

Proteolytic effect of *Loxosceles intermedia*
(brown spider) venom proteins on EHS-basement
membrane structures

Efeito proteolítico do veneno de
Loxosceles intermedia (aranha marrom) sobre
estruturas da membrana basal de EHS

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Loxoscelism is mainly characterized by two typical clinical signals, *i.e.*, a dermonecrotic lesion and systemic effects. The dermonecrotic lesions appear at the bite site, with erythema, edema, and a local dermal haemorrhage, that can evolve to the formation of necrotic sore of difficult cicatrization with degenerative implications for the affected tissue (FORRESTER, BARRET & CAMPBELL, 1978; FUTRELL, 1992). Systemic effects are characterized by fever, malaise and bleeding that can evolve to hemolysis, thrombocytopenia, disseminated intravascular coagulation, and renal failure (KURPIEWSKI *et al.*, 1981; BASCUR, YEVENES & BORGIA, 1982; REES *et al.*, 1988; TAMBOURGI *et al.*, 1995).

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Dermonecrotic lesions, thrombocytopenic activity, disseminated intravascular coagulation and renal failure are events that could be ascribed to the presence of proteolytic enzymes that degrade extracellular matrix molecules like fibronectin and fibrinogen (FEITOSA *et al.*, 1998; VEIGA *et al.*, 1998, *in press*) and especially the basement membrane of subendothelial capillary cells with a consequent reduced stability of the vessel wall, changing the normal surroundings for platelet adhesion and aggregation or altering the integrity of the glomerular basement membrane involved in the filtering of molecules from blood to urine (FARQUHAR *et al.*, 1991; ROHRBACH & TIMPL, 1993), as demonstrated in haemorrhage produced by snake bites (BARAMOVA *et al.*, 1986; HITE *et al.*, 1992). The existence of proteolytic enzymes such as metalloproteases, hyaluronidases and sphingomyelinase has been demonstrated to be present in brown spiders venom (FORRESTER, BARRET & CAMPBELL, 1978; KURPIEWSKI *et al.*, 1981; FEITOSA *et al.*, 1998; REKOW, CIVELLO & GREEN, 1983). Renal failure with proteinuria provoked by brown spider bites represents a potential action of currently unknown venom principles on renal tissue and on the renal extracellular matrix (with special emphasis on the glomerular basement membrane that acts on renal physiology like a selective barrier, establishing a filtering action between blood and urine formation).

ECM, which is structurally separated into basement membrane, connective matrix and plasma matrix, is remarkable for its complex structure consisting of secreted proteins and glycoconjugates that create a molecular network when tridimensionally assembled (YURCHENKO & SCHITTNY, 1990). ECM interactions with receptor molecules on the cell surface support several biological processes such as cell adhesion, locomotion and differentiation, playing an important physiological role in the homeostatic functions of tissues (ALBELDA & CLAYTON, 1990; MEREDITH, FAZELI & SCHWARTZ, 1993; VEIGA *et al.*, 1997). Basement membranes (a specialized kind of ECM) acts like a biomolecular filter separating many specialized tissues such as muscle, epithelial, endothelial, and nervous tissue from the respective connective tissues (MARTIN & TIMPL, 1987). Although they are widely disseminated in dif-

ferent tissues of organism, their molecular features are highly conserved. Basement membranes are composed of four main molecules, *i. e.*, laminin, type IV collagen, entactin and heparan sulfate proteoglycan (YURCHENCO & SCHITTNY, 1990; TIMPL, 1996). Basement membranes promote cell differentiation and neurite outgrowth, are involved in angiogenesis, platelet adhesion, blood-urine filtration in the kidney glomerulus where urine is formed, and also perform several other functions (FARQUHAR, 1991; ROHRBACH & TIMPL, 1993; TIMPL *et al.*, 1987). The action of molecular constituents of brown spider venom on basement membranes could explain the effects observed in *Loxoscelism*, with emphasis on haemorrhagic processes (subendothelial blood vessel basement membrane) and renal failure (glomerular basement membrane). In the present study, we checked the possibility that *L. intermedia* venom could act directly on basement membrane structures. Engelbreth-Holm-Swarm (EHS) tumor was used, a sarcoma producing large amounts of basement membrane that has been used for the last years as the most useful model to study this specialized ECM because of the easy extraction of basement membranes and their constituents and also because of the conserved characteristics of the latter, which render them similar to normal adult mammalian basement membrane structures (TIMPL *et al.*, 1987; TIMPL *et al.*, 1979). The EHS tissues were fixed in modified Karnovsky's fixative (KARNOVSKY, 1965) for 1 h. After fixation, the tissues were washed in 0.1 M cacodylic acid buffer, pH 7.3, and incubated with PBS or *Loxosceles intermedia* venom/PBS (100 µg/ml) overnight with shaking at 37°C and postfixed in 1 % OsO₄ in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h. They were then dehydrated in ethanol, critical-point dried, sputter-coated with gold and examined with a MEV XL-30 Philips scanning electron microscope. EHS tissue incubated with PBS served as negative controls. Figure 1A shows an EHS tissue that was incubated with PBS (arrow points the basement membrane that is a capsule of tumor), and Figure 1B shows an EHS tissue that was incubated with brown spider venom. A clearly visible disruption of the basement membrane (arrow) can be seen in EHS sections treated with venom, indicating that some of the constituents of the base-

ment membrane are degraded by the enzymes found in venom.

For transmission electron microscopy the tissues were fixed in modified Karnovsky's fixative (KARNOVSKY, 1965) for 2 h. After fixation, the tissues were washed in 0.1 M cacodylic acid buffer, pH 7.3, postfixed in 1 % OsO_4 in 0.1 M cacodylic acid buffer, pH 7.3, for 1 h, dehydrated with ethanol and propylene oxide, and embedded in Epon. Thin sections and ultrathin sections were then cut with a diamond knife on an LKB ultramicrotome. Ultrathin sections were incubated with PBS (Figure 2A) or *Loxosceles intermedia* venom/PBS (100 $\mu\text{g}/\text{ml}$) (Figure 2B) overnight at 37°C under humidified conditions. After incubation, these ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a JEOL-JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 KV. We can see extensive destruction of the basement membrane treated by venom, compared with negative control (arrows).

Paraffin-embedded EHS-tissue sections mounted on glass slides were deparaffinized in xylene overnight and rehydrated in a graded ethanol series and water (BEÇAK & PAULETE, 1976). The EHS-tissue sections were incubated with PBS (control) (figure 3A) or with *Loxosceles intermedia* venom (100 $\mu\text{g}/\text{ml}$) (Figure 3B) overnight at 37°C under humidified conditions. The EHS-sections were then washed with PBS and incubated in 3 % H_2O_2 at room temperature for 15 min to inhibit the activity of endogenous peroxidase, washed with PBS and nonspecific protein-binding sites were blocked with 1 % bovine serum albumin in PBS at room temperature for 30 min under humidified conditions. After washing in PBS, EHS-sections were incubated with a primary polyclonal anti-laminin antibody (Rb_1aLN) diluted 1:500 overnight at 4°C. Excess antibody was removed with PBS and incubated with goat anti-rabbit IgG peroxidase conjugate (Sigma) diluted 1:100. Following further washing in PBS, diaminobenzidine was used to visualize the immunoreactivity. Sections were washed in PBS and water, dehydrated in ethanol, cleared in xylene, and mounted in Entellan. We can see a reduction in the staining profile in EHS section treated with venom, compared to control, and a basement membrane disruptive effect (arrows).

Finally, in the figure 4, the major non-collagenous basement membrane molecules represented by the laminin-entactin dimer complex (0.5 mg diluted in 50 mM Tris-HCl buffer, pH 7.3, containing 1 mM CaCl_2 and 1 mM MgCl_2) purified from EHS tumors produced in 2-month-old C57-BL/10 female mice as described (23) were incubated with 100 μg of *Loxosceles intermedia* venom for a period of 16 hours at 37°C (lane 1), or were incubated with PBS under the same experimental conditions as negative control (lane 2); Lane 3 shows the electrophoretic positions of the major proteins of venom. These samples were analysed by linear gradient 3-15 % SDS-PAGE under reducing conditions and stained with Coomassie Blue R for visualization (LAEMMLI, 1970). Figure shows that the $\alpha 1$ laminin chain (open arrow) and the $\beta 1/\gamma 1$ laminin chains that comigrate (closed arrow) were not cleaved by the venom. However, the protein pattern of the entactin chain (arrowhead), a molecule of 150 kDa, was reduced after venom treatment, and fragments of approximately 100 kDa (minus signal) and 50 kDa (plus signal) can be seen. Entactin interacts with the other three major molecules of the basement membrane (laminin, type IV collagen and heparan sulfate proteoglycan), and has the important function of stabilizing this ECM structure.

Based on these results and on the cleavage of entactin by the brown spider venom constituents, we may propose that the basement membrane-disrupting effect of the venom is a possible and plausible mechanism for haemorrhage and renal failure, which, together with other anticoagulant properties of the venom (FUTRELL, 1992; KURPIEWSKI *et al.*, 1981; FEITOSA *et al.*, 1998) provides a plausible mechanism for the toxicity of brown spider venoms.

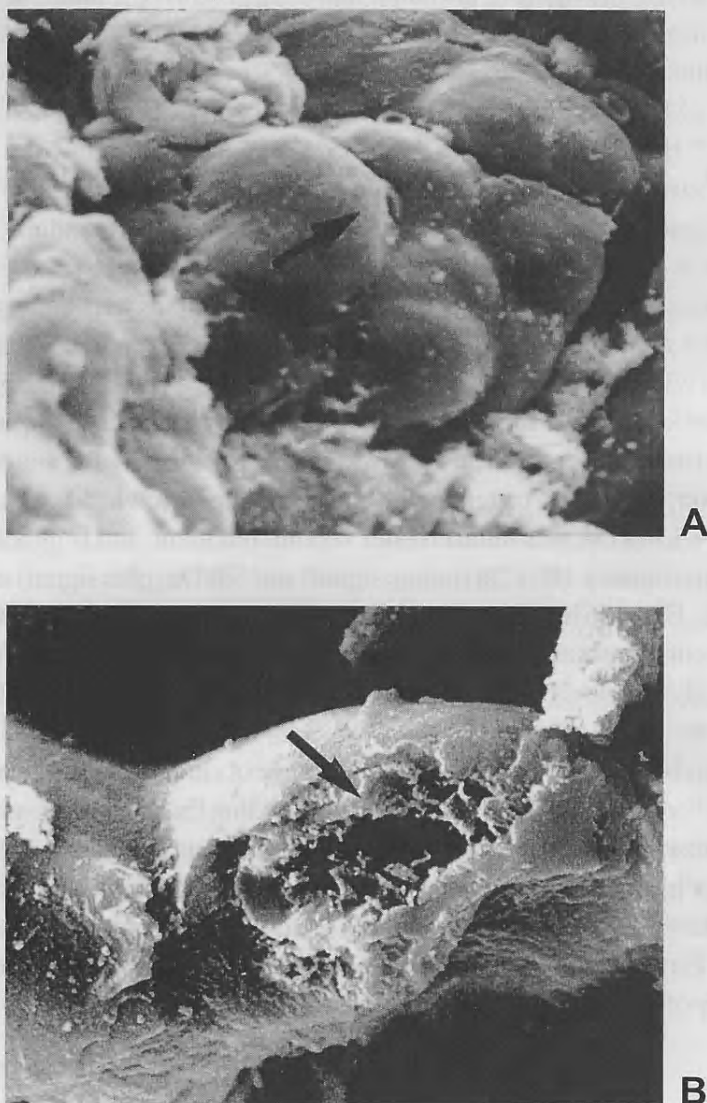


Fig. 1. Effects of *Loxosceles intermedia* venom on EHS visualized by scanning electron microscopy. A, EHS basement membrane (arrow) incubated with PBS (negative control) overnight at 37°C; B, EHS basement membrane treated with *L. intermedia* venom under the same experimental conditions as described above. The arrow points at an area where the basement membrane was disrupted by the action of the venom.

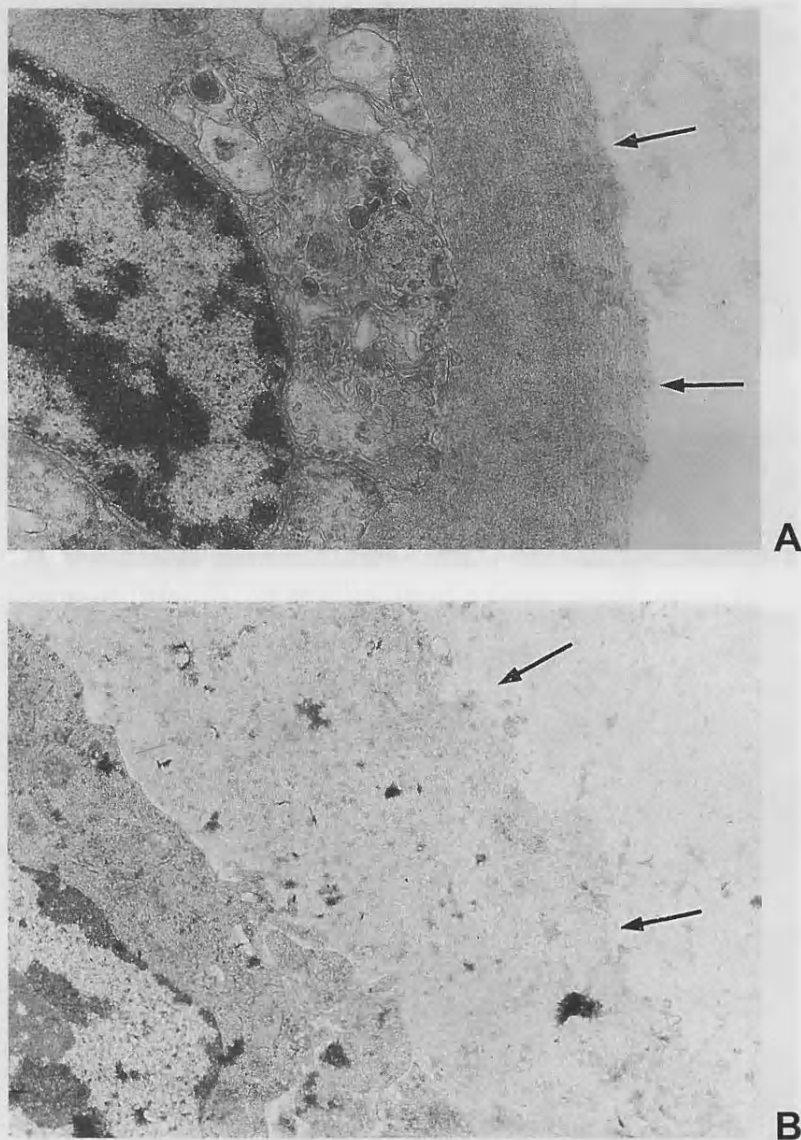


Fig. 2. Effects of *Loxosceles intermedia* venom on EHS visualized by transmission electron microscopy. A, EHS basement membrane treated overnight with PBS (negative control) at 37° C. The arrow indicates the intact basement membrane; B, EHS basement membrane that was incubated with *L. intermedia* venom under the same experimental condition. The arrow points at the basement membrane that was clearly fragmented by the venom.

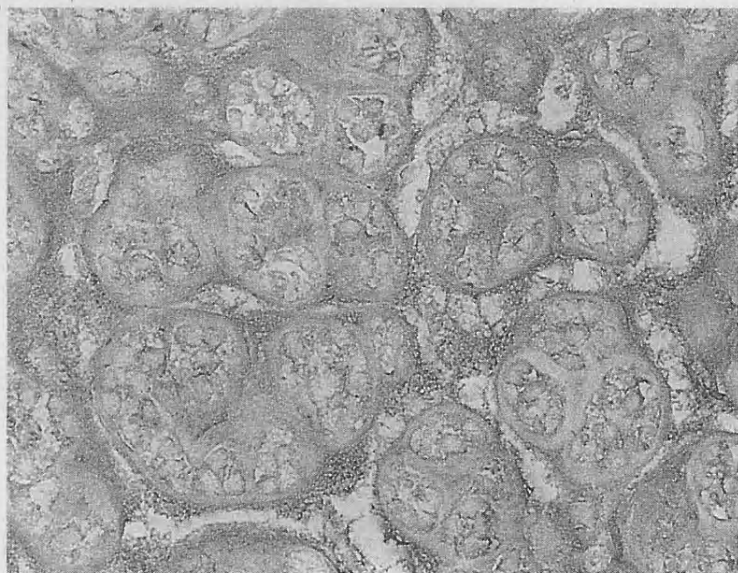
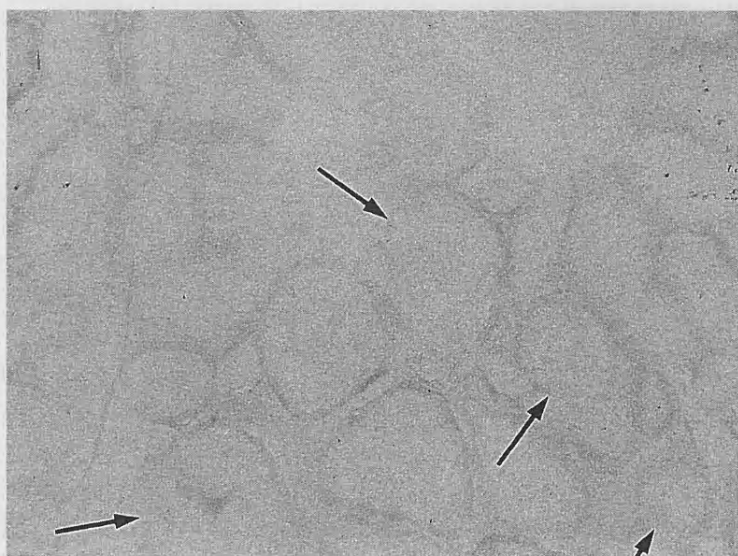
**A****B**

Fig. 3. Effects of *Loxosceles intermedia* venom on EHS visualized by light microscopy. A, EHS basement membrane incubated with PBS at 37° C as control and visualized by an immunohistochemistry using a rabbit polyclonal antibody against laminin; B, EHS basement membrane treated overnight with *L. intermedia* venom under the same experimental conditions. The arrows point at the basement membrane that was fragmented by the venom.

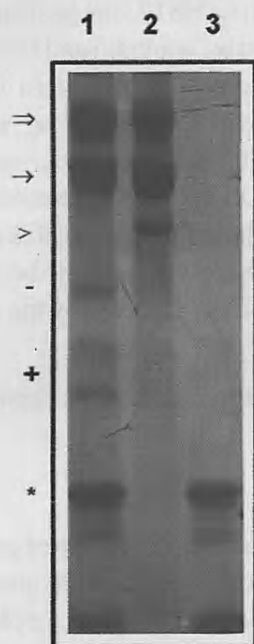


Fig. 4. Action of *Loxosceles intermedia* on purified laminin-entactin dimer complex. Laminin-entactin complex purified from EHS tumor was incubated with *Loxosceles intermedia* venom or PBS for 16 hours at 37° C. Lane 1 shows the complex treated with venom; Lane 2 shows the complex treated with PBS (negative control); and Lane 3 shows only venom. The open arrow points at the $\alpha 1$ laminin chain; the closed arrow points at the $\beta 1/\gamma 1$ laminin chains that comigrate; the arrowhead indicates entactin; the minus signal indicates the entactin fragment (100 kDa) that was produced by the action of the venom; plus signal indicates entactin fragments of approximately 50kDa (an asterisk indicates the venom profile).

SUMMARY

The envenomation induced by bites of spiders of the genus *Loxosceles* (brown spider) is known as Loxoscelism and is remarkable for a dermonecrotic lesion and systemic effects. These events are probably attributable to the presence of proteolytic enzymes in brown spider venom that degrade extracellular matrix (ECM) constituents. The objective of the present study was to determine whether brown spider venom can act on the basement membrane, a specialized kind of ECM. Using

Engelbreth-Holm-Swarm (EHS) tissue sections treated with brown spider venom and analysed by scanning and transmission electron microscopy and light microscopy, we detected a clearly visible destruction of the basement membrane structure. Using purified laminin-entactin complex, two major constituents of basement membranes, treated with venom and analysed by SDS-PAGE, we detected a partial degradation of the entactin molecule. The degradation of this molecule and the basement membrane disruption activity appear to be a plausible mechanism for some of the systemic effects triggered by the venom, as renal failure and haemorrhage.

Key words: *Loxocles*, loxoscelism, venom proteins.

RESUMO

O envenenamento induzido por acidentes com aranhas do gênero *Loxosceles* (aranha marrom) é conhecido como Loxoscelismo e é característico por uma lesão dermonecrótica e efeitos sistêmicos. Estes eventos são atribuídos provavelmente à presença de enzimas proteolíticas no veneno desta aranha que degrada constituintes da matriz extracelular (MEC). O objetivo do presente estudo foi determinar o quanto o veneno da aranha marrom pode agir na membrana basal, um tipo especializado de MEC. Usando cortes de tecido de Engelbreth-Holm-Swarm (EHS) tratados com o veneno da aranha marrom e analisados por microscopia eletrônica de transmissão, varredura e microscopia de luz, detectamos uma destruição visível da estrutura da membrana basal. Usando complexo laminina-entactina purificado (dois dos principais constituintes das membranas basais) tratados com veneno e analisados por SDS-PAGE, detectamos uma degradação parcial da molécula de entactina. A degradação desta molécula e a atividade de rompimento da membrana basal aparenta ser um mecanismo plausível para alguns dos efeitos sistêmicos ativados pelo veneno, como a deficiência renal e hemorragia.

PALAVRAS CHAVE: *Loxocles*, loxoscelismo, veneno.

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