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Review

Biotechnological potential of Phospholipase D for *Loxosceles* antivenom development

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ABSTRACT

Loxoscelism is one of the most important forms of araneism in South America. The Health Authorities from countries with the highest incidence and longer history in registering loxoscelism cases indicate that specific antivenom should be administered during the first hours after the accident, especially in the presence or at risk of the most severe clinical outcome. Current antivenoms are based on immunoglobulins or their fragments, obtained from plasma of hyperimmunized horses. Antivenom has been produced using the same traditional techniques for more than 120 years. Although the whole composition of the spider venom remains unknown, the discovery and biotechnological production of the phospholipase D enzymes represented a milestone for the knowledge of the physiopathology of envenomation and for the introduction of new innovative tools in antivenom production. The fact that this protein is a principal toxin of the venom opens the possibility of replacing the use of whole venom as an immunogen, an attractive alternative considering the laborious techniques and low yields associated with venom extraction. This challenge warrants technological innovation to facilitate production and obtain more effective antidotes. In this review, we compile the reported studies, examining the advances in the expression and application of phospholipase D as a new immunogen and how the new biotechnological tools have introduced some degree of innovation in this field.

1. Introduction: *Loxosceles* genus and loxoscelism

The genus *Loxosceles* belongs to the spider family *Sicariidae*, which comprises three genera: *Hexophthalma*, with 6 species from Africa; *Sicarius*, with 21 species distributed in Central and South America; and *Loxosceles,* with 139 described species worldwide (Magalhães et al., 2017; World Spider Catalog, 2020). While most species of the genus *Loxosceles* have been described in Central America, South America and Africa, some in North America, the number of species recorded in Europe and Asia is still very limited (World Spider Catalog, 2020).

All *Loxosceles* species, commonly named "violin" spiders, are light to dark brown, uniformly colored, and have six sets of eyes displayed in a characteristic pattern of three pairs, and relatively long, slim legs (Magalhães et al., 2017; Vetter, 2008). They share a distinct mark that resembles a violin in the dorsal side of the cephalothorax, which is dark brown on light brown carapace (Fig. 1a and b). Traditionally, different groups of species have been recognized based on the morphological characters: in North America (*L. reclusa*) (Gertsch and Ennik, 1983), in South America (*L. gaucho*, *L. laeta*, *L. amazonica*, *L. spadicea, L. intermedia*) (Gertsch, 1967), in South Africa (*L. vonwredei*, *L. spinulosa*) (Newlands and Atkinson, 1988), and in the Mediterranean (*L. rufescens*) (Nentwig et al., 2017). The species group *amazonica* has been recently synonymized with the species group *rufescens* (Duncan et al., 2010; Fukushima et al., 2017; Valdez-Mondragón et al., 2019).

Accidents caused by these spiders, known as loxoscelism, are often intra or peridomiciliary and constitute a public health problem. Despite

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the widespread distribution of the species, clinical cases of *Loxosceles* spider bites are more commonly reported in the Americas, especially in Brazil, where the number of accidents has increased in the past few years (Ribeiro de Oliveira Mendes et al., 2020). In 2017, the Brazilian Ministry of Health computed 7992 cases of loxoscelism, nine of which resulted in deaths, and in Southern Brazil, the state of Paraná alone concentrated 4085 reports of brown spider bites and one death in the same year (Ribeiro de Oliveira Mendes et al., 2020). In Argentina, Brazil and Peru, countries that taken together represent the vast majority of reported loxoscelism cases around the world, the use of the specific antivenom to treat loxoscelism is recommended by their Ministries of Health (Instituto Nacional de Salud, 2004; Ministério da Saúde, 2001; Ministerio de Salud, 2012). In Europe, scarse cases have been reported, mostly showing only local manifestations (Atilla et al., 2004; Bajin et al., 2011; Farace et al., 2006; Jerusalem and Salavert Lletí, 2018; Morales-Moreno et al., 2016; Ribuffo et al., 2012; Rubenstein et al., 2016). The venoms of *Loxosceles* spiders from the Old and New Worlds are likely to produce comparable clinical pictures (Planas et al., 2015).

Loxoscelism is a toxic condition caused by the venom inoculated by the bite of the spider. The initial bite frequently occurs without immediate pain, and evident signs or symptoms are absent in the first few hours; thus, the clinical consultation with a specialist is frequently delayed for at least 24 h since the accident. As a consequence, the precise diagnosis of loxoscelism is difficult and usually presumptive, and the symptomatology is sometimes confused with bacterial or viral cutaneous infections, dermatitis, vasculitis, or diabetic ulcer (Vetter and Isbister, 2008). Most accidents caused by *Loxosceles* spider envenomation are characterized by dermonecrotic lesions with gravitational spreading, and hence, these accidents are often referred to as necrotic or gangrenous arachnidism. However, in over 10% of the cases, particularly when *L. laeta* is responsible for the accident, systemic evolution of the pathology is observed, which can result in a fatal outcome, mostly in children and elders. Venom toxins are responsible for many cellular changes that follow envenomation, either in humans or in animal models for experimental exposure. When rabbit skin is exposed to venom, it shows the same injuries seen in human biopsies: ~~a~~ dermonecrosis with massive infiltration of inflammatory cells into the dermis (Barbaro et al., 1992; Ospedal et al., 2002).

Less frequently, systemic manifestations take place, including renal alterations and hematological disturbances such as hemolysis, thrombocytopenia, and intravascular coagulation (Da Silva et al., 2004). There is no consensus on cutaneous loxoscelism treatment, several alternatives are mentioned in the literature and usually include analgesics, antivenoms, corticoids, dapsone and, eventually, antibiotics (Hogan et al., 2004; Instituto Nacional de Salud, 2004; Ministério da Saúde, 2001; Ministerio de Salud, 2012). Recent reports show a beneficial effect of tetracycline treatment on experimental models of both cutaneous and systemic loxoscelism, which suggests a potential implementation of this therapeutic agent in the not-too-distant future (Okamoto et al., 2017; Paixão-Cavalcante et al., 2007). Nevertheless, the experience gained after several decades of loxoscelism treatment in most of the countries with the highest incidence and longest historical records of loxoscelism shows consensus on their Health Authorities to indicate antivenom administration to patients with evident of cutaneous lesions, and it is the only available therapeutic tool today for treating systemic loxoscelism.

Antivenom has been obtained by immunizing horses with spider venoms since the early 1960s, and it has great efficiency if administered soon after the bite. Unfortunately, venom extraction for antivenom production from the spiders is a very laborious task with very low yields per spider. Therefore, the search for alternatives to complement or substitute the use of venom during antivenom production by recombinant alternatives has been the focus of many work during the last two decades.

1.1. Venom toxins

The genus *Loxosceles* has been extensively studied in the fields of pharmacology, biochemistry, immunology, and biotechnology, and some of the venom components have been well biochemically and biologically characterized (Chatzaki et al., 2012; Dantas et al., 2014; Kalapothakis et al., 2002; Silvestre et al., 2005). In nature, brown spiders are arthropods that use their venom for predation and defense. Less than 2 μL of a highly viscous, clear liquid containing about 50 μg of proteins can usually be extracted from the venom gland of an adult specimen. Nevertheless, *Loxosceles* spider venom is quite complex and can be classified as highly expressed (phospholipases, metalloproteases, and inhibitor cystine knot family peptides) and lowly expressed (serine proteases, protease inhibitors, hyaluronidases, allergen/like toxins, and histamine-releasing effectors) polypeptides. The toxicity profiles of *Loxosceles* venoms are similar between female and male specimens and between distinct species, such as *L. laeta*, *L. reclusa, L. intermedia, L. adelaida, L. similis,* and *L.gaucho* (Chaves-Moreira et al., 2017). Fractionation by gel filtration of whole venom suggested that most, if not all, relevant *in vivo* toxicity from brown spider venom comes from components with a molecular weight of about 35 kDa. This observation was later confirmed by recombinant expression and *in vivo* testing of various isoforms from the phospholipase D (PLD) present in this fraction. Thus, the protein family of PLD enzymes, the most abundantly expressed

Fig. 1. a: Loxosceles laeta male. b: Loxosceles laeta female.

proteins in *Loxosceles venoms*, are mostly responsible for the local and systemic effects of loxoscelism (Kalapothakis et al., 2007; Veiga et al., 2000).

Other abundant components of *Loxosceles* venom are a group of 20–30 kDa astacin-like metalloproteases, and a series of 5.6–7.9 kDa peptide members of the inhibitor cystine knot family (ICK) (De Castro et al., 2004; Matsubara et al., 2017, 2013). The first description of peptides from the ICK in *Loxosceles* venoms was reported in 2004 (De Castro et al., 2004). The authors isolated a group of small peptides from the venom of *L. intermedia* with insecticidal activities against *Spodoptera frugiperda* and *Spodoptera cosmioides* and named them LiTx1, LiTx2, and LiTx3. These peptides have molecular masses ranging from 5.6 to 7.9 kDa, and further analysis of the sequences suggested the presence of possible post-translational modification regions in the sequences of LiTx1-3, such as N-myristoylation, amidation, and casein kinase II phosphorylation sites and their activity against ion channels.

The less abundant components of this venom include other proteases, from the serine proteases family (between 85 and 95 kDa molecular weight) (Veiga et al., 2000) and also protease inhibitors named serpins (Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). Members of the Translationally Controlled Tumor Proteins (TCTP) family have also been detected in this venom (Gremski et al., 2010). These proteins act as histamine-releasing effectors, proposed to be related to erythema, diffusion of the venom, itching, pain, and less frequently, cases of hypersensitivity or even allergic reactions after venom inoculation (Boia-Ferreira et al., 2019; Sade et al., 2012). At least three hyaluronidase isoforms of 41–43 kDa have been detected in *Loxosceles* venom, some of them also recombinant expressed (da Silveira et al., 2007; Fernandes-Pedrosa et al., 2008; Gremski et al., 2010). Studies in rabbit skin with recombinant hyaluronidase showed induction of local toxicity when co-administered with recombinant dermonecrotic toxin (Ferrer et al., 2013). Finally, other minor components from *Loxosceles* venom also include hydrolases, lipases, c-type lectins peptidases, collagenases, alkaline phosphatases, 5-ribonucleotidases, and phosphohydrolases (Da Silva et al., 2004; Fernandes-Pedrosa et al., 2008; Gremski et al., 2010).

To sum up, the venom is a mixture of several hundred biologically active compounds that act synergistically, and the detailed mechanism of action remains unknown. The only consensus up to date is that Phospholipase D isoforms present in the venom are responsible for the pathology of envenomation in mammals.

1.2. Phospholipase protein family

The terminology employed for referring to the most relevant group of toxins in *Loxosceles* venoms has changed over time and deserves some attention. In 1976, toxins of around 34 kDa that affected rabbits and mice were isolated from *Loxosceles reclusa* venom (Geren et al., 1976). Later, in 1981, a fraction of around 31–37 kDa was isolated from the same venom which was able to kill mice, causes lysis of human erythrocytes, affects human plasma coagulation, and produces dermonecrotic activity in rabbits (Babcock et al., 1981). By fractionation of venom content based on gel filtration and SDS-PAGE, it was found that the most abundant and also relevant toxin in this venom of *Loxosceles gaucho* had a molecular weight of about 35 kDa (Barbaro et al., 1992; Geren et al., 1976). This fraction alone was able to produce necrotic lesions, inflammatory response, and platelet aggregation when inoculated to experimental animals, and thus it was named dermonecrotic toxin. In 1998 Tambourgi et al. purified a 35 kDa fraction from *Loxosceles intermedia* venom, which was resolved by reversed-phase chromatography into three peaks named P1, P2, and P3 (Tambourgi et al., 1998). Functional characterization allowed the authors to conclude that P1 and P2 were not only capable of inducing all the *in vivo* effects already described but also displayed Sphingomyelinase-D (SMase-D) enzymatic activity. Sphingomyelin (SM) or N-acyl-sphingosine-1-phosphorylcholine is a type of sphingolipid present in the plasma membranes of animal cells. Particularly in humans, SM comprises nearly 85% of all the sphingolipids in the outer leaflet of mammalian plasma membranes, having important functions as a structural membrane component. SMase-D (EC number 3.1.4.41) catalyzes the hydrolysis of SM, resulting in the formation of ceramide1-phosphate (CIP) and choline (Meeteren et al., 2004; Tambourgi et al., 2010). Then, many authors referred to these toxins both as SMase-D or dermonecrotic toxins. Between 2002 and 2004, the amino acid sequences of SMase-D from several *Loxosceles* species were reported and cloned, and recombinant versions of these proteins were obtained, thus allowing further insight into their functional properties (Fernandes Pedrosa et al., 2002; Kalapothakis et al., 2002; Ramos-Cerrillo et al., 2004). It was then observed that these proteins were able to hydrolyze not only SM but also other phospholipids such as lysophosphatidylcholine (LPC) (Lee and Lynch, 2005). LPC hydrolysis in the presence of Mg^{2+}-cofactor releases lysophosphatidic acid (LPA), triggering LPA signaling pathways. Further on, they appeared to act *in vivo* on a broad range of bioactive lipids to exert their toxicity (Chaim et al., 2011). To account for a more generalized description of their enzymatic activity, the alternative Phospholipases D (PLD) name was proposed for referring to these toxins. Moreover, some authors have recently proposed that the enzymatic activity of these proteins *in vivo* should not be hydrolysis of their phospholipidic substrates but transphosphatidylation, forming cyclic phosphate products which exert their biologic effects (Lajoie et al., 2013). Based on cDNA sequence similarity to material extracted from venom glands, Kalapothakis et al. suggested a new and somehow more integrative nomenclature for these toxins: LoxTox (Kalapothakis et al., 2007). Therefore, many proteins with a high degree of homology, sharing important structural and functional motifs, could be grouped together without the need to analyze their enzymatic or biological activity. These changes in terminology significantly increased the number of molecules recognized as members of this group: from the initial one to three isoforms identified per species based on biological or enzymatic criteria, proteomic and RNA sequence homology studies were able to incorporate up to 23 isoforms from a single spider species to this protein family group (Dantas et al., 2016; Fernandes Pedrosa et al., 2002; Kalapothakis et al., 2007, 2002; Ramos-Cerrillo et al., 2004). Moreover, based on sequence similarity, Binford et al. proposed that new integrative gen grouping could be extended to all the members of the *Sicaridae* family, creating the SicTox gene family (Binford et al., 2009). Although both gene grouping proposals seem plausible, considering a historical perspective and for the sake of simplifying the comparison among the different studies reviewed in this manuscript, we will keep the traditional PLD naming for these proteins.

1.3. Immunotherapeutics treatment for envenomation

Loxosceles antivenom showed clinical effectiveness when properly applied and its use is recommended by several ministries of health (Instituto Nacional de Salud, 2004; Ministério da Saúde, 2001; Ministerio de Salud, 2012; Pauli et al., 2009, 2006). However, its real usefulness is controversial (Hogan et al., 2004; Isbister et al., 2003; Isbister and Fan, 2011; Isbister and White, 2004; Swanson and Vetter, 2005; White et al., 2003). This controversy is logical and due to various reasons: available studies on human beings are conflicting and mostly no placebo-controlled, the criteria to apply the antivenoms is not unanimous (doses, routes, time of application after the bite), the specie of spider and the site of bite, the characteristic of patients and the criteria to evaluate the results of the treatment, which are not unanimous (Isbister and Fan, 2011; Pauli et al., 2006). Nevertheless, the most important point is the lack of clinical trials designed *ad hoc* (Isbister and Fan, 2011). Experimental studies despite very different results and methodologies show that the antivenoms are able to limit cutaneous lesions caused by the venom and to rescue mice from lethal challenge-doses (de Roodt et al., 2007; Gomez et al., 1999; Pauli et al., 2009, 2006). However, experiments must not be directly extrapolated to the clinical usefulness of this or another antivenom (Isbister and Fan,

2011; Theakston and Reid, 1983; World Health Organization, 2017, 1981).

Since 1954, when it was developed in Brazil and after the beginning of their industrial production in 1961 (Furlanetto, 1961; Isbister and Fan, 2011), this antivenom is broadly used and at present is produced by sanitary official institutions from South American countries (Argentina, Brazil and Peru) and by a private Laboratory in Mexico (Cabrerizo et al., 2009; Fan et al., 2019; Temprano et al., 2017). In these South American countries the spider bites are of mandatory notification to national authorities and represent around 1,200 to 23,000/year, being those caused by *Loxosceles* from 400 to over 7,000 cases/year, while the lethality vary from 0.001–1.5% (de Roodt et al., 2007, 2002b; Ministerio da Saúde, 2020; Ministério da Saúde, 2001; Ministerio de Salud, 2012; Vargas, 2017). Data from Brazil in the last years indicates around 7,000 cases/year of accidents by *Loxosceles* (Ministerio da Saúde, 2020; Ministério da Saúde, 2001) and the antivenom use vary, considering available data, from 11.9% to 70% of the cases, with a median of 51% (Ministerio de Saúde 2001). The lethality ranges from 0.1% to 1.5%, depending on the region and the species of *Loxosceles*, being the most severe cases those due to *Loxosceles laeta*, the species of higher sanitary importance in Peru, Chile, Brazil and Argentina (Gonçalves-de-Andrade and Tambourgi, 2003; Ministério da Saúde, 2001; Pauli et al., 2006). In Argentina, with over 1,200 spider bites/year and 0.15% of lethality (de Roodt et al., 2017; Ministerio de Salud, 2013), considering that *Loxosceles* would be responsible of around 30% of spiders bites (de Roodt et al., 2002b), 300–400 *Loxosceles* accidents/year could be estimated, with a use of antivenom over 50% (de Roodt, unpublished data). In Peru, with around 1,400 *Loxosceles* bites/year, the national lethality by in the period 2011–2017 was around 0.001–0.5% (Vargas, 2017) but unfortunately we could not obtain information regarding the use of antivenom. In Mexico the accidents are not of mandatory report and possibly 100 cases/year occurs (Secretaría de Salud de México, 2017) with no official data or epidemiological reports available regarding antivenom use or deaths.

Data regarding *Loxosceles* bites or treatments in other countries are fragmentary or absent, since these accidents are not reported or are only partially reported to sanitary authorities. In Chile, where the exact number of cases in the country is not known and where the anvinenom is not used (Ministerio de Salud, 2016) the mortality reported was around 3% (Harz-Fresno et al., 2015; Ministerio de Salud, 2016; Schenone et al., 1989) with a 20–25% of lethality in the systemic envenomation (del Puerto et al., 2018). In Chile like in Argentina and Peru, *Loxosceles laeta* would be the most important species of *Loxosceles* involved with accidents.

Despite the scarcity of available information from clinical studies, there is a consensus in the countries where the antivenom is used that an early start of antivenom treatment helps to prevent dermonecrotic lesions from extending and limits systemic compromise (Ministério da Saúde, 2001; Ministerio de Salud, 2012; Pauli et al., 2006; Tambourgi et al., 2010). The active pharmaceutical ingredients of antivenom are immunoglobulins (Peruvian antivenom) or their F $(ab')_2$ fragments purified from plasma of animals (equines) hyperimmunized with different *Loxosceles* venoms or with recombinant PLDs (Mexico). The high homology present in their venoms and PLDs is responsible of an important immunological cross reactivity that turn the available therapeutic antivenoms able to be used in non-specific envenoming since this cross reactivity is able to provide enough cross-protection levels to pass *in vivo* tests employed for antivenom quality assessment (Barbaro et al., 2005; de Roodt et al., 2007) and their use as therapeutic tools, as these are used since several years ago in different South American countries.

The lower mortality reported in countries where the antivenom is used empirically suggests its usefulness, at least to treat systemic envenoming, when is properly applied (Instituto Nacional de Salud, 2004; Ministério da Saúde, 2001; Ministerio de Salud, 2012). Nevertheless, other factors should be in addition considered before to affirm this, and more information is necessary to clarify these differences. By this reason, well-designed clinical trials would clarify these points and would help to adjust the protocols for the different types of envenoming, turning the empiric knowledge in scientific knowledge.

Venom availability poses a severe restriction for antivenom production. While about 50 μg of proteins are extracted in every 0.15–0.45 μl drop of venom after electrostimulating each spider, mg quantities are required per horse in every hyperimmunization schedule (de Roodt et al., 2002a; Morgan, 1969; Ospedal et al., 2002). For this reason, efforts were undertaken to achieve reduced immunization schemes such as in anti-*L. laeta* venom production (de Roodt et al., 2002a). As already discussed, PLD proteins are responsible for venom toxicity. Between 2002 and 2004, the first complete amino acid sequences from members of this family were reported for several *Loxosceles* species (Fernandes Pedrosa et al., 2002; Kalapothakis et al., 2002; Ramos-Cerrillo et al., 2004). Soon afterward, several groups directed their efforts toward the recombinant expression of these proteins focused on reducing the amount of *Loxosceles* venom needed for antivenom production. A PLD from *L. laeta* (clone H17) was the first toxin of this class to be cloned and expressed in recombinant form in *Escherichia coli* (Fernandes Pedrosa et al., 2002). This recombinant PLD, with a molecular weight of 33 kDa, showed dermonecrotic and complement-dependent hemolytic activities. Noteworthy, antiserum raised against this recombinant protein was able not only to recognize a 32-kDa protein in crude *L. laeta* venom but also to block the dermonecrotic reaction caused by whole *L. laeta* venom. Thus, this study conclusively confirmed the principal role of PLD proteins in the pathology of *Loxosceles* spider envenomation. The number of reports on the recombinant PLD expression from different *Loxosceles* species soon started to grow (Table 1). Between 2002 and 2004, the first reports appeared for *L. intermedia*, in 2005 for *L. reclusa*, in 2006 for *L. boneti,* and finally in 2013 for *L. gaucho* (Araujo et al., 2003; Kalapothakis et al., 2002; Lee and Lynch, 2005; Magalhães et al., 2013; Olvera et al., 2006; Tambourgi et al., 2004). Brown spiders express several highly homologous isoforms of PLD with an identity varying from 40 to 90% (de Roodt et al., 2007; Tambourgi et al., 2010). According to sequence identity, biochemical activity, and molecular modeling, these proteins are grouped into two classes: Class I enzymes possess a single disulfide bridge and contain a variable loop whereas members of Class II enzymes contain an additional intra-chain disulfide bridge that links a flexible loop with a catalytic loop (Murakami et al., 2006). Both classes exhibit differences in their toxic potential, and Class II enzymes are less toxic than Class I enzymes (de Santi Ferrara et al., 2009). At first glance, the fact that over 20 PLD isoforms can be identified in a single venom gland could be discouraging when the objective of obtaining an antivenom from recombinant expression of a single clone is set. Nevertheless, recombinant PLD successfully replaced whole venom for equine hyperimmunization during antiserum production (De Almeida et al., 2008; Duarte et al., 2015; Olvera et al., 2006), and the antivenom produced using these recombinant enzymes as immunogen showed clinical efficacy and is currently in use in Mexico (Gómez-Rivera et al., 2014; Grashof et al., 2020; Sánchez Villegas et al., 2014a, 2014b; Sánchez Villegas and Rodríguez Álvarez, 2015). Antivenom produced from recombinant PLD conferred full protection against dermonecrotic effects of venom from the same *Loxosceles* species and at least partial cross-protection from venoms of different species. Unfortunately, the toxicity of recombinant PLD to horses is still present, thus limiting the amount of immunogen and the frequency of hyperimmunization plans. To avoid PLD's toxicity and with the initial aim on obtaining a potential vaccine candidate, several groups directed their efforts toward the construction of recombinant chimeric proteins expressing only selected B cell epitopes from PLD (Calabria et al., 2019; de Almeida Lima et al., 2018; Felicori et al., 2009; Figueiredo et al., 2014; Souza et al., 2018). In these chimeric proteins, as opposed to full recombinant PLD proteins, the lack of enzymatic activity results in an absence of toxicity. Their potential use for reducing the use of venom for antivenom production was also evaluated, as mentioned in Table 1 (Calabria et al., 2019; de Almeida Lima et al., 2018; Figueiredo et al., 2014; Souza et al., 2018).

Table 1
Recombinant PLD expression from different *Loxosceles* species.

References	Immunogen (annotation)	Main observations about protection
Fernandes Pedrosa et al. (2002)	*L. laeta*: Smase I (AY093599)	Previous incubation of *L. laeta* venom with sera from rabbit hyperimmunized with SmaseI completely neutralized otherwise dermonecrotic effects observed after intradermal inoculation of this venom in rabbits.
Kalapothakis et al. (2002)	*L. intermedia*: recLiD1 (fused to β-galactosidase)	No neutralization test performed.
Araujo et al. (2003)	*L. intermedia*: Li-rec (fused to β-galactosidase)	Previous incubation of *L. intermedia* venom with sera from rabbit hyperimmunized with Li-rec neutralized 25 LD_{50} *L. intermedia* venom per ml of hyperimmunized sera in a murine lethality model. Partial active protection against dermonecrotic effects after intradermal *L. intermedia* venom inoculation was observed in rabbit hyperimmunized with Li-rec.
Tambourgi et al. (2004)	*L. intermedia*: rP1 (AY304471) and rP2 (AY304472)	Unpublished experiments are mentioned, where sera from rabbit hyperimmunized with rP1 and rP2 were able to neutralize dermonecrotic effects of *L. intermedia* venom.
Lee and Lynch (2005)	*L. reclusa*: Smase D (AY862486)	*In vivo* protection tests were not reported.
Felicori et al. (2006)	*L. intermedia:* recLiD1 (unfused to β-galactosidase)	Partial (75%) protection against lethality from intraperitoneal inoculation of 2.5 LD_{50} *L. intermedia* venom in mice previously immunized with recLiD1.
Olvera et al. (2006)	*L. laeta*: Ll1N (DQ369999), Ll2C (DQ3370000); *L. boneti*: Lb1C (AY559845); *L. reclusa*: Lr1N (AY559846).	Previous incubation with sera from rabbit hyperimmunized with recombinant PLD from *L. reclusa* and *L. boneti* conferred cross neutralization of lethal effects from intraperitoneal inoculation of the venom from these two *Loxosceles* species in mice. In contrast, no cross protection was observed for sera from rabbit hyperimmunized with recombinant PLD between these two *Loxosceles* species and *L. laeta*. Previous incubation of *L. reclusa*, *L. boneti* and, to a lesser extent, *L. laeta* venoms with an antivenom, prepared from plasma of horses hyperimmunized with a combination of Lb1C, Lr1N, Ll1N, and Ll2C, neutralized the lethal effects of intraperitoneal inoculation of these venoms in mice.
De Almeida et al. (2008)	*L. intermedia*: rP1 and rP2; *L. laeta*: Smase I	Passive protection conferred by administration of an antivenom based on plasma from horses hyperimmunized with a combination of rP1, rP2, and Smase I was equal to or better than that conferred by a commercial antiarachnidic serum (Butantan) in an *in vivo* model of dermonecrosis when venoms of *L. laeta*, *L. intermedia* or *L. gaucho* were intradermally inoculated in rabbit.
Felicori et al. (2009)	Six antigenic peptides from *L. intermedia* LiD1	Variable levels of active protection against the dermonecrotic effects of intradermal inoculation of recLiD1 were conferred by immunizing rabbit with recLiD1 with or without the peptide mixture. The level of protection was directly proportional to the ratio of number of recLiD1 doses to number of peptide mixture doses in the hyperimmunization schedule.
Dias-Lopes et al. (2010)	A 27-mer peptide from *L. intermedia* LiD1	Partial protection against lethality after subcutaneous inoculation of LiD1 in mice previously immunized with the 27-mer peptide. Partial protection against dermonecrosis after intradermal inoculation of LiD1 in rabbit previously immunized with the 27-mer peptide.
de Moura et al. (2011)	Two mimotopes (peptides analogous to conformational epitopes) from LiD1	Partial protection against dermonecrotic and hemorrhagic activities (60% and 80%, respectively) from *L. intermedia* venom was observed in rabbit previously immunized with the two mimotopes. Almost complete (90%) protection was observed against both detrimental effects in rabbit previously immunized with *L. intermedia* venom.
Catalán et al. (2011)	*L. laeta*: SMasaD Ll1 (DQ369999) and SMasaD Ll2 (DQ370000), both fused to SUMO protein	Partial active protection against dermonecrotic effects of *L. laeta* venom inoculation in rabbits hyperimmunized with each recombinant protein.
Magalhães et al. (2013)	*L. gaucho*: LgRec1 (JX866729)	Previous incubation of *L. gaucho* venom with sera from rabbit hyperimmunized with LgRec1 conferred partial (70%) protection from local reaction (edema, erythema, ecchymosis, and paleness) and complete (100%) protection from dermonecrosis in a rabbit model of envenomation.
Mendes et al. (2013)	Chimeric protein (rCpLi) displaying 2 linear and 1 conformational epitopes from LiD1	Previous incubation of rLiD1 with IgG purified from rabbit immunized with the chimeric protein neutralized the dermonecrotic and hemorrhagic effects after intradermic inoculation in rabbits.
Figueiredo et al. (2014)	rCpLi	Sera from horses hyperimmunized with a mixture of *L. intermedia*, *L. gaucho* and *L. laeta* venoms conferred protection from dermonecrotic effects of intradermal inoculation of venom from the three Loxosceles species in rabbit. This protection was similar to that obtained with sera from horses where the antivenom mixture doses were partially replaced with rCpLi. When rCpLi was the only immunogen administered to the horses, protection was poor.
Duarte et al. (2015)	*L. intermedia* (recLiD1)	Previous incubation of 2.5 LD_{50} *L. intermedia* venom with 100 μl of antivenom prepared from plasma of horses hyperimmunized with recLiD1 conferred complete protection from lethal effects after intraperitoneal inoculation in mice. Similar exposure of 2.5 LD_{50} *L. laeta* venom with 100 μl of the same antivenom conferred partial (75%) protection from lethality in mice.
(de Almeida Lima et al., 2018)	Chimeric protein (rMEPLox) introducing 3 linear epitopes from a *Loxosceles* venom metalloprotease (LALP-1), 2 linear epitopes of a hyaluronidase (LiHYAL) and 1 linear epitope from SMase I to rCpLi	Complete protection form dermonecrotic effects from intradermal inoculation of *L. intermedia* venom in rabbit previously immunized with rMEPLox. Previous incubation of 2.5 LD50 *L. intermedia* venom with 100 μl sera from rabbit immunized with rMEPLox protected mice from lethal effects of intraperitoneal inoculation of the venom (DE_{50} not calculated).
Souza et al. (2018)	Chimeric protein (Lil) expressing 2 linear epitopes present both in *L. intermedia* and *L. gaucho* PLDs and 2 linear epitopes present in *L. laeta* PLDs.	Previous incubation of *L. intermedia* venom with sera from rabbit hyperimmunized with Lil conferred partial neutralization (37%) of dermonecrotic effects from intradermal inoculation against *L. intermedia* venom to rabbit. Identical incubation of *L. intermedia* venom with sera from rabbit hyperimmunized with a mixture of *L. intermedia*,*L. gaucho* and *L. laeta* venoms conferred almost complete (99%) protection against the same detrimental effects.
Calabria et al. (2019)	Chimeric protein (LgRec1ALP1) expressing hydrophilic regions of PLD and metalloproteases from *L. gaucho*.	Previous incubation of *L. gaucho*, *L. intermedia* and *L. laeta* venom with antibodies from mice hyperimmunized with LgRec1ALP1 neutralized (100%, 79%, and 68% for each venom, respectively) their dermonecrotic effects from intradermal inoculation of these venoms in rabbit.

When the results obtained between chimeric proteins displaying selected PLD epitopes against full-length PLD recombinant proteins are compared, two immediate conclusions can be extracted. Full replacement of venom in equine immunization schedules only leads to comparable results to full venom immunization schedules when full-length PLD isoforms and not when chimeric proteins are used (De Almeida et al., 2008; Figueiredo et al., 2014; Olvera et al., 2006). Nevertheless, chimeric proteins are able to reduce the need for whole venom by up to 67% (Figueiredo et al., 2014). Chimeric proteins might bring an opportunity to simultaneously raise an immune response to epitopes from PLD isoforms of different *Loxosceles* species together with those from other venom components. Thus, chimeric proteins displaying epitopes from the *Loxosceles* metalloproteases LALP-1 and LgALP-1, the hyaluronidase LiHYAL together with conformational and/or linear epitopes from the PLD isoforms of one or more *Loxosceles* species were constructed and used to immunize different animals of experimentation (Calabria et al., 2019; de Almeida Lima et al., 2018; Souza et al., 2018). The sera from the immunized animals proved to be effective for the neutralization of selected catalytic activities (sphyngomielinase, fibrinogenase and hyaluronidase activities) in the venom. Nevertheless, the formulations based on these chimeric proteins prove to be poorly immunogenic: about ten times as much the mass of chimeric proteins as compared to venom was required in immunization schedules to achieve at least partial protection from the pathological effects of venom inoculation in animal models (Calabria et al., 2019; Souza et al., 2018). Thus, at current state-of-the-art, recombinant PLD isoforms are the main immunogen in second-generation antivenoms production, which could allow partial or potentially complete venom replacement.

2. Discussion

Within the venom-dependent approaches, it is very important to investigate novel delivery systems, adjuvants, and detoxification strategies of the whole venom or individual toxins to enhance immune responses and antibody neutralization capacity.

As already discussed, PLD isoforms are the only recombinant antigens from *Loxosceles* venom, at least to our knowledge, that have been used for venom replacement during antivenom production. So far, the recombinant expression of PLD isoforms has been performed exclusively in traditional prokaryote systems. The incorporation of these methodologies to antivenom production requires bioreactors, bacterial homogenizers, dedicated facilities, and specifically trained personnel. *E. coli* is still the workhorse of gene expression, but unfortunately, many recombinant toxins expressed as inclusion bodies require exhaustive *in vitro* refolding steps to recover their biological activities. Although many authors report the successful expression of soluble recombinant PLD isoforms, others found it to be a hard task, observing that the insoluble fractions of PLD are poorly immunogenic (Catalán et al., 2011; Felicori et al., 2006; Fernandes Pedrosa et al., 2002; Kalapothakis et al., 2002; Magalhães et al., 2013; Olvera et al., 2006). Eukaryotic systems are usually less prone to express heterologous eukaryotic proteins as insoluble fractions. Nevertheless, expression in eukaryotic cell lines is significantly more expensive than expression in prokaryotes. But other promising biotechnological platforms, like lepidopteran larvae, deserve attention. Insect larvae are reared and inoculated with recombinant baculoviruses in insectaria under conditions similar to those required for the venomous arthropods used in venom production. Micro to milligram expression of different recombinant proteins per larvae have been reported with this system (Targovnik et al., 2016). Once the proteins are expressed, they are easily recovered from larval extracts by simply use of blenders and filtrating the homogenate. Downstream processing from this step-on is similar for both prokaryotic and larvae expression systems. We have indeed successfully expressed microgram amounts per larvae of an isoform of PLD in both *S. frugiperda* and *Rachiplusia nu* with conserved dermonecrotic activity (unpublished results). Estimating the costs for producing PLD isoforms in prokaryotic and larvae expression systems is suggestive. For this estimation we considered the following assumptions: we centered our calculations in the potential demand of Brazil, the country with the highest reported incidence of loxoscelism. The amount of antigen needed was estimated from the available information from various immunization schedules reported in the literature. A 40% yield can be considered acceptable for F $(ab')_2$ based antivenom production. Taking also into account the number of vials that are indicated for treatment by the National Health authorities of that country and a 5% severe outcome after *Loxosceles* envenomation, about 440 mg of recombinant PLD isoforms would be needed to satisfy the needs of an yearly production of antivenoms to treat all loxoscelesic accidents in that country. Average reported yield for prokaryotic expression systems is near 10 mg/L, then 44 L of cultured bacteria would satisfy the yearly demands from this country. This could be achieved in laboratory to pilot scale industrial microbiology facilities. In our hands, about 27,000 larvae would be required to match this yearly recombinant protein needs. Oral infection with occlusion bodies (OB) through their diet is perfectly suited to perform this task. Thus, upstream processing in this simple platform offers an easy-to-implement alternative for regular insect producing facilities at significantly lower costs when compared to laboratory or pilot scale industrial microbiology facilities.

Another relevant aspect of recombinant PLD expression deserves further attention. Horses, like humans, are particularly susceptible to the toxic effects of PLD (Olvera et al., 2006). This is a relevant aspect to be considered for antivenom production. The chemical or genetic modification of specific histidine and/or glutamic residues on PLD sequence completely abolishes their enzymatic activity, and thus their toxic potential (de Andrade et al., 2006; Vuitika et al., 2016). Noteworthy, circular dichroism studies show that this loss of activity occurs without altering PLD's native $(\alpha/\beta)_8$ TIM-like barrel structure and as such, immunogenic potential. This opens the door to the recombinant expression of genetically inactivated PLD isoforms for the production of antivenoms, which would have several advantages: minimizing animal suffering, increasing productivity, shortening the time of antibody production (as compared with the present methods of immunization, especially in naïve horses), and minimizing animal tissue damage. The recombinant expression of these atoxic PLD variants has so far been focused on characterization and structure elucidation studies but not at the scale of the immunogen production process. The sole available reference, at least to our knowledge about the use of site-directed genetically detoxified PLD for antivenom production is reported in a patent, with successful results according to the authors (Olvera Rodriguez et al., 2012).

3. Conclusion

Loxosceles antivenom production may have several drawbacks, particularly the complexity of the procedure to obtain venom and its low yield. In fact, a high amount of spider venom is needed for immunizing horses during antivenom production and for antivenom control. Using recombinant immunogen, the amount of required venom for the control steps would drastically drop, highly improving production efficiency. Therefore, more studies focusing on the recombinant production of some toxins like PLD are imperative. New recombinant immunogens produced at mg to gram levels by simple low-cost technologies, with high immunogenicity and low toxicity, are expected to change anti-loxosceles venom production in the not-too-distant future.

Ethical statement

The authors declare no conflict of interest.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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