralmente, não há dor no momento da picada, porém com evolução para dor local, ardência, prurido, edema, mal-estar e febre. Algumas horas

após o acidente pode-se constatar o aparecimento de uma área de vermelhidão com zonas pálidas centrais, chamadas de placa marmórea (CHAVES-MOREIRA *et al.*, 2016).

De três a cinco dias após o evento, ocorre acúmulo de leucócitos polimorfonucleares e necrose na região da picada. A lesão dermonecrótica pode evoluir, em certos casos, para a formação de uma escara de difícil cicatrização, podendo originar sequelas deformantes (BOIA-FERREIRA *et al.*, 2019; FUTRELL, 1992).

O loxoscelismo sistêmico ou cutâneo-visceral é a forma mais grave do acidente e atinge a menor parte dos pacientes. Dentre os sintomas e sinais clínicos passíveis de serem encontrados neste quadro, podem-se citar: “*rash*” cutâneo, petéquias, equimose, astenia, anemia hemolítica, trombocitopenia, coagulação intravascular disseminada, hemoglobinúria, episódios eméticos, alterações sensoriais, cefaleias, obnubilação, convulsões e coma (NGUYEN E PANDEY, 2019). A lesão dermonecrótica também pode estar presente (PETERSON, 2006; DA SILVA *et al.*, 2004; FUTRELL, 1992). Os diversos sinais clínicos decorrentes do acidente podem se instalar de duas a até 72 horas após a picada (DE OLIVEIRA-MENDES *et al.*, 2019; PETERSON, 2006; FUTRELL, 1992). Além disso, pode ocorrer também insuficiência renal aguda (IRA) de etiologia multifatorial, associada à diminuição da perfusão renal, alterações vasculares nos pulmões e fígado (LUNG, MALLORY, 2000). A IRA, associada à obstrução da luz tubular é a principal causa de morte nos acidentes loxoscélicos (MINISTÉRIO DA SAÚDE, 2001).

Fatores como a espécie da aranha causadora do acidente, seu sexo, o estágio de desenvolvimento e a quantidade de veneno inoculado, podem estar relacionados diretamente com a gravidade do acidente e o desenvolvimento do quadro clínico (MINISTÉRIO DA SAÚDE, 2001; GONÇALVES DE ANDRADE *et al.*, 1999; SEZERINO *et al.*, 1998). Além disso, características genéticas do indivíduo acidentado, estado nutricional da vítima, local da picada, susceptibilidade ao veneno e o tempo que o acidentado leva para obter tratamento correto também são fatores fundamentais para determinar o progresso do acidente (COSTA *et al.*, 2020; BARBARO, EICKSTEDT, MOTA, 1994; BARRETO, CARDOSO, DE CILLO, 1985). Sendo assim, considera-se que os efeitos do envenenamento, especialmente os sistêmicos, podem ser potencialmente fatais, devendo ser clinicamente abordados de forma rápida e objetiva (PETERSON, 2006).

O que torna o quadro clínico observado no loxoscelismo danoso ao acidentado é justamente a ação do veneno da aranha sobre os tecidos. Sabe-se que o veneno é uma mistura complexa de proteínas de ação tóxica ou enzimática de baixa massa molecular, sais inorgânicos e pequenos peptídeos (FINGERMAN *et al.*, 2020; GREMSKI *et al.*, 2014; GREMSKI *et al.*, 2010; KALAPOTHAKIS *et al.*, 2007; BARBARO *et al.*, 2005; MACHADO *et al.*, 2005; DA SILVA *et al.*, 2004; DA SILVEIRA *et al.*, 2002; VEIGA *et al.*, 2000; FEITOSA *et al.*, 1998; PATEL *et al.*, 1994; FUTRELL, 1992). Estudos de identificação e caracterização das toxinas presentes no veneno de *Loxosceles* revelaram a presença de toxinas dermonecróticas da família das fosfolipases-D (30-35 kDa), metaloproteases (20-35 kDa), hialuronidasas (41-43 kDa), serinoproteases (85-95 kDa), além de lipases, fosfatase alcalina e 5'-fosfohidrolase ribonucleotídica (MEDINA SANTOS *et al.*, 2019; DANTAS *et al.*, 2016).

Dentre estes componentes, é sabido que algumas moléculas possuem papel fundamental na lesão tecidual, como é o caso da fosfolipases-D, toxina mais bem caracterizada do veneno (DA SILVA *et al.*, 2021; BUCH *et al.*, 2015). A toxina interage com a membrana celular e hidrolisa lipídios, o que desencadeia reações celulares e teciduais culminando na lesão dermonecrótica e demais complicações sistêmicas (APPEL *et al.*, 2008; DA SILVEIRA *et al.*, 2007b; CHAIM *et al.*, 2006; DA SILVEIRA *et al.*, 2006; DE ANDRADE *et al.*, 2005). A hialuronidase, por sua vez, é considerada um dos fatores responsáveis pelo espalhamento gravitacional das lesões e edema, uma vez que possui ação sobre moléculas da matriz extracelular, especialmente o ácido hialurônico (DE BONA *et al.*, 2021; FERRER *et al.*, 2013). As metaloproteases do tipo astacina, que degradam gelatina, fibronectina, fibrinogênio e entactina (MORGON *et al.*, 2016; DA SILVEIRA *et al.*, 2007b; VEIGA *et al.*, 2000, 2001a, b; FEITOSA *et al.*, 1998;) podem estar envolvidas nos distúrbios hemorrágicos observados em alguns acidentes, agindo como fatores de espalhamento sistêmico e no espalhamento gravitacional da dermonecrose, atuando em conjunto com a hialuronidase (COSTA *et al.*, 2020; TREVISAN-SILVA *et al.*, 2010; DA SILVEIRA *et al.*, 2007b; DA SILVA *et al.*, 2004; DA SILVEIRA *et al.*, 2002; FUTRELL, 1992). Toxinas inseticidas, que são peptídeos similares às notinas (moléculas que formam o nó inibidor de cisteína, chamadas ICKs) são as toxinas mais abundantes no transcriptoma de *L. intermedia* (GREMSKI *et al.*, 2010). Proteínas da família das TCTP também foram identificadas na glândula de veneno de *L. intermedia*. Esta proteína está relacionada com os efeitos

histaminérgicos do veneno (BOIA-FERREIRA *et al.*, 2019; SADE *et al.*, 2012; CHAIM *et al.*, 2011; GREMSKI *et al.*, 2010).

Os estudos de caracterização das fosfolipases-D (PLD) dos venenos de diferentes espécies de *Loxosceles* mostraram que estas enzimas possuem massas moleculares muito próximas entre si, e têm sido consideradas como isoformas ou homólogos de uma mesma enzima. Tal fato reflete um processo evolucionário comum entre as espécies de aranhas-marrons (TAVARES *et al.*, 2011).

As fosfolipases-D realizam a hidrólise da esfingomielina resultando na formação de ceramida-1-fosfato (C1P) e colina, subprodutos envolvidos em importantes fenômenos biológicos (DA SILVA, *et al.*, 2021; KITATANI, IDKOWIAKBALDYS, HANNUN, 2008; MONTES *et al.*, 2008; HANNUN, LUBERTO, 2004). Autores como LEE e LYNCH (2005) e VAN MEETEREN e colaboradores (2004), a partir de estudos com fosfolipases recombinantes obtidas de *L. reclusa* e *L. laeta*, respectivamente, mostraram que estas toxinas são capazes de hidrolisar não somente a esfingomielina, mas um amplo espectro de fosfolipídios, como por exemplo, lisofosfolipídios, lisofosfatidilinositol, lisofosfatidilglicerol, ácido lisofosfatídico, ácido fosfatídico cíclico e lisofosfatidilcolina (LPC). Este último fosfolipídio é atualmente reconhecido como mediador lipídico, atuando diretamente sobre determinados receptores relacionados com a sinalização intracelular via proteína G, sendo encontrados em diferentes tipos celulares. Assim, por meio da geração do ácido lisofosfatídico, produto da degradação da LPC, a fosfolipase-D poderia estar envolvida com fenômenos relacionados à agregação plaquetária, hiper- permeabilidade vascular e com aumento da resposta inflamatória (ANLIKER, CHUN, 2004a; ANLIKER, CHUN, 2004b; HASERUCK *et al.*, 2004).

A utilização da soroterapia como método de tratamento de pacientes picados por animais peçonhentos data das últimas décadas do século XIX, e a produção de soros ainda é baseada nos preceitos e metodologias descritos por Vital Brazil (BRAZIL, 1903). Tem como finalidade a neutralização da maior quantidade possível de veneno circulante, reduzindo o risco de envenenamento sistêmico e complicações potencialmente fatais como hemólise, insuficiência renal e coagulação intravascular disseminada, além de diminuir o dano local e encurtar o tempo de convalescença. Todos os soros antiveneno utilizados no Brasil são produzidos por quatro laboratórios oficiais: Instituto Butantan (São Paulo), Fundação Ezequiel Dias (Minas Gerais), Instituto Vital Brazil (Rio de Janeiro) e Centro de Produção e Pesquisa de

Imunobiológicos – CPPI (Paraná). Porém, o soro antiloxoscélico trivalente (contendo anticorpos específicos frente as três espécies de maior importância médica no Brasil (*L. gaucho*, *L. intermedia* e *L. laeta*) disponível no Sistema Único de Saúde (SUS) é produzido apenas pelo CPPI.

O soro antiloxoscélico é uma solução injetável de fração F(ab')₂ de imunoglobulinas específicas purificadas, a ser administrada por via intravenosa, conferindo imunidade passiva, e é produzido a partir do processamento do plasma sanguíneo de equinos devidamente imunizados com os antígenos totais dos venenos das aranhas-marrons: *L. intermedia*, *L. gaucho* e *L. laeta*. Cada mililitro do soro contém imunoglobulinas suficientes para neutralizar 15 Doses Mínimas Necrosantes (DMN) de veneno de *L. intermedia* (AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA, 2010). PAULI e colaboradores (2009) avaliaram a eficácia da utilização do Soro Antiloxoscélico Trivalente produzido pelo CPPI na neutralização e diminuição da ação do veneno loxoscélico sobre coelhos, fazendo com que houvesse diminuição da área necrótica e menor tempo para recuperação e cicatrização das regiões afetadas. Além disso, a lesão dermonecrotica mostrou-se 90% menor se comparada com a dos animais controle, quando o soro foi aplicado até 6 horas pós-envenenamento e 30% menor quando administrado até 48 horas após a inoculação de veneno loxoscélico.

Buscando uma menor dependência do veneno bruto na produção do soro utilizado por acidentados (e consequente menor necessidade de captura de animais nos seus ecossistemas e manipulação laboriosa para obtenção do antígeno), além de visar a produção de um soro mais eficiente e com menores custos, diversos pesquisadores vêm testando, ao longo dos anos, alternativas na produção do soro Antiloxoscélico a partir de novas estratégias de imunização com diferentes antígenos produzidos em ambiente laboratorial. Tais abordagens fazem o uso de toxinas recombinantes (DA SILVA *et al.*, 2021; LIMA *et al.*, 2018; OLIVEIRA *et al.*, 2016; DUARTE *et al.*, 2015; DE ALMEIDA *et al.*, 2008; FELICORI *et al.*, 2006; OLVERA *et al.*, 2006; ARAÚJO *et al.*, 2003), epítomos/peptídeos sintéticos (BERMÚDEZ-MÉNDEZ *et al.*, 2018; DIAS-LOPES *et al.*, 2010; FELICORI *et al.*, 2009) e quimeras (LIMA *et al.*, 2018; FIGUEIREDO *et al.*, 2014). A capacidade imunogênica destes antígenos, bem como o potencial de reconhecimento dos venenos brutos ou determinadas toxinas, e a redução das lesões dermonecroticas proporcionadas pela utilização dos soros produzidos a partir da imunização de animais com estas moléculas foram observados com sucesso em vários trabalhos em diferentes graus (CALABRIA *et al.*, 2019;

BERMÚDEZ MÉNDEZ *et al.*, 2018; LIMA *et al.*, 2018; OLIVEIRA *et al.*, 2016; DUARTE *et al.*, 2015; FIGUEIREDO *et al.*, 2014; MAGALHÃES *et al.*, 2013; MENDES *et al.*, 2013; DE MOURA *et al.*, 2011; OLIVERA *et al.*, 2006; ARAÚJO *et al.*, 2003; PEDROSA *et al.*, 2002). De Almeida *et al.*, 2008, utilizaram proteínas recombinantes biologicamente ativas de fosfolipases-D de *L. intermedia* e *L. laeta* na imunização de equinos a fim de obterem um novo soro Antiloxoscélico. O soro obtido foi capaz de neutralizar de maneira mais eficaz os venenos de *L. laeta* e *L. intermedia*, diminuindo as extensões das lesões provocadas em coelhos, quando comparados com a diminuição provocada quando o soro Antiaracnídico (que contém anticorpos contra *Phoneutria nigriventer*, *Tityus serrulatus* e *L. gaucho* e que também é distribuído na Rede Pública de Saúde do Brasil) foi utilizado. A capacidade de neutralização dos efeitos do veneno de *L. gaucho* foi similar quando os dois soros foram comparados. Já Calabria *et al.* (2019) produziram um imunógeno recombinante híbrido, composto de regiões hidrofílicas de fosfolipases-D e metaloproteases do tipo astacina de *L. gaucho*, sendo denominado LgRec1ALP1. A imunização em camundongos produziu anticorpos neutralizantes e o soro foi eficiente em neutralizar as atividades fibrinogenolíticas, dermonecrótica e de agregação plaquetária causadas pelos venenos de *L. gaucho*, *L. intermedia* e *L. laeta*. Anteriormente, Figueiredo *et al.* (2014) imunizaram equinos com proteína quimera associada ou não ao veneno total de *Loxosceles spp.* A proteína quimera (rCpLi) combinava três epítomos de uma proteína recombinante produzida anteriormente (rLiD1). Os soros obtidos a partir das imunizações em equinos foram efetivos em neutralizar a atividade dermonecrótica dos venenos loxoscélicos em coelhos.

Análises estruturais realizadas nos últimos anos vêm evidenciando o papel de alguns resíduos de aminoácidos nas fosfolipases-D. Os aminoácidos D32, E34 e E91, por exemplo, atuam na coordenação de um íon magnésio presente nas enzimas. Já H12 e H47 são responsáveis diretos pela atividade catalítica das fosfolipases-D, estando conservado em todas as isoformas de fosfolipases-D de aranhas-marrons, enquanto que W230, Y228 e Y229, presentes em um exossítio conservado, atuam na ligação das enzimas com o substrato fosfolipídico (POLLI *et al.*, 2021). Vuitika *et al.* (2016; 2013), Da Silva *et al.* (2021) e Polli *et al.* (2021) demonstraram que a substituição dos resíduos citados por alanina manteve as conformações das enzimas próximas ao original, além de apresentarem atividade residual em ensaios *in vitro* (atividade fosfolipásica) e de não apresentarem atividade biológica em ensaios *in vivo*.

(atividade dermonecrótica), além de serem reconhecidas por soros antiveneno total e soros anti-proteínas recombinantes. Tal abordagem se mostrou promissora do ponto de vista de utilização destas enzimas mutadas como antígenos em imunizações visando a produção de um soro de segunda geração, visto que sua produção em laboratório poderia solucionar questões como obtenção de veneno para produção de soro e captura *in situ* e manutenção de aranhas *ex situ* na cadeia produtiva do soro Antiloxoscélico produzido atualmente.

Neste sentido, este trabalho procurou obter e analisar diferentes soros antiloxoscélicos produzidos a partir da imunização de coelhos com as fosfolipases-D recombinantes mutadas nos resíduos de aminoácidos citados, sendo estes substituídos por Alanina (H12A-H47A, D32A-E34A, W230A).

3. JUSTIFICATIVA

Nos últimos anos, o conhecimento sobre as toxinas do veneno da aranha-marrom e seus efeitos foi aprofundado. Muito já se sabe sobre a composição proteica do veneno das aranhas do gênero *Loxosceles* e sua principal toxina dermonecrótica, a fosfolipase-D. Essa toxina faz parte de uma família de enzimas com diversas isoformas e é encontrada em diferentes proporções nas espécies. Pesquisadores já obtiveram isoformas recombinantes de fosfolipases-D presentes no veneno de *L. intermedia* (DA SILVA *et al.*, 2021; VUITIKA *et al.*, 2016; VUITIKA *et al.*, 2013; CHAVES-MOREIRA *et al.*, 2009; APPEL *et al.*, 2008; DA SILVEIRA *et al.*, 2007a; CHAIM *et al.*, 2006; DA SILVEIRA *et al.*, 2006), *L. gaucho* (MACHADO *et al.*, 2005), *L. laeta* (DE SANTI-FERRARA, 2009) dentre outras espécies do gênero (VETTER, 2011). A caracterização bioquímica e biológica dessas toxinas recombinantes mostrou que as enzimas ativas são capazes de reproduzir todos os sinais e sintomas desenvolvidos após a picada da aranha-marrom, como dermonecrose com espalhamento gravitacional, infiltração leucocitária, edema, eritema, aumento da permeabilidade vascular, agregação plaquetária, hemólise, letalidade e falência renal (VUITIKA *et al.*, 2016; CHAVES-MOREIRA *et al.*, 2011; CHAVES-MOREIRA *et al.*, 2009; PALUDO *et al.*, 2009; APPEL *et al.*, 2008; KUSMA *et al.*, 2008; DA SILVEIRA *et al.*, 2007a; CHAIM *et al.*, 2006; DA SILVEIRA *et al.*, 2006). Recentemente, por meio da técnica de mutagênese sítio-dirigida, foram obtidas de forma recombinante dez fosfolipases-D mutantes com redução drástica na atividade enzimática e biológica de algumas destas moléculas. Alguns mutantes recombinantes da toxina dermonecrótica presente no veneno de *L. intermedia* apresentaram apenas efeitos tóxicos residuais sobre coelhos ou não mostraram atividade enzimática, porém ainda apresentaram forte imunogenicidade e reconhecimento pelo soro antiveneno total e antitoxina recombinante LiRecDT1 (DA SILVA *et al.*, 2021; VUITIKA *et al.*, 2016).

Acredita-se que com a produção de um soro antiloxoscélico de segunda geração contra as toxinas dermonecróticas biologicamente inativas (mutantes) de *L. intermedia*, *L. gaucho* e de *L. laeta* poderá se obter uma concentração ainda maior de anticorpos neutralizantes, o que poderá propiciar aos pacientes picados por aranhas-marrons um tratamento mais específico e eficaz.

A estratégia adotada na utilização de uma toxina sem atividade enzimática, mas com atividade antigênica tornará viável a utilização de maiores concentrações do

antígeno nos animais produtores de soro, sem efeitos tóxicos, portanto com ganho no aspecto de bem-estar animal, e assim se obter maiores títulos de anticorpos nos soros antiloxoscélicos, que poderão ter maior poder neutralizante.

Além disso, um dos fatores limitantes para a produção do soro antiveneno de primeira geração consumido atualmente no Sistema Único de Saúde – SUS é a dificuldade na obtenção de grandes quantidades de veneno total para a imunização de animais. Porém essa limitação pode ser transposta com a utilização das toxinas recombinantes, as quais podem ser produzidas em larga escala utilizando modelos heterólogos. Além disso, já foi demonstrado que o soro antitoxina recombinante de *L. intermedia* produzido em coelhos possui reatividade cruzada contra o veneno total e toxinas recombinantes de outras espécies de aranha-marrom (BUCH *et al.*, 2015; DE ALMEIDA *et al.*, 2008), o que reforçaria e justificaria a utilização desta metodologia.

O desenvolvimento de um soro específico mais potente e mais eficaz mesmo quando aplicado muito tempo após a exposição ao veneno seria clinicamente mais adequado, uma vez que dificilmente o acidentado identifica o aracnídeo, já que a picada é indolor e há a dificuldade de se fazer o diagnóstico preciso pelas equipes médicas nas primeiras horas pós-picada. Uma das hipóteses trazidas neste trabalho é de que títulos maiores de anticorpos específicos contra as fosfolipases-D possam ser alcançados em imunizações onde se associam toxinas dermonecróticas recombinantes e veneno total. Além disso, o uso de toxinas dermonecróticas recombinantes mutadas sem atividade biológica em altas concentrações poderá diminuir ou mesmo não causar danos aos animais produtores de soro, o que representa um avanço significativo no seu bem-estar.

Portanto, o desenvolvimento de um novo Soro Antiloxoscélico representa um grande impacto sobre a Saúde Pública brasileira, uma vez que, sendo distribuído gratuitamente pelo SUS, auxiliaria na diminuição dos danos causados pelo Loxoscelismo no país, em especial no Estado do Paraná, onde se concentra o maior número de acidentes com aranhas-marrons.

4. OBJETIVOS

4.1 OBJETIVO GERAL

Desenvolver um novo Soro Antiloxoscélico mais potente, mais específico e mais eficaz em neutralizar os efeitos do envenenamento por aranha-marrom por meio da imunização de coelhos com antígenos recombinantes sem atividade biológica.

4.2 OBJETIVOS ESPECÍFICOS

- Clonar, expressar e purificar na forma recombinante fosfolipases-D selvagens ativas de aranhas-marrons (*L. intermedia*, *L. gaucho* e *L. laeta*).
- Produzir na forma recombinante e biologicamente inativa fosfolipases-D com mutações sítio-dirigidas no sítio catalítico e domínios acessórios (H12A-H47A, D32A–E34A, W230A) dos venenos das três espécies de maior importância médica do Brasil (*L. intermedia*, *L. gaucho* e *L. laeta*).
- Imunizar coelhos com os venenos brutos de *L. intermedia*, *L. gaucho* e *L. laeta*, com toxinas dermonecróticas recombinantes mutadas das três espécies e com os venenos enriquecidos com essas toxinas dermonecróticas mutadas.
- Determinar em coelhos a potência e efetividade dos soros antiloxoscélicos produzidos e comparar as alterações cutâneas (edema, hemorragia local, permeabilidade vascular, dermonecrose e infiltração leucocitária) e sistêmicas (índices hematimétricos) causadas pelo envenenamento em coelhos, aplicação dos soros e imunizações.

5. ARTIGO SUBMETIDO DURANTE O DOUTORADO

Production and functional evaluation of anti-*Loxosceles* sera raised by immunizations of rabbits with mutated recombinant phospholipases-D.

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Abstract: Loxoscelism is the clinical condition triggered after the bite of spiders of the genus *Loxosceles*. The main species involved in accidents in South America are *L. intermedia*, *L. laeta* and *L. gaucho*. The only specific treatment is the anti-*Loxosceles* serum produced with crude venoms. As phospholipases D (PLDs) trigger most of the effects observed in accidents, we developed and evaluated second-generation sera using mutated PLDs as antigens. Three isoforms of PLDs with site-directed mutations without biological activities were used for rabbit immunizations: D32A-E34A (*L. gaucho*), W230A (*L. intermedia*), and H12A-H47A (*L. laeta*). Sera were produced using crude venoms of three species of *Loxosceles* enriched with mutated recombinant PLDs (MIX) or using only mutated PLDs (REC). Immunizations stimulated immune system from the second immunization with a higher antibody production in the REC group. *In vivo* neutralization assays demonstrated that both sera reduced edema and dermonecrosis caused by *Loxosceles intermedia* crude venom. Follow-up of animals during the immunization protocols and in the neutralization assays demonstrated that the mutated proteins and the sera are safe. Results demonstrate the potential of using mutated recombinant PLDs in total or partial replacement of *Loxosceles* venoms in animal immunizations to produce anti-*Loxosceles* sera for treatments of Loxoscelism.

Keywords: brown spider, venom, loxoscelism, serum therapy, phospholipases D

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1. Introduction

Accidents caused by bites of spiders of the genus *Loxosceles*, popularly known as Brown spiders, have been reported in different regions of the world [1,2]. In Brazil, it is considered that *Loxosceles intermedia*, *L. gaucho* and *L. laeta*, found especially in the South and Southeast regions, are the species of greatest medical and epidemiological importance [1,3].

The clinical picture developed by patients bitten by Brown spiders is known as Loxoscelism [1,4], and can be classified into two variants named Cutaneous Loxoscelism, seen in most victims, and Systemic Loxoscelism, which has lower incidence, but it is more severe [1,5]. In the cutaneous form, also designated as Dermonecrotic Loxoscelism or Gangrenous Arachnidism, some clinical signs can be observed at the bitten site and at the surrounding areas such as edema, erythema, ecchymosis, formation of a marble plaque

and dermonecrosis, with gravitational spreading [1,4,6,7]. On the Systemic Loxoscelism, the presence of thrombocytopenia, hemoglobinuria, disseminated intravascular coagulation, intravascular hemolysis and acute renal failure can be seen, which can lead to death of some patients [1,4,6–10].

It is known that the venom is a complex mixture of low molecular mass components (3 to 45 kDa) with toxic or enzymatic activities [1,4]. Identification and characterization studies of toxins present in *Loxosceles* venoms have revealed the presence of phospholipases D (30 – 35 kDa), astacins (metalloproteases) (20 – 35 kDa), knottins (Inhibitor Cysteine Knot peptides) (3 – 10 kDa), hyaluronidases (41 – 43 kDa), serine proteases (85 – 95 kDa), allergen factor (42 kDa), TCTP (translationally controlled tumor protein) (22 kDa), and serpin (serine proteases inhibitor) (43 kDa) [1,11–16].

The best characterized class of toxins corresponds to the phospholipases D (PLDs), which are also known as Dermonecrotic Toxins. Transcripts related to PLDs represent about 20% of toxin-encoding transcripts in the venom gland of *L. intermedia* [1,17,18]. These enzymes are highly conserved among different species of the genus and play a fundamental role in the pathophysiology of envenoming [1,18]. The biological significance of PLDs can be seen in several studies that demonstrate that both the purified native PLDs from crude venoms, as well as their recombinant counterparts, could reproduce most of the toxic effects observed in accidents. PLDs target cell membranes, and prompt the cleavage of phospholipids, releasing biochemical mediators. This molecular and cellular mechanism results in dermonecrosis and other systemic complications in the victims [18–26]. Through the description of the tridimensional structure of Brown spider venom PLDs using crystallography, the essential domains and amino acid residues involved in the enzymatic activity of these molecules were identified. In the catalytic site it was described the presence of amino acid residues His 12 and His 47 conserved in all phospholipase D isoforms of *Loxosceles* spp. described to date. Other amino acid residues such as Glu 32, Asp 34 and Asp 91, participate in the coordination of the magnesium ion which is very important for the enzyme activity. Also, the amino acid residues Y228, Y229 and W230 found in an exosite are related to the binding of the enzyme to its substrates (phospholipids) [1,18,27–30].

Through site-directed mutations in the wild-type PLDs it was possible to obtain several soluble PLDs recombinant isoforms (H12A, H12A-H47A, E32A-D34A, W230A, Y228A-Y229A-W230A) of *L. intermedia*, *L. gaucho* and *L. laeta* venoms. Such mutations resulted in molecules without activities, incapable of degrading sphingomyelin and lysophosphatidylcholine or developing dermonecrosis in rabbits, in addition to the lack of hemolytic activity and no increase of vascular permeability [29,31,32]. Considering that all mutated phospholipase D isoforms of the three Brown spider species studied maintained their conformational characteristics (determined by circular dichroism and docking analysis), as well as were recognized in ELISA and western blotting studies by using anti-*Loxosceles* crude venoms and anti-wild-type recombinant PLD antibodies [29,31,32], the possibility of using these antigens on immunization protocols to produce a new serum for the treatment of loxoscelism was raised.

Although there are some difficulties in diagnosing accidents caused by *Loxosceles* spiders, especially in the first hours, and considering that there is certain heterogeneity on the approach and dispensing of drugs to the injured patients [33], it is known that serum therapy with anti-*Loxosceles* serum is the available methodology with the greatest therapeutic potential, since it has specific antibodies capable of neutralizing Brown spider venom toxins [34]. However, the production of anti-*Loxosceles* serum using crude venoms has critical points that can even be considered limiting to the manufacture of the product, such as the need to capture thousands of spiders from the natural environment, *ex situ* maintenance under laboratory conditions and venom extraction of a great number of spiders, which makes the work risky, laborious, and costly, in addition to the low yield in the venom extractions. Besides that, the need to immunize horses with the crude venom,

which potentially causes damage and suffering to animals is another critical point regarding the production chain. In this sense, some research groups have been working in recent years to obtain recombinant molecules without biological activities, but with preserved conformational characteristics and immunogenic properties, which can be used in immunization protocols aiming at the development of a new generation serum [31,35–37].

Therefore, we present in this work the production of a new anti-*Loxosceles* toxin serum produced in rabbits immunized with mutated isoforms of PLDs present in the *Loxosceles intermedia* (LiRecDT1 W230A), *L. laeta* (LiRecDT1 H12A-H47A) and *L. gaucho* (LgRecDT1 D32A-E34A) venoms. Results concerning the pre-clinical neutralizing capacity and analysis of possible toxic effects caused during their use as antigens to immunization protocols are also shown.

2. Materials and Methods

2.1. Preparation of venom solutions

The solutions of *L. intermedia*, *L. laeta* and *L. gaucho* venoms were prepared and provided by CPPI (Production and Research Center of Immunobiological Products, State Department of Health, Piraquara, Paraná, Brazil). Adult spiders' specimens were collected from natural areas in the states of Paraná and Santa Catarina (authorization numbers 028/2021 – PR and 002/2021 – SC) and maintained at CPPI. Venoms were extracted by electrostimulation (15 V) applied to the spiders' cephalothorax, near the chelicerae, followed by collection with a micropipette [38]. After that, the venom's samples were crystallized, diluted in 0.85 % saline, filtered (0.22 µm) and maintained at -20 °C until use. The final protein contents of venom's solutions were determined by the Coomassie blue method (Bio-Rad, Hercules, USA) as described by Bradford [39].

2.2. Animals

Adult New Zealand rabbits (~2.5 kg) were acquired from the Canguiri Experimental Farm of Federal University of Paraná, and randomly selected for immunization protocols and in vivo experiments. All procedures involving animals were carried out in accordance with Brazilian Federal laws, following the Institutional Ethics Committee for Animal Studies Guidelines from the Federal University of Paraná (certificate no. 1238).

2.3. Production and characterization of recombinant Phospholipases D (PLDs)

2.3.1. Recombinant expression and purification

Wild-type PLDs designated as LiRecDT1 (from *L. intermedia*), LiRecDT1 (from *L. laeta*) and LgRecDT1 (from *L. gaucho*) [20,31], and PLDs with site-directed mutations designated as LiRecDT1 H12A-H47A (from *L. laeta*), LgRecDT1 E32A-D34A (from *L. gaucho*) and LiRecDT1 W230A (from *L. intermedia*) [29,31] were expressed as described next. Wild-type and mutated constructions based on pET-14b vectors were transformed into *Escherichia coli* BL21(DE3)pLysS, which were plated on to Luria-Bertani (LB) agar plates containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. Single colonies of PLDs were used to inoculate 10 mL of liquid LB broth containing the antibiotics specified and grown overnight (16 h) at 37 °C, followed by dilution of this culture in 1 L of liquid LB plus antibiotics. The culture was allowed to grow at 37 °C under agitation (180 rpm) and monitored until the optical density (550 nm) reached between 0.4 and 0.6. The inducer (isopropyl β-D-thiogalactoside) was then added to a final concentration of 0.05 mM and the culture was incubated at 30 °C for additional 3.5 h under vigorous shaking. Subsequently, cells were harvested and resuspended in binding buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH 8.0, 500 mM NaCl, 10 mM imidazole) under gentle agitation. Lysozyme was then added to a final concentration of 1 mg/mL and this suspension was stored at -20 °C. After at least 16 hours the suspension was thawed, submitted to ultrasonic cell lysis and centrifuged (9000 × g, 30 min at 4 °C). The resultant supernatant was then filtered (0.22 µm) and subjected to purification of the recombinant PLDs using a HisTrap™ HP 1 mL column coupled to an ÄKTA™ pure chromatography system (GE

Healthcare Life Sciences). Column equilibration and washing steps were performed with washing buffer (20 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, pH 7.5) and elution was executed with a linear gradient of elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 7.5). Purified PLDs were dialyzed against Phosphate-Buffered Saline (PBS) (100 mM NaCl, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, pH 7.4) and analyzed using a 12.5% SDS-PAGE under reduction conditions.

2.3.2. Sphingomyelinase activity

The enzymatic activity of wild-type and mutated recombinant PLDs was quantified using the AmplexTM Red Assay Kit (Thermo Fisher Scientific, Waltham, USA). Ten micrograms of mutated PLDs and the wild-type proteins LiRecDT1 and LgRecDT1 were incubated with the AmplexTM Red reagent and sphingomyelin mixture in reaction buffer (Tris-HCl 100 mM pH 7.4 containing 10 mM MgCl₂) for 30 min at 37 °C. After incubation, fluorescence was determined on a fluorimeter (Tecan Infinite M200, Männedorf, Switzerland) using excitation wavelength at 540 nm and emission at 570 nm [23,26].

2.3.3. In vivo dermonecrosis

Mutated toxins (50 µg/each) diluted in PBS were intradermally injected into a shaved area of the rabbits' skin (n = 3). Wild-type recombinant isoform LiRecDT1 (10 µg) was used as positive control and PBS represented the negative control. The injection areas were observed and photographed at 0, 3, 6 and 24 h post-injection. Signs for edema, bruise and necrosis were analyzed and documented [29].

2.4. Production of sera and neutralization assays

2.4.1. Immunization protocols

Twenty New Zealand rabbits were divided into four groups and used to produce the sera as described in table 1. Immunizations were performed at intervals between two and three weeks in the first and second immunizations, and intervals between four and six weeks in subsequent immunizations. Injections were divided in three subcutaneous (s.c.) injection sites per animal [40]. Samples of 60 mL of blood were collected 15 days after the last injection and processed (Harlow and Lane) to produce hyperimmune sera.

Table 1: Protocol to produce sera

Group name	Antigen	1 st injection	2 nd injection	3 rd injection	4 th injection
AV	Venom pool *	7.5 µg of pool + CFA	10 µg of pool + IFA	10 µg of pool + IFA	15 µg of pool + IFA
REC	Recombinant proteins' pool **	100 µg of pool + CFA	200 µg of pool + IFA	400 µg of pool + IFA	800 µg of pool + IFA
MIX	Venom pool * + Recombinant proteins' pool **	2.5 µg of venom pool + 97.5 µg of recombinant protein's pool + CFA	2.5 µg of venom pool + 197.5 µg of recombinant protein's pool + IFA	2.5 µg of venom pool + 397.5 µg of recombinant protein's pool + IFA	2.5 µg of venom pool + 797.5 µg of recombinant protein's pool + IFA
C	Negative control	0.85% saline + CFA	0.85% saline + IFA	0.85% saline + IFA	0.85% saline + IFA

* Venoms of *L. gauchoi*, *L. intermedia* and *L. laeta* – 1:1:1

** Mutated recombinant proteins LiRecDT1 W230A, LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A – 1:1:1

CFA: Complete Freund's Adjuvant

IFA: Incomplete Freund's Adjuvant

2.4.2. Hematological and biochemical parameters

Hematological and biochemical parameters of blood samples collected from the rabbits used to produce the sera were analyzed to verify an eventual toxicity derived from the immunization. For this, the samples were collected before each immunization step and submitted to different analyses. Red and white blood cell counts, hemoglobin concentration and red blood cell indices were determined by using a hematology analyzer (Mindray Headquarters, Shenzhen, China) according to [41]. Biochemical parameters included aspartate aminotransferase, creatinine, muscular creatine kinase, urea, total plasma protein and fibrinogen; these parameters were quantified by means of commercial kits from Bioclin (Belo Horizonte, MG, Brazil) applied in BS-200 Chemistry Analyzer (Mindray Headquarters, Shenzhen, China) and according to [42]. The statistical analyses were performed by two-way ANOVA with Tukey's post-test and the significance was established at $p < 0.05$ using the software GraphPad Prism 8.0.2.

2.4.3. Clinical follow-up of immunized rabbits

Various parameters such as body weight, rectal temperature, edema, erythema, ecchymosis, and necrosis formation were checked from the time of immunization through the next seven days. Erythema and ecchymosis were classified from 1 to 4 according their intensity, in which "1" represents a less intense reaction and "4" indicates a more intense sign (adapted from [43]). The injection area with the presence of lesion or with the more intense lesion was used for this analysis. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by a post hoc Tukey test.

2.4.4. Immunoassays

ELISA assays were adapted from [44] and performed to evaluate the production of antibodies during the immunization process as well as the profile of conformational epitopes recognized. Briefly, 96 well MaxiSorp plates (Nunc – ThermoFisher, Waltham, MA, EUA) were coated with 600 ng of *L. intermedia* venom diluted in bicarbonate buffer (NaHCO_3 0.02 M pH 9.6). After washing with saline containing 0.05% of Tween 20, the plates were blocked with 2% powdered casein-PBS for 1 h at 37 °C. Sera from immunized rabbits were then added at 1:400 dilutions and incubated at 37 °C for 1 h. To exclude non-specific reactions, pre-immune sera and controls without primary antibodies or antigens were also tested. After washing step, the plates were incubated for 1 h at 37 °C with anti-rabbit IgG conjugated with horseradish peroxidase (Sigma- Aldrich A0545) diluted 1:5000. Plates were washed again, and colorimetric reactions were carried out in the absence of light for 30 min at 25 °C using 0.4 mg/mL of o-phenylenediamine and 4 $\mu\text{L/mL}$ H_2O_2 in citrate buffer (50 mM NaH_2PO_4 , 24 mM sodium citrate, pH 5.0). The reactions were stopped by adding 20 μL of 1 M H_2SO_4 per well. Absorbance values were measured on a plate reader (Meridian ELX 800) at 490 nm. Samples were analyzed in triplicates and statistical analyses were performed using two-way analysis of variance (ANOVA) followed by a post hoc Tukey test. The significance was established at $p < 0.05$ using the software GraphPad Prism 8.0.2 (San Diego, CA, USA).

For the western blotting experiments, 10 μg of venom samples were analyzed in SDS-PAGE 12.5% under reducing conditions and transferred onto nitrocellulose membranes. The membranes were then incubated with the produced rabbit sera diluted 1:1000. Alkaline phosphatase-coupled anti-rabbit IgGs (1:5000) (Sigma-Aldrich A3812) was used as secondary antibodies and the reaction was developed with BCIP/NBT (Promega) as substrate.

2.4.5. Neutralization assays

Samples of 3.05 μg of *L. intermedia* venom corresponding to 1 MND (Minimum Necrotizing Dose, which is the amount of venom that induces a lesion of approximately 1 cm^2) were injected in the external ear (pinna) of rabbits ($n=5$). Right after venom injection, 1 mL of the tested serum was administered via the marginal ear vein of the opposite ear.

After 0, 24, 72 and 240 h of serum inoculation, the lesions were photographed and the diameters of the dermonecrotic lesions at the venom injection sites were measured with a Vernier caliper to determine the area. Also, the body temperature of the rabbits was measured and blood samples were collected from the central ear vein to verify the hematological and biochemical parameters described in 2.4.2 and 2.4.3.

Alternatively, samples of 3.05 µg (1 DMN) or 6.1 µg (2 DMN) of *L. intermedia* venom were incubated with each serum (total volume: 100 µL) for 1 h at 37 °C, and the mixture was injected (i.d.) in a back shaved area of rabbits' skin. Animals were evaluated after 0, 24, 72 and 240 h of injections when the diameters of dermonecrotic lesions and edema near the inoculation sites were measured with a Vernier caliper to determine the areas. Erythema and ecchymosis were classified according to their intensity, as described in 2.4.3.

2.4.6. Histological analysis

Skin samples from rabbits exposed for 48 h to the incubation mixture of 2 DMN of *L. intermedia* venom plus different sera were fixed in ALFAC solution (ethanol 85%, formaldehyde 10% and glacial acetic acid 5%) at 4 °C for 16 h. After, the samples were dehydrated in a graded series of ethanol and embedded in paraffin for 2 h at 58 °C. Materials were then cut in thin sections (4 µm) and stained with Hematoxylin and Eosin (De Bona, 2020). Images were observed under a light microscope Olympus BX41 and photographed with a DP 72 camera (Olympus, Tokyo, Japan).

3. Results

3.1. Mutated recombinant PLDs are devoid of enzymatic and dermonecrotic activities

PLDs from different species belonging to the genus *Loxosceles* generally share key amino acid residues for catalysis and binding to substrates. In order to produce inactive toxins that keep the three-dimensional conformation of wild-type PLDs, Vuitika [29] and Silva [31] performed site-directed mutagenesis protocols targeting some of these PLDs' amino acids of from three *Loxosceles* species, and generated the constructions LiRecDT1 H12A-H47A (from *L. laeta*), LgRecDT1 E32A-D34A (from *L. gaucho*) and LiRecDT1 W230A (from *L. intermedia*). Here, these constructions were expressed in *E. coli* cells from BL21(DE3)pLysS strain and purified by affinity chromatography. As observed in figure 1A, all recombinant isoforms were produced and purified as soluble proteins and appear as ~35 kDa polypeptides in the 12.5% reducing SDS polyacrylamide gel shown. The sphingomyelinase activity of the recombinant proteins was quantified by the Amplex™ Red Assay, and results showed that all mutated isoforms have no enzymatic activity on sphingomyelin, while such activity is highly detected when the wild-type isoform LiRecDT1 is tested (Figure 1B). These enzymatic profiles observed for the isoforms corroborate with their in vivo activity observed after 24 h of injection in the skin of rabbits (Figure 1C). The macroscopic lesion observed near the injection site of the wild-type isoform LiRecDT1 displays the usual signs triggered by *Loxosceles* PLDs, such as ecchymosis, erythema, necrosis, and gravitational spreading of the lesion. Conversely, it is possible to observe that the mutated isoforms did not trigger any of these signs, as the macroscopic aspect of the injection area is quite similar to what was observed for the negative control (Figure 1C).

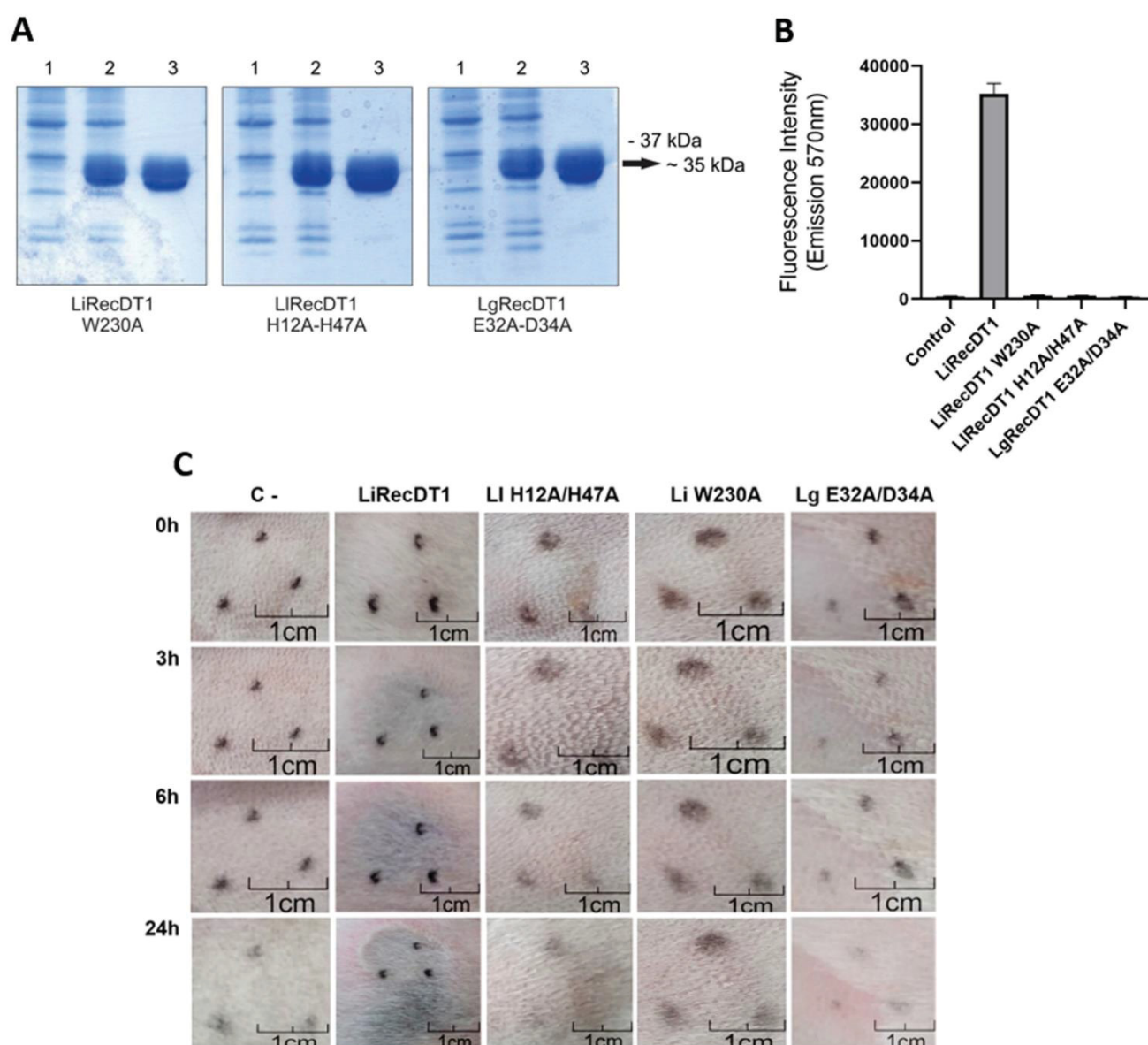


Figure 1: Properties of mutant phospholipases D (PLDs). (A) Recombinant expression and purification of PLDs analyzed by 12.5% SDS-PAGE under reducing conditions and stained with Coomassie blue dye. Lanes 1 show *E. coli* BL21(DE3)pLysS culture collected by centrifugation and resuspended in Laemmli sample buffer prior to induction with IPTG. Lanes 2 show the supernatant of cell lysates after induction with IPTG. Lanes 3 show purified eluted recombinant proteins through Ni²⁺-NTA agarose column. The position of the closest molecular weight standard and an arrow indicating the recombinant PLDs are depicted on the right of the figure. (B) Sphingomyelinase D activity of wild type (LiRecDT1) and mutated (LiRecDT1 W230A, LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A) PLDs evaluated by Amplex Red Assay. The product of reaction was measured on a fluorimeter using excitation wavelength at 540 nm and emission at 570 nm. Control reaction was performed without any toxin. Values represent average $\pm$ SD of three independent experiments carried out in triplicate. (C) Macroscopic analysis of rabbit skin exposed to wild-type (10 μ g) and mutated (50 μ g) PLDs. Photographs show the injection area immediately after inoculation (0 h) and after 3, 6 and 24 h following injection. Black dots marked in the skin surround the local of injection.

3.2. Sera production: effects of immunizations

For the sera production, rabbits were immunized with four antigen preparations as previously described: (1) whole venom of *L. intermedia*, *L. laeta* and *L. gaucha*, and this group was named AV (derived from antivenom); (2) recombinant mutated proteins LiRecDT1 H12A-H47A, LgRecDT1 E32A-D34A and LiRecDT1 W230A, which was named

REC (derived from recombinant); (3) both the venom of the three *Loxosceles* species and the three recombinant mutated proteins, and this group was named MIX.; (4) PBS (with adjuvant), which was named C- (derived from negative control). Macroscopic and clinical signs were monitored during the seven days following all immunizations. One of the macroscopic signs observed after immunizations was local edema, and it was detectable generally one day after the injections (data not shown). All animals administered with the antigens – whole venom, recombinant proteins or both antigens (mix) – developed edema in at least one site of injection, while 80% of rabbits (n=4) from the C- group showed this sign. This reaction was triggered after immunizations with either CFA or IFA. Erythema was also observed after immunization in all animals from all groups, however, the intensity of erythema in the C- group was significantly lower than that from the three other groups that received the specific antigens (Figure 2A). In addition, the intensity of erythema detected in the animals immunized with the recombinant proteins (REC) was significantly higher than that from the other groups. Ecchymosis was also detected in all groups, but not in all immunizations. This sign was mostly observed between the first and third days after injection and was significantly more intense in the animals from REC group when compared to C- and AV (Figure 2B). All these signs – edema, erythema and ecchymosis – progressively decreased in the days following the immunizations. Necrosis resulting from the immunizations was not observed in any of the treatments tested. The rectal temperature of immunized rabbits was the clinical sign checked in the days following immunizations. Results indicate that none of the injections induced fever, as the average temperatures of all animals did not exceed 40 °C during all experiment (data not shown).

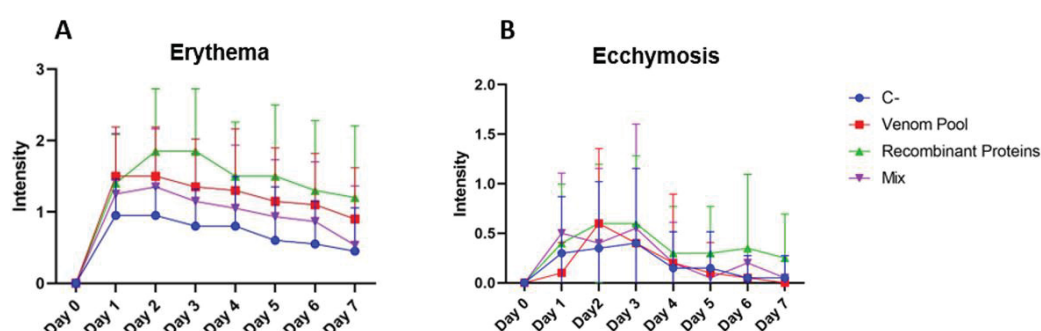


Figure 2: Monitoring of antigen injection sites of immunized rabbits. Erythema (A) and ecchymosis (B) were checked for seven days (X axis) following the immunizations with different antigens. Venom pool: pool of *L. intermedia*, *L. laeta* and *L. gaucho* venoms + adjuvant. Recombinant proteins: pool of mutated PLDs + adjuvant. Mix: venom pool + recombinant protein's pool + adjuvant. C-: saline + adjuvant. The intensity of erythema and ecchymosis (Y axis) were classified from 1 to 4 according to the intensity, in which "1" represents a less intense reaction and "4" indicates a more intense sign (adapted from [43]). Each point represents the averages of intensities measured after immunizations. For erythema, results were statistically significant for Venom pool, Recombinant proteins (ANOVA, $p < 0.0001$ for both) and Mix (ANOVA, $p = 0.0111$) comparing with C- group.

Hematological and biochemical parameters were also monitored in blood samples collected right before all immunizations and fifteen days after the last treatment injection. Red blood cells' count, hematocrit and hemoglobin concentration remained within the reference values throughout the immunization process, in all groups, with no significant differences among them (Figure 3A). Serum fibrinogen levels also remained normal during the process in all groups and with no significant differences among them, although it is possible to observe a slight increase of fibrinogen levels in the AV and MIX groups after the first immunization (Figure 3A). In addition, no difference among groups was observed regarding the platelets' count, although the C- group presented a close to the limit decrease between the second and fourth immunizations, which returned to normal values at the end of process (Figure 3A). The hematological parameters concerning white blood

cells reveal that the total leukocyte count did not vary significantly among groups during the immunization process. However, this leukocyte count decreased in all groups at the end of the protocol, especially in the MIX group, in which, at this point, a leukocyte count below the reference values was observed (Figure 3A). On the other hand, relative lymphocyte and heterophil counts revealed significant differences among groups. Before the second and fourth immunizations, the C- group statistically differed from the MIX and REC groups (Figure 3A). These differences are due to the high counts of lymphocytes in the C- animals, which balances with the low counts of heterophiles in the same group. Among the biochemical parameters evaluated during the process (Figure 3B), creatinine kinase (CK) from all groups and urea from C- and MIX groups (mainly after the third immunization) presented values above the limits for rabbits. Significant differences can be observed among some groups at certain steps of the treatment process (Figure 3B). However, as little variation exceeding the reference values were observed, no important physiological outcomes can be inferred.

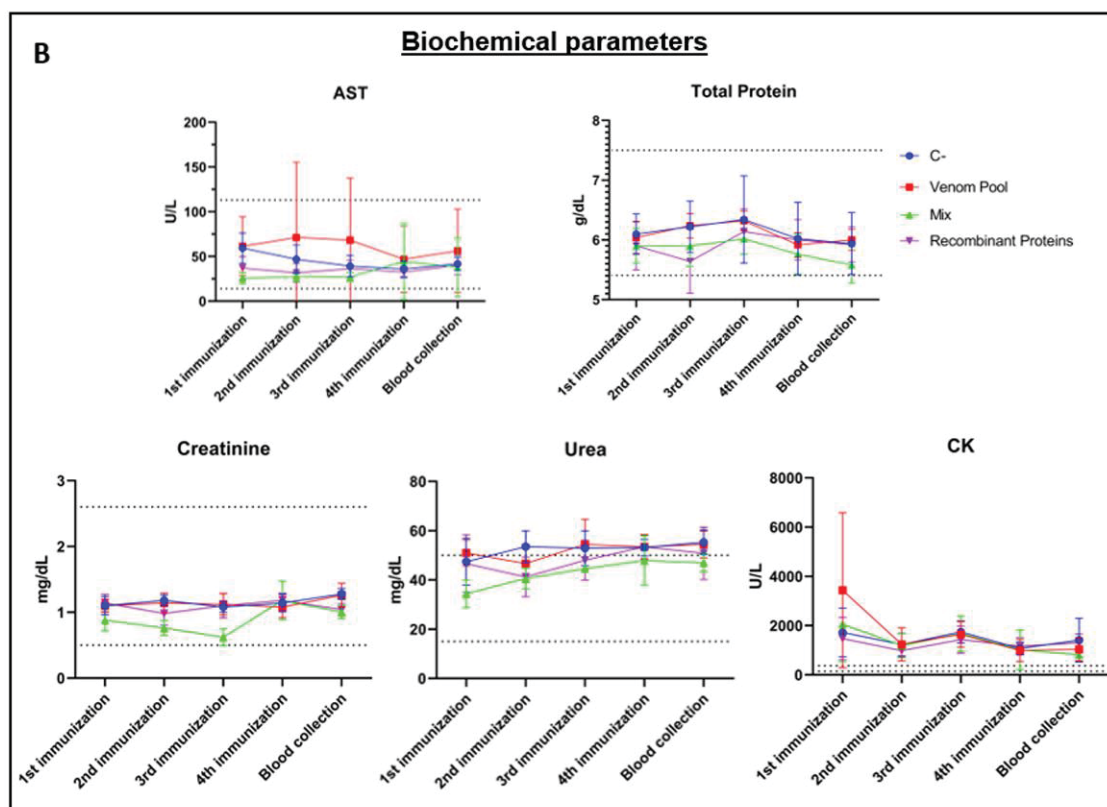
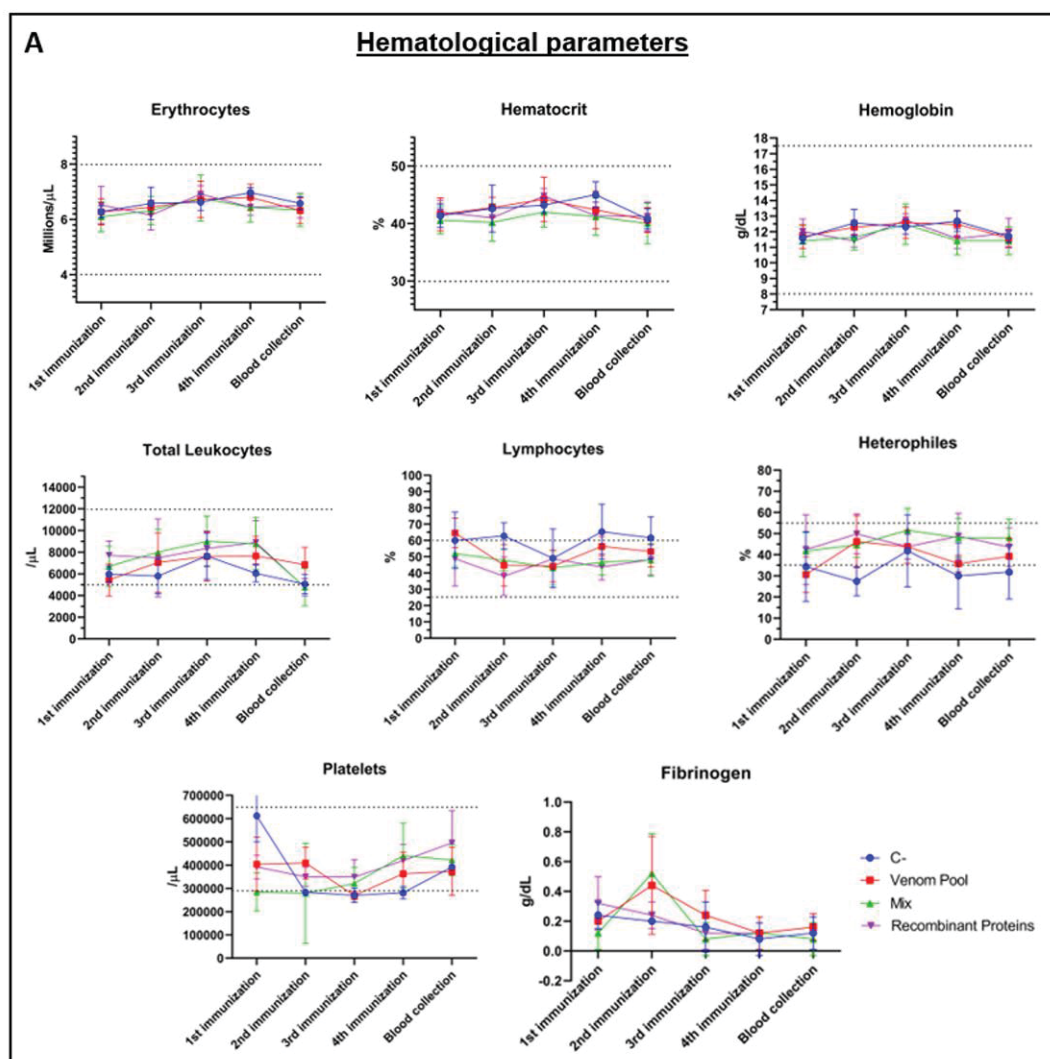


Figure 3: Monitoring of blood parameters of immunized rabbits. Hematological (A) and biochemical (B) parameters were verified from blood samples collected immediately before each immunization step. Venom pool: pool of *L. intermedia*, *L. laeta* and *L. gaucho* venoms + Complete Freund's Adjuvant (CFA) in the 1st immunization and Incomplete Freund's Adjuvant (IFA) in the following immunizations. Recombinant proteins: pool of mutated PLDs + CFA in the 1st immunization and IFA in the following immunizations. Mix: venom pool + a recombinant protein's pool + CFA in the 1st immunization and IFA in the following immunizations. C-: saline + CFA in the 1st immunization and IFA in the following immunizations. AST: aspartate aminotransferase. CK: muscular creatine kinase. Lymphocytes: relative lymphocyte count. Heterophiles: relative heterophile count. For the relative lymphocyte count, results were statistically significant for Recombinant proteins (ANOVA, $p = 0.0002$) and Mix (ANOVA, $p = 0.0015$) comparing with C- group. For the relative heterophile count, results were statistically significant for Recombinant proteins (ANOVA, $p = 0.0006$) and Mix (ANOVA, $p = 0.0002$) comparing with C- group. For AST, results were statistically significant for Recombinant proteins (ANOVA, $p = 0.0355$) and Mix (ANOVA, $p = 0.0141$) comparing with Venom pool group. For Total protein, results were statistically significant for Mix comparing with C- group (ANOVA, $p = 0.0428$). For creatinine, results were statistically significant for Mix comparing with the other groups (ANOVA, $p < 0.0001$). For Urea, results were statistically significant for C- (ANOVA, $p < 0.0001$) and Venom pool (ANOVA, $p = 0.0002$) comparing with Mix group.

3.3. Evaluation of the produced sera

The production of antibodies resulting from the immunization process and their recognition profile were analyzed in vitro using ELISA and western blotting analyses. The four produced sera (AV, REC, MIX and C) were evaluated and compared during all the immunization protocol, as blood samples of all animals were collected right before each immunization until the bleeding for sera production (blood collection). First, an ELISA was performed to quantify the recognition of native epitopes (conformational and linear) from venom antigens by the produced sera. As depicted in figure 4A, there was an increase in the production of specific antibodies after the second immunization in the AV, REC and MIX groups, with a tendency for curves to grow until the fourth inoculation and stabilization of the antibody's levels when the bleeding step for sera production was performed. After the second immunization, all groups immunized with specific antigens presented a significant higher production of antibodies when compared to the C- group. Furthermore, REC sera showed a higher response when compared to AV and MIX groups. Finally, a western blotting analysis was performed to verify the recognition profile of linear/denaturation-resistant epitopes from venom proteins by the produced sera. As displayed in figure 4B, all specific sera recognized the proteins of different *Loxosceles* venoms with molecular weights between 25 and 35 kDa. Nevertheless, it is possible to observe that the bands produced by AV serum are less intense than those from REC and MIX sera.

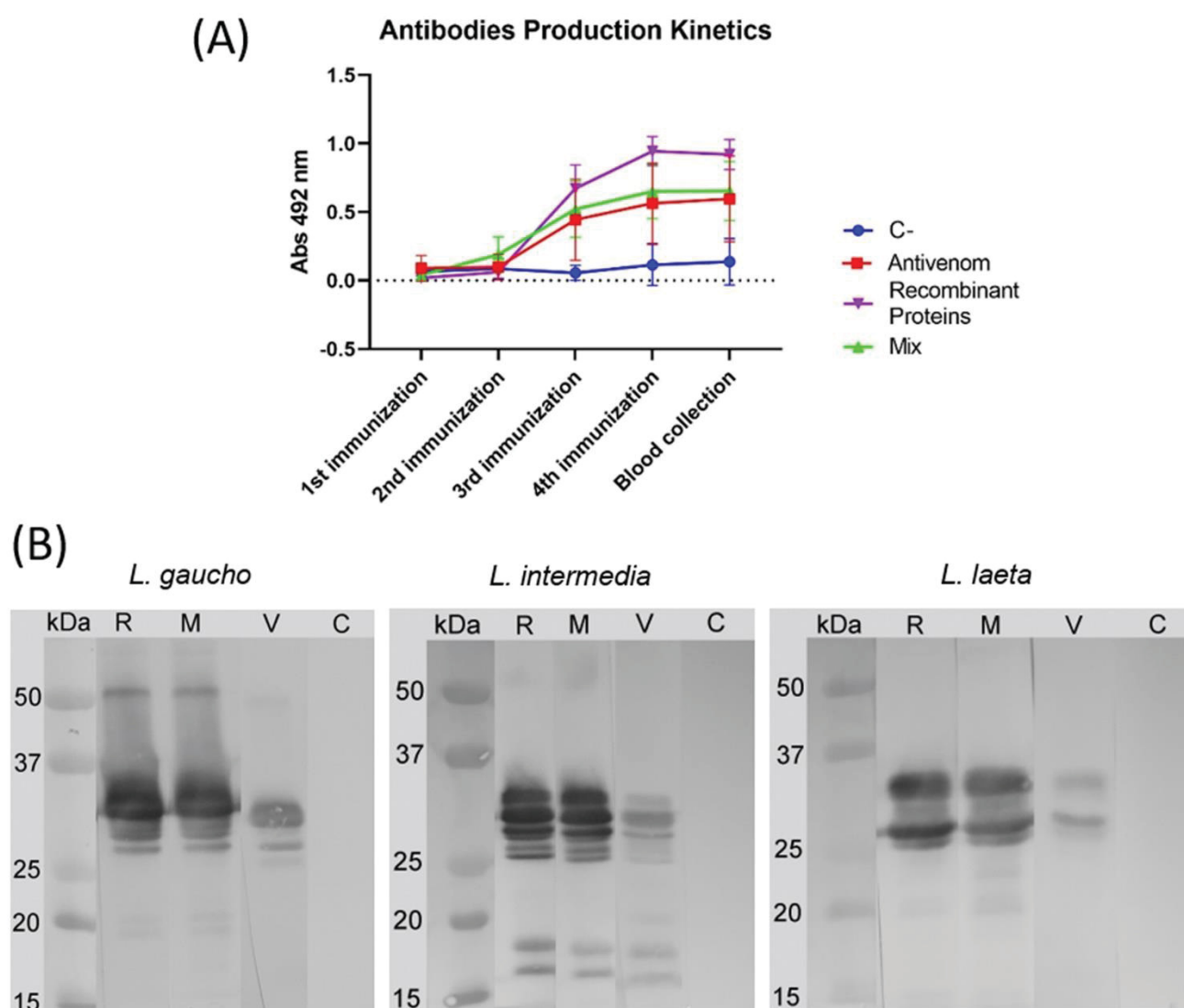


Figure 4: Reaction of produced sera with *Loxosceles* venoms. (A) ELISA for antibody capture assay with plates coated with *L. intermedia* venom (600 ng/well) and reacted with sera collected immediately before each immunization step (1:400 dilution). Antivenom: AV serum. Recombinant proteins: REC serum. Mix: MIX serum. C-: C serum. A pre-immune serum was used as control (data not shown). Samples were analyzed in triplicates and each point represents the mean $\pm$ SD. Results were statistically significant for all groups comparing with C- (ANOVA, $p < 0.0001$). Results of Recombinant proteins' group were statistically different from all other groups (ANOVA, $p < 0.0001$). No significant difference was found between Antivenom and Mix groups. (B) Western blotting showing immunological cross-reactivity among *Loxosceles* venoms and produced sera. Samples of venom (10 µg) from *L. gaucho*, *L. intermedia* and *L. laeta* were probed with the produced sera (1:1000 dilution). R: REC serum. M: MIX serum. V: AV serum. C: C serum. kDa: Molecular weight marker. A pre-immune serum was also used as control (data not shown). Molecular protein standards are shown on the left of figures.

3.4. Neutralization of *in vivo* toxicity

In order to verify if the produced sera are able to neutralize the main local signs of cutaneous loxoscelism, two neutralization assays were performed. The first one examined the effects of a *Loxosceles* venom pre-incubated with the produced sera in rabbits' dorsum skin (Figures 5 and 6). The second assay evaluated if *Loxosceles* venom inoculated intradermally in the external ear of rabbits could be neutralized by the produced sera injected intravenously (Figure 7).

In the pre-incubation experiment performed in the skin of rabbits, two different amounts of *L. intermedia* venom were tested (1 DMN and 2 DMN) and analyzed separately (Figures 5 and 6). When the development of dermonecrosis was examined, all specific sera significantly decreased the necrosis triggered by 1 or 2 DMN of venom when compared to the negative control serum (C-). After 72 h, the findings for the 1 DMN treatment showed that the MIX serum was able to reduce the development of the dermonecrotic lesion in 48.75% when compared to the C- serum. Also, AV and REC serum reduced the dermonecrotic reaction in 58.56% and 63.39% respectively when compared to the negative control (Figures 5A and B). The decrease in the development of dermonecrotic lesion with 2 DMN of venom by the specific sera was 41.81% for the AV serum, 53.6% for the REC serum and 56.33% for the MIX serum (Figures 6A and B). Edema formation was also evaluated in this experiment. The measurements showed that all specific sera groups presented a significant minor edema formation when compared to the C- group for both 1 and 2 DMN treatments (Figures 5B and 6B). In the groups that were tested with 1 DMN of *L. intermedia* venom, AV serum prevented the edema formation in 81.03% when compared to C- serum, which was similar to the reductions observed for the MIX and REC groups: 81.39% and 82.5%, respectively (Figure 5B). Although the prevention of edema by the specific sera was lower when 2 DMN of venom was tested, it was still significant: measurements indicate a reduction of 64.33% by AV serum, 71.96% by MIX serum and 77.41% by REC serum (Figure 6B). No ecchymosis was observed in the groups that were tested with AV, REC or MIX sera and 1 DMN of venom (Figures 5A and B). Conversely, the specific sera were not as effective in the prevention of ecchymosis triggered by 2 DMN of venom, although these groups still present significant differences with the C- group (Figure 6B). After statistical analyses regarding the prevention of dermonecrosis, edema and ecchymosis, no significant differences were found among the neutralization indices of AV, MIX and REC sera for both 1 and 2 DMN treatments. Finally, none of the sera proved to be efficient in reducing the onset of erythema (Figures 5A, 5B, 6A and 6B).

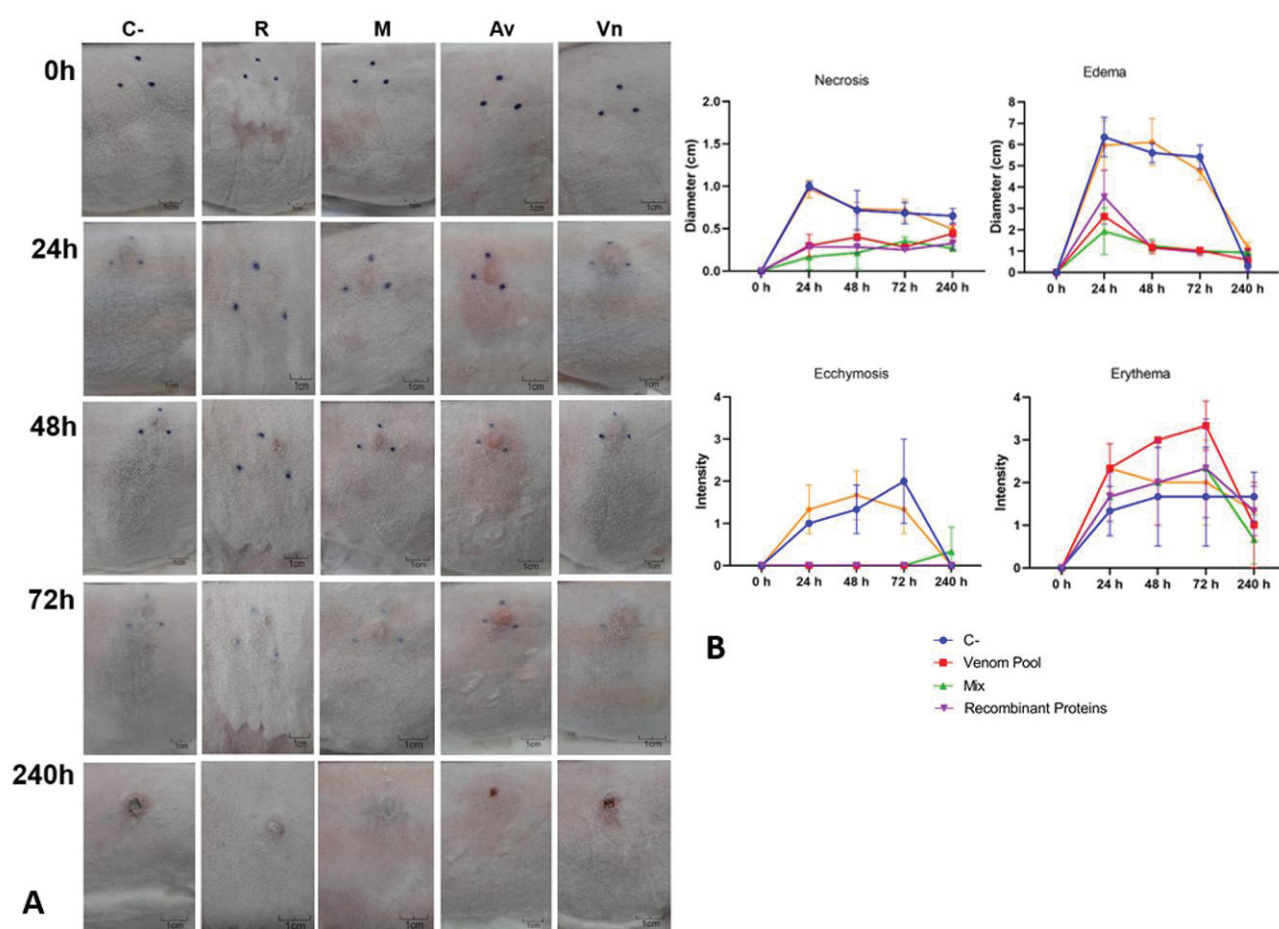


Figure 5: Neutralization of cutaneous loxoscelism triggered by 1 MND of venom tested with pre-incubation. One MND (3.05 µg) of *L. intermedia* venom previously incubated with each serum was injected (I.D.) in the back skin of rabbits (groups of five animals). (A) Macroscopic aspect of injected area after the indicated time courses. C-: C serum. R: REC serum. M: MIX serum. Av: AV serum. Vn: 1 MND of *L. intermedia* venom in the absence of serum. Photographs show a representative response of the animals. Black dots marked in the skin surround the local of injection. (B) Skin necrosis, edema, ecchymosis and erythema were checked after various time courses (X axis) following injections of 1 MND of venom pre-incubated with each serum. The intensity of such signs (Y axis) were classified from 1 to 4 according to the intensity, in which “1” represents a less intense reaction and “4” indicates a more intense sign (adapted from [43]). Each point represents the average of intensities of each group measured at the indicated times $\pm$ SD. Antivenom: AV serum. Recombinant proteins: REC serum; Mix: MIX serum. C-: C serum. *L. intermedia* venom: 1 MND of *L. intermedia* venom in the absence of serum. For necrosis, edema and ecchymosis, results of the groups AV, MIX and REC were statistically different from C (ANOVA, $p < 0.0001$). No significant difference was found among groups when erythema was analyzed. In all analyses, no significant difference was found among AV, MIX and REC.

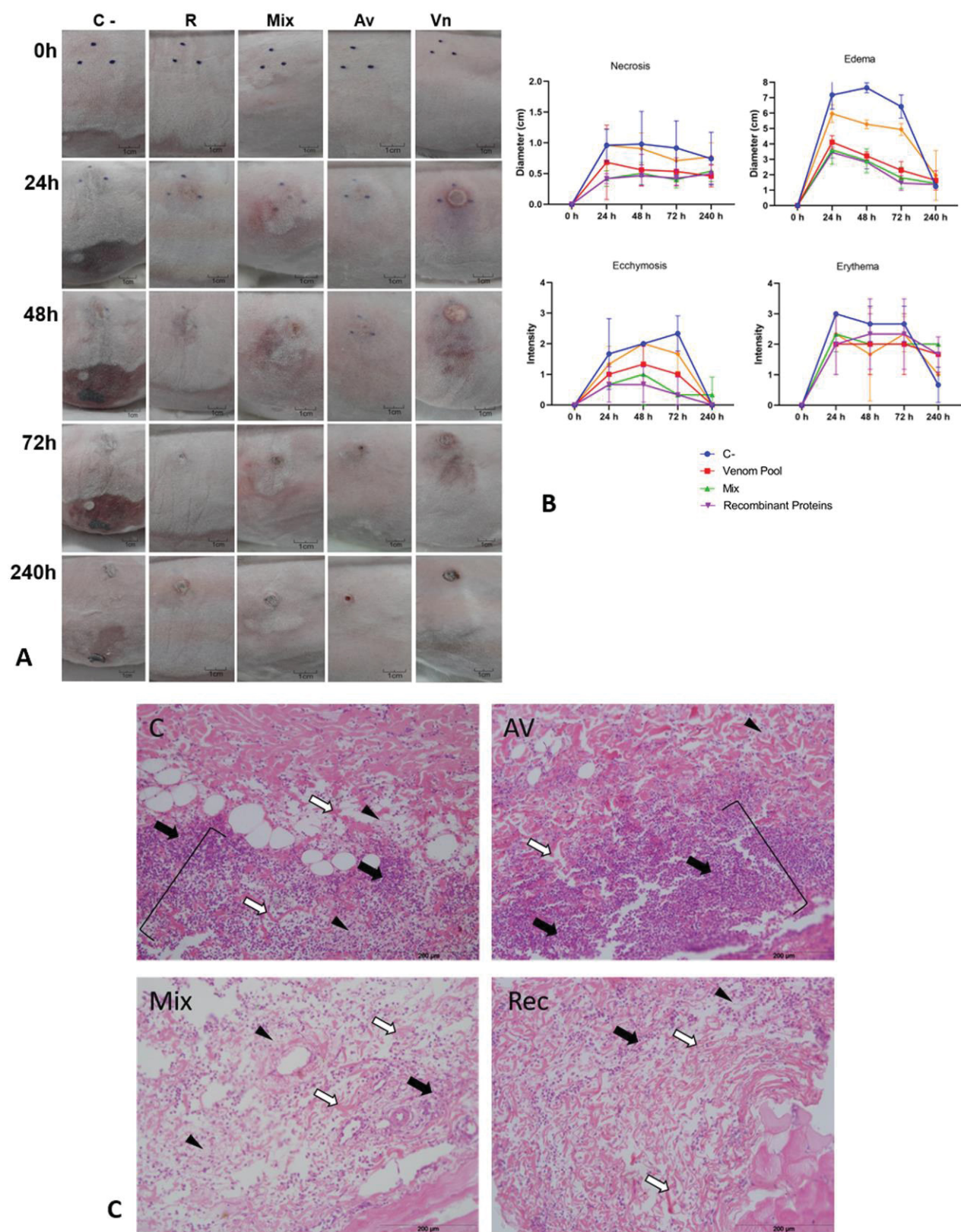


Figure 6: Neutralization of cutaneous loxoscelism triggered by 2 MND of venom tested with pre-incubation. Two MND (6.10 µg) of *L. intermedia* venom previously incubated with each serum were injected (I.D.) in the back skin of rabbits (groups of five animals). (A) Macroscopic aspect of injected area after the indicated time courses. C-: C serum. R: REC serum. M: MIX serum. Av: AV serum. Vn: 2 MND of *L. intermedia* venom in the absence of serum. Photographs show a representative

response of the animals. Black dots marked in the skin surround the local of injection. (B) Skin necrosis, edema, ecchymosis and erythema were checked after various time courses (X axis) following injections of 2 MND of venom pre-incubated with each serum. The intensity of such signs (Y axis) were classified from 1 to 4 according to the intensity, in which “1” represents a less intense reaction and “4” indicates a more intense sign (adapted from [43]). Each point represents the average of intensities of each group measured at the indicated times $\pm$ SD. Antivenom: AV serum. Recombinant proteins: REC serum; Mix: MIX serum. C-: C serum. *L. intermedia* venom: 2 MND of *L. intermedia* venom in the absence of serum. For necrosis, edema and ecchymosis, results of the groups AV, MIX and REC were statistically different from C (ANOVA, $p < 0.005$ for necrosis and ecchymosis, $p < 0.0001$ for edema). No significant difference was found among groups when erythema was analyzed. In all analyses, no significant difference was found among AV, MIX and REC. (C) Histopathological analyses of rabbit skin after 48 h of injection with the incubation mixture of 2 DMN of venom plus each serum. Tissue sections were stained with hematoxylin and eosin (HE). The images presented are representative and show the lower layer of the dermis, which usually concentrates the most evident changes in this tissue after *Loxosceles* venom I.D. injection. Scale bars are indicated in the figures. C: C serum. AV: AV serum. Mix: MIX serum. REC: REC serum. Closed arrows point out to leukocytes infiltrating the connective tissue. Square brackets in the upper panels delimit a massive infiltration of inflammatory cells that appear as a palisade of polymorphonuclear leukocytes. Disorganization of collagen bundles (open arrows) and fibrin deposition (arrowheads) are also present.

Histopathological analyses of rabbits' skin submitted to the pre-incubation experiment with 2 DMN of *L. intermedia* venom have shown that some usual injuries were observed when the venom was inoculated, such as collagen fibers disorganization and presence of fibrin in the connective tissue. These findings were present in all samples, including those in which a specific serum was pre-incubated with the venom (Figure 6C). However, it is possible to observe that the accumulation of polymorphonuclear cells was less pronounced in MIX and REC group (Fig. 6C – lower panels) when compared to the massive infiltration of inflammatory cells that appear as a palisade of polymorphonuclear cells in the lower layer of the dermis seen for the AV and C- treatments (Fig. 6C – upper panels).

The neutralization experiment performed in the external ear of rabbits aimed to evaluate the neutralization capacity of the produced sera by performing a required step in the quality control of the traditional serum currently used in the treatment of the victims. As demonstrated in figures 7A and 7B, AV and REC sera were efficient in neutralizing the dermonecrotic effect of *L. intermedia* venom (1 DMN) when compared to the neutralization observed for the C- group. However, this neutralization was not observed when the MIX serum was tested. The development of dermonecrotic lesions evaluated 72 h post-injection was reduced in 56.42% by REC serum, 59.74% by the AV serum and 29.21% by the MIX serum (Figures 7A and 7B). To verify if serum administration triggered toxic effects in the tested animals, blood samples were collected after 0, 24, 72 and 240h post-inoculation. The graphs presented in figure 8 demonstrate that the animals administered with any of the sera tested did not present hematological (Figure 8A) or biochemical (Figure 8B) indices exceeding the reference values determined for rabbits. In addition, no significant differences in parameters were detected when the specific sera were compared to each other, except for a slight difference in red blood cell count for the AV and REC groups ($p = 0.0254$).

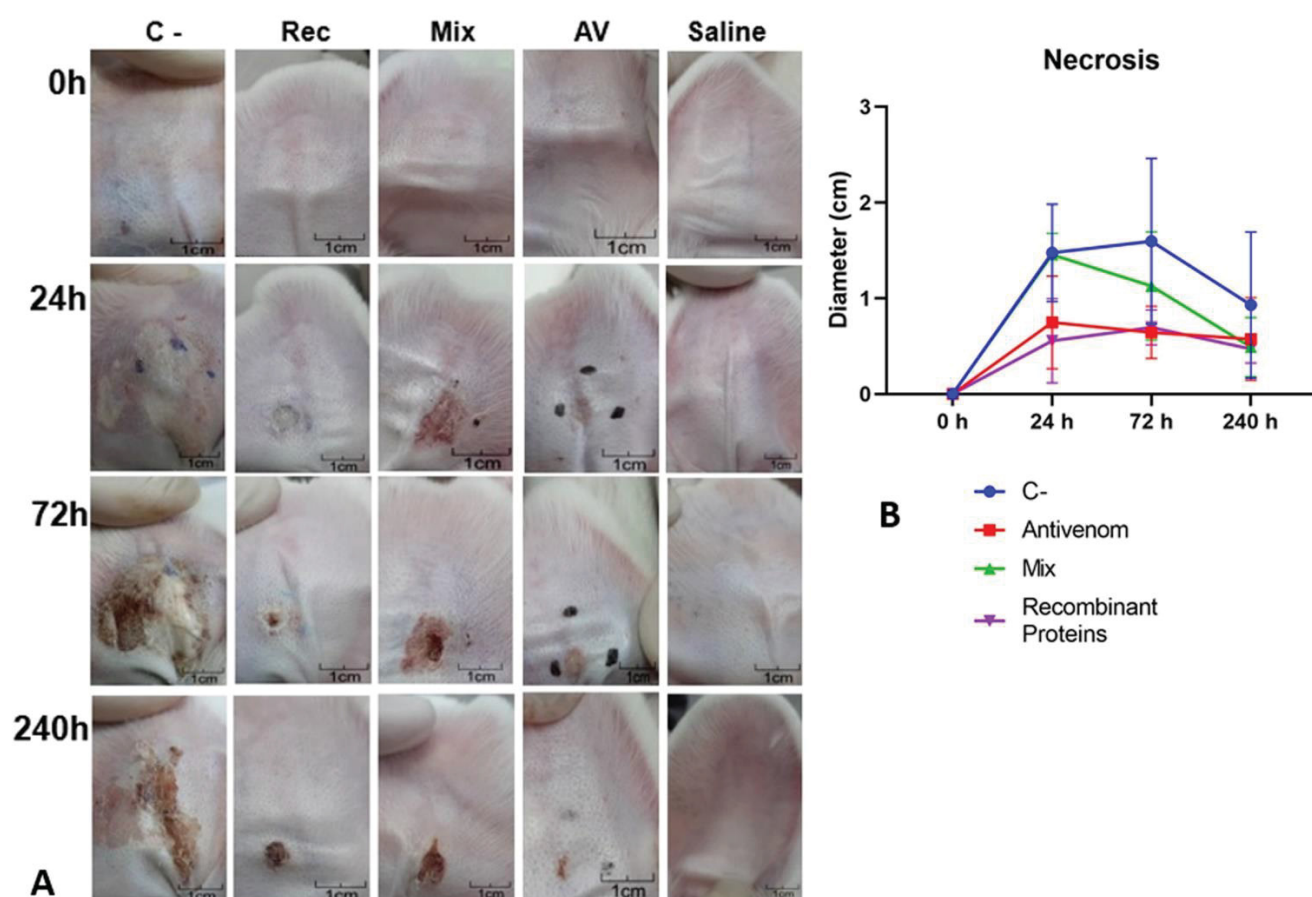


Figure 7: Neutralization of cutaneous loxoscelism in the external ear of rabbits. One MND (3.05 μ g) of *L. intermedia* venom was injected (I.D.) in one ear of rabbits (groups of five animals). Immediately after venom injection, 1 mL of the tested serum was administered via the marginal ear vein of the opposite ear. (A) Macroscopic aspect of the area injected with venom after the indicated time courses. C-: C serum. Rec: REC serum. Mix: MIX serum. Av: AV serum. Saline: Inoculation of saline instead of venom. Photographs show a representative response of the animals. Black dots marked in the skin surround the local of injection. (B) Skin necrosis was checked after various time courses (X axis) after injections of 1 MND followed by inoculation of the tested sera. The intensity of necrosis (Y axis) were classified from 1 to 4 according to the intensity, in which “1” represents a less intense reaction and “4” indicates a more intense sign (adapted from [43]). Each point represents the average of intensities of each group measured at the indicated times. Antivenom: AV serum. Recombinant proteins: REC serum; Mix: MIX serum. C-: C serum. Results of the groups AV (ANOVA, $p = 0.0005$) and REC (ANOVA, $p = 0.0017$) were statistically different from C at 72 h. No significant differences were found when MIX group was compared to C and neither among AV, REC and MIX at 72 h.

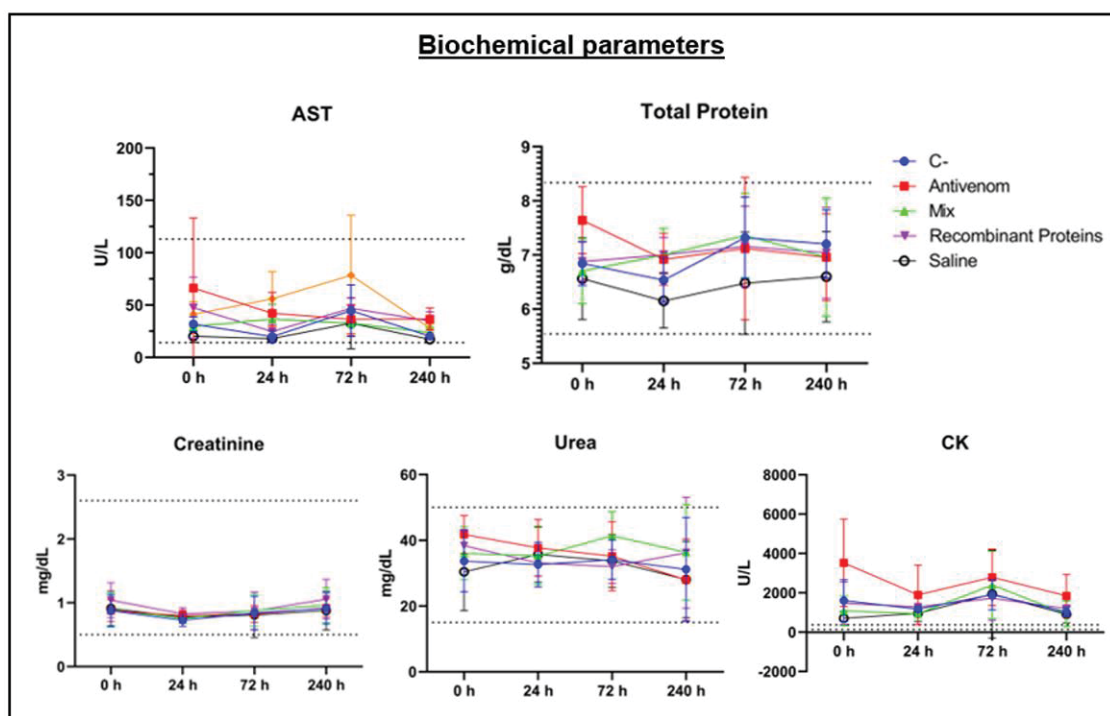
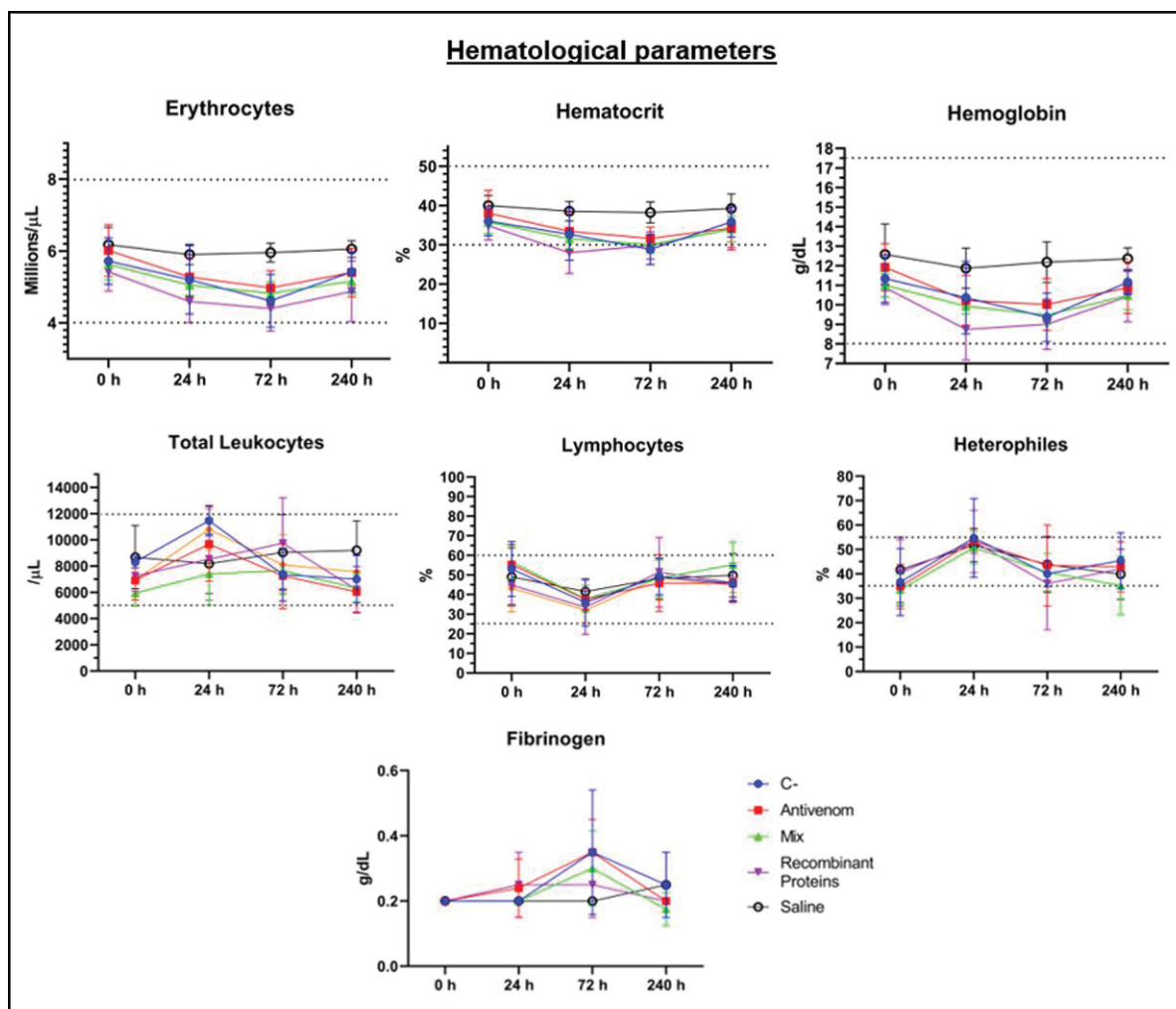


Figure 8: Monitoring of blood parameters of rabbits submitted to the neutralization test in the external ear. Hematological (A) and biochemical (B) parameters were verified from bloods samples collected in different time courses (X axis). C-: C serum. Antivenom: AV serum. Mix: MIX serum. Rec: REC serum. Saline: Inoculation of saline instead of venom. AST: aspartate aminotransferase. CK: muscular creatine kinase. Lymphocytes: relative lymphocyte count. Heterophiles: relative heterophile count. For all parameters analyzed, no significant differences were found among the control groups (C and Saline) and AV, REC and MIX groups.

4. Discussion

The current approach based on serum to treat victims bitten by spiders of the genus *Loxosceles* is controversial from the efficacy standpoint, since it has been reported that the serum was not able to prevent the development of signs of the loxoscelism. Also, the current serum therapy is often target of criticism due to technical aspects related to the production, which involves animal suffering used to raise the serum, the risk brought to personnel during the extraction of venom to be used in the immunization and the inconvenient process of maintaining many spiders at the laboratory to extract this venom. The new approach based on mutated recombinant PLDs herein presented is promising since it overcomes all the issues mentioned above. SDS-PAGE analysis of the whole venom of *Loxosceles* spiders indicates the presence of two major groups of toxins in mass in approximately equivalent amounts [17,45]. These two groups are related to the PLDs (31–35 kDa) and the insecticide peptides of the knottin family (6–10 kDa). The knottin peptides are innocuous to the victims, since they are specific to insects that are predated by the spiders in the natural environment. Thus, when whole venom is used to raise the therapeutic serum, the immune system of the victims directs part of its machinery to elicit antibodies against molecules that are not detrimental. This may account to the low efficacy of the current serum therapy. The serum that this work studies has the advantage of being focused on the PLDs, which are the class of toxins that are responsible for the major signs triggered by the envenoming. This approach may be clinically more effective since the antibodies raised were all directed against the PLDs. Furthermore, because the mutated PLDs are engineered to stimulate the production of neutralizing antibodies but to not induce harm in the immunized animal, the problem regarding the injuries in those animals is solved. Finally, since the mutated PLDs are produced by means of molecular biology techniques, there will be no need to collect and keep spiders as well as no need to perform hazardous venom extraction.

The techniques for producing mutated and wild-type recombinant proteins (LgRecD32A-E34A, LiRecW230A, LiRecH12A-H47A and LiRecDT1) analyzed in this work have been successfully used by several authors over the last few years [20,29,31,32]. Native amino acids were replaced by a residue of alanine (D32A-E34A, W230A e H12A-H47A), a non-polar amino acid capable of influencing the functionality of wild-type proteins. The residue of alanine incorporated does not alter PLDs' native conformation, which was proved by circular dichroism and docking analysis [29,31,32].

Through *in vitro* analysis, it was observed that the wild-type recombinant PLD (LiRecDT1) degraded sphingomyelin, while the mutated toxins did not show any significant activity. Likewise, the *in vivo* assay showed that LiRecDT1 caused extensive dermonecrosis on rabbit skin, whereas the mutated proteins did not lead to the development of lesions, except for LiRecDTH12A-H47A, which caused a slight residual inflammatory reaction close to 24 h after treatment injection. Such results were previously reproduced by several authors [20,29,31,32] and indicate the success on the amino acid replacements in the mutated proteins, preventing their biochemical and functional activities.

With the evidence that the mutated proteins do not have biochemical and functional activities, but have their structural characteristics preserved, different groups of rabbits were immunized in order to produce sera to be evaluated. Studies available on the literature that aimed at the clinical follow-up of animals producing antivenom serum are scarce

[46–48]. Therefore, a greater knowledge of this subject becomes essential for the improvement of the processes. Thus, in this work, we produced two hyperimmune sera using recombinant PLDs D from *Loxosceles* spider venoms, which were evaluated for their efficiency, as well as their antigens toxicity during the protocols for obtaining the sera. From the results herein generated, it is believed that the greater intensities of erythema and ecchymosis during the immunization protocols using recombinant mutated proteins alone may be directly related to the large mass of antigens applied to the animals, plus the adverse effects of adjuvant [49]. On the other hand, the signs of erythema, ecchymosis, and the increase in serum fibrinogen after the first immunization observed in the animals that received crude venom or a mixture of crude venom and mutated PLDs (AV and MIX) can be explained by the action of the crude *Loxosceles* venom toxins administered to these animals, in addition to the adjuvant effects [34,50].

The hematocrit, erythrocyte count and hemoglobin concentration in the sample of the tested animals were not altered, suggesting that there was no hemolysis caused by the toxins in the conditions used [51]. This finding is probably related to the low concentration of molecules that was gradually inoculated into the animals, not inducing systemic changes [25,52]. The differences between the lymphocytes and heterophils curves observed throughout the immunization protocols are coherent, considering the cellular dynamics of the immune response and the inflammatory profile triggered by some of the toxins present in the venoms [53].

In the analysis of serum protein concentrations, there was a significant difference between the MIX treatment and the negative control, which despite receiving only adjuvants in the composition of the immunogens, had a higher serum protein concentration. This fact may be related to the pro-inflammatory effects triggered by adjuvants and to an eventual greater susceptibility of individuals in this group to the adjuvant side effects [49].

Liver function was verified by measuring the concentration of the enzyme aspartate aminotransferase (AST), since the enzyme alanine aminotransferase (ALT), also commonly analyzed in other animals, has little tissue specificity in rabbits [54]. Despite the hepatotoxic effect of *Loxosceles* venom reported by CHRISTOFF [55], all groups had enzyme concentrations within the reference values for the animals tested [56], not indicating a liver damage resulting from immunization processes under the conditions used.

Values above the reference levels for creatine kinase (CK) were found in all analyzed points, indicating lesions in skeletal muscle resulting from immunizations. This fact was also observed in immunizations in other species [46,57] and is directly correlated with the adverse effects of the use of oily adjuvants. These adjuvants can cause local reactions, derived from the damage to cells, and even granulomas at the site of inoculation or which arise from animal manipulations [49,58].

Based on the concentrations of urea and creatinine found and considering that the concentration of crude venom applied to the animals was small, it can be stated that the renal function was not affected during the immunization processes, although the nephrotoxic capacity of Brown spider venoms has already been demonstrated both experimentally [20] and clinically from injured patients [10].

There are reports in the literature indicating that the application of large doses of antigens can lead to a greater production of antibodies, showing a possible relationship between the two variables [59–61]. However, this premise cannot be taken as a rule. In the ELISA graph (Figure 4A), it is observed that the sera obtained from MIX and AV groups presented similar curves, although the concentration of venom of the three species of Brown spiders injected in the animals belonging to the MIX group was up to six times lower than that used for the AV group. On the other hand, the serum obtained by using only recombinant proteins showed a significant difference when compared to the others. In that case, the animals received 2.5 µg more of mutated recombinant proteins than the Mix group. This probably increased the stimulus for clonal expansion of B lymphocytes

against the antigens [53], since they are in greater concentration and have smaller heterogeneity when compared to the MIX group, which has three venoms and three mutated proteins.

In the western blotting assays, it was verified that sera obtained from MIX and REC groups presented more intense reactions on the 25–35 kDa region. This fact may be related to the immunizations since these groups received mutated phospholipases on a large concentration, causing the production of antibodies to be greater than that of the AV group, whose animals received small concentrations of phospholipases of the three species of Brown spiders [59–61]. The recognition of bands with low molecular weight is probably due to the presence of degraded phospholipases in the venom analyzed. Serum cross-reactivity, as observed in this trial, has been described by several authors, using different anti-*Loxosceles* sera and venoms [15,37,62]. This characteristic is essential and desirable for the development of a product with great capability of neutralization and recognition of venoms from a large number of species.

In the *in vivo* incubation test for serums and toxins, after 72 h of inoculation of the mixture, the sera produced from immunizations with the REC treatment had a superior capability to reduce edema and dermonecrotic lesions (except for the group MIX when 1 MND of venom was used), when compared to serum raised from the AV preparation. The residual reactions observed are due to the action of non-neutralized toxins, possible other than PLDs. In the neutralization assay similar to that done here, Leal [63] verified that individual sera obtained using recombinant phospholipases D (recLoxtox s1A) or (recLoxtox s11A), and the mixture of both sera, significantly reduced the appearance of dermonecrotic lesions in rabbits exposed to the venom, suggesting, as well as what was seen in the results described here that the presence of specific antibodies against dermonecrotic toxins (PLDs) are essential and play a role on the positive evolution of the clinical picture of Loxoscelism. All sera produced in this work were efficient in reducing the swollen areas after venom exposure. As reported by Leal [63] that obtained a significant reduction in the diameter of edema formed after inoculation of crude venom, the same data was acquired herein from animals immunized with the recombinant PLDs. These results suggest that specific antibodies for neutralization of certain PLDs epitopes can contribute to the reduction of edema formation.

Recombinant proteins previously described, such as LgRec1, LgRecDT1 and *Loxosceles gaucho* crude venom itself, have the capacity to form ecchymosis in rabbits [31,64,65]. Thus, the neutralization of this clinical sign, especially for the groups that received 1MND of venom, was consistent with the hypothesis of reduced disruption of the vessels' wall through the neutralization of venom toxins by antibodies from this second-generation serum using recombinant PLDs.

Erythema is also a common sign observed in experimental or clinical Loxoscelism [31,64]. However, on the work described here, none of the sera produced were efficient in preventing the erythema followed venom exposure. Knowing that the signs observed in Loxoscelism are due to the synergism action of the components of the venoms [63], it is suggested that other toxins such as metalloproteases and hyaluronidases act as spreading factors [66], as well as pro-inflammatory agents that may be involved with the formation of erythema and ecchymosis.

Currently, during the production of commercial anti-loxoscelic serum, the plasma of immunized horses is tested through neutralizing assays performed in the ear of rabbits in order to determine which horses produced a minimum amount of antibodies capable of neutralizing the action of the venom. The same protocol is used in evaluating the final product. The test consists of an intradermal application of one MND of *Loxosceles intermedia* crude venom in the inner portion of the ear of rabbits. In the opposite ear, the plasma or serum is injected intravenously. The evaluation of the effectiveness of the serum takes place 72 h after its application (Official Protocol for Anti-*Loxosceles* serum production, CPPI, Brazil, 2014, 2017). This test was also carried out with sera produced in this work to assess the quality of the sera produced (quality control step). There was no difference

when the produced sera were compared 72 h after the inoculation of the preparations. The reduction of lesions was less significant when compared to the incubation assay performed. It is believed that this is due to at least one of the following factors: i) in the incubation assay, longer exposure times of antibodies and antigens allow a better recognition of epitopes and a greater number of molecular bindings. In the ear serum neutralization test, the sera were administered in the opposite ear that received the venom, causing the molecules to travel a long way through blood vessels until they found the venom antigens; ii) mechanism of action of the venom toxins. Although the clinical signs of *Loxoscelism* appear a few hours after the bite, the venom when in contact with its substrates acts promptly, and it is expected that the activation of leukocytes occurs within 4 hours [34,67]. This fact can be commonly observed in the quality control assays of the serum currently produced. The hypothesis that justifies the lower efficacy of the serum obtained for the group MIX in this trial, derives from the data reported by Figueiredo [44] who proposed that genetic and individual conditions of each animal used to produce antibodies can generate different sera since they do not come from isogenic strains. Such events are also observed on the production of commercial serum, which can show differences regarding the potency for each batch due to variations on the production of antibodies from the same animal over a period.

The anti-*Loxosceles* serum, if injected on the first six hours after the accidents can reduce up to 90% of the dermonecrotic lesion [34]. It is believed that this difference with the sera produced on this project is due to the fact that the processed serum tested by Pauli [34] has a very high concentration of antibodies, being able to neutralize at least 15 times the MND. In addition, anti-*Loxosceles* serum-producing horses are immunized more frequently over the course of a year (about four immunizations cycles, whereas the animals tested here have gone through only one), which may influence a greater production of antibodies.

Considering that 1MND of *L. intermedia* venom is not capable of causing serious alterations in rabbits as evidenced by the negative control group, any possible alteration in the test results would indicate serum toxicity. The animals that received the sera did not show non-standard clinical indices for the species [56], except for the enzyme creatine kinase (CK), used as a marker for muscle damage. It is considered that high concentrations of CK may be related to the handling of animals, resulting stress and injuries caused by containment, application of antigens, application of serums and blood collections.

As final conclusions, after the experimental protocol carried out in this work, we can suggest that sera produced from immunizations with mutated recombinant proteins without biological activity proved to be as efficient as the traditional anti-*Loxosceles* serum currently used, obtained from immunizations with the crude venoms of the three species of Brown spiders of major medical importance in Brazil and South America *L. gaucha*, *L. intermedia* e *L. laeta*. The results observed and gathered herein indicated no significant differences between the serum based on mutated PLDs and the serum obtained using crude venom regarding the development of dermonecrotic lesions and edema in the immunized animals. The results presented show potential use of these antigens to produce a second-generation of anti-*Loxosceles* serum, also because the use of mutated PLDs minimize the need for capture and maintenance of specimens of *Loxosceles spp ex situ* for the venom extraction step, which is characterized by being laborious, low yield, requiring thousands of spiders to complete the equine immunization cycles, and finally, the danger of spider bites during manipulation. This step could be replaced completely or partially by the production of recombinant proteins at the laboratory.

5. Conclusions

The results presented here, although suggestive and technically robust, need additional evidence. Other important data should be reported in future studies aiming a change in the current production model to obtain anti-*Loxosceles* serum, such as the veri-

fication of the minimum concentration of antigens capable of stimulating an adequate immune response (cost-benefit), evaluation of immunization in horses instead of rabbits and the assessment of suitable hyperimmune plasma processing methodologies. Nevertheless, the data presented here highlight that mutated recombinant PLDs are promising tools to produce anti-*Loxosceles* serum with higher efficacy and other operational advantages over the traditional approach.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Federal University of Paraná (UFPR) (protocol code 1238; date of approval: 12/11/2018).

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the amount of data.

Conflicts of Interest: The authors declare no conflict of interest.

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6. DISCUSSÃO

As seguintes mutações das fosfolipases-D das três espécies de aranhas-marrons foram obtidas: D32A-E34A de *L. gaucho* (LgRecD32A-E34A), W230A de *L. intermedia* (LiRecW230A) e H12A-H47A de *L. laeta* (LiRecH12A-H47A). Igualmente, foi obtida a toxina recombinante selvagens de *L. intermedia* LiRecDT1, a fim de servir como controle positivo em alguns experimentos. As técnicas para obtenção de proteínas recombinantes, em especial as citadas, vêm sendo utilizadas com sucesso por diversos autores ao longo dos últimos anos (POLLI *et al.*, 2021; DA SILVA *et al.*, 2021; VUITIKA *et al.*, 2016; CHAIM *et al.*, 2011, 2006).

Os aminoácidos alterados nas mutações identificam a enzima alterada (D32A-E34A, W230A e H12A-H47A), os quais foram substituídos pela alanina, um aminoácido apolar e normalmente utilizado para analisar a função de determinados resíduos de aminoácidos de proteínas de interesse. Esta substituição altera a funcionalidade das proteínas originais, sem alterar sua conformação geral (CHAIM *et al.*, 2011), o que é essencial do ponto de vista do seu reconhecimento pelo organismo receptor e produção de anticorpos.

A constatação de presença ou ausência de atividade fosfolipásica das isoformas selvagem de fosfolipase-D, bem como das toxinas com mutações sítio-dirigidas foi verificada a partir da realização de ensaios de atividade fosfolipásica *in vitro* (kit Amplex™ Red - Thermo Fisher Scientific, Waltham, USA) e atividade dermonecrótica *in vivo*.

A toxina selvagem recombinante de *L. intermedia* (LiRecDT1) apresentou alta emissão de fluorescência, o que indica a degradação de esfingomiélin pela referida enzima, tendo uma atividade significativa quando comparada com o controle negativo.

Por outro lado, as toxinas com mutações sítio-dirigidas LgRecD32A-E34A, LiRecW230A e LiRecH12A-H47A não apresentaram atividade significativa quando comparadas com o controle negativo, o que indica que as alterações nos aminoácidos foram bem-sucedidas no sentido de evitar a sua atividade bioquímica.

No ensaio de atividade dermonecrótica, os animais que receberam o controle positivo LiRecDT1 desenvolveram lesões dermonecróticas extensas, conforme já demonstrado por CHAIM *et al.* (2006), apresentando sinais clínicos a partir de três

horas pós-inoculação da toxina, com grande evolução até as 24 horas do experimento, onde verifica-se, inclusive, que a lesão se estendeu até a região abdominal.

Já as toxinas com mutações sítio-dirigidas não provocaram lesões dermonecroticas, edema, eritema ou equimose nos animais que as receberam, apresentando aspecto macroscópico similar ao do controle negativo. Tais resultados corroboram com os achados de VUITIKA *et al.* (2016), em que a inatividade ou atividade residual de algumas mutações de LiRecDT1 foram demonstradas *in vivo* e *in vitro*.

Com as evidências de que as proteínas mutadas não apresentam atividade biológica, procedeu-se à imunização de diferentes grupos de coelhos a fim de produzir os soros antiveneno e antitoxinas recombinantes a serem avaliados.

As imunizações foram analisadas quanto a presença de edemas, eritemas, equimoses, necroses e temperatura dos animais ao longo dos sete dias subsequentes às aplicações e quanto a parâmetros hematimétricos (hemograma completo com fibrinogênio, aspartato amino transferase - AST, creatinoquinase - CK, creatinina e ureia).

Todos os grupos apresentaram edemas, eritemas e equimoses em diferentes intensidades, com regressão progressiva, e as temperaturas retais permaneceram dentro dos limites para a espécie. Os achados clínicos citados (formação de edemas, eritemas, equimoses e acompanhamento da temperatura) são compatíveis ao já demonstrado pela literatura, em que relatos do desenvolvimento de reação inflamatória decorrente da utilização de adjuvantes, especialmente os oleosos, como é o caso do Adjuvante de Freund, são citados (DEEB *et al.*, 1992). Acredita-se que as maiores intensidades de eritemas e equimoses apresentadas pelo grupo Proteínas Recombinantes (*Recombinant Proteins*, conforme artigo de ANTUNES *et al.*, 2022) possam estar diretamente relacionado à grande massa de antígenos aplicados nos animais deste grupo, que chegou a 800 µg na quarta imunização. Além disso, os próprios efeitos adversos da utilização dos adjuvantes também contribuem para o aparecimento de sinais diretamente relacionados à inflamação. Contudo, deve-se ponderar que parte destes sinais também podem ter como cofatores a própria ação do veneno loxoscélico total presente nos grupos *Pool* de Veneno (*Venom Pool*) e *Mix*, conforme artigo de ANTUNES *et al.*, 2022), já que é conhecida a sua ação inflamatória (LAUSTSEN *et al.*, 2016), mesmo que este esteja sendo liberado em pequenas

quantidades e de maneira gradativa no organismo devido à ação do adjuvante, prevenindo, assim, o rápido catabolismo do antígeno (HARLOW E LANE, 1988).

Amostras de sangue de todos os animais foram colhidas imediatamente antes de cada imunização, para a quantificação e acompanhamento de índices hematimétricos (hemograma completo com fibrinogênio, aspartato amino transferase - AST, creatinoquinase - CK, creatinina e ureia) a fim de detectar eventuais alterações sistêmicas decorrentes dos processos de imunização.

A permanência dos valores de hematócrito, contagem de eritrócitos e concentração de hemoglobina dentro dos limites estabelecidos para a espécie durante o processo de imunização sugerem que a característica hemolítica dos venenos de aranhas-marrons (DE SOUZA *et al.*, 2008), que eventualmente buscou-se observar nos grupos que receberam solução de veneno destes animais (grupos *Pool* de Veneno e *Mix*), não se mostraram presentes possivelmente pela abordagem de imunização realizada com a utilização de Adjuvante de Freund, em que uma concentração relativamente baixa de moléculas capazes de provocar este efeito (CHAVES-MOREIRA *et al.*, 2009) foram gradualmente apresentadas ao sistema imune dos animais (COX E COULTER, 1997), não sendo capazes de provocarem alterações sistêmicas detectáveis nos exames.

Estudos disponíveis na literatura que visam o acompanhamento clínico de animais produtores de soros antiveneno são escassos. Em um destes trabalhos, PARRA *et al.* (2009) demonstraram que equinos submetidos à imunização com veneno crotálico apresentavam queda nas contagens de eritrócitos e de hemoglobina, porém, segundo valores de referência determinados por GARCIA-NAVARRO (2005), mantiveram-se dentro dos limites da espécie. De maneira similar, ÂNGULO *et al.* (1997) verificaram queda nos valores de hematócrito durante a imunização de cavalos destinados à produção de soro antiveneno polivalente (contra os venenos de *Bothrops asper*, *Crotalus durissus durissus* e *Lachesis muta stenophrys*) com valores abaixo dos níveis aceitos para a espécie (GARCIA-NAVARRO, 2005) nas duas últimas imunizações.

Outro ponto observado nas imunizações foi o aumento de fibrinogênio sérico nos animais dos grupos Antiveneno Total e Mix após a 1ª imunização. Tal fato pode ser explicado devido à presença de solução de veneno de aranhas do gênero *Loxosceles spp.* na emulsão aplicada nos animais destes grupos. A ação das enzimas ativas do veneno, ainda que em baixa concentração, provoca reação

inflamatória, havendo a produção de citocinas, como as interleucinas IL-1 e IL-6, que podem estimular a produção de fibrinogênio (PAULI *et al.*, 2009).

Sabe-se que animais submetidos ao envenenamento por *L. intermedia* e *L. gaucho* apresentam queda abrupta na contagem de plaquetas nas primeiras 24 horas de ação do veneno (TAVARES *et al.*, 2004; DA SILVA *et al.*, 2003), com os valores retornando próximos aos obtidos antes da inoculação por volta de 10 dias após a exposição (PAULI *et al.*, 2009), o que sugere a relativa proximidade entre os valores obtidos neste ensaio no momento da 1ª imunização e na 2ª nos grupos Antiveneno Total, Mix e Proteínas Recombinantes, onde há um intervalo de colheita de amostras de cerca de três semanas (HARLOW E LANE, 1988). Contudo, o grupo Controle Negativo apresentou queda na contagem de plaquetas entre a 2ª e 4ª imunizações, porém os valores mantiveram-se muito próximos à normalidade, não sugerindo alterações fisiológicas significativas e retornando a valores normais no momento da colheita de sangue.

Em todos os grupos observou-se uma queda na contagem de leucócitos totais no momento da colheita de sangue final. De acordo com GARCIA-NAVARRO (2005), a dinâmica de leucopenia pode estar diretamente relacionada com a diminuição do número de linfócitos e heterófilos, contudo, esta queda não foi observada em todos os grupos.

A diminuição na contagem de linfócitos observada após a primeira imunização (exceto no grupo Controle Negativo) contrasta com o apresentado por Waghmare *et al.* (2009), em que equinos imunizados com diferentes venenos de serpentes exibiram leucocitose e linfocitose após as inoculações.

Os contrastes entre as curvas de linfócitos e heterófilos, em que no mesmo ponto analisado, uma apresenta valores maiores e o outro menores, eram esperados, uma vez que se tratam de contagens relativas. Sabe-se que linfocitoses podem ocorrer naturalmente após vacinações ou imunizações (GARCIA-NAVARRO, 2005), porém os aspectos individuais dos animais pertencentes a este grupo, bem como a sua espécie, o veneno utilizado e a cinética de exposição ao veneno também devem ser considerados, como fatores para eventual maior reatividade aos componentes do produto inoculado.

A elevação na contagem de heterófilos observada após a primeira imunização também foi constatada por DEEB *et al.* (1992) em que coelhos imunizados com

diferentes adjuvantes exibiram aumento na contagem de heterófilos no dia seguinte à aplicação.

Na análise das proteínas totais, não foram observadas diferenças significativas entre os valores das imunizações e os valores pré-ímmes dentro de um mesmo grupo, assim como constatado por WAGHMARE *et al.* em 2009. Contudo, na análise entre grupos, verificou-se diferença significativa entre o grupo Mix e o grupo Controle Negativo. Este último, apesar de receber apenas os adjuvantes na composição do imunógeno, apresentou maior concentração média de proteína sérica. Supõe-se que o aumento na concentração de proteína sérica neste grupo possa estar relacionado aos efeitos pró-inflamatórios desencadeados pelos adjuvantes (DEEB *et al.*, 1992) e por uma eventual maior susceptibilidade dos indivíduos deste grupo aos efeitos do produto utilizado.

A função hepática pode ser verificada em diversos animais, inclusive no ser humano, através da mensuração das enzimas Alanina Aminotransferase (ALT) e Aspartato Aminotransferase (AST). Contudo, a enzima alanina aminotransferase possui pouca especificidade tecidual em coelhos (MEREDITH e RAYMENT, 2000), não sendo, portanto, a mais indicada para a análise do funcionamento do fígado. Conforme observado nos resultados, todos os grupos apresentaram concentrações enzimáticas dentro dos valores de referência para a espécie (CARPENTER, 2018), não indicando, desta maneira, qualquer tipo de lesão hepática decorrente dos processos de imunização, revelando-se um processo seguro para o órgão, apesar do efeito hepatotóxico do veneno de aranha-marrom (CHRISTOFF *et al.*, 2008). Porém, sugere-se que as diferenças significativas observadas quando o grupo *Pool* de Venenos foi comparado com os grupos Proteínas Recombinantes (*Recombinant Proteins*) e *Mix* possa ser devido justamente à presença de venenos de aranhas-marrons em maior concentração no imunógeno do primeiro grupo.

A creatina quinase pode se apresentar em três formas de isoenzimas, de acordo com a sua localização/distribuição: CK-MM (músculo esquelético), CK-BB (cérebro) e CK-MB (coração). Em coelhos, as principais formas circulantes no sangue periférico são CK-MM e CK-BB (MCLAUGHLIN e FISH, 1994). Desta maneira, esta enzima é rotineiramente utilizada como indicador de lesões musculares. Os valores acima dos níveis de referência para a espécie em todos os pontos analisados indicando, portanto, lesões em músculo esquelético decorrentes das imunizações. Níveis elevados de CK também foram observados por PADULA *et*

al. (2016) na imunização de alpacas e por ÂNGULO *et al.* (1997) em equinos. Tal fato, correlaciona-se diretamente com os achados clínicos apresentados em outros estudos que demonstraram os efeitos adversos da utilização de adjuvantes oleosos, tais como o Adjuvante de Freund, capaz de provocar reações locais, com danos às células musculares, e até mesmo granulomas nos pontos de inoculação (BENNETT *et al.*, 1992; DEEB *et al.*, 1992).

A fim de analisar a função renal dos animais imunizados, as concentrações de ureia e creatinina foram mensuradas. Os exames bioquímicos mostraram níveis de creatinina dentro dos limites para a espécie. De maneira similar, WAGHMARE *et al.* (2014) não observaram alterações relevantes nos níveis de creatinina sérica em equinos imunizados com diferentes adjuvantes durante a produção de soro antiofídico polivalente.

Já a concentração de ureia ficou pouco acima dos limites nos grupos Controle Negativo, *Pool* de Venenos e Proteínas Recombinantes.

Tomando por base as concentrações de ureia e creatinina verificadas durante as imunizações em coelhos elevando em consideração que a concentração de veneno ativo de aranhas do gênero *Loxosceles* aplicada nos animais é pequena, pode-se afirmar que, neste ensaio, a função renal não foi afetada em grau significativo durante os processos de imunização, muito embora a capacidade nefrotóxica dos venenos de aranhas-marrons já tenha sido demonstrada tanto no campo experimental (CHAIM *et al.*, 2006), quanto em achados clínicos em pacientes acidentados (NGUYEN e PANDEY, 2019).

A cinética da produção de anticorpos dos animais imunizados foi analisada *in vitro* por meio da técnica de ELISA. Todos os grupos (exceto o Controle Negativo) apresentaram aumento na produção de anticorpos após a segunda imunização, com aumento da curva até a quarta imunização e tendência de estabilização no momento da colheita de sangue final, tanto nos grupos que receberam grandes massas de proteínas mutadas (grupos Proteínas Recombinantes e Mix), quanto o grupo *Pool* de Venenos, que recebeu doses baixas de solução de veneno loxoscélico. Há relatos na literatura indicando que a aplicação de grandes massas/doses de antígenos no organismo pode levar a uma maior produção de anticorpos e consequente proteção, evidenciando uma eventual relação entre as duas variáveis (ROMSTAD *et al.*, 2013; KATARE *et al.*, 2005; PAY *et al.*, 1987). Contudo, não se pode tomar esta premissa como regra. Analisando as curvas de absorbância do teste de ELISA durante o

processo de imunização visando a produção dos soros em estudo neste trabalho, observamos que os grupos *Mix* e *Pool* de Venenos apresentaram curvas muito similares, sem diferença significativa, ainda que nas imunizações do Grupo *Mix* a massa aplicada de veneno total das três espécies de aranhas-marrons tenha sido até seis vezes inferior ao do Grupo *Pool* de Venenos na 4ª imunização. Por outro lado, a curva relativa ao grupo Proteínas Recombinantes apresentou diferença significativa frente aos outros grupos, com absorbâncias médias maiores, em especial na 4ª imunização e sangria de produção. Neste caso, em que os animais imunizados receberam 2,5 µg a mais de proteínas recombinantes mutadas que o Grupo *Mix*, a hipótese para explicar uma maior produção de anticorpos seria o estímulo da expansão clonal de linfócitos B frente aos antígenos apresentados (ABBAS, 2011), que apesar de estarem em maior massa, possuem uma heterogeneidade menor quando comparados ao imunógeno do Grupo *Mix*, que possui três venenos e três mutações de proteínas recombinantes.

Ensaio de ELISA vêm sendo amplamente utilizados para atestar interações entre proteínas e anticorpos produzidos por grupos de pesquisa a partir do reconhecimento de epítomos conformacionais e não desnaturados (KARIM-SILVA *et al.*, 2020; COSTA *et al.*, 2020; MEDINA-SANTOS *et al.*, 2019). DA SILVA e colaboradores (2021), por exemplo, produziram sete proteínas mutantes recombinantes de *L. gaucho* e *L. laeta*, dentre elas a LgRecD32A-E34A e LIRecH12A-H47A - utilizadas neste trabalho para a produção dos soros – e verificou-se a existência de reatividade cruzada em ensaio de ELISA, ou seja: soro antiveneno de *L. gaucho* reconheceu as proteínas recombinantes mutadas de *L. laeta*. De maneira similar, as proteínas mutantes de *L. gaucho* foram reconhecidas pelo soro antiveneno de *L. laeta*. Tal fato evidencia a conservação de epítomos entre espécies e mutações, sendo um indicativo robusto para a utilização destas proteínas no desenvolvimento de um soro de segunda geração.

A fim de verificar a capacidade de reconhecimento de epítomos lineares ou resistentes à desnaturação, foram realizados ensaios de Western Blotting com os diferentes soros produzidos frente os venenos de *L. intermedia*, *L. laeta* e *L. gaucho*. Todos os soros (exceto o Controle Negativo) foram capazes de reconhecer todos os venenos, especialmente proteínas na faixa de 25 kDa a 35 kDa, sendo que o Soro Antiveneno (obtido a partir das imunizações do grupo *Pool* de Veneno) apresentou bandas menos intensas quando comparado com os soros *Mix* e Proteínas

Recombinantes. Tal fato pode estar relacionado diretamente às imunizações, nas quais nestes últimos dois grupos citados, foram utilizadas fosfolipases-D recombinantes mutadas em grandes massas, fazendo com que a produção de anticorpos frente às fosfolipases, que se encontram justamente nesta faixa de massa molecular, fosse maior que a do soro Antiveneno, cujos animais receberam pequenas massas do veneno das três espécies de aranhas-marrons (ROMSTAD *et al.*, 2013; KATARE *et al.*, 2005; PAY *et al.*, 1987). Como também foi observado nos ensaios de ELISA, constatou-se reatividade cruzada dos soros frente os venenos loxoscélicos. Este efeito vem sendo descrito ao longo dos anos por diversos autores, seja na utilização dos soros atualmente aplicados no tratamento de acidentes loxoscélicos frente a diferentes venenos de aranhas do gênero *Loxosceles*, bem como de soros produzidos a partir de imunizações com proteínas recombinantes e quimeras de toxinas específicas do veneno (DA JUSTA *et al.*, 2020; CALABRIA *et al.*, 2019; MEDINA-SANTOS *et al.*, 2018; BARBARO *et al.*, 2005). Esta avaliação torna-se essencial, em especial no estudo de toxinas recombinantes com potencial para utilização em imunizações visando a produção de soro, uma vez que se torna desejável um produto com grande capacidade de neutralização e de reconhecimento dos venenos do maior número de espécies possível.

No ensaio *in vivo* de incubação, os soros foram testados frente a 1 DMN e 2 DMN de veneno de *L. intermedia*. Em ambos os casos, os soros produzidos a partir de imunizações com proteínas recombinantes mutadas foram capazes de diminuir os diâmetros das lesões dermonecroticas e edemas em níveis superiores aos obtidos pelo soro Antiveneno (exceto o Soro Mix frente a 1 DMN de veneno) no tempo 72 horas pós inoculação. Optou-se por realizar a análise neste período de tempo, pois é o tradicionalmente utilizado em ensaios de controle de qualidade na produção do Soro Antiloxoscélico. As reações observadas são decorrentes da ação das toxinas não neutralizadas. A análise da capacidade de anticorpos em neutralizarem os efeitos tóxicos de proteínas recombinantes de enzimas da família das fosfolipases de aranhas-marrons vem sendo amplamente estudada ao longo dos anos. Leal e colaboradores (2020), por exemplo, produziram, a partir de imunizações em coelhos, anticorpos contra duas isoformas de toxinas fosfolipásicas do veneno de *L. similis* (recLoxtox s1A e recLoxtox s11A). Em ensaio de incubação similar ao apresentado neste trabalho, os autores verificaram que os soros anti-recLoxtox s1A e a combinação anti-recLoxtox s1A/s11A reduziram de maneira significativa o

aparecimento das lesões dermonecroticas nas 48 horas observadas, sugerindo, assim como nos resultados aqui apresentados, que a presença de anticorpos específicos contra toxinas dermonecroticas são primordiais e possuem protagonismo na evolução positiva dos quadros clínicos de loxoscelismo. Recentemente, Calabria e colaboradores (2019) avaliaram os efeitos *in vivo* em coelhos após a incubação de venenos loxoscélicos com anticorpos produzidos frente à uma proteína recombinante híbrida, que combinava porções de fosfolipases-D e metaloproteases tipo astacina, denominada LgRec1ALP1. Os anticorpos produzidos foram capazes de prevenir a formação de dermonecrose quando exposto ao veneno de *L. gaucho* e a diminuir em 79% e 68% o tamanho das lesões dermonecroticas, quando incubado com os venenos de *L. intermedia* e *L. laeta*, respectivamente. A maior eficácia deste soro pode estar relacionada à presença de maiores concentrações de anticorpos frente às metaloproteases, uma vez que também desempenham importante papel no envenenamento e considerando que são o segundo maior grupo identificado em transcritos de *L. gaucho*, espécie utilizada como modelo para obtenção das proteínas híbridas (CALABRIA *et al.*, 2019). Já Lima *et al.* (2018), produziram proteínas quiméricas a partir da combinação de epítomos lineares de algumas das principais toxinas do veneno loxoscélicos (LALP-1, LiHYAL e SMase D) com proteína quimérica composta por epítomos lineares e conformacionais previamente produzidos (rCpLi), gerando uma nova proteína recombinante (rMEPLox). Anticorpos produzidos em coelhos contra esta proteína apresentaram reatividade em ensaios de ELISA e *immunoblot* frente a diversos venenos de aranhas do gênero *Loxosceles*. Estudos *in vitro* e *in vitro* também demonstraram que os anticorpos obtidos foram capazes de neutralizar as atividades esfingomielinásicas, hialuronidásica e metaloproteásicas do veneno de *L. intermedia*. A avaliação dos efeitos da exposição dos venenos loxoscélicos após incubação com soros antiproteínas recombinantes não é realizada apenas em coelhos e não visa unicamente verificar o potencial de proteção contra a formação de dermonecroses. Em 2003, Araújo *et al.* verificaram que o soro anti-Li-rec poderia neutralizar até 2,5 DL50 de veneno de *L. intermedia* por mL de soro em camundongos. Tal dado já apresentava o potencial de utilização de proteínas recombinantes na produção de soros no tratamento de acidentes, uma vez que davam indicativos quanto à segurança e eficiência dos anticorpos.

Todos os soros foram eficientes na diminuição das áreas edemaciadas, tanto frente a 1 DMN quanto a 2 DMN de veneno loxoscélico. Outros autores observaram

que a atividade edematogênica decorrente da ação dos venenos é um fator de difícil neutralização, mesmo quando os soros antiproteínas recombinantes mostravam sucesso na diminuição das lesões necróticas (DUARTE *et al.*, 2015; MENDES *et al.*, 2013). Por outro lado, Leal e colaboradores (2020) obtiveram significativa redução no diâmetro de edemas formados quando utilizados os soros anti-recLoxtox s1A, anti-recLoxtox s11A e anti-recLoxtox s1A/s11A (provenientes de imunizações com fosfolipases recombinantes). Tais resultados sugerem que apesar da patofisiologia da edematogênese no loxoscelismo ainda não estar completamente desvendada, anticorpos específicos para a neutralização de determinados epítomos das fosfolipases podem contribuir para a diminuição da formação de edemas, embora saiba-se que outros componentes do veneno podem contribuir para o seu aparecimento (BARBARO *et al.*, 2010).

Equimoses, um dos sinais clínicos possíveis de serem identificados decorrentes do rompimento de pequenos vasos pela ação do veneno loxoscélico (DA SILVA *et al.*, 2021; TAVARES *et al.*, 2016), também foram mensuradas, bem como a capacidade dos soros em preveni-las ou diminuí-las. Não foram observadas equimoses nas primeiras 72 horas pós-inoculação nos grupos testados com 1 DMN, porém o mesmo nível de sucesso não foi verificado nos grupos que receberam 2 DMN de veneno.

Proteínas recombinantes já descritas previamente, como a LgRec1, apresentaram capacidade de formar equimose (MAGALHÃES *et al.*, 2013) em coelhos, portanto, a neutralização observada especialmente nos grupos que receberam 1 DMN de veneno mostrou-se condizente com a hipótese de redução do rompimento da parede de vasos através da neutralização das toxinas do veneno pelos anticorpos dos soros de segunda geração.

O eritema, assim como a equimose, pode ser um sinal clínico observado no loxoscelismo cutâneo (DA SILVA *et al.*, 2021), apresentando-se como uma área ruborescida decorrente da vasodilatação em processos inflamatórios. Proteínas recombinantes da família das fosfolipases também demonstraram ser capazes de provocar tal sinal clínico, como a LgRec1, LIRecDT1 e LgRecDt1 (DA SILVA *et al.*, 2021; MAGALHÃES *et al.*, 2013). Nenhum dos soros se mostrou eficiente na prevenção do aparecimento de eritemas, não havendo diferenças significativas quando comparados com o Controle Negativo nas duas massas de veneno utilizadas. Barbaro *et al.* (2010) sugeriram que outros componentes do veneno

de *Loxosceles* podem estar envolvidos na formação de edemas. Sabendo que os sinais observados no loxoscelismo são decorrentes da ação sinérgica dos componentes dos venenos (LEAL *et al.*, 2020), a mesma hipótese pode ser levantada no que se refere à formação de eritemas e equimoses.

De maneira a complementar os achados clínicos apresentados até aqui, as análises histopatológicas de peles de coelhos submetidos ao experimento de incubação de 2 DMN de veneno de *L. intermedia* com os diferentes soros produzidos mostraram que alterações esperadas foram observadas, tais como: desorganização de fibras de colágeno e presença de fibrina no tecido conectivo. Estes achados foram identificados em todas as amostras, mesmo quando os soros foram incubados com o veneno. Entretanto, foi possível observar que o acúmulo de células polimorfonucleares foi menos evidente quando os soros Mix e Proteínas Recombinantes foram utilizados, quando comparados com a grande infiltração de células inflamatórias na camada mais inferior da derme dos grupos tratados com soro Antiveneno e Controle Negativo.

Durante a produção do Soro Antiloxoscélico distribuído ao Sistema Único de Saúde –SUS para tratamento de pacientes acidentados por picadas de aranhas do gênero *Loxosceles*, o plasma de equinos imunizados é testado por ensaios de soroneutralização na orelha de coelhos a fim de determinar quais cavalos produziram uma quantidade mínima de anticorpos capazes de neutralizar a ação do veneno que justifique a colheita de seu sangue e posterior processamento industrial. O mesmo protocolo é utilizado na avaliação do produto final, já processado. O ensaio consiste na aplicação intradérmica de 1 DMN de veneno de *L. intermedia* na porção interna da orelha de coelhos. Na orelha oposta aplica-se na Veia Auricular Marginal uma diluição pré-determinada de plasma equino ou soro finalizado, dependendo da etapa de produção (CENTRO DE PRODUÇÃO E PESQUISA DE IMUNOBIOLOGICOS, 2019; CENTRO DE PRODUÇÃO E PESQUISA DE IMUNOBIOLOGICOS, 2017). A avaliação da eficácia dos soros dá-se 72 horas após a sua aplicação. Este ensaio foi realizado com os soros produzidos neste trabalho (Soro Antiveneno, Soro Mix, Soro Proteínas Recombinantes e Soro Controle Negativo) a fim de verificar a capacidade de neutralização de cada um em uma simulação desta etapa de controle de qualidade. Não houve diferença significativa na comparação entre os soros Antiveneno Total, Mix e Proteínas Recombinantes, contudo, a redução das lesões neste ensaio foi menor que a observada no ensaio de

incubação. Acredita-se que a menor eficiência dos soros neste ensaio seja devido aos seguintes fatores: i) exposição dos anticorpos aos antígenos: no ensaio de incubação, anticorpos e antígenos permaneceram na mesma solução durante uma hora. Um tempo maior de exposição facilita o reconhecimento dos epítomos e possibilita um maior número de ligações. Por outro lado, no ensaio de soroneutralização em orelha de coelhos, os soros, apesar de terem sido aplicados imediatamente após a aplicação dos venenos, foram administrados via endovenosa na orelha oposta à que recebeu o veneno. Sendo assim, as moléculas precisam percorrer um grande trajeto por vasos sanguíneos até atingirem o local da aplicação; ii) ação do veneno: embora os sinais clínicos do loxoscelismo apareçam algumas horas após a picada, o veneno, ao entrar em contato com os seus substratos age prontamente (PAULI *et al.*, 2009), levando ao eventual aparecimento de pequenas lesões, ainda que o soro tenha sido administrado imediatamente após a aplicação do veneno. Tal fato pode ser comumente observado nos ensaios de controle de qualidade dos soros produzidos atualmente.

A hipótese para justificar a menor eficiência do Soro Mix neste ensaio segue o proposto por Figueiredo e colaboradores (2014). O autor afirma que fatos similares a este possam estar relacionados com as condições genéticas e individuais de cada animal, uma vez que não provêm de linhagens isogênicas. Tais acontecimentos também são observados na produção de soros comerciais, que podem apresentar diferenças de potências a cada lote, devido às variações na produção de anticorpos do mesmo animal ao longo de um determinado período.

A capacidade de neutralização do Soro Antiloxoscélico utilizado no SUS foi comprovada quando utilizado até 48 horas após a picada, podendo reduzir até 90% da lesão dermonecrotica se aplicado nas primeiras seis horas pós-acidente (PAULI *et al.*, 2009). Acredita-se que tal diferença para com os soros produzidos neste projeto se deva ao fato de que o soro processado industrialmente possui uma concentração de anticorpos muito alta, sendo capaz de neutralizar, no mínimo, 15 vezes a DMN. Além disso, os equinos produtores de soro são imunizados com maior frequência ao longo do período de um ano (passam por cerca de quatro ciclos de imunizações, enquanto os animais testados aqui passaram por apenas um), o que pode influenciar de maneira direta em uma maior produção de anticorpos.

Visando avaliar eventuais efeitos tóxicos causados pelos soros de segunda geração produzidos a partir de imunizações com as proteínas recombinantes mutadas, foram realizadas colheitas de sangue dos animais no Tempo 0

(imediatamente antes da aplicação do veneno e soro), 24 horas, 72 horas e 240 horas pós-aplicação. Para isso, optou-se por aplicar 1 DMN de veneno de *L. intermedia* neste ensaio, visto que, sabidamente, esta massa não é capaz de causar alterações sistêmicas graves em coelhos, conforme evidenciado especialmente pelo grupo Controle Negativo, no qual foi observado apenas o aparecimento de dermonecrose no local da aplicação. Portanto, qualquer eventual alteração nos resultados dos exames hematimétricos poderia ser decorrente da utilização dos soros. Os animais que receberam os soros não apresentaram índices fora dos padrões determinados para a espécie (CARPENTER, 2018), indicando não-toxicidade dos soros produzidos e preservando a atividade de sistemas vitais, como o renal e hepático e não evidenciando diferença significativa quando comparados entre si. Apenas a contagem de eritrócitos na comparação entre os soros Antiveneno e Proteínas Recombinantes, foi significativa ($P=0,0254$), porém os valores permaneceram dentro do esperado para coelhos. Uma observação deve ser feita com relação à enzima creatinoquinase (CK), utilizada como marcador para dano muscular: todos os grupos apresentaram curvas similares, com todos os tempos analisados acima dos valores de referência para a espécie (CARPENTER, 2018). Contudo, pondera-se que este acontecimento pode ter relação com a manipulação dos animais e decorrente estresse e lesões ocasionadas pela contenção, aplicação de veneno, aplicação dos soros e colheitas de sangue.

7. CONCLUSÕES

Avaliando os resultados apresentados neste trabalho, pode-se considerar que os soros produzidos a partir de imunizações com proteínas recombinantes mutadas sem atividade biológica cumprem o papel proposto na hipótese formulada de serem tão eficientes quanto o soro antiloxoscélico tradicional utilizado atualmente, obtido de imunizações com os venenos totais das três espécies de aranhas-marrons de maior importância médica no País: *L. gaucho*, *L. intermedia* e *L. laeta*, não apresentando diferenças significativas quando comparados entre si e com o Soro Antiveneno, diminuindo lesões dermonecróticas e edemas.

Os resultados apresentados mostram potencial de utilização destes antígenos na produção de um soro antiloxoscélico de segunda geração também pelo fato de minimizarem a necessidade de captura e manutenção de espécimes de *Loxosceles ex situ* para a etapa de extração de veneno, que se caracteriza por ser laboriosa e de pouco rendimento, necessitando de milhares de aranhas para que os ciclos de imunizações dos equinos sejam completos. Tal etapa poderia ser substituída por completo ou em partes pela produção das proteínas recombinantes em laboratório. Ademais, as proteínas recombinantes mutadas mostraram-se como sendo antígenos seguros nas imunizações de animais.

Apesar das evidências concretas que indicam sucesso na ação dos soros obtidos a partir de imunizações com as toxinas utilizadas neste trabalho e do seu futuro promissor como ferramenta de Saúde Pública, sugere-se que outros dados importantes devem ser obtidos em outros projetos a fim de dar maior robustez na proposta de troca do modelo de produção atual para o sugerido nesta tese, tais como: verificação da massa mínima de antígenos capaz de estimular resposta imune adequada (custo-benefício), avaliação da produção em equinos e avaliação do produto final processado industrialmente, que se caracteriza por possuir uma potência maior.

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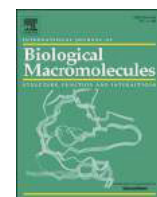
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9. OUTROS ARTIGOS PUBLICADOS DURANTE O DOUTORADO



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A protective vaccine against the toxic activities following Brown spider accidents based on recombinant mutated phospholipases D as antigens

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ABSTRACT

Accidents involving Brown spiders are reported throughout the world. In the venom, the major toxins involved in the deleterious effects are phospholipases D (PLDs). In this work, recombinant mutated phospholipases D from three endemic species medically relevant in South America (*Loxosceles intermedia*, *L. laeta* and *L. gaucho*) were tested as antigens in a vaccination protocol. In such isoforms, key amino acid residues involved in catalysis, magnesium-ion coordination, and binding to substrates were replaced by Alanine (H12A-H47A, E32A-D34A and W230A). These mutations eliminated the phospholipase activity and reduced the generation of skin necrosis and edema to residual levels. Molecular modeling of mutated isoforms indicated that the three-dimensional structures, topologies, and surface charges did not undergo significant changes. Mutated isoforms were recognized by sera against the crude venoms. Vaccination protocols in rabbits using mutated isoforms generated a serum that recognized the native PLDs of crude venoms and neutralized dermonecrosis and edema induced by *L. intermedia* venom. Vaccination of mice prevented the lethal effects of *L. intermedia* crude venom. Furthermore, vaccination of rabbits prevented the cutaneous lesion triggered by the three venoms. These results indicate a great potential for mutated recombinant PLDs to be employed as antigens in developing protective vaccines for Loxoscelism.

1. Introduction

The genus *Loxosceles* comprises about 150 species of spiders that are adapted to diverse environmental conditions [1,2]. Due to the significant number of cases reported, accidents with *Loxosceles* spiders are of public health concern in certain regions of Brazil, Argentina, Chile and Peru [3–5]. In South America, the species of greatest medical importance are *Loxosceles intermedia*, *L. laeta* and *L. gaucho* [2,5–8].

Accidents involving *Loxosceles* spiders, loxoscelism, can trigger clinical manifestations that include cutaneous and systemic changes. Cutaneous loxoscelism is characterized by lesions at the bite site or in its vicinity, and consists of edema, erythema, ecchymosis, ischemia,

massive inflammatory response and dermonecrosis with gravitational spreading. Secondary infections can aggravate the skin lesion, whereas tissue loss and disfiguring scars can be perceived as a result of the injury exacerbation [2,5,7–9]. Although less frequent, systemic loxoscelism is potentially more severe and may lead to death. Disseminated intravascular coagulation, intravascular hemolysis (which may progress to hemolytic anemia), and acute renal failure are the principal systemic symptoms reported [2,5,7–11].

The venoms of *Loxosceles* spiders are a complex mixture of peptides, proteins, and glycoproteins, with the prevalence of low molecular mass molecules ranging from 3 to 45 kDa [5,12]. Several families of molecules have already been described, but three of them stand out for their high

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expression: Phospholipases D, Inhibitor Cystine Knot peptides (Knottins) and Astacins (metalloproteases). Phospholipases D, which represent about 20% of the transcripts coding for toxins in *L. intermedia* venom gland [13], are also known as Dermonecrotic toxins, as they are involved in the development of necrotic skin lesions. These enzymes are highly conserved at both intra-species and inter-species levels [5,14]. Native isoforms of phospholipases D purified from crude venoms or expressed as recombinant molecules reproduce, under laboratory conditions, most of the toxic effects caused by crude venoms [5,14–18]. Although originally designated as sphingomyelinases-D [19–21], these enzymes are currently classified as phospholipases D due to the broad spectrum of phospholipids that they cleave, such as sphingomyelin, lysophosphatidylcholine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylethanolamine, lyso-PAF and phosphatidic acid [18,22,23].

PLDs comprise a single polypeptide chain that forms a structural organization composed of eight beta-strands interspersed with eight alpha-helices, resulting in a structure that resembles an $\alpha_8\beta_8$ barrel [24,25]. Based on this data, the existence of 3 conserved domains was proposed, which were referred to as the catalytic, flexible, and variable domains. His12 and His47 amino acid residues are located in the catalytic site and are conserved in all Brown spider phospholipases D isoforms. Glu32, Asp34 and Asp91 residues, in turn, coordinate a magnesium ion. Lastly, aromatic amino acid residues (Y228, Y229 and W230) are present in a conserved exosite, which is potentially involved with the binding of these enzymes with their phospholipid substrates [5,14,24–28].

By performing site-directed mutations in the cDNA of a wild-type PLD regarding the mentioned key amino acid residues, it was possible to produce isoforms devoid of toxic activities. The substitutions H12A, H12A-H47A, E32A-D34A, W230A and Y228A-Y229A-W230A performed in *L. intermedia*, *L. laeta* and *L. gaucho* phospholipases D generated recombinant isoforms lacking sphingomyelin degrading activity and did not induce dermonecrosis, hemolysis or increase capillary permeability [15,26,28]. However, all the mutated PLD isoforms of *L. intermedia*, *L. laeta* and *L. gaucho* venoms retained their native structural conformation, surface electrostatic charges, solvent exposure domains, topology, and immunological properties, and maintained their antigenic features and conservation of epitopes [26,28].

Despite the severity of loxoscelism and the high number of accidents reported, a standard and definitive treatment is not yet established. Serum therapy has a great therapeutic potential; however, it still has limitations, such as collateral effects, involve laborious and expensive procedures regarding spiders' capture and venom extraction, in addition to the undesirable animal suffering in the production process. Efforts have been made to generate non-toxic immunogens to produce therapeutic antivenoms or protective vaccines to address accidents involving *Loxosceles* spiders [28–33]. In this work, we tested three mutants of PLDs from the venoms of *Loxosceles intermedia* (LiRecDT1 W230A), *L. laeta* (LiRecDT1 H12A-H47A) and *L. gaucho* (LgRecDT1 E32A-D34A) as immunogens in a vaccine protocol to investigate the protection elicited against toxic effects caused by the crude venoms. In addition, functional and structural features of these three recombinant proteins were assessed to analyze their toxicity and to confirm whether they maintain similar antigenic and structural features compared to their native counterparts.

2. Materials and methods

2.1. Animals, venom and antivenom polyclonal antibodies

Swiss mice (~20 g) were supplied by the Animal House of the Biological Sciences Center of the Federal University of Paraná. Adult New Zealand rabbits (~2.5 kg) were obtained from the Canguiri Experimental Farm of Federal University of Paraná. Animals were randomly selected for immunization protocols and in vivo experiments. All procedures involving animals were carried out in accordance with

“Brazilian Federal Laws”, following the Institutional Ethics Committee for Animal Studies Guidelines from the Federal University of Paraná (certificate no. 1205). *Loxosceles intermedia*, *L. laeta* and *L. gaucho* venom samples were provided by Production and Research Center of Immunobiological Products (CPPI, Brazil). Specimens were captured from different areas of Paraná and Santa Catarina states with authorization of the responsible Federal Agency (Ministry of Environment, IBAMA, Brazil). After extraction by electric stimulation, the samples of venom collected were crystallized and then stored at -20 °C in the dark till further use. Saline solution 0.85% was used to resuspend venom samples. The protein content of venom samples, as well as of recombinant proteins samples, was determined by the Coomassie Brilliant Blue method (BioRad, Hercules, USA) as described by Bradford [34]. The production of rabbit polyclonal antibodies against *L. intermedia*, *L. laeta* and *L. gaucho* crude venoms was performed following the protocol of Harlow and Lane [35] with minor modifications [36].

2.2. Production and characterization of wild-type and mutated recombinant phospholipases D

2.2.1. Expression and purification

Wild-type PLDs LiRecDT1 [37], LiRecDT1 and LgRecDT1 [28], and mutated isoforms LiRecDT1 W230 [26], LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A [28] were expressed and purified as described in the cited references, with minor modifications. Briefly, the constructions in pET-14b plasmid were transformed into *Escherichia coli* BL21(DE3) pLysS and positive selected colonies were inoculated into 10 mL liquid LB broth containing ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL) at 37 °C for 16 h. This culture was inoculated in 1 L of LB broth with antibiotics and grown at 37 °C until an optical density between 0.4 and 0.6 was attained. Isopropyl β-D-thiogalactoside (IPTG) inducer was then added (0.05 mM) and the culture was grown for additional 3.5 h at 30 °C. Following centrifugation, the resultant bacterial pellet was resuspended in the binding buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH 8.0, 500 mM NaCl, 10 mM imidazole). Samples of this resuspended pellet were saved for SDS-PAGE analysis. Next, 1 mg/mL of lysozyme was added, and the suspension was maintained at -20 °C for at least 16 h. The frozen resuspension was then thawed, subjected to sonication lysis, and centrifuged (9000 ×g, 30 min at 4 °C). Purification of all recombinant PLDs was performed by affinity chromatography in Ni²⁺-NTA resin using the ÄKTA™ pure system (GE Healthcare Life Sciences). Filtered supernatant (0.22 µM) was applied to a HisTrap™ HP 1 mL column, which was previously equilibrated with washing buffer (20 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole, pH 7.5). Washing steps were performed using the same buffer. Finally, elution was executed with a linear gradient of elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 7.5). Fractions (1 mL) containing eluted PLDs were dialyzed with Phosphate-Buffered Saline (PBS) and analyzed using a 12.5% SDS-PAGE.

2.2.2. Enzymatic activity

The Amplex™ Red Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used to determine the phospholipase activity of wild-type and mutated recombinant PLDs. Ten micrograms of wild-type recombinant proteins and 100 µg of mutated recombinant isoforms were incubated with sphingomyelin (SM: Egg Sphingomyelin, Chicken) and the Amplex™ Red reagent mixture in reaction buffer (Tris-HCl 100 mM pH 7.4 containing 10 mM MgCl₂). Negative control consisted of Amplex™ Red reagent with SM, without any PLD isoform. After incubation (30 min, 37 °C), the reactions were transferred to 96-well black fluorimeter plates and the fluorescence was measured using excitation wavelength at 540 nm and emission at 570 nm on a fluorimeter (Tecan Infinite M200, Männedorf, Switzerland). All treatments were performed in triplicate and the results represent an average of three experiments ± s. d.

Table 1
Erythrocytic and leukocyte counts after immunizations with PLDs.

	Erythrogram			Leukogram	
	Non-vaccinated	Vaccinated		Non-vaccinated	Vaccinated
RBC	6.5 ± 0.3	6.6 ± 0.2	WBC	7.37 ± 0.7	6.70 ± 0.6
Hb	12.2 ± 0.5	12.5 ± 0.3	He	2.97 ± 0.4	3.38 ± 0.6
PCV	38.9 ± 1.5	40.8 ± 0.8	Ly	3.85 ± 0.6	2.81 ± 0.2
MCV	60.4 ± 0.7	61.2 ± 0.4	Eo	0.05 ± 0.03	0.12 ± 0.04
MCHC	31.6 ± 0.2	30.9 ± 0.2	Ba	0.18 ± 0.04	0.17 ± 0.05

RBC: red blood cell ($\times 10^9/L$); Hb: hemoglobin (g/dL); PCV: packed cell volume (%); MCV: mean cell volume (μm^3); MCHC: mean cell hemoglobin concentration (g/dL); WBC: white blood cell ($\times 10^9/L$); He: heterophile ($\times 10^9/L$); Ly: lymphocyte ($\times 10^9/L$); Eo: eosinophil ($\times 10^9/L$); Ba: basophil ($\times 10^9/L$). Results are expressed as mean $\pm$ SEM of nine animals in each group. No differences were found between vaccinated and non-vaccinated animals ($p < 0.01$, parametric t-test).

2.2.3. In vivo dermonecrosis

The dermonecrotic activity of mutated recombinant PLDs was evaluated after intradermal (i.d.) injections of toxins at a back shaven area of rabbit's skin. Each animal ($n = 3$) received a wild-type PLD (LiRecDT1, LiRecDT1 or LgRecDT1) as positive control (10 μg), the mutated isoforms (LiRecDT1 W230A, LiRecDT1 H12A-H47A or LgRecDT1 E32A-D34A) (50 μg) and PBS solution as negative control. Photographs were taken at 0 and 24 h post-injection and the skin was evaluated for the presence (+) or absence (-) of edema, erythema, bruise, and necrosis.

2.2.4. Histological analysis

Skin samples from rabbits exposed to different PLDs were fixed in ALFAC fixative solution (ethanol 85%, formaldehyde 10% and glacial acetic acid 5%) for 16 h at 4 °C, dehydrated in a graded series of ethanol and embedded in paraffin for 2 h at 58 °C. The materials were processed in thin sections (4 μm) and stained with Hematoxylin and Eosin. Images were analyzed and photographed under a light microscope Olympus BX41 with DP 72 camera for image capture (Olympus, Tokyo, Japan).

2.2.5. Immunoreactivity with antivenom antibodies

Wild-type and mutated recombinant PLDs (2.5 μg) were analyzed through SDS-PAGE (12.5% under reducing conditions) and the proteins were transferred onto nitrocellulose membranes using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell System (Bio Rad) for 30 min at 25 V. Next, membranes were incubated with antivenom sera containing polyclonal antibodies (1:1000) produced as described ahead. Control of antivenom specificity was performed using pooled pre-immune sera. Alkaline phosphatase-coupled anti-rabbit IgGs (1:5000) (Sigma-Aldrich, A3812) were used as secondary antibodies and the reactions were developed using BCIP/NBT (Promega, Madison, WI, USA).

2.3. Structural analyses

The three-dimensional structure of LiRecDT1 (PDB ID: 3RLH) was used to create the models of LiRecDT1 W230A, LgRecDT1 and LgRecDT1 E32A-D34A. To create the model of LiRecDT1 H12A-H47A, the three-dimensional structure of *L. laeta* PLD (PDB ID: 2FR9) was used. The structural models were built using SWISS - MODEL and the electrostatic potential was calculated using the program APBS [38] according to the molecular electrostatic potential ranging from -5.0 to 5.0 and implemented in PYMOL [39].

2.4. Vaccination and analysis of protective response

2.4.1. Immunization protocols

The antigen formulation included equal volumes of recombinant PLDs LiRecDT1 W230A, LiRecDT1 H12-H47A and LgRecDT1 E32-D34A, and aluminum hydroxide 4% (m/v) as adjuvant [40]. Following

collection of pre-immune sera, a group of nine rabbits received an initial intramuscular (i.m.) injection of a pool sample containing 300 μg of PLDs in aluminum hydroxide adjuvant (1:1 v/v). Two booster injections (i.m.) were administered at intervals of 30 days with increasing doses of the PLDs pool (600 μg and 1000 μg). As negative control (non-vaccinated), twelve rabbits received solutions containing the adjuvant and PBS according to the same protocol for the vaccinated groups. Blood samples were collected 15 days after the third dose and processed to detect antibodies and to perform neutralization assays.

Mice ($n = 24$) were vaccinated using the same antigens and adjuvant administered in the rabbits. Animals received three intraperitoneal injections of increasing doses of the PLDs pooled (30, 60 and 100 μg) with an interval of 21 days. Non-vaccinated animals ($n = 24$) received only PBS and aluminum hydroxide, following the same immunization protocol.

2.4.2. Immunoassays to detect antibodies for mutated recombinant PLDs

Antibody-capture ELISA was performed as described by Figueiredo et al. [32], with minor modifications. MaxiSorp plates (Nunc – ThermoFisher, Waltham, MA, USA) were coated overnight at 4 °C with 100 μL of *L. intermedia*, *L. laeta* or *L. gaucho* venom (600 ng/well) in bicarbonate buffer (NaHCO₃ 0.02 M pH 9.6). After blocking (2% powdered casein-PBS, 1 h 37 °C) and washing (0.05% Tween 20-PBS) steps, sera from vaccinated and non-vaccinated rabbits were added at 1:400 dilutions and incubated for 1 h at 37 °C. Plates were washed and incubated with anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich A0545) diluted 1:5000 for 1 h at 37 °C. After washing step, reactions were developed using 0.4 mg/mL of o-phenylenediamine and 4 $\mu L/mL$ H₂O₂ in citrate buffer (50 mM NaH₂PO₄, 24 mM sodium citrate, pH 5.0). Reactions were performed in the absence of light for 30 min, at 24 °C, and stopped with 1 M H₂SO₄ (20 μL /well). Absorbance values were determined at 490 nm on a plate reader (Meridian ELX 800). To discard nonspecific reactions, pre-immune sera and controls without antigens or primary antibodies were tested. All samples were analyzed in triplicates. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by a post hoc Tukey test. The significance was established at $p < 0.1$ using the software GraphPad Prism 8.0.2 (San Diego, CA, USA).

For western blotting, samples of 3 μg of each *Loxosceles* spp. venom were subjected to SDS-PAGE (12.5%) under reducing conditions. Gels were transferred onto nitrocellulose membranes, which were incubated with the sera of vaccinated and non-vaccinated rabbits (1:2000). Control of sera specificity was performed using pooled pre-immune sera. Alkaline phosphatase-coupled anti-rabbit IgGs (1:5000) (Sigma-Aldrich A3812) and BCIP/NBT (Promega) were used for detection.

2.4.3. Neutralization assays

Based on immunologic assays, the sera from three rabbits with higher antibody titles were pooled and used for neutralization assays. Likewise, sera from rabbits were randomly chosen and pooled. Groups of three rabbits were injected on the shaved back skin with 6.10 μg of *L. intermedia* venom (corresponding to 2 MND – Minimum Necrotizing Dose, which was previously determined in CPPI with the same lot of venom) pre-incubated (1 h at 37 °C) with 100 μL of sera from vaccinated rabbits. As a control, the same quantity of venom was pre-incubated with 100 μL sera from non-vaccinated animals. Photographs and measurements of cutaneous lesions and edema at the affected area were taken at 0, 24, 48, 72 and 96 h. The largest horizontal and vertical diameters of swollen areas were measured with a Vernier caliper to determine the area. The cutaneous lesion areas were analyzed using Image J by delimiting the specific area. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by post hoc Sidak test. The significance was established at $p < 0.1$ using the GraphPad Prism 8.0.2.

Table 2
Biochemical parameters after immunizations with PLDs.

Biochemical parameters					
	Non-vaccinated	Vaccinated		Non-vaccinated	Vaccinated
tB	0.08 ± 0.0	0.06 ± 0.0	Alb	4.5 ± 0.1	4.4 ± 0.1
dB	0.03 ± 0.0	0.03 ± 0.0	Glob	1.3 ± 0.1	1.5 ± 0.1
iB	0.06 ± 0.0	0.03 ± 0.0	BUN	41.8 ± 0.8	40.9 ± 1.9
AST	74.3 ± 23.8	64.74 ± 17.9	Creat	0.99 ± 0.1	0.99 ± 0.0
ALT	67.9 ± 5.1	70.9 ± 10.8	Lac	7.5 ± 0.6	8.3 ± 0.7
ALP	73.5 ± 5.3	69.1 ± 7.1	LDH	672.8 ± 105.1	584.9 ± 76.6
GGT	12.9 ± 0.8	11.5 ± 1.0	CK-MM	2620.6 ± 671.1	1954.8 ± 270.3
Prot	5.8 ± 0.1	6.0 ± 0.2			

tB: total bilirubin (mg/dL); dB: direct bilirubin (mg/dL); iB: indirect bilirubin (mg/dL); AST: aspartate aminotransferase (U/L); ALT: alanine aminotransferase (U/L); ALP: alkaline phosphatase (U/L); GGT: gamma-glutamyltransferase (mg/dL); Prot: total protein (g/dL); Alb: albumin (g/dL); Glob: globulin (g/dL); BUN: blood urea nitrogen (mg/dL); Creat: creatinine (mg/dL); Lac: lactate (mmol/L); LDH: lactic acid dehydrogenase (U/L); CK-MM: muscle creatine kinase. Results are expressed as mean ± SEM of nine animals in each group. No differences were found between vaccinated and non-vaccinated animals ($p < 0.01$, parametric t -test).

2.4.4. Protection assays

Dermonecrosis and local edema triggered by crude venoms were determined by challenging vaccinated rabbits with different *Loxosceles* venoms. Fifteen days after the last immunization protocol, vaccinated and non-vaccinated rabbits were inoculated (i.d.) with 10 µg of *L. intermedia*, *L. laeta* or *L. gaucho* venom (approximately 3.3 MND of *L. intermedia* venom) or PBS. Photographs and measurements of edema and dermonecrosis at the affected area were taken at 0, 3, 6, 24, 48, 72 and 96 h.

Lethality assay was performed using vaccinated and non-vaccinated mice 10 days after the last immunization dose. Animals (eight vaccinated and eight non-vaccinated) were inoculated (i.p.) with *Loxosceles intermedia* venom (10 µg) and survival was then assessed at 24, 48 and 72 h after injection. Results show the total number of animals tested in three independent experiments.

2.4.5. Hematological and biochemical parameters of vaccinated rabbits

In vivo toxicity of mutant PLDs was evaluated after the vaccination protocol in rabbits by analyzing hematological parameters (red and white blood cell counts, hemoglobin concentration and red blood cell indices) by using a hematology analyzer (Mindray Headquarters, Shenzhen, China) according to Wintrobe and Greer [41]. Biochemical parameters were also analyzed according to McPherson et al. [42] and included total bilirubin, direct bilirubin, indirect bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total serum protein, albumin, globulin, blood urea nitrogen, creatinine, lactate, lactic acid dehydrogenase and muscular creatine kinase by means of commercial kits from Bioclin (Belo Horizonte, MG, Brazil) applied in BS-200 Chemistry Analyzer (Mindray Headquarters, Shenzhen, China). Statistical analyses were performed using parametric t -test. The significance was established at $p < 0.01$ using the software GraphPad Prism 8.0.2. Results are available as complementary Tables 1 and 2.

3. Results

3.1. Mutated phospholipases D of *Loxosceles intermedia*, *L. laeta* and *L. gaucho*

Fig. 1A presents images of the three species of Brown spiders endemically encountered in South America, which are involved in

accidents and which are of clinical significance (*Loxosceles intermedia*, *L. laeta* and *L. gaucho*). These spiders exhibit sexual dimorphism, but animals of both sexes are potentially venomous. The alignment of amino acid sequences regarding three native phospholipases D (Fig. 1B and C) shows great identity, especially in the regions which are of significance for catalytic activity and also those which are involved in substrate binding. These amino acid residues were replaced by alanine residues in the mutant isoforms and are shown in colored squares. The mutated phospholipases D at positions W230A for *Loxosceles intermedia*, H12A-H47A for *L. laeta* and E32A-D34A for *L. gaucho* were expressed in a heterologous system using *Escherichia coli* BL21(DE3)pLysS and purified by affinity chromatography. All recombinant isoforms were produced as soluble proteins and have molecular masses of approximately 35 kDa, as previously described in the literature [14,28] (Fig. 1D).

3.2. Biological activities of recombinant mutated PLDs isoforms

Fig. 2 shows the biological activity of the recombinant mutated PLDs that was assessed by subcutaneous injections on the back of rabbits followed by macroscopic observation of cutaneous lesion and histopathological analysis of the inoculated area. PLDs containing any of the mutations studied strongly inhibited the development of dermonecrosis and gravitational spreading of lesion (Fig. 2A), when compared with the recombinant wild-type isoforms. Macroscopical visualization also allowed the detection of other signs, i.e., edema, erythema, and bruise. Images show that these signs are absent in the rabbits' skin after mutated PLDs were inoculated; on the other hand, control animals injected with the wild-type PLDs exhibited the deleterious signs. Histopathological analyses of rabbits' skin also show that usual injuries observed when active PLDs are inoculated, e.g., massive polymorphonuclear cells accumulation, collagen fibers disorganization, hemorrhage, and presence of fibrin in the connective tissue (Fig. 2B – left panel) are not present when mutated isoforms are injected (Fig. 2B – right panel). Histological sections of the skin inoculated with mutated PLDs reveal a normal tissue architecture, evidencing a lack of deleterious activity by these proteins (Fig. 2B – right panel).

3.3. Functional and immunological properties of recombinant mutated PLDs isoforms

The biochemical activity of recombinant PLDs was evaluated by their ability to degrade sphingomyelin using the Amplex™ Red Assay. Fig. 3A shows that the PLDs containing any of the three mutations had their degrading activity on sphingomyelin completely abolished. Next, by means of immunoblotting assays, we evaluated the antigenic properties of the PLDs isoforms. Wild-type and mutated PLDs were analyzed using hyperimmune sera against the three *Loxosceles* venoms. As shown in Fig. 3B, these hyperimmune sera recognized the recombinant isoforms, indicating that mutations did not change linear epitopes present in the mutated toxins compared to the wild-types. However, it is worth it to note that, in general, the reactions were visually more intense between the hyperimmune serum and toxins related to the same species (for example, hyperimmune serum raised against *L. intermedia* whole venom recognized *L. intermedia* recombinant PLDs better than it recognized *L. laeta* or *L. gaucho* PLDs).

3.4. Predictions of three-dimensional structures and surface charge profile of wild-type and mutated phospholipases D

Using data from crystallographic studies of a PLD isoform from the venom of *L. intermedia* [25] and a PLD from *L. laeta* [24], comparative predictions of the structural and topographic organizations of wild-type and mutated PLDs of the three venoms were performed. Fig. 4 indicates that none of the mutations changed the PLDs three-dimensional structure. These results were strengthened by comparative analyzes of PLDs surface charges. As depicted in Fig. 5, although positive amino acid

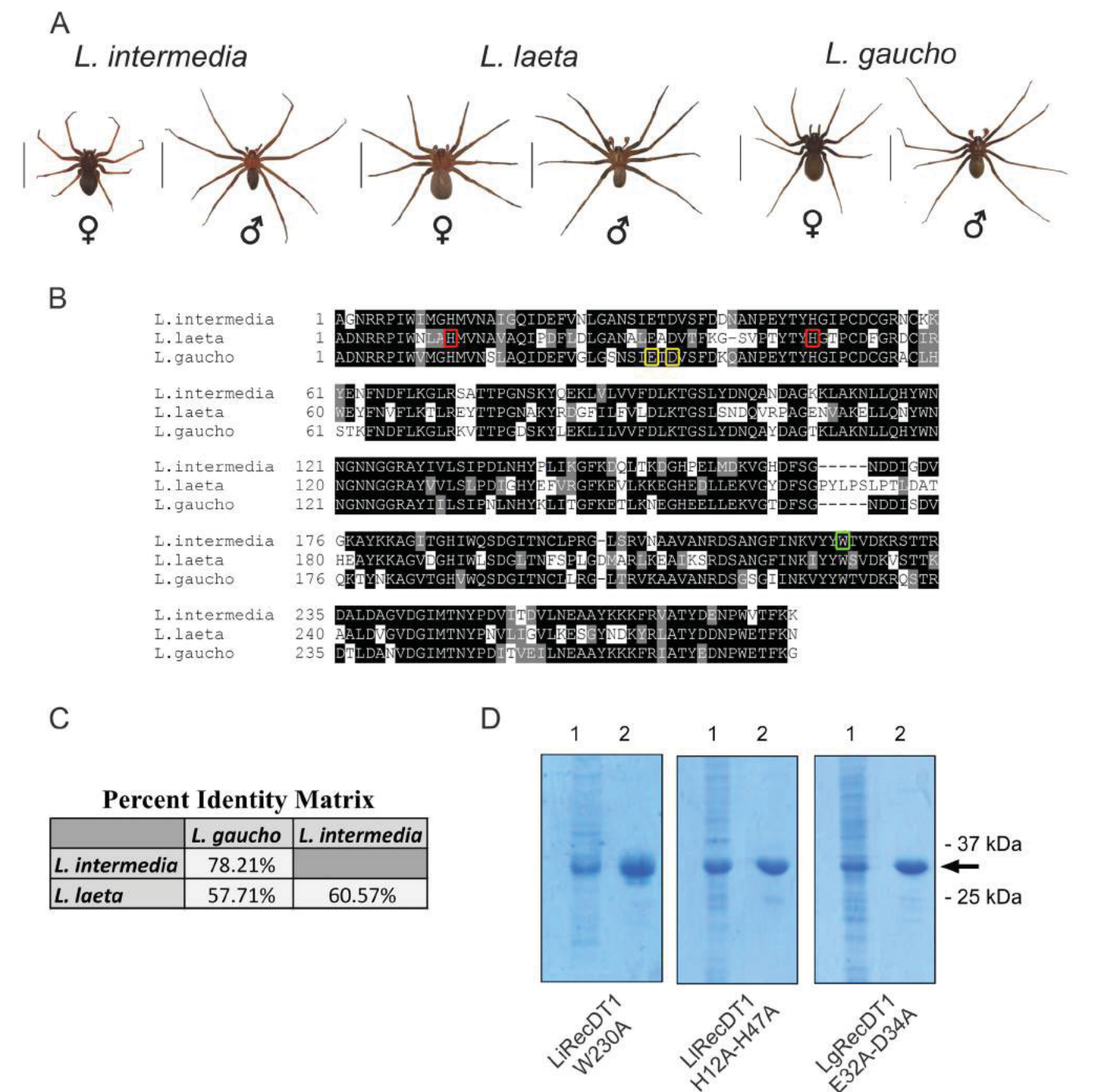


Fig. 1. *Loxosceles* spiders, sequence data and recombinant expression results. (A) Adult Brown spiders. *Loxosceles intermedia*, *Loxosceles laeta* and *Loxosceles gaucho* female and male specimens. Scale bar: 1 cm. (B) Multiple sequence alignment of the phospholipases D of *L. intermedia* (GenBank accession number ABA62021.1), *L. laeta* (AAM21154.1) and *L. gaucho* (AFY98967.1) used as templates to perform the site-directed mutations. Amino acid residues indicated by colors (Histidine in red, Glutamic Acid and Aspartic Acid in yellow and Tryptophan in green), were replaced by alanine residues. Sequences were aligned using the CLUSTAL X2 program [64]. Amino acid identities are shown in gray. (C) Percent identity matrix showing the sequence similarities among PLDs aligned in (B). Values given by CLUSTAL X2 program [64]. (D) Analysis of protein expression and purification of mutated PLDs (SDS-PAGE 12.5% under reducing conditions). Proteins were stained by Coomassie blue dye. Lanes 1 represent cultures induced with 0.05 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and lanes 2 show the protein profile of toxins after chromatography purification. Arrow indicates the apparent electrophoretic mobility of PLDs (~35 kDa). Positions of molecular mass standards are shown on the right.

(H12A-H47A) or negative amino acid (E32A-D34A) residues were replaced by alanine, which is hydrophobic, the surface charge densities of both wild-type and mutated isoforms did not differ significantly.

3.5. Immunogenicity of recombinant mutated phospholipases D

Since the recombinant mutated PLDs showed no toxic activities, nor had their structures altered after mutations, a vaccination protocol was performed in rabbits using a mixture of three recombinant mutated isoforms as antigens. One mutated PLD from each venom was used and

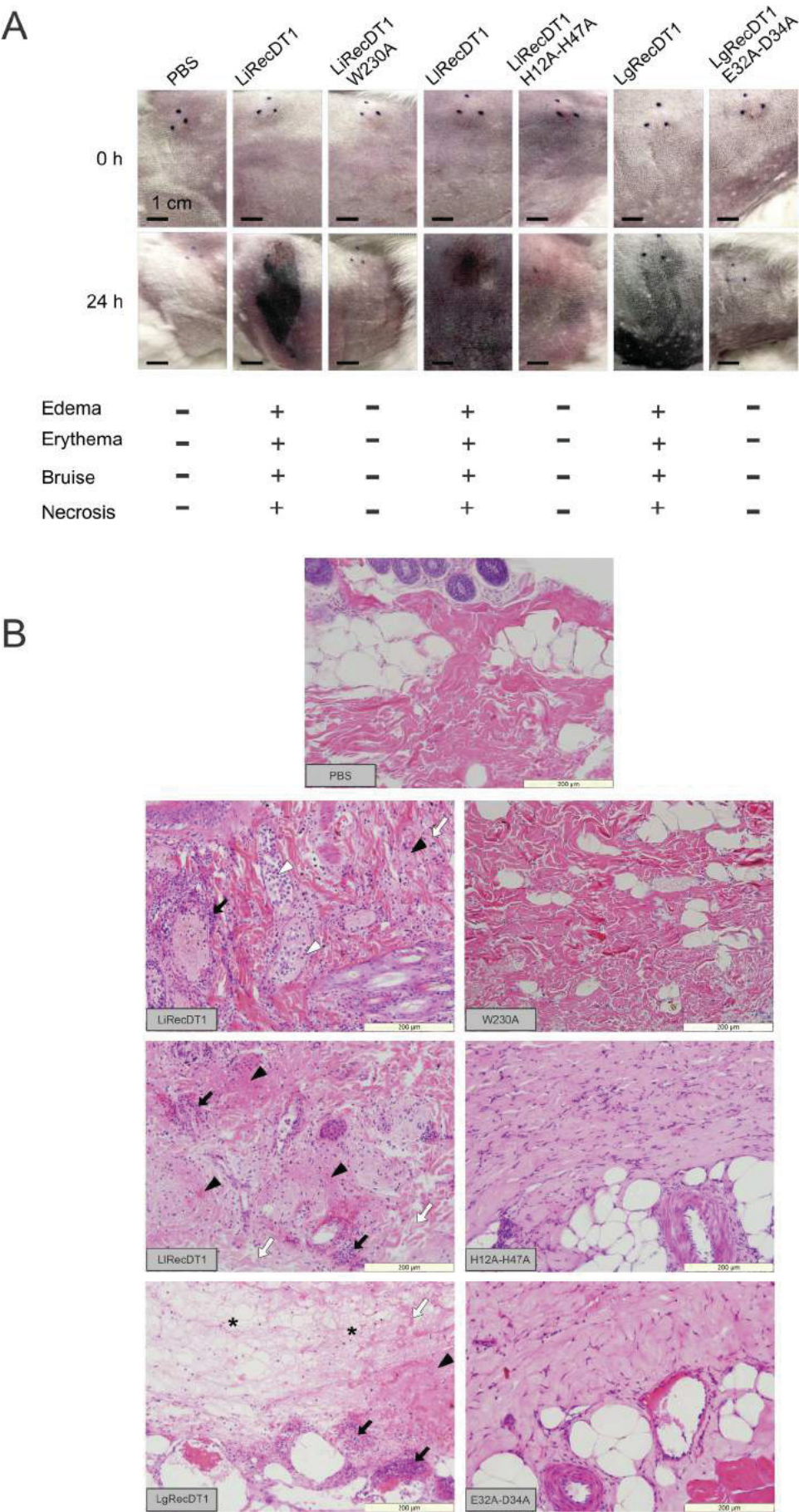


Fig. 2. Biological activity of mutant phospholipases D. (A) Macroscopic analysis of rabbit skin exposed to wild-type (10 µg) and mutated (50 µg) PLDs. Injection area was photographed immediately after inoculation (0 h) and 24 h following injection. The black dots show the local of the injection and scale bars correspond to 1 cm. The indication (+) means “presence”, while the indication (–) means “absence” of each listed signal 24 h after exposure. (B) Histopathological analyses of rabbit skin exposed to *Loxosceles* wild-type recombinant PLDs LiRecDT1, LiRecDT1 and LgRecDT1 (10 µg) and their mutated counterparts LiRecDT1 W230A, LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A (50 µg). Tissue sections were stained with hematoxylin and eosin (HE). Scale bars are indicated in the figures. Top image displays rabbit skin inoculated with PBS (negative control) showing the appearance of normal dermis. Images in the left depict the skin tissue sections 24 h after inoculation of wild-type PLDs (isoforms are indicated in the images). Closed arrows point out to leukocytes infiltrating the connective tissue. Inflammatory cells (leukocytes) are accumulated in (open arrowheads) and around blood vessels (closed arrows). Disorganization of collagen bundles (open arrows) and fibrin deposition (asterisks) are also present. Tissue damage is also observed by the presence of hemorrhage (closed arrowheads). Images in the right show skin tissue sections exhibiting normal architecture 24 h after the inoculation of mutated PLDs.

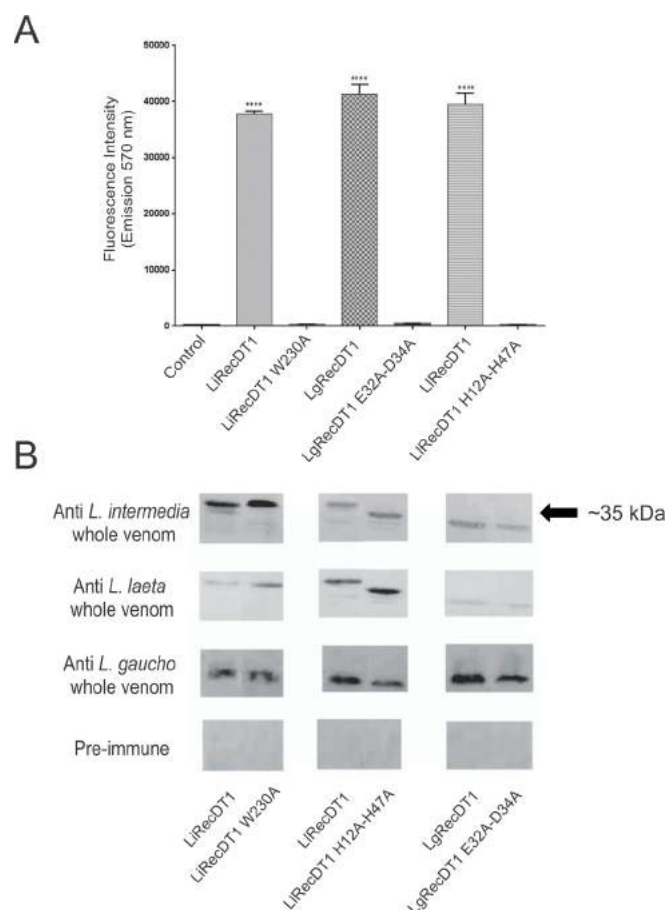


Fig. 3. Enzymatic and antigenic properties of mutant phospholipases D. (A) Sphingomyelinase D activity of wild-type (LiRecDT1, LiRecDT1 and LgRecDT1) and mutated (LiRecDT1 W230A, LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A) PLDs was evaluated with Amplex Red Assay. The product of reaction was fluorometrically measured using excitation at 540 nm and emission at 570 nm. Negative control was performed by incubation of Amplex Red reagent mixture without PLDs. The values represent average $\pm$ SD of three independent experiments carried out in triplicate ($***p < 0.0001$). (B) Immunological cross-reactivity among recombinant toxins and antibodies raised against crude venom native toxins evaluated by western blotting. Purified toxins samples were submitted to SDS-PAGE, transferred onto nitrocellulose membranes, and exposed to hyperimmune sera produced in rabbits against whole venoms of *L. intermedia*, *L. laeta* or *L. gaucho* (1:1000). Pre immune sera (1:1000) produced no visible reactions. Arrow points to 35 kDa molecular mass position.

each isoform contained a different mutation. Therefore, the isoforms LiRecDT1 W230A from *L. intermedia*, LiRecDT1 H12A-H47A from *L. laeta* and LgRecDT1 D32A-E34A from *L. gaucho* were chosen and then used in equal parts to produce a pool. Two weeks after the last immunization, the sera were collected and tested by western blotting and antibody-capture ELISA against crude venoms of the three investigated spiders. Fig. 6A (ELISA) and Fig. 6B (immunoblotting) show that despite individual differences among animals, all vaccinated rabbits produced polyclonal antibodies that recognized native toxins in the crude venoms. Fig. 6B demonstrates that the antibodies recognized venom proteins with approximately 35 kDa, which corresponds to the expected electrophoretic mobility for venom PLDs.

3.6. Mutated PLDs are safe immunogens for rabbits

Vaccinated rabbits were subjected to hematological and serum biochemical analyses before and after the immunization protocol to verify the toxicity of mutated PLDs in vivo, and the values are presented

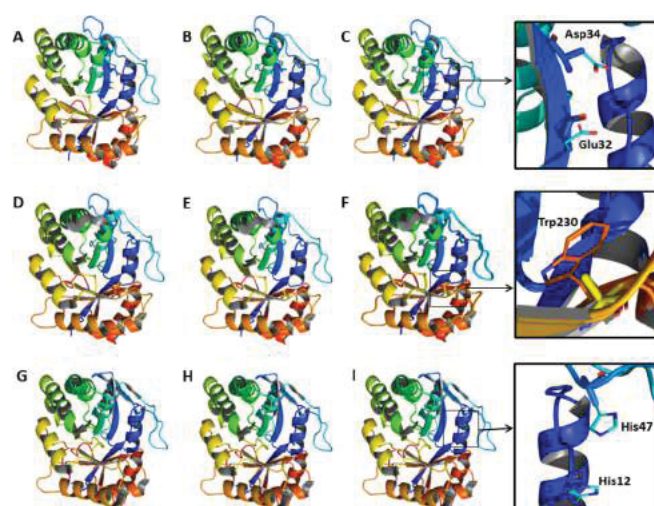


Fig. 4. Structural analysis of recombinant wild-type (WT) and mutated *Loxosceles* phospholipases D. Structures of *L. intermedia* wild-type (WT) PLD (PDB ID: 3RLH), *L. laeta* WT PLD (PDB ID: 2FR9) and *L. gaucho* WT PLD, are presented. LiRecDT1 W230A, LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A (mutated PLDs) are also displayed. Ribbon views of the general structures of *L. gaucho* WT (A), LgRecDT1 E32A-D34A (B) and the superposition of structures (C); *L. intermedia* WT (D), LiRecDT1 W230A (E) and the superposition of structures (F); *L. laeta* WT (G), LiRecDT1 H12A-H47A (H) and the superposition of structures (I). Squares on the right depict a zoom on mutated residues, which are presented in atom colors. Original amino acid residues are presented as thin sticks while alanine residues are displayed as thick sticks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

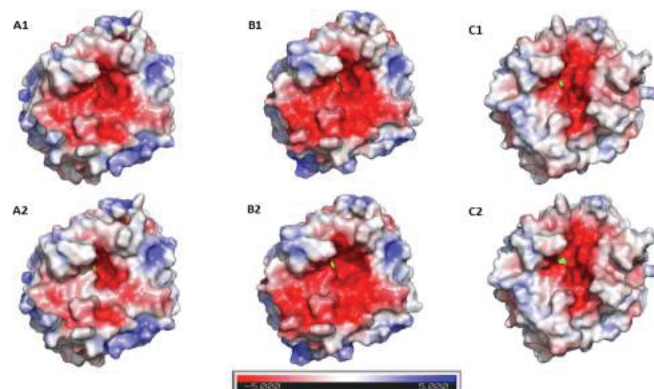


Fig. 5. Electrostatic surface colored by charge, from red (-5 kV) to blue (+5 kV) of recombinant wild-type (WT) and mutated PLDs. *L. gaucho* WT (A1) and LgRecDT1 E32A-D34A (A2), *L. intermedia* WT (B1) and LiRecDT1 W230A (B2), *L. laeta* WT (C1) and LiRecDT1 H12A-H47A (C2) PLDs. The Mg^{2+} ion is shown as a green sphere. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in Table 1. Red and white blood cells' counts after the vaccination protocol are not statistically significant from those acquired before the first immunization with mutated PLDs, as well as the hemoglobin concentration and red blood cell indices. These results show that the mutated toxins used to immunize the animals did not cause any detectable damage in blood cells or an impairment in their production. In addition, the tested serum biochemical parameters, which mainly detect kidney and liver damage, did not change significantly after the vaccination protocol, indicating that the mutated toxins administered in the immunizations did not induce any detectable injury in these organs (Table 2).

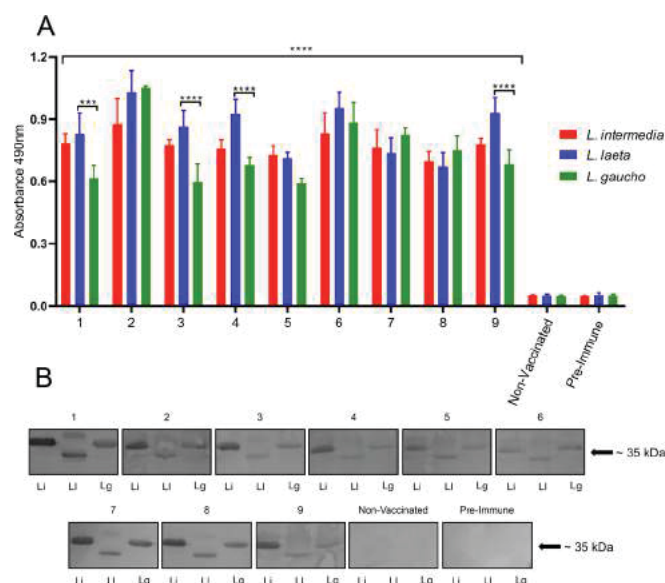


Fig. 6. Immunological cross-reactivity between sera of vaccinated rabbits and native *Loxosceles* venom toxins. (A) ELISA for antibody capture assay was performed by coating the plates with *L. intermedia*, *L. laeta* and *L. gaucho* crude venoms (600 ng/well). Venom samples were then reacted with sera (1:400 dilution) (animals 1 to 9 tested – depicted in X axis) raised in animals vaccinated with the pool of the recombinant mutated PLDs. Sera of non-vaccinated animals and a pre-immune serum were used as controls and one representative reaction was chosen to compose the graph. Each point represents the mean $\pm$ SEM (** p < 0.001, **** p < 0.0001); the major bar above the graph (****) shows the statistical difference between hyperimmune serum and non-immune and pre-immune sera. (B) Western blotting of *L. intermedia* (Li), *L. laeta* (Ll) and *L. gaucho* (Lg) crude venoms (3 μ g) probed with sera (1:2000 dilution) of rabbits vaccinated with recombinant mutated PLDs as previously described. Sera of non-vaccinated rabbits and a pre-immune serum were used as controls.

3.7. Sera of rabbits immunized with mutated PLDs neutralize toxic activities caused by *L. intermedia* venom

The hyperimmune sera obtained using mutated PLDs were pooled and evaluated for their ability to neutralize toxic effects of *L. intermedia* crude venom. For this purpose, the pooled serum was pre-incubated (1 h at 37 °C) with 2 MND of crude venom and mixtures were injected into the skin of rabbits. As displayed in Fig. 7, pre-incubation with hyperimmune serum significantly blocked the development of the cutaneous lesion and edema caused by the crude venom. Quantification of areas containing edema and cutaneous lesion confirmed the macroscopic aspects by showing statistically significant reduction for both parameters.

3.8. Induction of protective activity in mice by vaccination of animals with mutated phospholipases D

As initial tests indicated that the vaccination protocol was able to raise neutralizing antibodies, the protective potential of these antigens against experimental loxoscelism was investigated. Ten days after the last immunization, as described in material and methods, mice were challenged with 10 μ g of *Loxosceles intermedia* crude venom that was intraperitoneally injected and the lethality was evaluated for up to 72 h. As shown in Table 3, the vaccination protocol protected animals, increasing the survival rate more than 79% after 72 h.

3.9. Induction of protective activity in rabbits by prior immunization with mutated phospholipases D

Two weeks after the last immunization, as described in material and methods, rabbits were challenged by injecting the crude venoms of *L.*

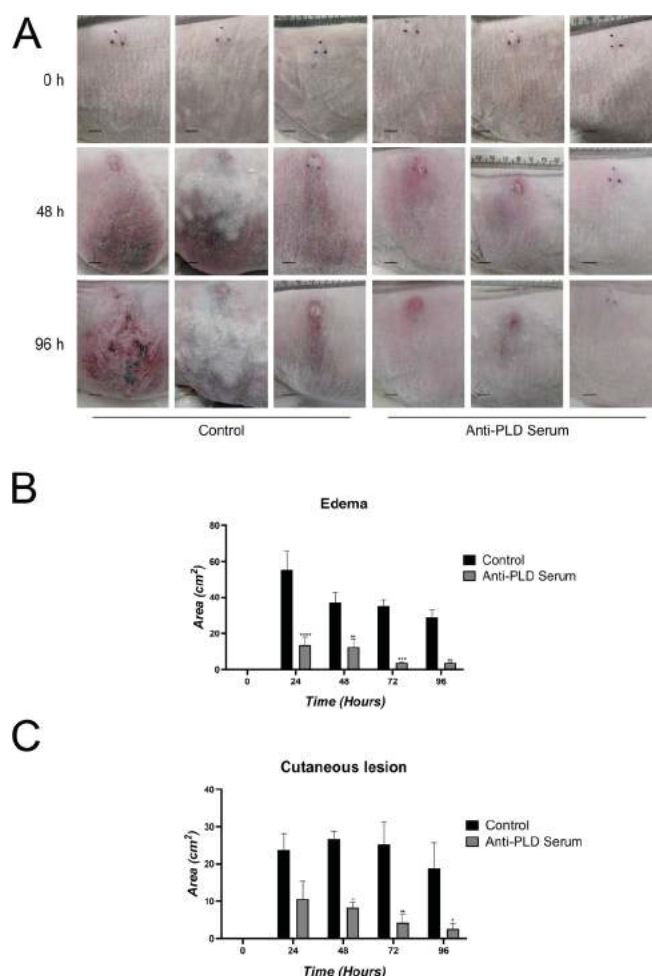


Fig. 7. Edema and cutaneous lesion neutralization by anti-mutated PLDs pooled sera. Groups of three rabbits were injected with 2 MND (6.10 μ g – I.D. in the skin) of *L. intermedia* venom, which was previously incubated with 100 μ L of anti-mutated PLDs pooled sera. The same amount of venom was pre-incubated with 100 μ L of a pool of non-vaccinated animals' sera as control. (A) Macroscopic aspect of injected area. Three dot points indicate the region of injections. Scale bars correspond to 1 cm. Photographs show a representative response of the animals after 48 h and 96 h. Additional images of other treatment periods are available as a supplementary figure (1). (B) Edema areas were measured, and results are presented as the mean $\pm$ SEM related to three animals tested (n = 3) (** p < 0.01, *** p < 0.001, **** p < 0.0001). (C) Cutaneous lesion areas were measured, and results are shown as the mean $\pm$ SEM related to three animals tested (n = 3) (* p < 0.1, ** p < 0.01).

intermedia, *L. laeta* and *L. gaucho* and the macroscopic lesions were evaluated for up to 96 h. As shown in Figs. 8 and 9, the vaccination protocol protected the animals, extensively preventing the development of skin lesions such as dermonecrosis with gravitational spread, which is the typical sign of cutaneous loxoscelism. Images shown in Fig. 8 are representative from three animals per challenge (vaccinated and non-vaccinated). Additionally, swollen areas were also measured and the results demonstrate that the edema was scarce in the vaccinated rabbits (Fig. 9). As observed, the greatest reduction of edema was in the animals challenged with *L. gaucho* venom (almost 97% of reduction after 96 h), but inhibition of edema was observed in the vaccinated rabbits challenged with all of the three crude venoms.

4. Discussion

Accidents caused by Brown spiders often lead to serious complications [2,5,10], currently, loxoscelism has no efficient specific

Table 3
Mice lethality after exposure to *L. intermedia* crude venom.

Time	Lethality	
	Non-vaccinated	Vaccinated
0 h	0/24	0/24
24 h	16/24	1/24
48 h	22/24	5/24
72 h	24/24	5/24
Protection after 72 h (%)		79.17%

pharmacological treatment targeting the venom toxins. Although there is no consensus on the current protocols, the most common treatment aims to reduce the intense inflammatory response triggered after envenoming, which arises from the action of phospholipases D present in the venom. These enzymes act on phospholipids of host tissues, giving rise to biologically active derivatives that activate leukocytes (mostly neutrophils) [43–45]. The consequent massive inflammatory response results in skin necrosis [43]. In this sense, the treatment of cutaneous loxoscelism is based on anti-inflammatory drugs and analgesics to control the pain at the bite site. The only specific treatment that inactivates the venom toxins is the serum therapy, which is based on neutralizing polyclonal antibodies produced in horses immunized with crude venoms. The adverse reactions, the ineffectiveness associated with late application — especially regarding the management of skin lesion — and the suffering of animals used to produce the therapeutic sera are convincing reasons that should cause their production and use to be gradually reduced [5,7,46].

In this work, our objective was to use non-toxic recombinant mutated PLDs from the three main species involved in accidents in South America (*L. intermedia*, *L. laeta* and *L. gaucho*) as antigens in pre-clinical trials of a protective vaccine. Phospholipases D were chosen to elicit protective antibodies among the various toxins present in the venoms because of several aspects. First, PLDs are highly expressed toxins [13,47,48]. In addition, PLDs can be easily produced in large amounts by bacterial expression models, which are low-cost, rapid and easy-to-handle systems [14]. Another advantage is that both native and recombinant PLDs have similar biochemical properties [14]. Finally, among the various *Loxosceles* toxins, the PLD family is highly antigenic and is recognized by sera raised against different crude venoms [5,14,30,49–53], in addition to being responsible for most of the deleterious effects followed accidents with *Loxosceles* spiders. The chosen PLDs isoforms are from clinically important species and have substantial differences in their amino acid sequences, which led us to the decision to use a mixture of antigens, thus resulting in a greater variety of epitopes. Although the mutated PLDs used herein (LiRecDT1 W230A, LiRecDT1 H12A-H47A and LgRecDT1 E32A-D34A) had altered residues in conserved regions, the lack of an epitope in one of the isoforms was compensated by the presence of the same epitope in the other two isoforms in order to minimize the absence of putative vital epitopes that may generate neutralizing antibodies. The mutated PLDs were expressed in a bacterial system and yielded large amounts of soluble proteins purified in a one-step process, which facilitated the testing procedures regarding the application of these antigens. The electrophoretic mobility of mutated PLDs did not undergo major changes when compared with their wild-type isoforms, even when charged residues (H12, H47, E32 and D34) were replaced by alanine. Mutations also reduced the enzymatic activity of PLDs on degrading sphingomyelin to very low levels. In addition, the ability of mutated PLDs to trigger cutaneous loxoscelism was decreased to residual levels, which was confirmed by the histopathological analysis of the skin. These results confirmed previous literature data, which indicated that the toxic activities of phospholipases D from *Loxosceles* spiders are dependent on their catalytic activity [15–17,28,54,55], and that the mutations carried out resulted in inactive phospholipases D isoforms [26,28]. It is interesting to note that the edematogenic activity of the mutated PLDs was also inhibited, confirming literature that shows

that this activity is correlated with the enzymatic activity of phospholipases D [5,14].

To serve as a vaccine, mutations performed in antigens need to be efficient in generating sera that neutralizes the toxic activities, but ideally, they cannot change the repertoire of antigenic determinants. As described herein, all phospholipases D, wild-type and mutated, were recognized by polyclonal sera against the crude venoms of *Loxosceles intermedia*, *L. laeta* and *L. gaucho*. Thus, despite the inactivation of the toxic properties, all mutated PLDs maintained their antigenic characteristics. However, it is noteworthy that the immunological reactivity of recombinant PLDs with their homologous sera was greater for both wild-type and mutated isoforms. If the goal is to achieve a protective effect to the endemic loxoscelism, these data indicate that the use of at least one PLD from each species that prevail in South America will probably elicit a more complete immunological response.

In addition to the linear epitopes, the preservation of conformational epitopes is essential to confer specific interactions between antigens and antibodies' paratopes [56]. Thus, it is expected that the mutated PLDs with potential applicability as antigens retain the solubility, as well as the secondary and tertiary structures of their wild-type counterparts. For the mutated PLDs studied herein, the secondary structures were previously determined by circular dichroism spectroscopy [26,28] and results indicated that the absence of biochemical and biological activities was not due to a loss of their spatial structure. The comparative analysis between the molecular models of mutant PLDs and their wild-type isoforms strengthened these data, showing that the mutations did not induce significant changes in the structural organization of these molecules. Furthermore, in silico data regarding the topologies, surface electrostatic charges and solvent exposure profile pointed out that the substitution of the charged amino acid residues H12, H47, E32 and D34 by alanine residues (nonpolar and uncharged) did not cause major changes. As shown by previous studies, conformational epitopes are essential for the generation of neutralizing antibodies [57,58], and that is why such findings are in line with the potential use of mutated PLDs as effective antigens.

As mutated PLDs were confirmed as non-toxic and structurally related to wild-type molecules, they were used as antigens in a vaccination protocol performed in rabbits. This protocol comprised equal amounts of the non-toxic PLDs of *L. intermedia*, *L. laeta* and *L. gaucho* and included aluminum hydroxide as adjuvant, which is inexpensive and thoroughly used in humans [40]. The general health status of the rabbits was periodically evaluated by a veterinarian, and no alterations on clinical aspects were observed over the vaccination process. In addition, biochemical and hematological parameters confirmed that the immunization protocol was innocuous to the animals. Evaluation of sera from vaccinated rabbits indicates that the protocol gave rise to the production of antibodies that recognized toxins from three crude venoms, confirming the humoral immunogenic potential of these molecules. The ELISA immunoassay indicated that the mutated PLDs share conformational antigenic identities with venom toxins of studied spiders, and immunoblotting confirmed that the antibodies raised against native toxins were able to recognize the mutated PLDs in their typical electrophoretic profile (~35 kDa). Overall, the reactions observed were quite similar and they highlight the protective effect of the antibodies raised against the mutated toxins. These data altogether corroborate with a previous study that showed the great immunogenic potential of *Loxosceles* PLDs [53], and suggest the great potential of these toxins attenuated by mutations to be used as tools to produce immunobiological products.

The neutralization ability of antibodies produced by vaccinated rabbits was tested and showed that the preincubation of hyperimmune sera with *L. intermedia* crude venom reduced the development of signs of loxoscelism, i.e., skin lesion and edema. The challenge of vaccinated rabbits was performed with ten micrograms of each venom (*L. intermedia*, *L. laeta* and *L. gaucho*), which is a higher amount than the usual minimum necrotizing dose as previously shown. As described, the

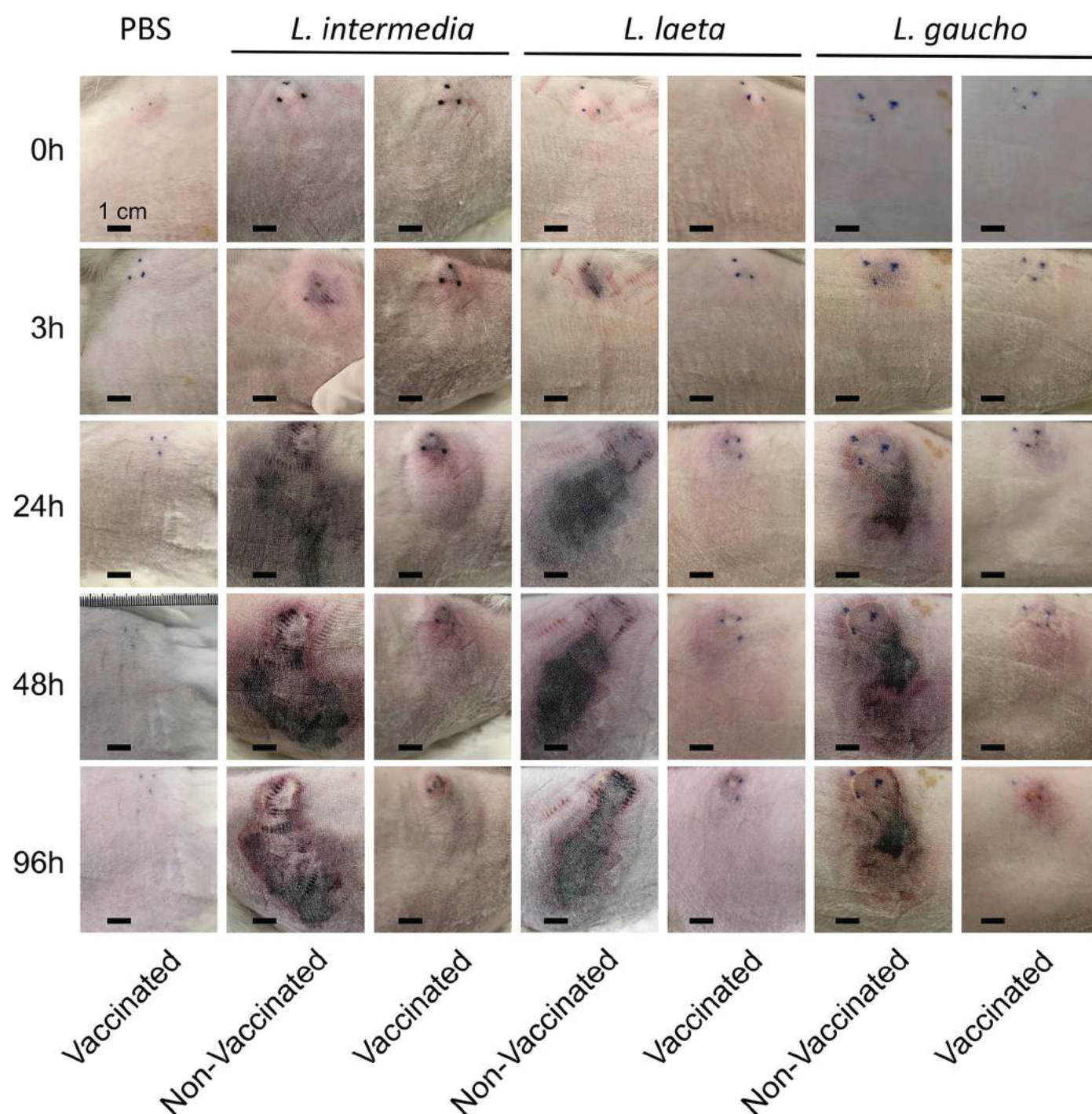


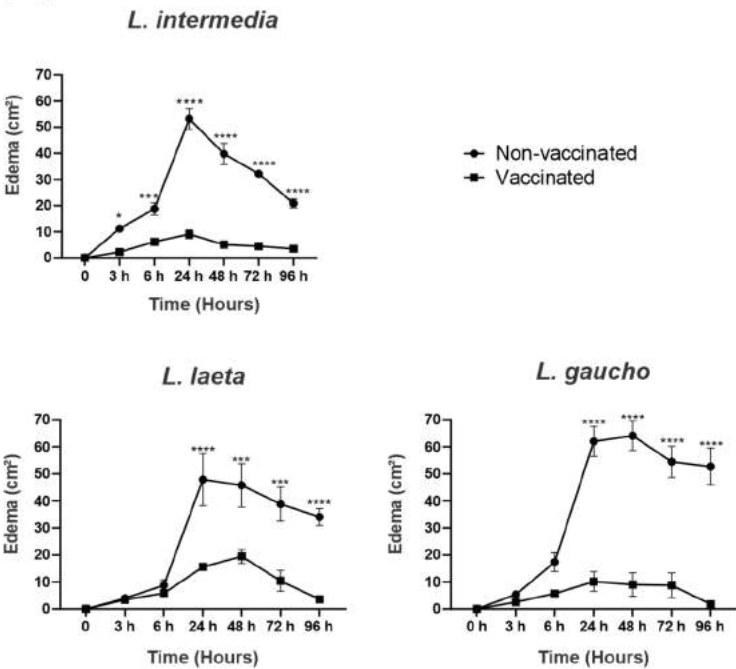
Fig. 8. Macroscopic visualization of lesions in the skin of rabbits exposed to *L. intermedia*, *L. laeta* or *L. gaucho* venoms. Rabbits immunized or non-immunized with the pool of mutated recombinant PLDs were intradermally inoculated with crude venoms from different *Loxosceles* species (10 µg) or PBS (negative control) 15 days after the last immunization protocol. Venoms were injected in the center of the triangle indicated by three dots. Images are representative of an experiment performed with 3 animals. Additional images of other tested animals are available as supplementary figures (2, 3 and 4). Scale bars are shown at left of each picture and represent 1 cm.

vaccination protocol reduced the development of the skin lesions induced by the venoms, determined by macroscopic necrosis and edema. The immunization protocol prevented the development of much of the edema induced by crude venoms, thus protecting animals from around 90% of the swelling triggered in the non-vaccinated rabbits. Various studies have observed that edema is more difficult to neutralize than other signs such as dermonecrosis and hemorrhage [29,59,60]. In addition, since dermonecrosis is the hallmark sign of loxoscelism and a matter of great concern for clinicians, the protection induced by the

vaccination protocol tested herein is remarkable, since the protection against dermonecrosis was above 95% for the three venoms after 24 h of the challenge (97% for *L. intermedia* venom, 97.3% for *L. laeta* venom and 95.8% for *L. gaucho* venom).

Although systemic disorders develop in only a few cases of loxoscelism, they are usually worrisome since most cases of death after Brown spider envenoming occur due to this. Animals such as mice and rats are good models to study the systemic effects of Brown spider venoms, such as nephrotoxicity [15,16]. The vaccination scheme using

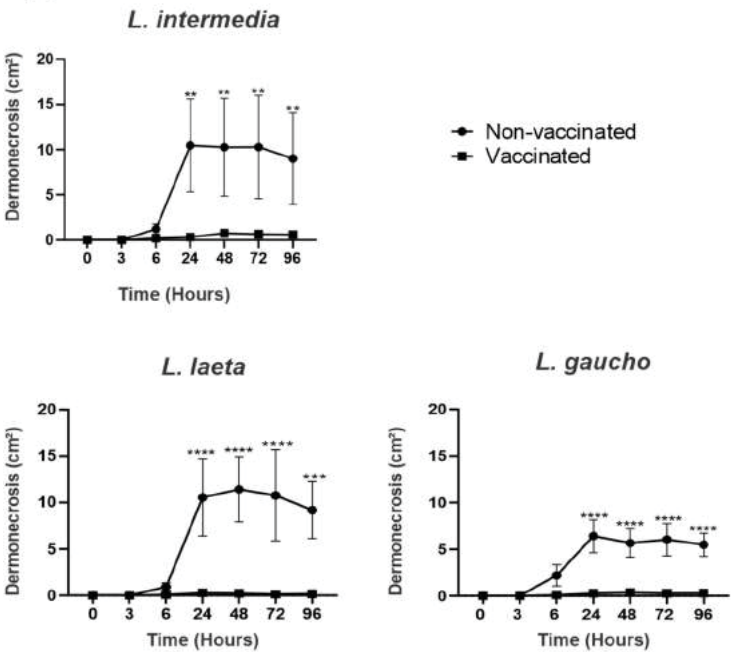
A



Inhibition of edema (%)

	3 h	6 h	24 h	48 h	72 h	96 h
<i>L. intermedia</i>	80,2	67,1	82,9	87,2	86,0	83,3
<i>L. laeta</i>	-	-	67,5	57,5	73,2	89,7
<i>L. gaucho</i>	-	-	83,6	85,9	83,9	96,6

B



Inhibition of dermonecrosis (%)

	24 h	48 h	72 h	96 h
<i>L. intermedia</i>	97,0	92,8	93,9	93,4
<i>L. laeta</i>	97,3	98,1	98,8	98,1
<i>L. gaucho</i>	95,8	93,8	95,5	94,8

Fig. 9. Prevention of dermonecrosis and edema caused by *L. intermedia* venoms in vaccinated rabbits. Rabbits immunized or non-immunized with mutated PLDs were challenged with *L. intermedia*, *L. laeta* or *L. gaucho* crude venoms (10 µg). Results shown in the graphs represent the mean area of the dermonecrotic (A) or edematous (B) region ($\pm$ SEM, n = 3) at time intervals (* p < 0.1, ** p < 0.01, *** p < 0.001 and **** p < 0.0001). Tables below the graphs display the percentages of relative inhibition compared to the control (100% of dermonecrosis or edema) with vaccinated animals (statistically significant points). Values highlighted in gray depict the greatest reductions (%).

mutated PLDs protected mice from the lethal effect triggered by *L. intermedia* venom, reducing the mortality rate in approximately 80% of the animals challenged with the whole venom. It is already known that the Brown spider venom is lethal to mice mainly due to its nephrotoxicity, and that PLDs have a key role in this pathological process [15,16,61–63]. Thus, it is reasonable to suggest that the specific antibodies raised against the inoculated mutated PLDs neutralized the activity of native PLDs present in *L. intermedia* venom and abolished their toxicity to the kidneys.

The results described in this work will guide further pre-clinical studies in order to establish a solid foundation for future clinical studies. Although envenomation caused by Brown spiders is very frequent in South America, it is still considered as a neglected disease and therapeutic interventions remain to be developed. Our findings reveal the great potential of the recombinant mutated PLDs to the development of a rational and efficient approach to manage accidents involving *Loxosceles* spiders.

5. Conclusion

In summary, our results describe the production of recombinant mutated isoforms of phospholipases D from the venoms of three endemic species in South America. These mutations decreased to residual levels the sphingomyelinase and dermonecrotic activities of these toxins, but did not change the antigenic and immunogenic features of these proteins. These non-toxic phospholipases D used as antigens in an immunization protocol established in rabbits generated antibodies that recognized native toxins in the crude venoms. This vaccination protocol protected rabbits from noxious activities of crude venoms such as the induction of dermonecrosis, gravitational spread and edema, which are harmful signs seen after envenoming. The vaccination of mice, in addition, protected these animals from the lethal effect of the venom injected. Data reported open up the possibility to use mutated molecules to produce protective vaccines to populations exposed in regions where accidents are recurrent and of public health concern. Moreover, results reported herein indicate that the mutated phospholipases D may be further studied as suitable candidates as antigens for antivenom production used against loxoscelism.

CRedit authorship contribution statement

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2021.10.005>.

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Article

Brown Spiders' Phospholipases-D with Potential Therapeutic Applications: Functional Assessment of Mutant Isoforms

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Abstract: Phospholipases-D (PLDs) found in *Loxosceles* spiders' venoms are responsible for the dermonecrosis triggered by envenomation. PLDs can also induce other local and systemic effects, such as massive inflammatory response, edema, and hemolysis. Recombinant PLDs reproduce all of the deleterious effects induced by *Loxosceles* whole venoms. Herein, wild type and mutant PLDs of two species involved in accidents—*L. gaucho* and *L. laeta*—were recombinantly expressed and characterized. The mutations are related to amino acid residues relevant for catalysis (H12-H47), magnesium ion coordination (E32-D34) and binding to phospholipid substrates (Y228 and Y228-Y229-W230). Circular dichroism and structural data demonstrated that the mutant isoforms did not undergo significant structural changes. Immunoassays showed that mutant PLDs exhibit conserved epitopes and kept their antigenic properties despite the mutations. Both in vitro (sphingomyelinase activity and hemolysis) and in vivo (capillary permeability, dermonecrotic activity, and histopathological analysis) assays showed that the PLDs with mutations H12-H47, E32-D34, and Y228-Y229-W230 displayed only residual activities. Results indicate that these mutant toxins are suitable for use as antigens to obtain neutralizing antisera with enhanced properties since they will be based on the most deleterious toxins in the venom and without causing severe harmful effects to the animals in which these sera are produced.

Keywords: *Loxosceles*; venom enzymes; phospholipases-D; mutant phospholipases-D; site-directed mutations

1. Introduction

Spiders of the *Loxosceles* genus are commonly referred to as brown spiders and have been broadly implicated in accidents with humans [1,2]. In Brazil, *Loxosceles* spiders are considered a public health issue, being responsible for approximately 8000 accidents each year over the period of 2017–2019 [3]. *Loxosceles intermedia*, *Loxosceles gaucho* and *Loxosceles laeta* are the most important species from this medical standpoint. Envenomation caused by *Loxosceles* spiders generates loxoscelism is the term used to describe the clinical findings observed following the bites, and is categorized into two variants: cutaneous

and systemic. Cutaneous loxoscelism is represented by local reactions seen near or at the bite site, such as swelling, erythema, ecchymosis, cutaneous rash, inflammatory response, and dermonecrotic lesion with gravitational spreading. Systemic loxoscelism can result in thrombocytopenia, intravascular hemolysis, acute renal failure, and in some cases, can lead to death in patients [1,2,4].

Loxosceles spiders' venoms have a colorless appearance and consist of a complex mixture enriched in proteins and glycoproteins with molecular masses ranging from 3–45 kDa [1,2,4]. Amongst the great diversity of toxins found in these venoms, the Phospholipases-D (PLDs) are undoubtedly the most well-studied and functionally well-characterized molecules. PLDs cause the majority of the pathological effects triggered by *Loxosceles* crude venoms such as massive inflammatory response and hemolysis, in addition to dermonecrosis, which is the reason why they are also known as dermonecrotic toxins. PLDs present in the venom of *Loxosceles* spiders were initially characterized as sphingomyelinases-D based on their ability to cleave sphingomyelin, resulting in choline and ceramide-1-phosphate [5,6]. Currently, these molecules are classified as a phospholipases-D due to the wide spectrum of lipids that they can cleave such as lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidyl-inositol, lyso- platelet activating factor (PAF), cyclic phosphatidic acid, in addition to sphingomyelin [7–9].

Molecular biology techniques enabled the identification and heterologous expression of several recombinant PLDs isoforms from *Loxosceles* spiders' venoms. These isoforms were characterized and contributed to unveil their participation on the pathophysiology of envenoming. *L. laeta* venom gland transcriptome showed that 16.3% of the ESTs encode PLDs [10]. Two PLDs from *L. laeta*, which were named SMase I and SMase II, were recombinantly obtained, and both displayed sphingomyelinase and complement-dependent hemolytic activities, in addition to triggering dermonecrosis in rabbit's skin [11,12]; furthermore, polyclonal antibodies raised against SMase I efficiently neutralized its dermonecrotic activity after toxin and serum were incubated prior to injection in rabbits [11]. Machado and colleagues [13] performed proteomic analysis of *L. intermedia*, *L. laeta*, and *L. gaucho* crude venoms by using two-dimensional electrophoresis, revealing spots corresponding to PLDs (30 to 35 kDa); these spots were further analyzed through a combination of mass spectrometry, immunoblotting and chromatography, which resulted in the identification of 11 *L. gaucho* PLDs isoforms. LgRec1, a *L. gaucho* PLD recombinantly obtained, elicited platelet aggregation, induced sphingomyelinase cleavage and prompted direct hemolytic activity; LgRec1 also provoked local reactions near the injection site (i.e., edema and erythema) and dermonecrosis in rabbit's skin, which were neutralized when LgRec1 was previously incubated with arachnidic antivenom, anti-*L. gaucho*, or anti-LgRec1 sera [14].

Structural analyses identified PLDs amino acid residues considered important for catalysis, substrate binding, and metal ion binding [15–19]. The mechanisms by which these PLDs interact and cleave their substrates have been described and are related to two theoretical models under discussion, which are the cleavage of phospholipids by hydrolysis or by transphosphatidylolation [2,9,15]. All of this information is relevant for extending biotechnological applications, such as the use of these molecules in the production of neutralizing antibodies for serum therapy or the diagnosis of loxoscelism [20–22].

In this work, we aimed to perform comparative biochemical and biological analyses of wild type and mutants of PLDs from *L. laeta* and *L. gaucho* venoms. Results herein obtained may provide rational parameters for the use of recombinant PLDs as antigens for the development of a second-generation anti-*Loxosceles* serum. In addition, we present relevant data for the production of other therapeutic inputs such as a protective vaccine to be used in areas where envenomation by *Loxosceles* spiders are epidemic.

2. Materials and Methods

2.1. Wild Type and Site-Directed Mutant PLDs

Wild type LfRecDT1 (GenBank: AY093599.1) and LgRecDT1 (GenBank: JX866729.1) constructions were obtained by using pET-14b plasmids as cloning vectors, according to the methods described by Chaim and colleagues [23]. Wild type constructions previously mentioned were used for the generation of site-directed mutations H12A-H47A, E32A-D34A, Y228A, and Y228A-Y229A-W230A. Initially, sense oligonucleotides responsible for introducing the intended mutations were designed using the QuikChange Primer Design tool (<https://www.agilent.com/store/primerDesignProgram.jsp> (accessed on 7 February 2021)) (Table 1). The mutated isoforms were produced using pET-14b wild type constructions as templates and the QuikChange® Multi Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) following manufacturers' instructions. For this, PCR reactions were performed in order to amplify the mutated sequences. Next, amplified sequences were incubated with the endonuclease DpnI, which recognizes and hydrolyzes methylated, non-mutated strands containing the 5'-Gm6ATC-3' target sequence. All of the mutated constructions were then transformed into chemically competent *Escherichia coli* XL10-gold bacterial strain (Agilent, Santa Clara, CA, USA). The colonies were subjected to plasmid mini-preparations using the WizardPlus SV Minipreps DNA Purification Systems® Kit (Promega, Madison, WI, USA), followed by sequencing reactions using the reagent BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Each clone was sequenced using the T7 sense primer or the T7 terminator primer by means of automated sequencing in the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Warrington, UK). The sequencing reactions were analyzed using ChromasPro (Version 2.6.4, Technelysium Pty Ltd., Brisbane, QLD, Australia) software to confirm the mutations produced. Comparison between wild type and mutated sequences was carried out using the ClustalW tool (<https://www.genome.jp/tools-bin/clustalw> (accessed on 7 February 2021)).

Table 1. Mutagenic oligonucleotides designed for the generation of site-directed mutations. The codons encoding residues of alanine are underlined.

Species	Mutation	Oligonucleotide Sequence (5'-3' Sense)
<i>Loxosceles laeta</i>	H12A	GTCCAATTTGGAACCTCGCTGCCATGGTGAACGCTGTTGCACA
	H47A	GCCTACTTACACTTACGCCGGAACGCCTTGCGACT
	Y228A	GGGATTCGGCAAATGGATTATCAATAAAATTGCCCTACTGGTCTGTAGACAA
	Y228A-Y229A-W230A	CGGCAAATGGATTATCAATAAAATTGCCGCGCCTCTGTAGA CAAAGTATCAACAACGAAGGC
	E32A-D34A	GGATCTTGGTGCAAACGCATTAGCCGCGGCCGTTACTTTTAAGGGATCAG TGCC
<i>Loxosceles gaucho</i>	H12A	CCTATATGGGTTATGGGTGCCATGGTTAACTCCCTCGCT
	H47A	AGCTAATCCTGAATACACATACGCCGGAATCCCTGCGATTGTGGA
	Y228A	GCGGGATCATTAACAAAGTGCCCTATTGGACAGTGGACAAACG
	Y228A-Y229A-W230A	GAAGCGGGATCATTAACAAAGTGCCGCGCCACAGTGGACAAAC GCCAATCGACAAG
	E32A-D34A	GCCTTGGATCGAATTCAATCGCCACAGCCGTGTATTGATAAGCAAGC

2.2. Recombinant Expression

The constructions containing the site-directed mutations were transformed into *Escherichia coli* BL21(DE3)pLysS and plated on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL). An isolated colony was inoculated into 10 mL of liquid LB broth containing the antibiotics mentioned above at 37 °C for 16 h. Subsequently, the 10-mL culture was inoculated in 1 L of liquid LB containing the antibiotics at 37 °C and the culture growth was monitored until the optical density (OD) at 550 nm reached between 0.4 and 0.6. The isopropyl β-D-thiogalactoside (IPTG) inducer

was then added to a final concentration of 0.05 mM and the culture was allowed to grow for additional 3.5 h at 30 °C, under vigorous shaking. Bacterial cells were finally harvested by centrifugation (3500× g, 10 min) and the resultant pellet was resuspended in 20 mL of binding buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH 8.0, 500 mM NaCl, 10 mM imidazole). After adding lysozyme (1 mg/mL), the suspension was stored at −20 °C [23,24].

2.3. Protein Purification

The frozen resuspension was thawed, subjected to mechanical lysis using a French pressure cell press (3 cycles of 5 min on ice-pressure within 5000 and 10,000 psi) and centrifuged (9000× g, 30 min at 4 °C). The resulting supernatant was collected and 2 mL of Ni²⁺-NTA agarose resin was added to it, followed by incubation under gentle agitation for 1 h at 4 °C. The batch was exhaustively washed with washing buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH 8.0, 500 mM NaCl, 20 mM imidazole) until the OD at 280 nm reached 0.01/0 or until the readings remained constant. The recombinant protein was eluted using elution buffer (50 mM NaH₂PO₄/Na₂HPO₄ pH 8.0, 500 mM NaCl, 250 mM imidazole), dialyzed against phosphate-buffered saline (PBS: 100 mM NaCl, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, pH 7.4) and the purity the eluate was analyzed by 12.5% SDS-PAGE under reducing conditions (β-mercaptoethanol 5%) [24].

2.4. Circular Dichroism

Circular dichroism spectra were acquired to compare the structure of the recombinant wild type proteins and their mutants. The purified proteins were dialyzed against phosphate buffer (20 mM NaH₂PO₄/Na₂HPO₄ pH 7.4 and 150 mM NaCl) at 4 °C and then diluted in the same buffer to a final concentration of 0.5 mg/mL. The spectra were acquired in a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan) using a 1 mm path-length cuvette. Each spectrum, recorded at 0.5 nm intervals, consists of an average of 8 measurements performed at a rate of 50 nm/min, with a response time of 8 s and a bandwidth of 1 nm. The temperature was maintained constant at 20 °C [24,25]. Measurements were performed in triplicate. Secondary structures of the recombinant proteins were predicted using the online tool K2D3 (<http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3> accessed on 7 February 2021).

2.5. Immunoassays

The interaction between wild type isoforms and mutated toxins with antibodies raised against *L. gaucho* or *L. laeta* crude venoms produced in rabbits were performed by both western blot and ELISA immunoassays. Initially, protein quantification was performed according to Bradford [26]. For the western blotting analysis, samples containing 2.5 µg of each protein were submitted to SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane using the Trans-Blot[®] SD Semi-Dry Electrophoretic Transfer Cell System (Bio-Rad, Hercules, CA, USA) for 30 min at 25 V. The membranes were incubated with hyperimmune sera raised against *L. gaucho* or *L. laeta* crude venom (1:3000). Control of antivenom sera specificity was performed using pre-immune serum. After incubation with the mentioned sera, membranes were treated with secondary alkaline phosphatase-conjugated anti-IgG antibody (1:5000) (Sigma-Aldrich, St. Louis, MO, USA). Finally, results were developed using BCIP/NBT substrate reaction (Promega, Madison, WI, USA). For the ELISA antibody capture assay, a 96-well microplate (Nunc MaxiSorp, Roskilde, Denmark) was coated with a solution containing 1 µg/mL protein (100 µL per well) in 0.02 M sodium bicarbonate buffer pH 9.6 overnight at 4 °C. After that, the plate was washed with PBS-Tween 20 solution (0.05%), blocked with PBS-casein (2%) for 1 h at 37 °C and the reactions were incubated for 1 h with hyperimmune sera against *L. gaucho* or *L. laeta* crude venom (1:10,000) at 37 °C. Control of antivenom sera specificity was performed with pre-immune serum. Thereafter, the plate was washed with PBS-Tween 20 solution (0.05%) and wells were incubated with secondary antibodies against IgG-rabbit conjugated with horseradish peroxidase (Sigma Aldrich, San Luis, MO, USA)

(1:5000) for 1 h at 37 °C. Finally, the colorimetric reactions were developed using a solution containing 0.4 mg/mL ortho-phenylene diamine (OPD) with 4 µL/mL H₂O₂ in citrate buffer for peroxidase pH 5.0 (50 mM NaH₂PO₄, 24 mM citric acid) for 30 min at 25 °C and reactions were then stopped with 50 µL of 1 M H₂SO₄. Absorbance values were measured at 490 nm in spectrophotometer (Meridian ELX 800) [23,27]. Each treatment was assessed in quadruplicate and the results are shown as mean ± SEM of three independent experiments.

2.6. Structural Analyses

The analysis of the three-dimensional structures was performed using the crystallographic structures of the PLDs of *L. intermedia* (Protein Data Bank (PDB) ID: 3RLH) and *L. laeta* (PDB ID: 2FR9), and the structural models of the mutants built using SWISS-MODEL based on their respective PDB IDs as a template. The native and mutants *L. gaucho* PLDs models were constructed using the *L. intermedia* PDB ID 3RLH as a template. The electrostatic potential was calculated using the program APBS according to the molecular electrostatic potential ranging from −5.0 to 5.0 [28] and implemented in PYMOL (version 2.4, GitHub, San Francisco, CA, USA) [29].

2.7. Sphingomyelinase Activity

Sphingomyelinase activity was determined using the Amplex Red Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). To measure the sphingomyelinase activity of the recombinant toxins, they were incubated (10 µg) in microtubes containing the Amplex Red reagent mixture in reaction buffer (Tris-HCl 100 mM pH 7.4 containing 10 mM MgCl₂), including the substrate sphingomyelin (SM: egg sphingomyelin, chicken). Wild type recombinant isoforms, LIRecDT1 and LgRecDT1, were used as positive controls and reactions containing only the Amplex Red reagent with the substrate SM (without any toxin) were used as negative controls. After incubation at 37 °C for 30 min, the reactions were transferred from the microtubes to a 96-well black fluorimeter plate and the fluorescence was measured on a fluorimeter (Tecan Infinite M200, Männedorf, Switzerland) using excitation wavelength at 540 nm and emission at 570 nm. All treatments were performed in triplicate and the results represent an average of three experiments ± SEM.

2.8. High Performance Thin Layer Chromatography (HPTLC)

The phospholipase activity of the recombinant PLDs was also assessed by HPTLC. For this, samples of 50 µg of each PLD were incubated with 1 mg/mL of sphingomyelin (SM) for 2 h at 37 °C. The organic phase was recovered using 1 mL of a water:butanol solution (1:1), from which 200 µL was collected and dried. Next, the organic phase was resuspended in chloroform and 20 µL was then applied to the base of the thin-layer chromatographic silica plate with capillary tubes. A solution containing chloroform:methanol:methylamine (65:35:10, v/v/v) was used as the mobile phase [24]. The samples were visualized as blue spots when the plates were sprayed with molybdenum blue dye. The results shown are from one experiment out of two independent experiments that were performed.

Densitometry of the digital images from the HPTLC plates were acquired using the GeneSnap software for G: Box Chemi XL (Syngene, Cambridge, UK) and quantified by the Quantity One software for Chemic Doc XRS (Bio-Rad, Hercules, CA, USA). The percentages of cleavage by LIRecDT1 and LgRecDT1 were considered as 100%. Results represent the densitometry of one experiment.

2.9. Animals

Adult Swiss mice weighing between 25 and 30 g were supplied by the Animal House of the Biological Sciences Center of the Federal University of Paraná and adult New Zealand rabbits weighing approximately 3 kg were acquired from the Canguiri Experimental Farm of Federal University of Paraná and kept at Production and Research Center of Immunobiological Products (CPPI, Brazil). Animal experiments were carried out in accordance to the Brazilian Guidelines for Care and Use of Animals for Scientific and

Teaching Purposes established by the National Council for Control of Animal Experimentation (CONCEA) and the International Guidelines for animal experimentation. The Ethics Committee for Animal Use from the Biological Sciences Sector of the Federal University of Parana (CEUA/BIO-UFPR) approved all of the protocols using animals in this work (Statement number 1112).

2.10. Hemolytic Activity

The hemolysis assay was conducted as described by Chaves-Moreira and colleagues [7], with modifications. Initially, 4 mL of rabbit blood was harvested with acidic EDTA Na⁺ 5% and centrifuged at $2000 \times g$ for 15 min, followed by aspiration and discard of the overlying platelet-rich plasma and buffy coat. The remaining erythrocytes were washed three times in Tris buffer sucrose (TBS: 250 mM sucrose, 10 mM Tris/HCl, pH 7.4). After washing steps, the erythrocytes were resuspended in TBS with 1 mM CaCl₂ to obtain an initial concentration of 5×10^8 cells/mL. Aliquots containing 200 μ L of the erythrocyte suspension were placed in microtubes, followed by addition of 200 μ L of a solution containing wild type or mutant PLDs (10 μ g) solubilized in TBS. Negative control reactions contained only cell suspensions just in TBS and positive control reactions were constituted by cell suspensions in distilled water and Triton X-100 0.1% (v/v). The reactions were incubated under gentle and constant agitation at 37 °C. After the treatment times (0, 3, 6, 12, and 24 h), the microtubes were centrifuged at 4 °C using a refrigerated microfuge (Centrifuge 5804 R, Eppendorf, Hamburg, Germany), for 3 min at $3500 \times g$. The absorbance of free hemoglobin in the supernatant was read at 550 nm in a spectrophotometer (Meridian ELx 800, BioTek Instruments, Winooski, VT, USA). Absorbance readings were converted to percent hemolysis considering the positive control absorbance as 100% lysis. Each treatment was assessed in triplicate and the results are shown as mean $\pm$ SEM of three independent experiments performed.

2.11. Vascular Permeability Assay

In order to evaluate alterations triggered by the recombinant PLDs in the microvascular permeability, the Miles assay was performed [24,30]. For this analysis, mice were intravenously injected with Evans Blue diluted in PBS (30 mg/kg), followed by intradermal administration of wild type or recombinant mutant PLDs samples (10 μ g) 5 min later at the dorsolateral region of the animals. Each treatment group consisted of five animals and the negative control was represented by animals that received the vehicle (PBS) without any toxin. After 1 h, the mice were euthanized using ketamine (30 mg/kg) and xylazine (4 mg/kg) and the dye leakage was visualized (bluish stain). A tissue patch of the dorsal skin from each animal was removed and photographed.

2.12. In Vivo Dermonecrosis

PLDs dermonecrotic activity was evaluated after intradermal application of 10 μ g of the wild type and recombinant mutant toxins at a dorsolateral shaven area of rabbits' skin. PBS solution was administered to animals of the control group. The test was performed in duplicate for each sample. Animals were monitored throughout the evolution of the dermonecrotic reaction and macroscopic images were taken after 0, 3, 6, and 24 h post toxin injection. After the maximum exposure time (24 h), the rabbits were euthanized with ketamine (240 mg/kg) and xylazine (28 mg/kg), followed by tissue excision for histopathological analysis purpose [24].

2.13. Histological Methods for Light Microscopy

Excised rabbit skin sections were fixed in Alcohol–Formalin–Acetic Acid (ALFAC) fixative solution (ethanol 85%, formaldehyde 10%, and glacial acetic acid 5%) for 16 h at 4 °C, dehydrated in a graded series of ethanol and embedded in paraffin for 2 h at 58 °C. The fixed samples were processed in thin sections (4 μ m), placed on glass slides for histological analysis, and stained with hematoxylin and eosin (H&E). The sections were

analyzed and photographed under a light microscope Olympus BX41 with DP 72 camera for image capture (Olympus, Tokyo, Japan).

2.14. Statistical Analyses

Values were expressed as mean and standard error of the mean. Statistical analysis of the ELISA and hemolytic assays were performed using two-way analysis of variance (ANOVA) followed by post-hoc Tukey test for average comparisons using the GraphPad Prism 6 program (GraphPad Software, San Diego, CA, USA), considering p value < 0.001 as statistically significant. Statistical analysis of the Sphingomyelinase activity assay was performed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test for average comparisons using the GraphPad Prism 6 program, considering p value < 0.05 as statistically significant.

3. Results

3.1. Analysis of Sequences Encoding Wild type PLDs, Site-Directed Mutagenesis, and Production of Mutant PLDs Isoforms

The cDNA-deduced amino acid sequences encoding the wild type PLDs of *L. laeta* (LlRecDT1) and *L. gaucho* (LgRecDT1) revealed 285 and 280 amino acid residues, respectively, while the degree of sequence identity between these PLDs is 56.84% (Figure 1A). In addition, the two sequences exhibit the conservation of important amino acid residues, which were identified by crystallographic findings [15,31] (Figure 1A). Therefore, specific primers (Table 1) allowed the obtainment of mutants regarding important amino acid residues important for the enzyme structure and that are involved in the functionality of the PLDs: LlRecDT1 H12A-H47A and LgRecDT1 H12A-H47A mutants produced contain mutations in amino acid residues essential for the PLD's catalytic activity (H12 and H47). LgRecDT1 E32A-D34A mutant obtained display mutations in amino acid residues required for the coordination of the magnesium ion (E32 and D34). LlRecDT1 Y228A and LgRecDT1 Y228A, as well as LlRecDT1 Y228A-Y229A-W230A and LgRecDT1 Y228A-Y229A-W230A mutants, were generated with mutations associated with three amino acids residues containing aromatic side chains and which are located at the PLD's binding site to lipid substrates (Y228, Y229, and W230) [2,15,20,31]. All selected amino acid residues were replaced by an alanine residue. The wild type and mutated PLDs sequences were cloned in pET-14b vector and sequencing analysis confirmed the constructions (Figure 1B,C). These constructions were then transformed into BL21(DE3)pLysS *E. coli* competent cells.

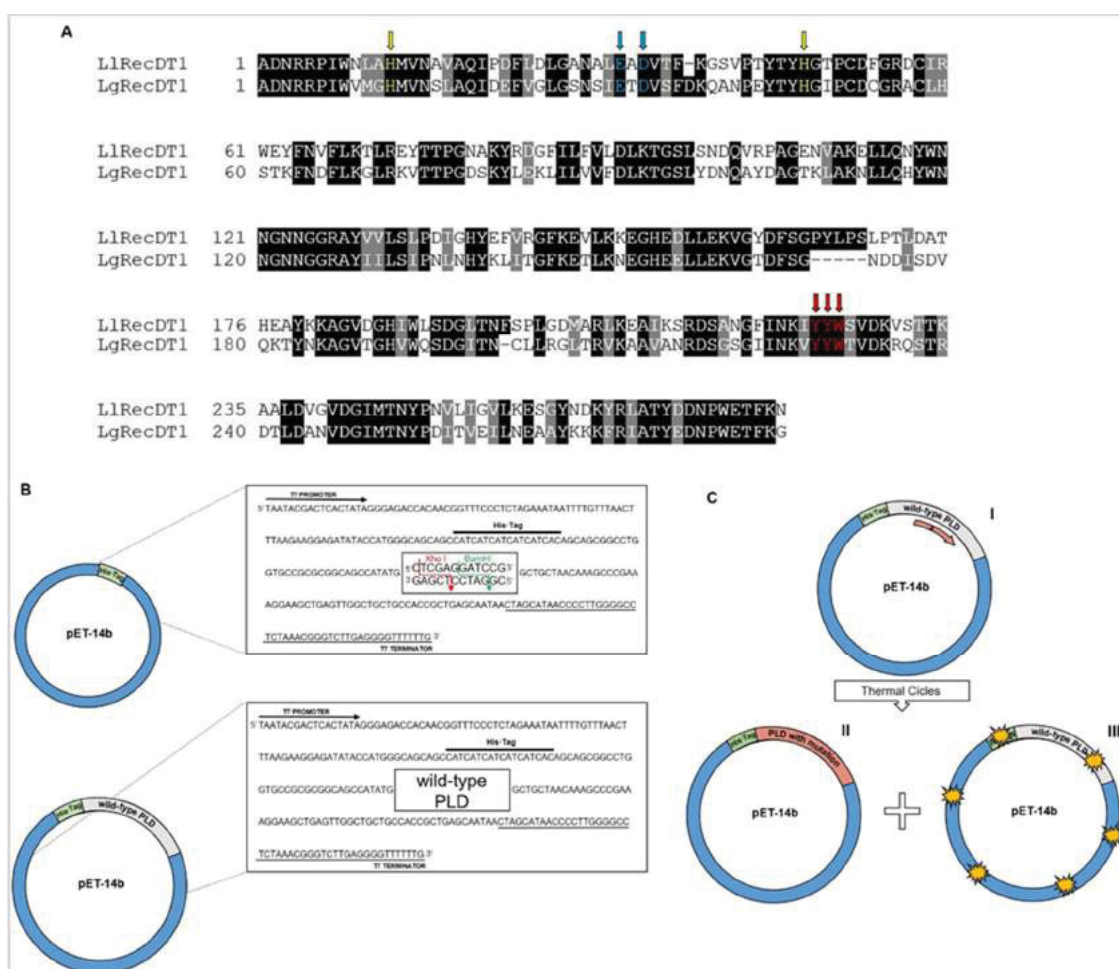


Figure 1. Overview of the wild type and mutant Phospholipases-D (PLDs)—sequence alignment and scheme of the constructions produced for heterologous expression. (A) Alignment of the cDNA-deduced amino acid sequences encoding the wild type PLDs of *L. gaucha* (LgRec1—GenBank: JX866729.1) and *L. laeta* (SMase I—GenBank: AY093599.1). Box shade representations show the conserved amino acid residues shaded in black and conservative substitutions are highlighted in gray. Yellow arrows indicate the sites of H12A-H47A mutations; blue arrows show the E32A-D34A mutation, only for *L. gaucha*; red arrows point out the site of both mutagenesis Y228A and Y228A-Y229A-W230A; (B) schematic representations of pET-14b vector (Promega) displaying the restriction sites for XhoI and BamHI enzymes and the N-terminal 6× His-tag, and the construction containing the wild type PLD sequence cloned in the vector; (C) schematic representation of the steps followed to obtain the constructions containing the sequence encoding mutant PLDs. The red arrow represents the mutagenic primer annealing with the wild type PLD coding sequence (I); after thermal cycles, the enzyme blend closes the nicks generating a construct with the specific mutation (II); and, prior to transformation in BL21(DE3)pLysS *E. coli*, the DpnI enzyme digests the methylated remaining wild type templates (III).

3.2. Expression and Purification of Recombinant PLD Wild type and Mutant Isoforms

Wild type and mutated PLDs were expressed in *E. coli* BL21 (DE3)pLysS and purified as soluble products using affinity chromatography (Figure 2A,B). The yield of all recombinant proteins ranged from 6 to 12 mg/L. Attempts to obtain the mutant isoform LlRecDT1 E32A-D34A were unsuccessful, resulting in aggregation and consequent production of an insoluble product (data not shown). As shown in Figure 2A,B, all expressed recombinant isoforms displayed molecular masses of approximately 35 kDa as predicted by theoretical analysis and described in the literature [11,14].

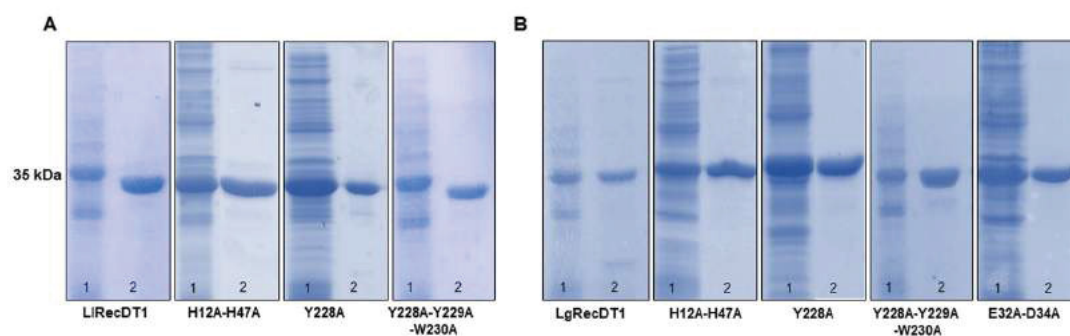


Figure 2. PLDs recombinant expression and protein purification analyzed by 12.5% SDS-PAGE under reducing conditions and stained with Coomassie blue dye. Lanes numbered as 1 represent samples after expression in *E. coli* BL21 (DE3)pLysS cells for 3.5 h at 30 °C with 0.05 mM isopropyl β -D-thiogalactoside (IPTG). Lanes numbered as two represent samples of purified PLDs (5 μ g) after purification by Ni-NTA affinity chromatography: (A) *L. laeta* PLDs—LiRecDT1 and its mutants; (B) *L. gaucha* PLDs—LgRecDT1 and its mutants.

3.3. Circular Dichroism Spectroscopy Analysis

In order to verify the secondary structure profiles and the overall folding of the recombinant toxins produced, normalized solutions of *L. laeta* and *L. gaucha* purified recombinant PLDs were analyzed by circular dichroism (CD) spectroscopy, followed by deconvolution analysis of the generated spectra. As shown in Figure 3A,B, the spectra obtained and deconvolution data indicated similar proportions of alpha-helices, beta-sheets, and other structures for all recombinant PLDs, pointing out that regardless the incorporated mutations, the mutant toxins maintain their native-like conformation when compared to their respective wild type isoforms [24].

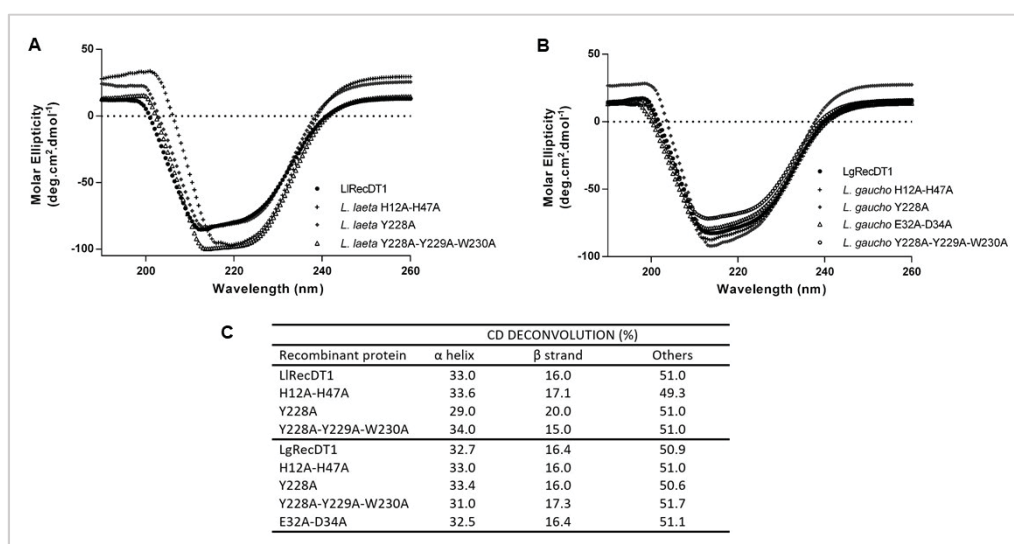


Figure 3. PLDs secondary structures analyzed by circular dichroism (CD). CD spectra were acquired using proteins samples at a concentration of 0.5 mg/mL in phosphate buffer solution, pH 7.4 at 20 °C: (A) LiRecDT1 and its mutants; (B) LgRecDT1 and its mutants; (C) spectra deconvolution showing the percentages of each secondary structure in the recombinant PLDs.

3.4. Molecular Modeling Analysis

Using as parameters *L. intermedia* and *L. laeta* PLDs crystallography data available [15,31,32], a molecular modeling analysis was performed to compare electrostatic potentials and solvent accessible surface areas between wild type PLDs and their mutants (Figure 4). Global Model Quality Estimation score and Qualitative Model Energy

Analysis scores presented values, which indicated high reliability in the generated models. Molecular modeling examination did not show significant differences in the topographic organization (Figure 4A,B) and charges densities (Figure 4C) when mutant PLDs were compared to their wild type counterparts. Together with CD data, these results reinforce that the mutations did not cause substantial structure changes.

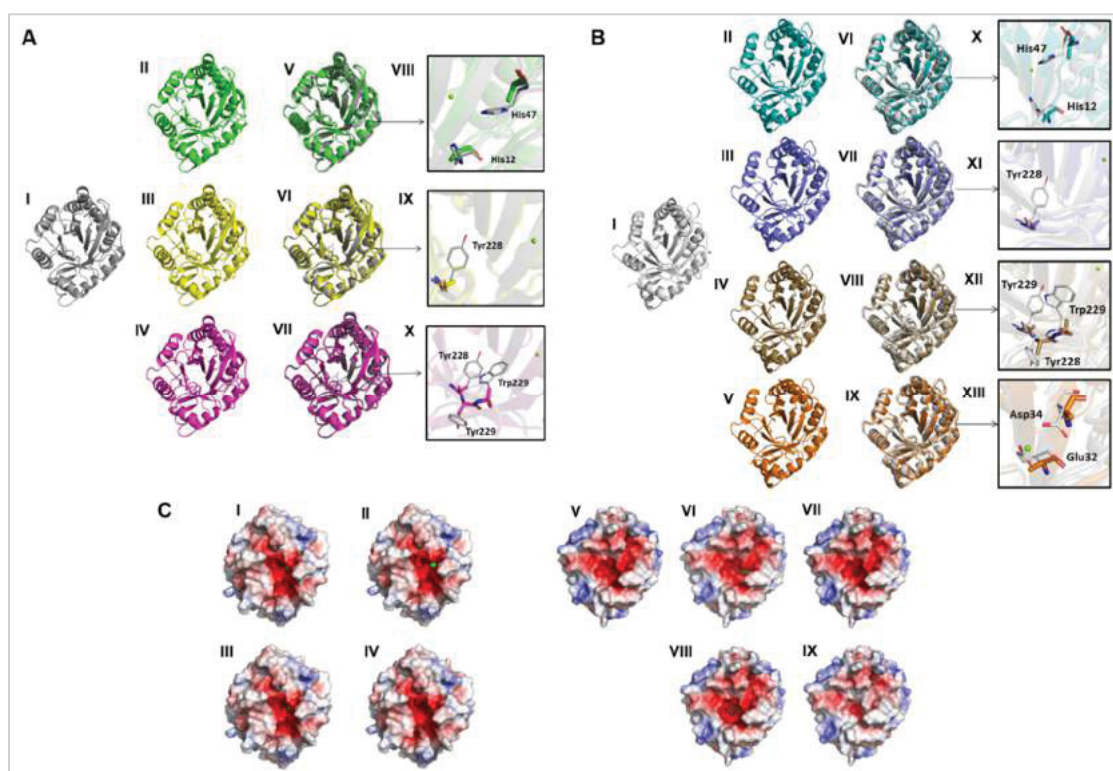


Figure 4. (A) Cartoon representation of the crystal structure of native PLD of *L. laeta* and molecular models of *L. laeta* mutants. The magnesium ion is represented by green spheres. (I) Crystal structure of the native *L. laeta* PLD in gray (Protein Data Bank ID: 2FR9); (II) structural model of *L. laeta* H12A-H47A in green; (III) structural model of *L. laeta* Y228A in yellow; (IV) structural model of *L. laeta* Y228A-Y229A-W230A in pink; (V) superposition of I and II; (VI) superposition of I and III; (VII) superposition of I and IV; (VIII) zoom on mutated residues with native H12 and H47 represented by lines with gray side chain and mutated A12 and A47 represented by sticks with green side chain; (IX) zoom on mutated residues with native Y228 represented by lines with gray side chain and mutated A228 represented by sticks with yellow side chain; (X) zoom on mutated residues with native Y228-Y229-W230 represented by lines with gray side chain and mutated A228-A229-A230 represented by sticks with pink side chain; (B) cartoon representation of the molecular model of native and mutant *L. gauchoi* PLD. The magnesium ion is represented by green spheres. (I) Structural model of native *L. gauchoi* PLD in gray; (II) structural model of *L. gauchoi* H12A-H47A in cyan; (III) structural model of *L. gauchoi* Y228A in blue; (IV) structural model of *L. gauchoi* Y228A-Y229A-W230A in brown; (V) structural model of *L. gauchoi* E32A-D34A in orange; (VI) superposition of I and II; (VII) superposition of I and III; (VIII) superposition of I and IV; (IX) superposition of I and V; (X) zoom on mutated residues with native H12 and H47 represented by lines with gray side chain and mutated A12 and A47 represented by sticks with cyan side chain; (XI) zoom on mutated residues with native Y228 represented by lines with gray side chain and mutated A228 represented by sticks with blue side chain; (XII) zoom on mutated residues with native Y228-Y229-W230 represented by lines with gray side chain and mutated A228-A229-A230 represented by sticks with brown side chain; (XIII) zoom on mutated residues with native E32-D34 represented by lines with gray side chain and mutated A32-A34 represented by sticks with orange side chain; (C) electrostatic potential visualization of *Loxosceles laeta* and *L. gauchoi* PLDs. Surface charge distribution around the surface of *L. laeta* native PLD (I) and its mutants H12A-H47A (II), Y228A (III), Y228A-Y229A-W230A (IV). The potentials red, white, and blue represent negative, neutral and positive charges, respectively. Electrostatic potential visualization of *L. gauchoi* PLDs. Surface charge distribution around the surface of *L. gauchoi* native PLD (V) and its mutants H12A-H47A (VI), Y228A (VII), Y228A-Y229A-W230A (VIII), and E32A-E34A (IX). The potentials red, white, and blue represent negative, neutral, and positive charges, respectively.

3.5. Immunological Cross-Reactivity between Recombinant PLDs and Native Venom Toxins

To examine the immunological cross-reactivity among antibodies against native venom toxins and recombinant PLDs, western blotting (WB), and ELISA assays were carried out. WB was performed to detect linear and denaturation-resistant epitopes while ELISA was conducted to identify conformational and non-denatured epitopes. Pre-immune serum in WB did not react with the recombinant PLDs (Figure 5A). In contrast, hyper-immune serum against *L. laeta* crude venom reacted with all of the wild type isoforms and their respective mutant isoforms (Figure 5B). The same findings were observed when a hyperimmune serum against *L. gaucho* crude venom was used (Figure 5C). The densitometry of the WB bands showed that, although the serum to *L. laeta* venom toxins cross-reacted with *L. gaucho* recombinant PLDs, the recognition of *L. laeta* recombinant toxins were qualitatively greater; serum against *L. gaucho* venom toxins also recognized *L. gaucho* recombinant PLDs in a greater extent when compared to *L. laeta* PLDs (Figure 5D,E). ELISA demonstrated that the recombinant proteins were recognized by the polyclonal sera raised against the crude venoms of *L. laeta* and *L. gaucho*, pointing out the epitopes' conservation among native toxins and recombinant isoforms (Figure 5F). The serum against the crude venom of *L. gaucho* recognized all of the recombinant proteins of *L. laeta*. Similarly, all of the recombinant proteins of *L. gaucho* were recognized by the serum raised against the crude venom of *L. laeta*. However, it is well evidenced that the recognition of recombinant proteins from each species was quantitatively higher when the serum raised against the crude venom of the same species was used (Figure 5F).

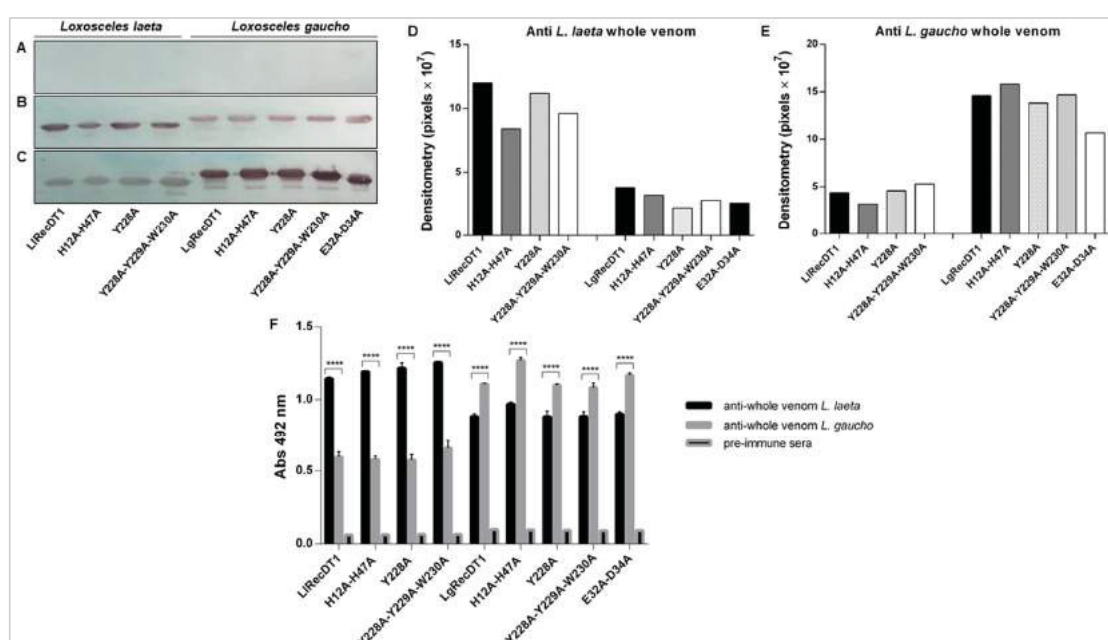


Figure 5. Immunoassays analyses—western blotting (WB) and ELISA. For WB analysis, samples of each purified PLD (2.5 μ g) were separated by 12.5% SDS-PAGE under reducing conditions, then transferred onto nitrocellulose membranes that were exposed to: (A) pre-immune serum or (B) hyperimmune serum raised against *L. laeta* crude venom or (C) hyperimmune serum raised against *L. gaucho* crude venom. All sera were obtained from rabbits and used at a 1:1000 dilution; (D) densitometry of the bands obtained in the WB showing reactivity between LIRecDT1 and its mutants with hyperimmune serum raised against *L. laeta* venom as well as cross-reactivity between LgRecDT1 and its mutants with the same serum; (E) densitometry of the bands obtained in the WB showing reactivity between LgRecDT1 and its mutants with hyperimmune serum raised against *L. gaucho* venom as well as cross-reactivity between LIRecDT1 and its mutants with the same serum; (F) antibody-capture ELISA assay was performed using samples of each purified PLD (1 μ g/mL) that were immobilized in the microplate wells and incubation with pre-immune and hyperimmune raised against *L. gaucho* and *L. laeta* crude venom obtained from rabbits at a 1:10,000 dilution. Each treatment was assessed in quadruplicate. The values are shown as averages $\pm$ SEM (standard error of the mean) of three independent experiments, with significance level at **** $p < 0.0001$ comparing pre-immune with antivenom sera raised against *L. laeta* and *L. gaucho* crude venom.

3.6. Sphingomyelinase Activity

Two experimental approaches were carried out in order to evaluate the sphingomyelinase activity of the wild type and mutants PLDs: spectrofluorimetric quantification of enzymatic products and detection of degradation products by HPTLC. As shown in Figure 6, the sphingomyelin-cleavage activity of isoforms with mutations in the catalytic site (H12A-H47A) or in the magnesium ion coordinating region (E32A-D34A), as well as the isoforms containing the triple mutation of aromatic amino acid residues (Y228A-Y229A-W230A) displayed only residual activity in both experiments when compared to the wild type controls (LlRecDT1 or LgRecDT1). Interestingly, the Y228A mutation did not give rise to inactive isoforms, as shown by the slight decrease on sphingomyelin cleavage on the Amplex Red assay and the maintenance of 42% and 34% percentage of sphingomyelinase cleavage for LlRecDT1 Y228A and LgRecDT1 Y228A, respectively. The difference in the cleavage activity between the assays reflects the specific sensitivity of the two methods used, but indicates that Y228A mutation did not abolish sphingomyelin-cleavage activity on the mutant PLDs.

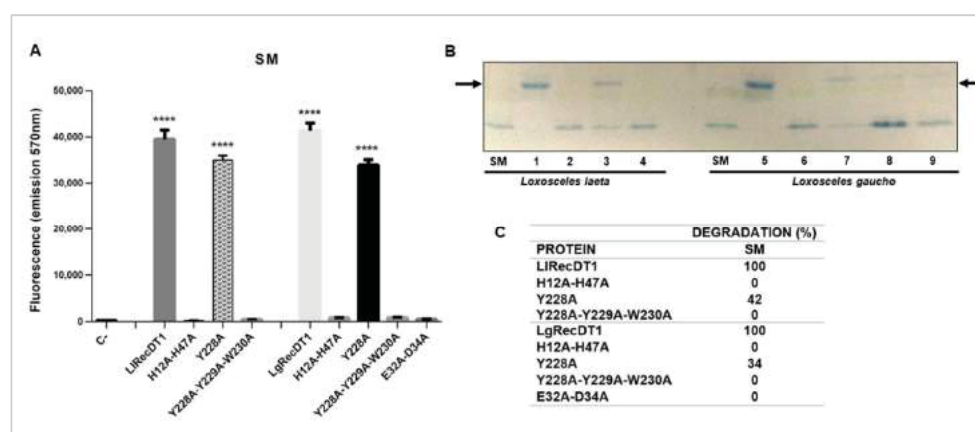


Figure 6. Comparative sphingomyelinase activity between the wild type PLDs (LgRecDT1 and LlRecDT1) and their mutant isoforms. **(A)** Amplex Red assay was performed at 37 °C for 1 h using 10 µg of each toxin and sphingomyelin (substrate). The fluorescent reaction product (resorufin) was detected on a fluorimeter using excitation wavelength at 540 nm and emission at 570 nm. LlRecDT1 and LgRecDT1 (wild type recombinant PLDs) were used as positive controls and reactions without any toxin were used as negative controls. The values represent averages of three experiments ($n = 5$) $\pm$ SEM and values with **** $p < 0.05$ were considered statistically significant; **(B)** HPTLC analysis was carried out using 50 µg of each PLD and 1 mg/mL of sphingomyelin (SM) as substrate (representative image of two independent experiments). Reactions containing sphingomyelin without any proteins were used as negative control. The lipids were extracted with butanol and applied to the plate for separation, followed by the visualization of bands stained with molybdenum blue. Arrows show bands corresponding to sphingomyelin cleavage products following protein treatments. Numbers shown in the figure represent: (1) LlRecDT1; (2) LlRecDT1 H12A-H47A; (3) LlRecDT1 Y228A; (4) LlRecDT1 Y228A-Y229A-W230A; (5) LgRecDT1; (6) LgRecDT1 H12A-H47A; (7) LgRecDT1 Y228A; (8) LgRecDT1 Y228A-Y229A-W230A; (9) LgRecDT1 E32A-D34A; **(C)** densitometry of the bands corresponding to sphingomyelin cleavage products after incubation with the recombinant PLDs. Quantification was performed in one HPTLC experiment. The percentage of cleavage by the wild type recombinant PLDs (LlRecDT1 and LgRecDT1) were considered as 100%.

3.7. Hemolytic Activity

The cytotoxic effects of recombinant toxins resulting in hemolysis on red blood cells were assessed according to previous works, which have shown that brown spider PLDs are hemolytic [7,33]. As shown in Figure 7, the wild type recombinant toxins from the two venoms exhibited high hemolytic activity, resulting in hemolysis percentages close to 100% in the 24 h treatment. Both Y228A-mutation isoforms presented slightly reduced hemolytic activity when compared to the wild type recombinant PLDs. H12A-H47A, Y228A-Y229A-W230A, and E32A-D34A mutations led to a massive inhibition of the hemolytic activity

when compared to their wild type counterparts. The observed hemolytic activity was time-dependent for both *L. laeta* and *L. gaucho* PLDs (Figure 7).

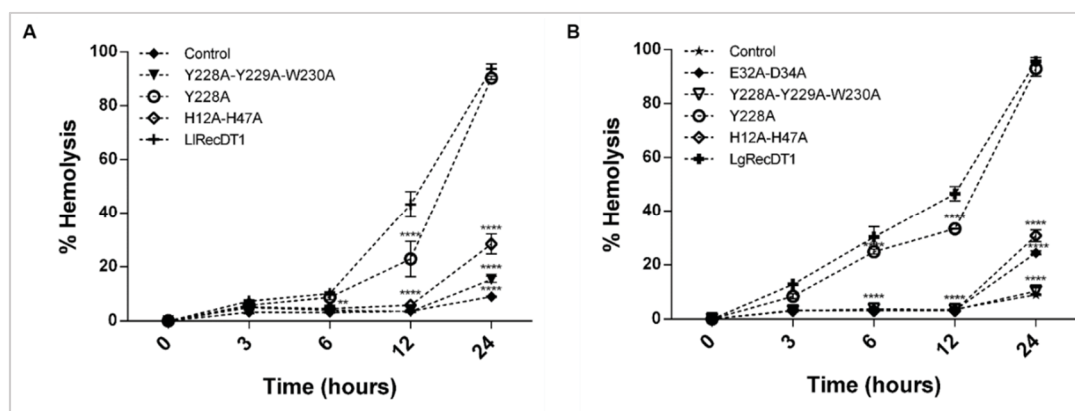


Figure 7. Hemolytic activity of recombinant PLDs on rabbit erythrocytes. PLDs samples (10 µg) in TBS were incubated with erythrocytes for 0, 3, 6, 12, and 24 h at 37 °C. Reactions containing cell suspensions without any toxin and reactions containing cell suspensions incubated with 0.1% Triton X-100 were used as negative and positive controls, respectively. The absorbance of supernatants was measured at 550 nm and the percentage of hemolysis was determined considering the absorbance values of the positive control as 100%. The values represent the averages of three experiments $\pm$ SEM and values with **** $p < 0.001$ were considered statistically significant; (A) hemolysis percentages for LIRecDT1 and its mutant isoforms; (B) hemolysis percentages for LgRecDT1 and its mutant isoforms.

3.8. Activity on Blood Vessel Permeability

In order to evaluate the activity of the PLDs on blood vessels properties, the Miles Assay was performed. As depicted in Figure 8, both wild type recombinant toxins caused a diffuse Evans Blue dye leakage to the extracellular compartment when compared to the PBS treatment (negative control). H12A-H47A, Y228A-Y229A-W230A, and E32A-D34A mutations resulted in residual capillary permeability, pointing out that these mutant PLDs did not induce severe alterations in the structure and functionality of blood vessel walls. In contrast, the Y228A mutated PLD was able to increase capillary permeability (Figure 8).

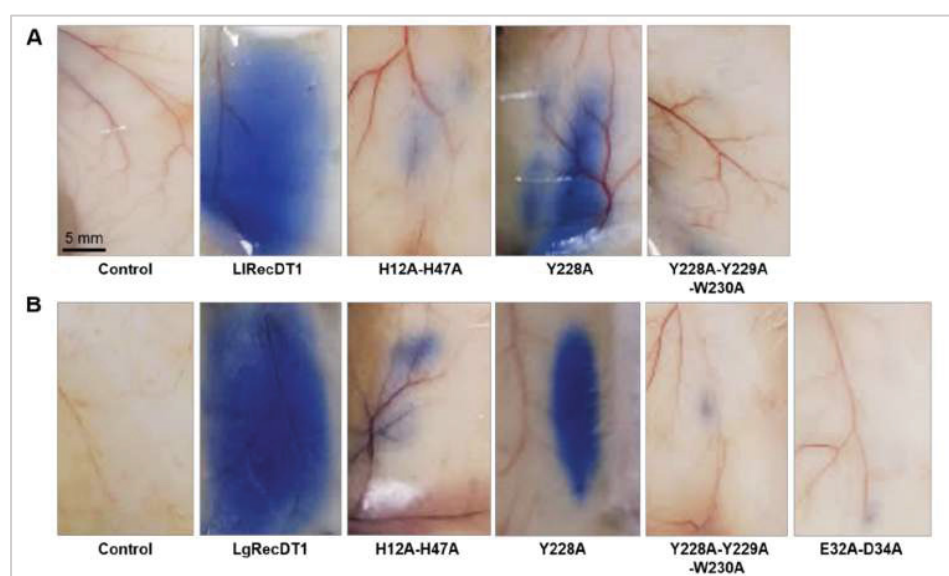


Figure 8. Vascular permeability assay. Mice ($n = 5$ for each treatment) were administered intravenously with Evans Blue (EB) dye (30 mg/kg) followed by intradermal injection of PLDs samples

(10 µg) diluted in PBS 5 min later. Dorsal skin tissue samples were removed after 1 h in order to visualize EB dye extravasation. PBS was used as negative control, corresponding to regular permeability level: (A) photographs show EB leakage after treatment with *L. laeta* wild type PLD and its mutant isoforms; (B) photographs show EB leakage after treatment with *L. gaucho* wild type PLD and its mutant isoforms.

3.9. Dermonecrotic Activity

The major sign of the cutaneous loxoscelism is the dermonecrotic lesion observed on the skin of injured patients, mainly triggered by the action of PLDs. To evaluate the necrotic lesion induced by the recombinant PLDs, toxins were injected on the skin of rabbits and the lesions were observed at a macroscopic level (0, 3, 6, and 24 h after treatments). Both wild type recombinant toxins provoked a range of local reactions near the injection site such as swelling, erythema, and tissue necrosis, which was detected by the darkening of the skin (Figure 9). H12A-H47A, Y228A-Y229A-W230A, and E32A-D34A mutations inhibited the assayed activity to macroscopically undetectable levels, similar to the negative control. The Y228A mutation, in its turn, was not able to inhibit the development of the lesion (Figure 9).

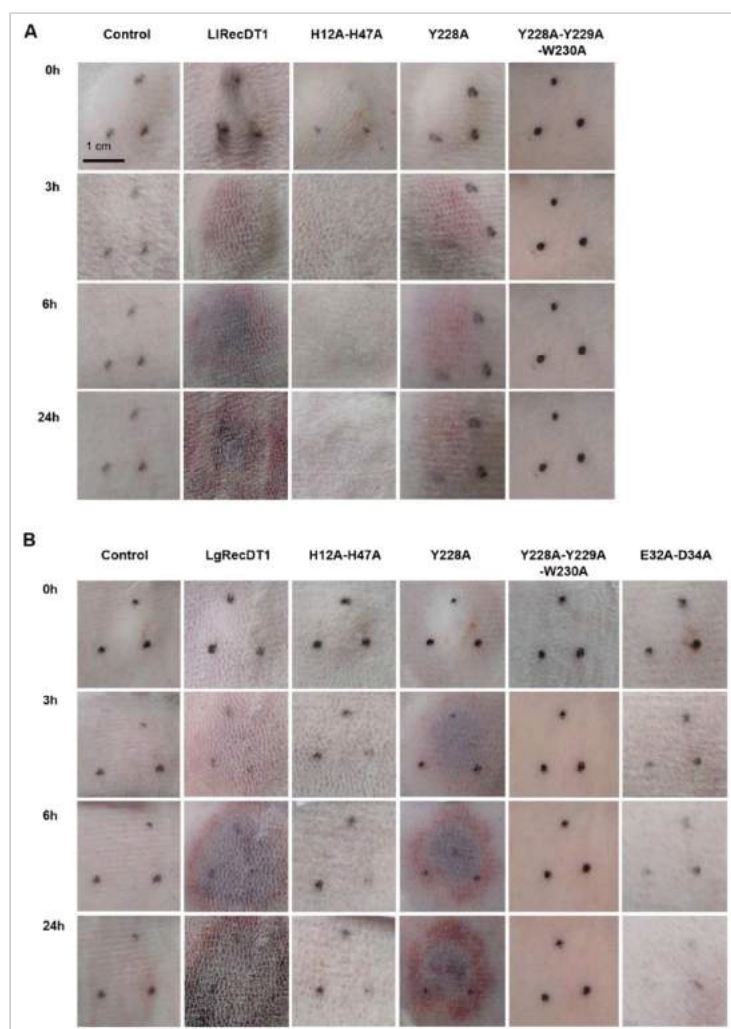


Figure 9. Macroscopic analysis of dermonecrotic lesions in rabbits after treatment with wild type PLDs and its mutant isoforms (10 µg). Macroscopic aspect of the lesions near the injection site was observed and photographed 0, 3, 6, and 24 h after treatments. PBS was used as negative control. Black dots show the local of the injection: (A) lesions induced by *L. laeta* PLDs; (B) lesions induced by *L. gaucho* PLDs.

3.10. Histopathological Changes

Skin sections excised from the areas of PLD injections were submitted to light microscopic analysis to evaluate histopathological alterations (Figure ??). Based on the acquired images, wild type recombinant PLDs (LIRecDT1 and LgRecDT1) induced a series of tissue impairments when compared to the negative control (PBS), such as disorganization of dermis collagen fibers, hemorrhage, fibrin network deposition, and presence of dense infiltrates of inflammatory cells in the dermis. Both LgRecDT1 Y228A and LIRecDT1 Y228A caused the same tissue alterations verified for the wild type recombinant PLDs, pointing out that the correspondent mutation did not generate a biologically inactive isoform. On the other hand, LgRecDT1 E32A-D34A and LgRecDT1 H12A-H47A did not trigger any noxious effects, which can be confirmed by the normal tissue structure observed in their respective histological sections; these results suggest that the E32A-D34A and H12A-H47A mutations generated biologically inactive PLDs (Figure ??).

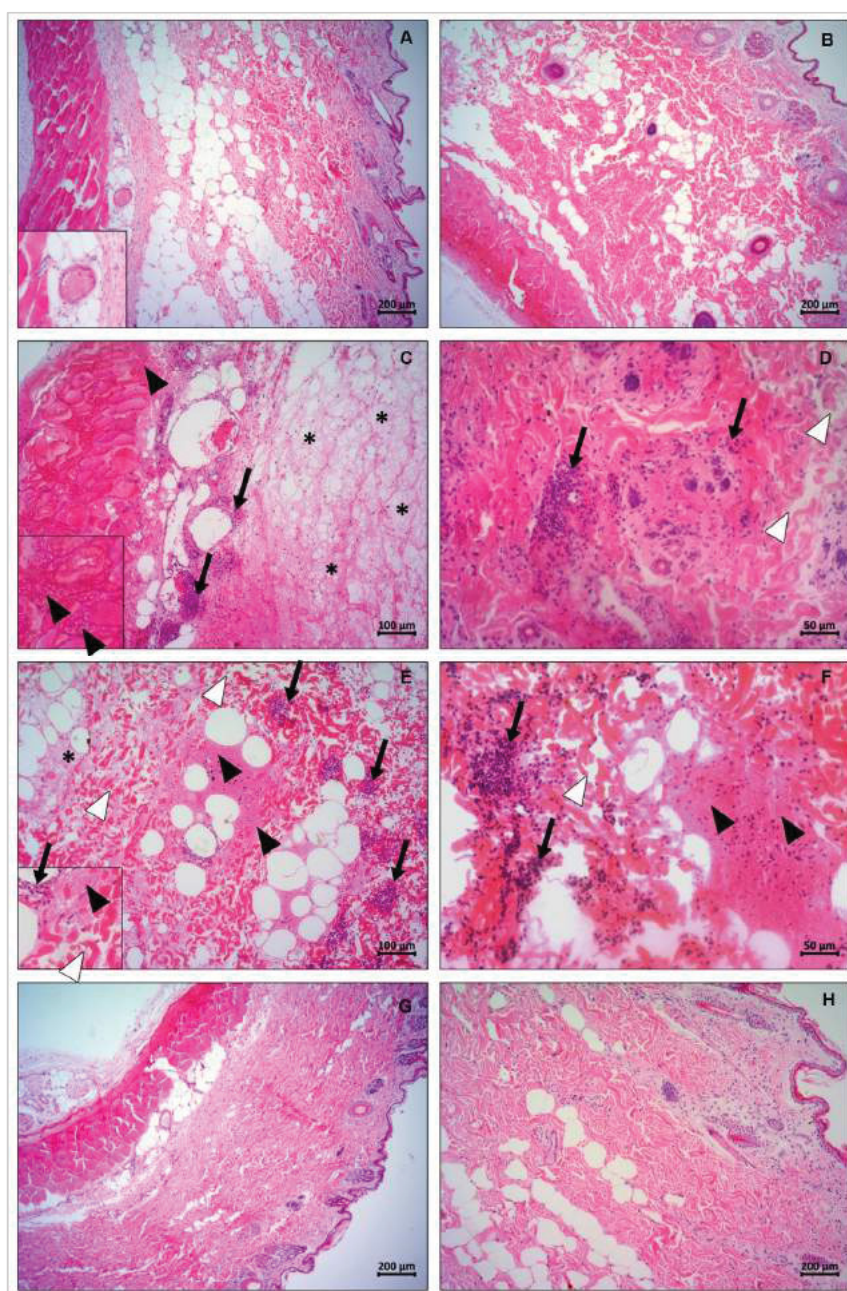


Figure 10. Histopathological analysis of the lesions in rabbits after treatment with wild type PLDs

and its mutant isoforms (10 µg). Histological images of rabbits' skin 24 h after injection of L1RecDT1, LgRecDT1, and their mutants: (A,B) normal aspect of the tissue after injection of PBS. In A, subfigure highlights an intact blood vessel (zoom in); (C,D) tissue damage after exposition to LgRecDT1 and L1RecDT1, respectively. Tissue impairment is observed by the presence of hemorrhage (black arrowheads), inflammatory cells (black arrows), disorganization of collagen fibers (white arrowheads), as well as fibrin network deposition (asterisks). In C, subfigure highlights the signs of hemorrhage (zoom in); (E) tissue aspect after injection with Y228A mutation of *L. gaucho*; disorganization of collagen fibers (white arrowheads), hemorrhage (black arrowheads), inflammatory cells (black arrows) and fibrin network deposition (asterisks) are present. Subfigure shows details in the disorganization of collagen fibers (white arrowheads), hemorrhage (black arrowheads) and inflammatory cells (black arrows) (zoom in); (F) inflammatory cells accumulation (black arrows), disorganization of collagen fibers (white arrowhead) and hemorrhage (black arrowheads) caused by Y228A mutation of *L. laeta*; (G,H) tissues with no effects after injection with E32A-D34A and H12A-H47A mutant PLDs of *L. gaucho*, respectively.

4. Discussion

Accidents caused by brown spider bites constitute a public health problem in many parts of the world, especially in South American countries such as Brazil, Argentina, Mexico, Peru, and Chile. In these countries, *Loxosceles* spiders are endemic and accidents with them have resulted in critical problems to exposed populations. In the United States of America, especially in the South and Center-South States, many accidents are also reported. In Brazil, these accidents prevail mainly in populations living in the southern states [1,2,34–37]. The treatment currently established for loxoscelism is essentially symptomatic for the cutaneous form and it aims to halt the robust inflammatory response, which is a key process associated to the pathophysiology of the accidents, as well as the dermonecrosis (aseptic coagulative necrosis). Treatments include steroidal anti-inflammatory drugs such as prednisolone or dapsone, in addition to analgesics as acetylsalicylic acid or paracetamol. Antibiotics, such as erythromycin or cephalosporins, can also be used to treat secondary infections, and antihistamines have also been prescribed to reduce allergic reactions or skin rash [1,2,34,35,38–40]. In the case of systemic loxoscelism, also known as viscerocutaneous form, treatments include steroidal anti-inflammatory drugs and antivenom serum therapy. In the most severe cases, antivenom serum therapy is widely adopted in countries such as Brazil, Argentina, Mexico, and Peru [1,2,34,35,38–40]. In some regions in Brazil, especially in the southeastern states, the antivenom serum therapy used involves the intravenous application of the polyvalent anti-arachnidic serum produced by the Butantan Institute in São Paulo. This anti-arachnidic serum is obtained by horse immunization with a mixture of the crude venoms from the spiders *Loxosceles gaucho* and *Phoneutria nigriventer*, as well as the crude venoms from the scorpions *Tityus serrulatus* and *Tityus bahiensis*. In Brazil's southern states, the serum therapy is based on the administration of the anti-*Loxosceles* serum, which is produced by the Production and Research Center of Immunobiological Products (CPPI) in Paraná state. The anti-*Loxosceles* serum is also obtained by horse immunization with the crude venoms from the brown spiders *L. intermedia*, *L. laeta*, and *L. gaucho*, which are the most important species from the medical perspective in South America [1,2,34,35].

The use of antivenom sera in the treatment of loxoscelism is controversial and it is not a practice performed in the United States of America, for example. The use of anti-*Loxosceles* serum therapy in cases of the systemic loxoscelism has been successful, which is supported by experimental and clinical data. A study has already shown that the pediatric mortality decreased after serum therapy [1,41]. Besides that, it has also been shown that anti-*Loxosceles* serum experimentally administered in rabbits reduced the noxious alterations often seen followed venom exposure, such as hematological disorders, extravascular deposition of fibrinogen and alterations in renal function [1,42]. On the other hand, experimental and clinical evidence supports that antivenom serum therapy is ineffective in the treatment of cutaneous loxoscelism for reasons that still remain unknown [42]. It is speculated that the mentioned ineffectiveness involves the mechanisms following cell/toxin

interactions, or simply because patients are slow to seek medical help. The early events of unregulated inflammatory response and dermonecrosis start to develop in the first 4–8 h after the accidents so that a delay in initiating the use of serum therapy no longer results in the neutralization of the venom toxins [1,2,42,43]. The ineffectiveness of the anti-*Loxosceles* serum therapy in cutaneous loxoscelism could be understood based on the cell biology mechanisms associated to the envenoming. One of the possible explanations lies in the inability of the anti-toxins antibodies to neutralize the toxins after activation or induction of molecular changes in the main target-cells (endothelial cells of the blood vessels, fibroblasts, platelets, and erythrocytes) [20]. Another possibility is that the dermonecrotic toxins (PLDs), which cause almost all of the effects triggered by crude venoms, represent between 15 and 20% of toxin-encoding transcripts in the venom-producing glands. This could generate a low proportional concentration of PLD-neutralizing antibodies in the serum when compared to the high proportional concentration of neutralizing-antibodies for other toxins, resulting in an ineffective serum. If the mentioned hypothesis is true, the production of polyclonal sera with crude venoms enriched with recombinant PLDs or sera obtained using only PLDs as antigens could have a higher neutralizing antibody titer and greater efficiency in serum therapy.

In this work, our main objective was to generate molecular constructions for recombinant expression of PLDs found in the venoms of *L. laeta* and *L. gaucho*, two species of brown spiders with medical importance and endemic to many South American countries. The mutant isoforms lacking biological activity can be used as antigens in high concentrations to obtain an improved serum or vaccine or in other biotechnological applications. Furthermore, these mutant toxins are valuable biotools that can be used to understand PLDs structure-function relationship. The standardization of the cloning, heterologous recombinant expression and purification processes of the recombinant PLDs described herein is a major step since it shows that these toxins can be easily obtained in large scale. PLDs present in the venoms of *L. laeta*, *L. intermedia*, and *L. arizonica* had their structures unveiled at tridimensional level by crystallography approaches [9,15,31,32]. These structural data enabled a better understanding of the mechanisms involved in the cleavage of lipid substrates as well as an identification of the amino acid residues located in functionally important intramolecular sites/regions. Based on the data about the amino acid residues involved in PLDs functionality, we designed constructions encoding wild type and mutant PLDs for both *L. laeta* and *L. gaucho*. The selected mutations comprise amino acid residues located in the catalytic site (H12A-H47A), in the regulatory exosite involved in substrate binding (Y228A and Y228A-Y229A-W230A) and in the magnesium ion coordinating site (E32A-D34A) [15,20,24,31]. With the exception of E32A-D34A mutation for *L. laeta*, all of the designed forms (wild type and mutants) were expressed and purified as soluble proteins (Figure 2). The production of L1RecDT1 E32A-D34A as a soluble protein was unsuccessful despite the numerous attempts (data not shown). The electrophoretic pattern of all wild type and expressed mutant PLDs showed no changes in the migratory profile (such as drag down, aggregations, or fragmentations) nor significant variations in the expected molecular masses (Figure 2), which suggests that mutations did not alter the physicochemical behavior of these molecules. In addition, circular dichroism data pointed out no significant changes in the overall structure of the recombinant PLDs (Figure 3). These data are in line with previous studies that generated mutated isoforms of *Loxosceles intermedia* PLDs [24,44] sharing the same secondary structures profile or spatial conformation/folding of the wild type forms. The comparative molecular modeling and the analysis of the surface electrostatic charges indicated that there are no significant differences between wild type and mutated isoforms for both PLDs of *L. laeta* and *L. gaucho*, except for a slight reduction in the surface negative charges of *L. gaucho* E32A-E34A (Figure 4C). These results strengthen the experimental data acquired in circular dichroism technique, pointing out that the spatial molecular structures did not undergo significant changes after mutations.

In the immunoassays, the highest recognition pattern of the recombinant toxins by the sera raised against the native toxins of their counterpart venom, although may result

from different affinities of the respective antibodies, suggests the existence of different epitopes between toxins from *L. gaucho* and *L. laeta* (Figure 5B–D). These data support the idea that protocols for the production of broad-spectrum neutralizing antivenom sera or protective vaccines using recombinant PLDs as antigens must contain recombinant toxins from both venoms, which can assure high neutralization or protection effectiveness following envenomation, especially in regions where both spider species are endemic such as South America.

One of the major challenges for the production of neutralizing antivenom sera and protective vaccines is the choice of antigens. Considering the production of neutralizing hyperimmune sera in animals, it is important that these antigens do not cause great toxic effects in the inoculated animals. The same mindset should be taken into account for antigens used for vaccines. For this reason, the constructions used in this work were designed to generate antigens that meet all of the desirable requirements, i.e., immunogenicity and low toxicity. As observed by spectrofluorimetry and HTPLC, the mutations H12A-H47A, E32A-D34A, and Y228A-Y229A-W230 were extremely efficient in inactivating the PLDs, resulting in only residual sphingomyelin cleavage. On the other hand, the mutation of a single tyrosine at position 228, for both *L. laeta* and *L. gaucho* PLDs, was not sufficient to inhibit their biochemical properties, indicating that enzymes containing this mutation are not useful for serum-development. Some previous data in the literature have shown that the biochemical activities of PLDs in spider venoms, such as sphingomyelin or lysophosphatidylcholine cleavage, and biological activities, such as dermonecrosis and hemolysis, are deeply dependent on the catalytic activities of these enzymes. Inactive or artificial mutant isoforms that do not degrade sphingomyelin also do not induce dermonecrosis or hemolysis [23,24,33,44]. When PLDs act upon sphingomyelin or lysophosphatidylcholine present in the membranes of target cells the lipid mediators formed after enzymatic cleavage constitute biologically active molecules and activate the molecular machinery responsible for the signs and symptoms observed after envenomation [20,45,46]. In this sense, after identifying the inhibition of sphingomyelin-cleavage activity in the mutant isoforms H12A-H47A, E32A-D34A, and Y228A-Y229A-W230, we performed biological analyses regarding well-described activities of these enzymes. The mutated PLDs H12A-H47A, E32A-D34A, and Y228A-Y229A-W230 resulted in loss of hemolytic activity, unchanged capillary permeability, strongly reduced signs of dermonecrosis, and abrogated inflammatory response when compared to the wild type isoforms, strongly supporting a correlation between catalytic activity, dependence on ion metal coordination and substrate binding ability with the noxious effects induced by these enzymes/toxins. These data strongly suggest that the conformational epitopes of mutated isoforms that can act as antigenic determinants have not undergone significant changes, when compared to antigenic determinants of wild type isoforms, enabling any of these mutated variants to be used as antigens in the production of neutralizing hyperimmune sera for treatment of injured victims or of protective vaccines.

5. Conclusions

Data shown here describe the production of soluble recombinant PLDs isoforms from *Loxosceles* spider venoms that can be produced in large quantities by using reasonably cost-effective techniques. Additionally, these recombinant mutant PLDs do not exhibit toxic activities, and maintain some of their immunogenic properties, which is desirable for immunotherapeutic purposes. As discussed throughout the text, these mutated recombinant toxins can be used in their pure forms and at high concentrations to generate high-titer hyperimmune sera, which hopefully will recognize wild type native PLDs present in the crude venoms. As a consequence of this recognition, the hyperimmune sera may neutralize the harmful activities caused by the envenomation. These mutant PLDs lacking biological activities are also potential biotools that could be used as antigens for the production of protective vaccines in regions where *Loxosceles* spiders are endemic and therefore cause a great deal of accidents.

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