ISJ 2: 152-158, 2005 ISSN 1824-307X

Review

Insights into brown spider and loxoscelism

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Accepted December 27, 2005

Abstract

Loxosceles is a genus of cosmopolitan spiders comprising several species, and popularly known as brown spiders or brown recluses. Brown spider bites can cause dermonecrotic lesions and systemic reactions known as loxoscelism. Systemic effects are less common but may be severe or even fatal in some patients. Systemic manifestations include intravascular hemolysis, disseminated intravascular coagulation and acute renal failure. A rapid diagnosis and an understanding of the venom's molecular activity are crucial for satisfactory treatment. Mechanisms by which venoms exert their deleterious effects are under investigation, and searches are underway for diagnostic envenomation assays. Molecular biology is being used to produce quantities of several of the most important venom molecules and has contributed to the study and understanding of their mechanisms of action.

Key words: brown spider; loxoscelism; venom; recombinant toxins; dermonecrosis

Introduction

More than 40,000 spider species exist, with probably 100,000 to be described, but only 3 taxa are recognized as dangerous, namely Theridiidae, Loxoscelidae and Ctenidae. Moreover, only the genera *Atrax*, *Lactrodectus* and *Loxosceles* are associated with human deaths (Escoubas *et al.*, 2000; Rash and Hodgson, 2002). Early European tales during the Middle Ages linked injuries or illness to spider bites (Schienle *et al.*, 2005). For example the tarantula bite was associated with a disease (tarantism) for which the cure was a frenetic dancing for 3-4 days. This energetic dance, called tarantella, is now a typical Italian dance (Isbister, 2004). Today, as a consequence of mistaken diagnoses of spider bites, scientists are looking for methods to characterize and identify spider bites and their manifestations as well as to better understand the biological and molecular mechanisms of envenomation.

The genus *Loxosceles* (variously known as the brown spider, brown recluse, fiddleback, or gaucho spiders) is important in these studies because of its commonness in and around human dwellings. Their bite is characterized by dermonecrosis and systemic effects known as loxoscelism (Hogan *et al.*, 2004).

The first case of documented loxoscelism occurred in 1879 in Tennessee. However, consistent data traced back about 50 years ago and were collected in Chile, then other observations were made in Brazil followed by the United States. These reports linked brown spider bite with necrotic skin lesions (Macchiavello, 1947; Atkin *et al.*, 1958; Sams *et al.*, 2001). Spiders' habits have caused a close association with humans, and the number of bites is increasing and has become a public health problem in Brazil, Chile and the United States (da Silva *et al.*, 2004). Most bites occur during sleep or dressing, and women are bitten more often than men. Thighs, trunk, hands and arms are more often bitten (Hogan *et al.*, 2004).

Loxosceles spiders

Loxosceles spiders are known as violin (fiddleback) spiders due to a characteristic violin

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shape on their cephalothorax (Futrell, 1992). They are also known as brown spiders because their colour varies from a pale (*L. laeta*) to a dark brown (*L. gaucho*). *Loxosceles* body length ranges from 8 to 15 mm with legs measuring from 8 to 30 mm (da Silva *et al.*, 2004). They are sedentary and nocturnal (Andrade et al., 1999) with a lifetime of 3 – 7 years (Andrade *et al.*, 2000). Brown spiders have three pairs of eyes (an important characteristic useful to identify the genus) (Vetter and Visscher, 1998). They build irregular, cottony webs (Futrell, 1992) and normally prefer dead scavenged prey rather than live preys (Sandidge, 2003). They can survive months without food or water and withstand temperatures ranging from 8 °C to 43 °C. They are not aggressive and prefer dark dry places (Futrell, 1992; Málaque *et al.*, 2002; Vetter and Barger, 2002; da Silva *et al.*, 2004). The sexes produce venom with differences in volume, toxicity and compounds proportion (Oliveira *et. al.*, 1999). Comparative analysis of sex and species in *L. laeta* and *L. intermedia* venom showed some biological activities (complement-dependent hemolysis and dermonecrosis) more prominent in venom from female spiders, especially from *L. laeta* (Oliveira *et al.*, 2005).

Epidemiology

Loxosceles spiders can be found distributed all over the world. In North America, the most important species are *L. reclusa*, *L. deserta*, *L arizona*, *L. rufences* (United States and Mexico) and *L. laeta* (Canada) (Sams *et al.*, 2001; Vetter and Bush, 2002a). Europe, Africa, Middle East, some parts of Asia, Israel, and Australia are hosts to some *Loxosceles* species (Futrell, 1992; Borkkan *et al.*, 1995; Young and Pincus, 2001; Nicholson and Graudins, 2003).

In Brazil, seven species have been described but three are the most implicated in human bites *L. intermedia, L. gaucho and L. laeta* (Sezerino *et al.*, 1998). From 1990 to 1993, the Brazilian Ministry of Health received 17.781 reports of spiders' bites, of which 36 % were due to *Loxosceles* (Sezerino *et al.*, 1998). In the metropolitan area of Curitiba, in the state of Parana (southern Brazil) about 3.000 brown spider bites are reported annually (Málaque *et al.*, 2002). In a retrospective study in Florianopolis, in the state of Santa Catarina, Brazil, 487 suspected cases of brown spider bites were found, 267 of which fulfilled the criteria for inclusion in the study (Sezerino *et al.*, 1998). In 359 cases of loxoscelism between January 1985 and December 1996 at Butantan Intitute, São Paulo, Brazil, 14 % of patients captured the spiders so that 28 were classified as *L. gaucho*, 5 as *L. laeta* and 18 as non-classified *Loxosceles* (Málaque *et al.*, 2002). More bites occur in warmer months (Schenone, 1996). In Curitiba, from 1998 to 2001 the incidence of *Loxosceles* bites was 1.4 cases per 1,000 habitants. Of these, 23 % were in the thigh, 16.7 % in the trunk, 14 % in the arm and 13 % in the lower leg. Only 1 % of cases were severe (Health Secretary, Curitiba, Parana, Brazil, 2002).

Pathophysiology of Loxoscelism

Dermonecrosis is the hallmark of loxoscelism (Fig. 1). Histopathology and clinical data are obtained from biopsies of human patients after brown spider bites. Rabbit skin artificially injected with *Loxosceles* venom is used for more controlled investigation since this animal model reproduces human skin lesions that follow envenomation (Ospedal *et al.*, 2002). Systemic effects, such as renal failure, are less common and are usually reproduced in mouse (Luciano *et al.*, 2004). Observation of human skin biopsies showed an inflammatory infiltrate, thrombosis, hemorrhage, dermatitis, erythema, induration of affected area and liquefactive necrosis of the epidermis and dermis consistent with pyoderma grangrenosum (Futrell, 1992; Yannias and Winkelmann, 1992). Symptoms in an experimental study in rabbits showed that after 4 h oedema, hemorrhage, degeneration of blood vessel walls, plasma exudation, thrombosis, neutrophil accumulation in and around blood vessels with an intensive diapedesis, a diffuse collection of inflammatory cells (polymorphonuclear leucocytes) in the dermis, and subcutaneous muscular oedema all occur. Over the following hours and up to 5 days after envenomation, the changes progressed to a massive neutrophil infiltration into the dermis and even into subcutaneous muscle tissue, destruction of blood vessels, thrombosis, hemorrhage, myonecrosis, and coagulative necrosis on the 5th day (Ospedal *et al.*, 2002). Neutrophil participation and the inflammatory response seem to be dependent on an endothelial cell agonist effect triggered by the venom that leads to an indirect and dysregulated neutrophil activation involved in dermonecrosis (Patel, 1994). Envenomation of rabbit skin with *L. reclusa* venom after 14 days results in a mixed inflammatory cell infiltrate, coagulative tissue necrosis, vasculitis and a dense band of neutrophils bordering the zone of necrosis (Elston *et al.*, 2000). *L. intermedia* venom damaged vessel endothelia, as shown by vessel instability, endothelium cell vacuolization in biopsies of rabbit skin (Veiga *et al.*, 2001a; Zanetti *et.al.*, 2002). *In vitro* experiments on rabbit aorta endothelium cell cultures showed cytotoxicity of *L. intermedia* venom associated with loss of cell adhesion to the culture substrate and the shedding of proteoglycans from the extracellular matrix and cell surface into the medium (Veiga *et al.*, 2001a). In human umbilical vein endothelial cell (HUVEC) cultures treated with *L. reclusa* venom, agonist activity ensued, inducing endothelial cell expression of E-selectin and the release of interleukin (IL)-8 and granulocyte macrophage colony-stimulating factor, resulting in dysregulated inflammatory response (Patel *et al.*, 1994). HUVEC exposed to *L. deserta* venom produced IL-8, growth-related oncogene-α and monocyte chemoattractant protein-1 via an NF-κB- dependent pathway (Desai *et al.*, 1999; Gomez *et al.*, 1999). *L. deserta* venom induces the expression of vascular endothelial growth factor (VEGF) in human keratinocytes, suggesting that keratinocyte-derived VEGF may contribute to vasodilatation, oedema and erythema in brown spider envenomation (Desai *et al.*, 2000). Primary cultures of keratinocytes exposed to 100 ng/ml of *L. gaucho* venom release tumour necrosis factor (TNF)-α into the medium after 6 h (Málaque *et al.*, 1999).

Mice injected with *L. reclusa* venom developed local hemorrhage after 6 h accompanied by blistering of the ear skin (Sunderkötter *et al.*, 2001).

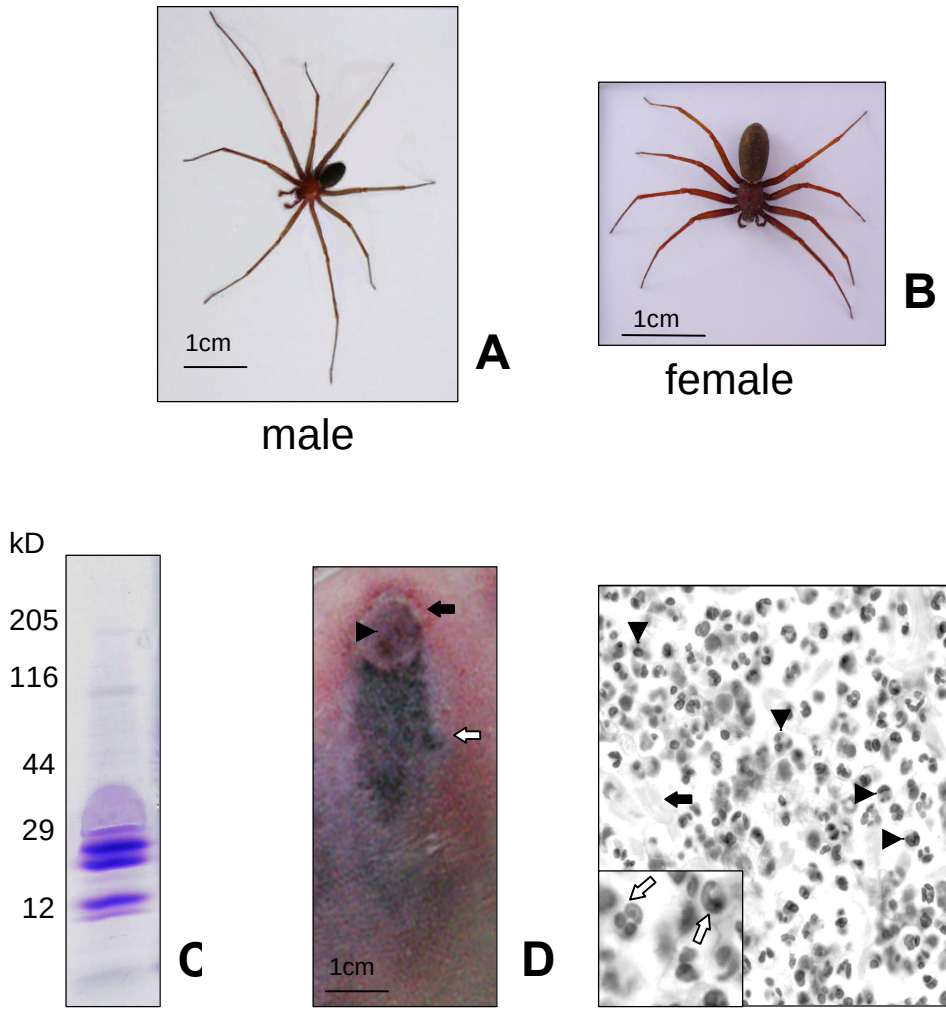

Fig. 1 Cellular and molecular aspects of brown spider and loxoscelism. (A) *Loxosceles intermedia* (brown spider) male. (B) *L. intermedia* (brown spider) female. (C) SDS-PAGE 3-20 % venom profile stained by Coomassie blue dye. (D) Dermonecrotic lesion on rabbit skin after 24 h post-*L. intermedia* venom (10 µg) exposure. Arrowhead indicates the site of venom injection with characteristic black and white eschar named marble plate. Black arrow points an erythema surrounding the lesion and white arrow shows the gravitational spreading of lesion (a hallmark of dermonecrotic loxoscelism). (E) Microscopical view of dermonecrotic lesion showing inflammatory leukocytes accumulated in the connective tissue (arrowhead) and disorganization of collagen fiber and oedema (black arrow) (magnification 400X). The inset shows inflammatory cells of the infiltrate represented by neutrophils (white arrow) (magnification 1.000X).

Histopathology showed a vasculitis reaction 2 h after exposure. The microscopical analysis of some mouse organs injected with different doses of *L. intermedia* venom revealed remarkable kidney alterations. Acute tubular necrosis accompanied by deposition of eosinophilic material inside the proximal and distal renal tubules was seen in several nephrons (Tambourgi *et.al.*, 1998). Mouse kidneys, treated with *L. intermedia* venom showed hyalinisation and erythrocytes in the Bowman's space, glomerular collapse, tubular epithelial cell cytotoxicity and deposition of eosinophilic material within the tubular lumen (Luciano *et al.*, 2004). Confocal microscopy observations of double staining immunofluorescence against type IV collagen or laminin and *L. intermedia* venom showed that toxins deposit and bind along the tubular and glomerular basement membrane of mice kidneys. Ultrastructural observations showed glomerular epithelial and endothelial cell cytotoxicity, the collapse and destruction of glomerular basement membrane and tubular epithelial cell degeneration. The basement membrane is a target for brown spider venom, as shown administrating *L. intermedia* venom to murine tumor Engelbreth-Holm-Swarm (EHS), which is rich in basement membrane molecules. *L. intermedia* venom degraded and fragmented the basement membrane (Veiga *et.al.*, 2000a). Venom

displays hydrolytic activity for entactin and heparan sulphate proteoglycan, two important constituents of basement membranes, while having no apparent activity on purified type IV collagen and laminin (Veiga *et al.*, 2000a, 2001a,b).

In the bone marrow and peripheral blood cells, *L. intermedia* initially causes a decrease in the number of nucleated red cells, bone-marrow depression of megakariocytes with thrombocytopenia in peripheral blood and decrease of platelet count (da Silva *et al.*, 2003). Neutropenia in peripheral blood and low neutrophil counts were observed as consequence of bone-marrow depletion, which may reflect an extensive neutrophil influx to the tissues. Eosinophils are apparently unaffected.

Brown spider venom toxins

L. intermedia and *L. laeta* have different protein patterns of glycosylation and the same is between sexes of the same species (Oliveira *et al.*, 2005). Hemolytic and dermonecrotic activities have been described for *L. similes* venom. Sphingomyelinase D molecules, with molecular mass ranging from 30 to 35 kDa and having hemolytic, necrotic and platelet aggregation activity were found in *L. reclusa*, *L. rufescens*, *L. gaucho*, *L. laeta* and *L. intermedia* venoms (Futrell 1992; Barbaro *et al.*, 1994; Mota and Barbaro, 1995; Tambourgi *et al.*, 1995; Barbaro *et al.*, 1996a,b, 1997). Three sphingomyelinase D isoforms were purified from *L. boneti* venom (Lb1, Lb2 and Lb3). Only Lb1 and Lb2 had dermonecrotic activity (Ramos-Cerrillo *et al.*, 2004). An alkaline phosphatase was described in *L. reclusa* venom (Futrell, 1992). Hyaluronidase (32.5 kDa) was found in *L. refescens* and *L. reclusa* (Futrell, 1992; Young and Pincus, 2001). *L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta* and *L. reclusa* venoms contained an enzyme of similar molecular size (44 kDa), which digested hyaluronic acid (Barbaro *et al.*, 2005). A 5'-ribonucleotide phosphohydrolase was found in *L. reclusa* venom (Futrell 1992). Loxnecrogin A (31.4 kDa) and Loxnecrogin B (31.6 kDa) with necrotic activity on rabbit skin were found in *L. gaucho* venom (Cunha *et al.*, 2003). *L. intermedia* has a range of proteases described in its venom: Loxolysin A (20-28 kDa) with fibronectinolytic and fibrinogenolytic activity; Loxolysin B (32-35 kDa) with gelatinolytic activity (Feitosa *et al.*, 1998); a serin protease (85 kDa) with gelatinolytic activity (Veiga *et.al.*, 2000b) and proteases able to hydrolyse entactin, heparan sulphate proteoglican and basement membrane (Veiga *et al.*, 2000b, 2001a,b). *L. rufescens* also has a broad molecular range of caseinolytic, gelatinolytic and fibrogenolytic metalloproteases (Young and Pincus, 2001). To test whether proteases in *L. intermedia* venom were due to natural constitution and not a digest fluid contamination, da Silveira *et al.*, (2002) compared the proteolytic activity of the venom obtained directly from venom glands with that obtained by electroshock. Both protein profiles showed very similar electrophoretic and enzymatic characteristics.

At present, a new generation of molecules developed through cloning techniques is under study. *L. intermedia* LiD1 recombinant protein (31.4 kDa) is a sphingomyelinase D family molecule without dermonecrotic activity but with antigenic activity (Kalapothakis *et al.*, 2002). *L. laeta* recombinant protein (33 kDa) is a sphingomyelinase isoform able to degrade sphingomyelin (Pedrosa *et al.*, 2002). This recombinant protein induced complement susceptibility, release of glycophorins and had dermonecrotic activity. *L. intermedia* recombinant protein (LiRecDT, 34 kDa) has dermonecrotic activity and was able to directly induce nephrotoxicity in mice (Chaim *et al.*, 2005). *L. laeta* recombinant phospholipase D generated lysophosphatidic acid and was hemolytic (Lee and Lynch, 2005).

Clinical features, diagnosis and treatment of brown spider bites

Diagnosis of loxoscelism is rarely based on spider identification and therefore clinical features, epidemiological and historical findings must be well known (Wright *et al.*, 1997; Vetter, 1999; Málaque *et al.*, 2002). Lesion recovery improves once the patient is treated. However, brown recluse bites have been misdiagnosed in North America because they occurred in regions of non-endemicity (Vetter, 1999; Nishioka, 2001; Vetter and Barger, 2002; Vetter and Bush, 2002a,b; Vetter *et al.*, 2003). A typical necrotic skin lesion begins soon after the spider bites the victim, followed by gravitational spreading (da Silva *et al.*, 2004). The bite is painless, hence the patient is often unaware that he has been bitten (Futrell, 1992), and the delay between the bite and when the victim pursues help makes the treatment less effective. From mild to severe pain begins 2-8 h after the bite. At the bite a small puncture wound may appear, associated with transient erythema with itching and swelling and mild to severe tenderness (Futrell, 1992; da Silva *et al.*, 2004). Blebs or blisters appear (12-24 h), may become hemorrhagic, and surrounded by a halo of ischemic tissue. In the following days, necrotized lesions become a dull blue-violet, the area of the gravitational spread turns blue, and the size of the blue area increases. Within three to seven days an eschar may form, after which the lesion hardens. The eschar may drop off leaving an ulcer that may require a skin graft (Schenone, 1996; Sezerino *et al.*, 1998; Málaque *et al.*, 2002; da Silva *et al.*, 2004).

Success of therapy depends upon a correct and rapid diagnosis, the volume of the venom injected, and the patient susceptibility to the venom (Futrell, 1992; da Silva *et al.*, 2004). Phentolamine, heparin, topical nitro-glycerine, cyproheptadine and hyperbaric oxygenation have been used for therapy, but the efficacy of these therapies is inconclusive and their use is not recommended (Futrell, 1992; Wendell, 2003; da Silva *et al.*, 2004). The established therapy is dapsone, acetylsalicylic acid (aspirin), antibiotics (erythromycin and cephalosporin), ice and elevation, avoidance of strenuous activity and heat and, when necessary, surgery. Early surgical excision has not been shown to be effective and often delays healing (Futrell, 1992; Merigian and Blaho, 1996; Goddard, 1998, Monteiro *et al.*, 2002; da Silva *et al.*, 2004). Serum anti-*Loxosceles* venom is used only in severe cases and effectiveness is doubtful especially against local manifestation. Systemic envenomation studies in

animals and humans have demonstrated that antivenom neutralizes the deleterious effects of the venom and reduces paediatric mortality (Isbister *et al.*, 2003). Effectiveness of antivenom to prevent dermonecrotic lesions seems to be time dependent and usually patient looks for medical help 4 h after the bite when lesions is already established (Ospedal *et al.*, 2002; Nicholson and Graudins, 2003). Some local and systemic noxious activities of the venom are attributed to proteolytic toxins that degrade fibrinogen, fibronectin, entactin and heparan sulphate proteoglycan and disrupt basement membrane structures, thereby causing local hemorrhage, gravitational spreading, disseminated intravascular coagulation and renal failure (Feitosa, *et al.*, 1998; Veiga *et al.*, 1999, 2000b, 2001a,b; Luciano *et al.*, 2004; Chaim *et al.*, 2005).

Biotechnological products from brown spider venom

Recently developed technologies are being used to produce biotechnological products from *Loxosceles* venom. ARACHnase (Hemostasis Diagnosis International Co., Denver, CO, USA), normal plasma containing *L. reclusa* venom, mimics a lupus anticoagulant and may provide a positive control for anticoagulant testing (McGlasson *et al.*, 1993). An antiserum against venoms of *L. gaucho*, *Phoneutria nigriventer*, *Tityus serrulatus*, and *Tityus bahiensis* that reacts with *L. intermedia* and *L. laeta* toxins is produced by The Butantan Institute, São Paulo, Brazil. The CCPI (Production Center of Immunobiologic Products, Parana, Brazil) has also produced antiserum using *L. intermedia* venom that is able to neutralize some activities of *Loxosceles* venom. *L. laeta* antiserum is produced by the National Institute of Health (Peru) (da Silva *et al.,* 2004). These antisera have all been used as bioproducts for serum therapy (Roodt *et al.,* 2002; Barbaro *et al.*, 1994; 1996a; Health Secretary, Curitiba, Parana, Brazil). Guilherme *et al.* (2001) produced monoclonal antibodies recognising *L. gaucho* venom toxins, which were able to neutralize the dermonecrotic effect and lethal activities of this species venom but not those of heterologous venoms.

Monoclonal and polyclonal antibodies are not only powerful tools for neutralizing the effects of venom; they are also useful for research. They can be used to purify toxins from venom by affinity chromatography. They can be used on location of specific toxins on cell and tissue treated with venom toxins. Immunofluorescence techniques such as confocal microscopy and flow cytometry are modern techniques based on antibody specific binding.

In contrast to the collection of snake venom, spiders provide very little venom, which limits the ability to study spider venom toxins. Protein cloning techniques are helping to solve this problem. After cloning it is possible to have milligrams of the same protein thereby improving the quality of research work and allowing more controlled experimental studies. Today several spider venom recombinant proteins are under investigation most of which are in the sphingomyelinase protein family (Kalapothakis *et al.,* 2002; Pedrosa *et al.,* 2002; Chaim *et al.,* 2005).

Future perspectives

Toxins from *Loxosceles* spiders are a group of proteins with a great range of different activities. Each toxin may be used to investigate molecular and cellular effects of venom. Also each of these proteins is a putative molecular model for drug design and to develop knowledge on some effects not yet fully understood such as the inflammatory reaction of dermonecrosis and platelet aggregation.

Acknowledgements

This work was supported by CNPq, CAPES, Fundação Araucária and Secretaria de Estado de Ciência, Tecnologia e Ensino Superior do Parana, Brazil.

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