Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

Activities against hemostatic proteins and adrenal gland ultrastructural changes caused by the brown widow spider *Latrodectus geometricus* (Araneae: Theridiidae) venom

Belsy Guerrero [a], Hector J. Finol [b], Matias Reyes-Lugo [c], Ana M. Salazar [a], Elda E. Sánchez [d], Amalid Estrella [e], Antonio Roschman-González [b], Carlos Ibarra [a], Ivan Salvi [c], Alexis Rodríguez-Acosta [e,*]

[a] Pathophysiology Laboratory, Medicine Experimental Center, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela
[b] Centre for Electron Microscopy of the Science Faculty, Universidad Central de Venezuela, Caracas, Venezuela
[c] Medical Entomological Laboratory, Tropical Medicine Institute, Caracas, Venezuela
[d] Natural Toxins Research Center (NTRC), Texas A&M University-Kingsville, MSC 158, Kingsville, TX 78363, USA
[e] Immunochemistry Section, Tropical Medicine Institute of the Universidad Central de Venezuela, Caracas, Venezuela

ARTICLE INFO

ABSTRACT

Brown widow spider (BrWS) (*Latrodectus geometricus*) venom produces intense systemic reactions such as cramps, harsh muscle nociceptive, nauseas, vomiting and hypertension. The proposed pathogenic mechanisms resulting in these accidents have principally been damages occurring at the nervous system. However, it is suspected that there is also damage of the adrenal glands, as a result of the experimental animal's clinical manifestations, which developed symptoms compatible with acute adrenal insufficiency. We have currently found that the adrenal gland is damaged by this venom gland homogenates (VGH) producing severe alterations on cortex cells resulting in death by acute adrenal insufficiency. In general, the ultrastructural study on the glands of mice under transmission electronic microscopy observations showed alterations in the majority of the intracellular membranes within 3 to 24 h. BrWSVGH also showed specific actions on extracellular matrix proteins such as fibronectin, laminin and fibrinogen. In addition, zymogram experiments using gelatin as substrates detected gelatinolytic activity. The molecular exclusion fractionation of crude BrWSVGH resulted in 15 fractions, of which F1 and F2 presented α/β-fibrinogenase and fibronectinolytic activities. Fractions F6, F14 and F15 showed only α-fibrinogenase activity; in contrast, the gelatinolytic action was only observed in fraction F11. Only metalloproteinase inhibitors abolished all these proteolytic activities. Our results suggest that adrenal cortex lesions may be relevant in the etiopathogenesis of severe brown widow spider envenoming. To our knowledge, this is the first report on adrenal gland damages, fibrinogenolytic activity and interrelations with cell-matrix adhesion proteins caused by *L. geometricus* VGH. The venom of this spider could be inducing hemostatic system damages on envenomed patients.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Brown widow spider venom (BrWSV) (*Latrodectus geometricus*) could contain toxins such as α-latrotoxin, similar to that of the black widow spider, that stimulate substantial neurotransmitter discharge when injected in mammals, followed by complete vesicle exhaustion and impedance of neuromuscular transmission at the neuromuscular connection (Orlova et al., 2000; Ushkaryov et al., 2008). Müller (1993) reported that black widow spider bites are a more severe envenomation than brown widow bites, characterized by broad muscle nociceptive and cramps, abdominal muscle hardness, copious sweating, elevated blood pressure and tachycardia. They proposed that symptoms and signs of brown widow spider bites were not as severe and have a tendency to be limited to the bite site and nearby tissues.

Some authors (Shukla and Broome, 2007) infer that despite the effects that *Lactrodectus* venom has on muscle activity, there are no long-term effects to the human body. However, as a result of the current findings, this venom produced intense ultrastructural damages on mice adrenal glands. Adrenal cellular activities occur within active and multi-molecular compartments whose characterization involves analysis of cell-matrix adhesion sites, which consist of numerous cytoskeletal and signaling proteins. These adhesions sites are extremely variable in their morphology, dynamics, and obvious function, yet their molecular diversity is not well known. In the present work, we studied the activity of brown widow spider venom gland homogenates (BrWSVGH) and some of its chromatographic fractions on extracellular matrix (ECM) proteins, with particular emphasis on fibronectin, laminin, collagen type IV, and fibrinogen, with the purpose to elucidate potential effects on the basal membrane as well as to comprehend the hemorrhagic symptomatology rarely observed in patients bitten by this spider. Three types of enzymes from animal venoms have been described affecting fibrinogen and

---

other hemostatic proteins: 1) thrombin-like enzymes; 2) plasminogen activators; and 3) fibrinolytic enzymes (Zhang et al., 1995; Markland, 1998a,b; Arocha-Piñango et al., 1999; Tatematsu et al., 2000; Champagne 2004; Swenson and Markland, 2005).

Fibrinogen-depleting agents reduce plasma fibrinogen levels; thus having potential in the treatment and prevention of acute strokes and heart attacks. No enzymes, including fibrinogenolytic enzymes, with these activities had been found in the venom of the *Latrodectus* genus. For these reasons, the therapeutic efficacy of *L. geometricus* fibrinogenases, when purified and characterized, could be an important finding.

2. Materials and methods

2.1. Reagents

Fibronectin was purified from human plasma following Lucena et al. (2006) method. Sepharose–Gelatine and Sepharose–Arginine gels were from Pharmacia Biotechnology (Uppsala, Sweden). Chromogenic substrates and bovine fibrinogen (10% w/w of plasminogen as contaminant demonstrated by immunoblotting) were obtained from Chromogenix AB (Mölndal Sweden). Superose H12-HR (GE Healthcare, USA) and broad molecular mass standards for PAGE-SDS (Bio-Rad Laboratories Ltd., CA, USA). Purified factor Xa, bovine alpha thrombin and plasmin were from American Diagnostica Inc. (Greenwich, CT, USA). ADP, collagen and thrombin as platelet aggregation inducers were purchased from Chrono-log (Havertown, USA). Laminin from Engelbreth–Holm–Swarm murine sarcoma basement membrane, collagen from human placenta, mouse sarcoma anti-human fibronectin, goat anti-rabbit IgG conjugated to horseradish peroxidase, apyrase, prostaglandin E1, aprotinin, benzamidine/HCl, 1-10 phenantroline, phenylmethylsulfonyl fluoride (PMSF), aprotinin, ethylene glycol-bis-N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA) and other chemical and solvent were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Animals and venoms

L. geometricus (Koch, 1841, Araneae, Theridiidae) spiders used in this work were captured in the field from El Hatillo, Miranda state, Venezuela and kept in the Medical Entomology Section "Pablo Anduze" at the Tropical Medicine Institute of the Universidad Central de Venezuela. Brown widow spider crude venom gland extract was prepared from female specimens. Venom glands were dissected from frozen spiders through the cephalothoraxes and a sagittal incision was done amid their eyes, and the complete venom gland was separated via chelicerae articulation and homogenized in cold lysis buffer (25 mM Tris/HCl, 100 mM NaCl, and pH 8.0). The venom gland homogenate (VGH) was cleared by centrifugation (20 min, 15,000 $\times g$) and assayed for biological activity using BALB/c mice (18–22 g) obtained from the Animal House of the National Institute of Hygiene "Rafael Rangel", Venezuela.

2.3. Ethical statement

All the experimental events concerning the use of live animals were done by specialized personnel. The Venezuelan pertinent regulations as well as institutional guidelines, according to protocols approved by the Tropical Medicine Institute of the Universidad Central de Venezuela and the norms obtained from the guidelines for the care and use of laboratory animals, published by the US National Institute of Health (NIH, 1985) were followed.

2.4. Protein concentration

Protein concentration of VGH was spectrophotometrically estimated by assuming that 1 unit of absorbance/cm of path length at 280 nm corresponds to 1 mg protein/mL (Stoscheck, 1990).

2.5. Polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Polyacrylamide gel electrophoresis was performed following Laemmli (1970) or Schägger and von Jagow (1987) methods, using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were visualized with silver stain. Molecular mass estimations were determined using commercial standard proteins (Bio-Rad).

2.6. Superose VGH fractionation

L. geometricus VGH was fractionated by molecular exclusion chromatography on a Superose 12 HR10/30 size exclusion column equilibrated with 50 mM ammonium acetate buffer pH 6.8, in a Biologic Work Station (Bio-Rad). The VGH was dissolved in the equilibrium buffer (3 mg/500 µL) and injected into the column. The elution was carried out with the same buffer at 0.5 mL/min flow rate and monitored at 280 nm.

2.7. Preparation of specimens for electron microscopy

Adult male mice were separated into a control group, in which mice were injected intraperitoneally (i.p.) with 0.1 mL of saline solution and the experimental group in which nine mice were injected i.p. with 40 µg of VGH in 200 µL of PBS. After 3, 6 and 24 h, three mice from each group were prepared for adrenal gland biopsies. The biopsies were obtained from control and experimental mice under anaesthesia. Samples were immediately *in situ* fixed with 3% glutaraldehyde and 1% OsO_4 (both fixatives diluted in 320 mM phosphate buffer saline, pH 7.4), dehydrated in ethanol and embedded in LX-112 resin (Ladd Research Inc.). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi HS-500 transmission electron microscope with an accelerating voltage of 100 kV.

2.8. Amidolytic activity

Amidolytic activity of VGH was measured by a micromethod of Guerrero and Arocha-Piñango (1992) using chromogenic substrates (Chromogenix-Instrumentation Laboratory, Milano, Italy). Briefly, in 96 wells polystyrene plates, a mixture of 80 µL of the recommended buffer for each substrate, 10 µL of venom sample and 10 µL of chromogenic substrate was placed in each well. The final concentrations for the substrates were 1.00 mM S-2302, 1.20 mM S-2288, 0.80 mM S-2251, 0.60 mM S-2238 and 0.30 mM S-2444. After incubation at 37 °C for 30 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as ΔA 405 UA/min. Specific activity was calculated as UA/min/µg.

2.9. Coagulant activity

Coagulant activity of VGH was tested by the Salazar et al. (2007) method. Briefly, 0.1 mL of 0.3% purified fibrinogen solution in Tris–HCl buffer, pH 7.4 (coagulation buffer) was incubated in a borosilicate tube at 37 °C for 1 min. Then 0.1 mL of coagulation buffer and 0.1 mL venom sample (diluted in coagulation buffer) or 0.1 mL thrombin solution (0.5 to 15 IU/mL) were added. The solution was thoroughly mixed manually in a 37 °C water bath and the clotting time recorded when the appearance of a clot was visually detected. Assays were carried out four times and the mean clotting time was calculated. The results were expressed in thrombin-like units by plotting the clotting times against a calibration curve prepared with a standard thrombin (National Institute for Biological Standards and Control, London, England).

2.10. Fibrinolytic activity

Fibrinolytic activity of VGH was studied by the fibrin plate method (Marsh and Arocha-Pinango, 1972). Briefly, fibrin plates were settled

using 3-cm diameter Petri dishes: 1.5 mL of a 0.1% plasminogen-rich fibrinogen (10% plasminogen as contaminant) in 5 mM imidazol saline buffer, pH 7.4 was clotted by adding 75 μL bovine thrombin (10 IU/mL, in 0.025 M CaCl$_2$). The mixture was incubated at room temperature for 30 min. Then, 10 μL (3–10 μg) samples were applied over the fibrin, and after 24 h incubation at 37 °C the diameter of the lysed areas was measured. Fibrinolytic activity was expressed as the diameter of the lysed area per μg of protein (mm^2/μg). Human plasmin, sct-PA and tcu-PA were used as positive controls.

2.11. Fibrino(geno)lytic activity

The activity on fibrinogen by proteinases present in *L. geometricus* crude VGH and fractions venom was tested. Briefly, bovine fibrinogen was incubated with venom at a 1 μg VGH/100 μg fibrinogen ratio, for 2 h and 24 h at 37 °C. The degradation of the fibrinogen molecule was visualized by SDS-PAGE on a 10% gel under reduced conditions by a Tris–Tricine-system (Schägger and von Jagow, 1987).

The fibrino(geno)lytic effect was also assayed in the presence of enzymatic inhibitors by incubation of crude venom for 30 min at 37 °C with a mixture of inhibitors. Serine proteinase inhibitors (SPI) were employed as a mixture of 10 mmol/L PMSF, 10 mmol/L benzamidine/HCl, and 100 IU/mL aprotinine (final concentrations). Metalloproteinase inhibitors (MPI) were used as a mixture of 10 mmol/L EGTA-Na and EDTA 10 mmol (final concentrations) (Girón et al., 2008).

2.12. Effects of crude VGH and fractions on ECM proteins

The effect of VGH and Superose 12 HR10/30 VGH fractions on ECM proteins was evaluated using purified fibronectin, laminin, and collagen type IV. The substrates were incubated at a 1 μg VGH/100 μg adhesive protein ratios, for 2 or 24 h at 37 °C, and then the changes in the molecules were visualized by SDS-PAGE under reduced conditions. These effects were also evaluated in the presence of the serine proteinase and metalloproteinase inhibitor mixtures.

2.13. Gelatin zymogram

To study the gelatinolytic activity of BrWSVGH and its fractions, gelatin zymography was carried out as described previously by da Silveira et al. (2002). Venom gland homogenate proteins were diluted in SDS sample buffer without reducing agents and electrophoresed on a 10% SDS-polyacrylamide gels co-polymerized with 1 mg/mL of gelatin. Following electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100 to remove the SDS and then incubated in zymography incubation buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 0.01 M CaCl$_2$, 0.01 M MgCl$_2$) at 37 °C for 18 h. The gels were then stained with Coomassie blue for 2 h and then destained. The presence of gelatinolytic activity was recognized as clear bands on a uniform black background. This gelatinolytic activity was also evaluated in the presence of the serine proteinase and metalloproteinase inhibitor mixtures.

2.14. Platelet aggregation assay

Platelet aggregation was estimated by turbidimetry using a dual-channel Chrono-log model 560 CA aggregometer (Havertown, USA). Platelet-rich plasma (PRP) was prepared by mixing fresh blood sample with trisodium citrate solution (3.8%, w/v) in a volume ratio of 9:1, followed by centrifugation at 190×g at 4 °C for 20 min to sediment leukocytes and erythrocytes. The platelet count was adjusted to 3.0×10^5 platelets/mL with platelet-poor plasma. Four hundred ninety microliters of citrated PRP was pre-incubated with 10 μg/10 μL of VGH in Tyrode's buffer or Tyrode's buffer alone for 4 min at 37 °C after which 5 μL of ADP (final concentration 10 μM), or 1 μL collagen (final concentration 2 μg/mL), or 1 μL ristocetin (final concentration 0.77 mg/mL) was added. The changes in light transmittance were continuously recorded for 8 min. The maximum aggregation response obtained after addition of inducer in the absence of VGH was taken as 100% aggregation. The inhibition percentage was calculated by comparing light transmittance obtained in the presence of VGH against the control sample.

3. Results

3.1. Chromatographic analysis

The VGH molecular exclusion chromatographic profile from a Superose 12-HR10/30 column is shown in Fig. 1B. Fifteen main chromatographic fractions were collected of which F1 and F2 presented α/β-fibrinogenase activity. Fractions F6, F14 and F15 showed only α-fibrinogenase activity. In contrast, only fraction 11 had gelatinolytic activity on a zymographic-gelatin gel.
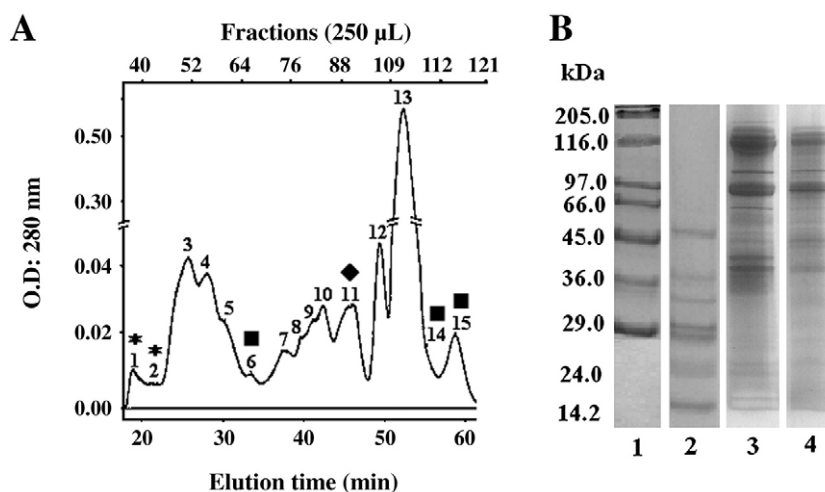

Fig. 1. (A) Molecular exclusion chromatography of *Latrodectus geometricus* venom. Venom was separated by a superose 12 HR10/30 column, equilibrated with 50 mM ammonium acetate buffer, pH 6.9 plus 125 mM NaCl. The flow rate was at 0.4 mL/min. Activities: (*): α/β-fibrinogenases; (■): α-fibrinogenase; (♦): gelatinase. (B) 10% SDS-PAGE of *Latrodectus geometricus* crude venom. Lanes: 1) high molecular weight markers; 2) low molecular weight markers; 3) crude venom under reducing conditions; and 4) crude venom under non-reducing conditions.
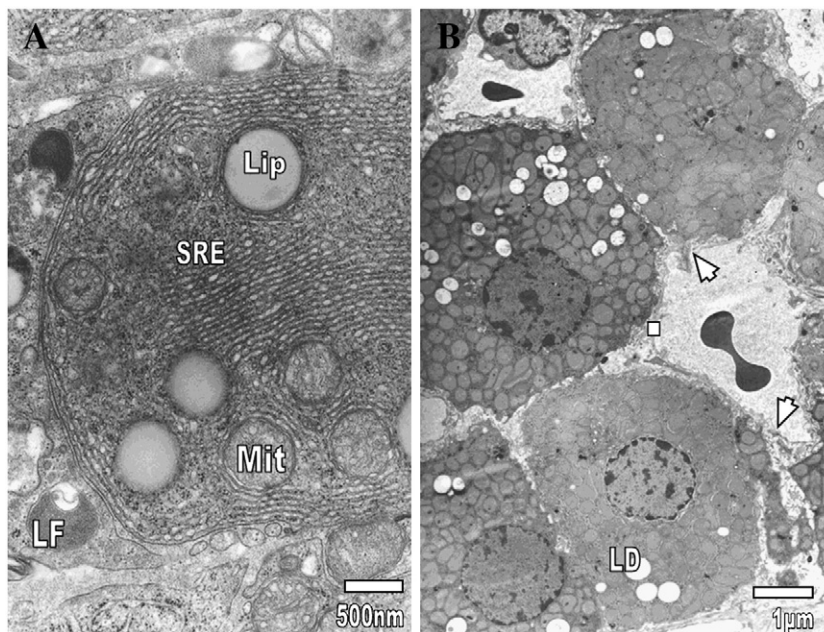
Fig. 2. Transmission electron micrographs (TEM) analysis of the control mouse adrenal gland after 24 h of an intraperitoneal (i.p.) saline solution injection. (A) The magnification analysis viewing the main cellular components displayed a normal ultrastructure with a smooth endothelial reticulum (SER), mitochondria (Mit), lipid (Lip) and lipofucsin drops (LF) (×26,000). (B) Capillary typical ultrastructure with normal endothelial wall (empty square), lipid droplets (LD) and fenestrae (arrows) were noticed (×26,000).

3.2. Electrophoretic analysis

The VGH electrophoretic profile on SDS-PAGE (Fig. 1A) demonstrated that the protein bands correspond to an ample range of molecular masses. There were a small number of proteins dispersed between 30 and 14 kDa. Additionally, under non-reducing conditions, there were extra bands between 120/115, 97/66, and 36/40 kDa. Under reducing conditions multiple bands between 25/27 and 30/120 kDa were visible.

3.3. Adrenal gland cortex alterations analyzed by transmission electron microscopy (TEM)

Normal controls of adrenal gland cortex in unstressed mice after 24 h of i.p. saline solution injections were analyzed by TEM. The samples displayed a normal ultrastructure with a smooth endothelial reticulum, mitochondria, lipid and lipofucsin drops and typical capillary ultrastructure with normal endothelial wall and fenestrae (Fig. 2A and B).

After 3 h of L. geometricus VGH injection, a change on erythrocytes density was seen. Mitochondria devoid of internal membrane and cristae and some with lipid impregnation were observed. Nuclear envelope loss was noticed. A myelinic figure was noticed. In the right top (high magnification) mitochondria devoid of cristae are noticed (Fig. 3A). The fenestrae (pinocytotic vesicles) of capillary endothelia disappeared. Mitochondria devoid of internal membrane and cristae were seen. A myelinic figure was detected. In the right top (high magnification) mitochondria devoid of cristae are noticed (Fig. 3B).

After 6 h of L. geometricus VGH injection, adrenal gland cortex showed electron dense inclusions with aspect of myelinic figures. Capillary without fenestrae and endothelium loss was also noticed. Swollen mitochondria without cristae were detected (Fig. 4A). Intensely damaged mitochondria without internal structures and autophagic vacuoles were seen. Electron dense inclusions were observed. The
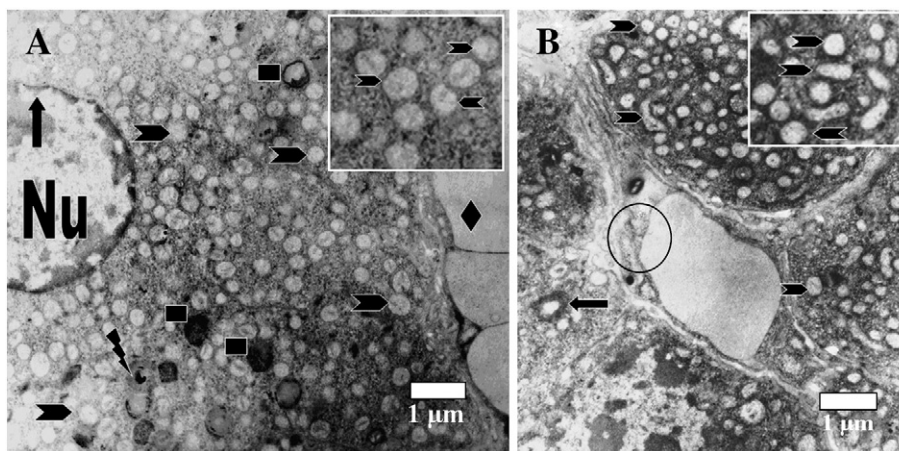

Fig. 3. TEM analysis of adrenal gland after 3 h of an i.p. injection of L. geometricus venom. (A) Lesions were characterized by a change on erythrocytes density (Er). Mitochondria devoid of internal membrane and cristae (arrowheads) and some with lipid impregnation (arrows) were observed. Nuclear (Nu) envelope loss was noticed. A myelinic figure (square) was noticed. In the right top (high magnification) mitochondria devoid of cristae (arrowheads) are noticed (×20,000). (B) The fenestrae (pinocytotic vesicles) of capillary endothelia (triangle) were absent. Mitochondria devoid of internal membrane and cristae (arrowheads) were seen. Myelinic figure (ray) was observed. In the right top (high magnification) mitochondria devoid of cristae (arrowhweads) are noticed (×20,000).
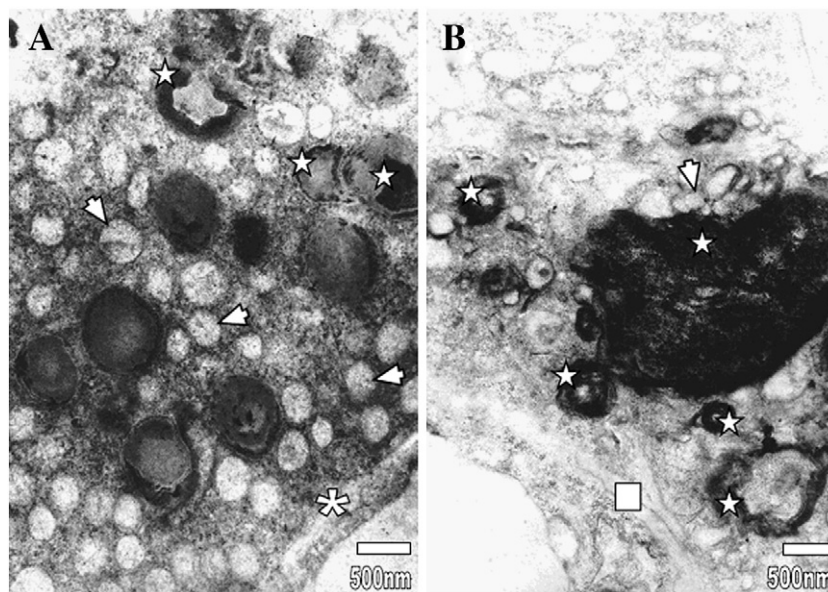
Fig. 4. TEM analysis of adrenal gland after 6 h of an i.p. injection of *L. geometricus* venom. (A) Electron dense inclusions with aspect of myelinic figures (stars) were seen. Capillary without fenestrae and endothelium loss (asterisk) was also noticed. Swollen mitochondria without cristae (arrowheads) were observed (×26,000). (B) The mitochondria intensely damaged without internal structures and autophagic vacuoles (arrowhead) were seen. Electron dense inclusions (stars) were observed. The capillary showed swollen endothelium without fenestrae or pinocytotic vesicles (empty square) (×26,000).

capillary displayed swollen endothelium without fenestrae or pinocytotic vesicles (Fig. 4B).

After 24 h of *L. geometricus* VGH injection, adrenal gland cortex presented mitochondria lacking internal membrane and cristae. Electron dense lipofucsin granules and some without lipid drops were observed. Intense damage of capillary endothelium was seen, showing disappearance of fenestrae and caveoles (Fig. 5A). Pleomorphic mitochondria with different electron density and lost of cristae were detected. Intense edema and capillary fenestrae lost were also seen. Intercellular spaces without defined membranes and nucleus capsule disappearance in some places were noticed. Nucleoli with differences in electron density were observed. Plasmatic membrane separation by edema was seen; however, smooth endoplasmic reticulum seemed to be normal (Fig. 5B).

3.4. Effects of L. geometricus venom on hemostatic components

3.4.1. Crude VGH

Brown widow spider VGH presented strong fibrinogenolytic activity. Degradation of the Aα-chain occurred primarily, followed by the Bβ-chain. At a molar ratio of 100:5 (fibrinogen:BrWSVGH), the SDS-PAGE results under reduced conditions showed a fast (30 min) degradation of the fibrinogen Aα chains and a slow (2 h) degradation of Bβ chains. After 24 h incubation, a full degradation of both chains was observed. Associated with the digestion of both fibrinogen chains, major fragments between M_r ~45 and 20 kDa were observed. The γg-chain was not degraded following prolonged 24 h incubation with VGH (Fig. 6). Degradation of fibrinogen with VGH in the presence of protease inhibitors was also evaluated in which metalloproteinase inhibitors
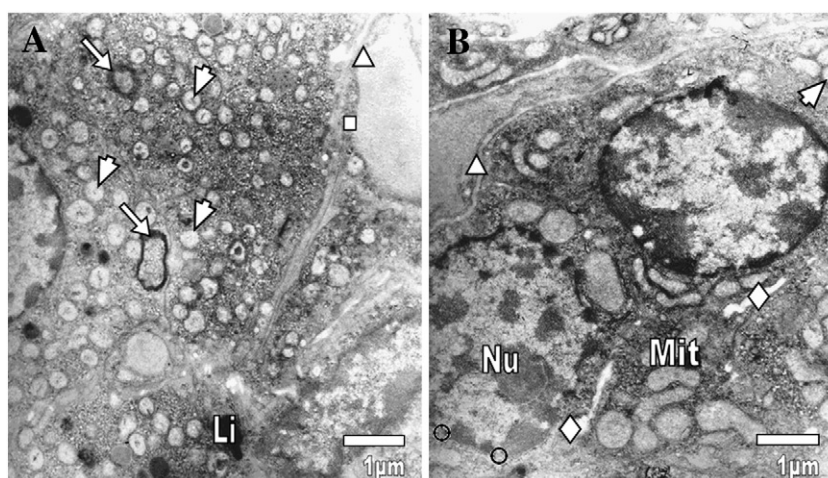

Fig. 5. TEM analysis of adrenal gland after 24 h of an i.p. injection of *L. geometricus* venom. (A) Mitochondria lacking internal membrane and cristae (arrowheads) were observed. Electron dense lipofucsin granules (Li) and some without lipid drops (arrow) were observed. Intense damage of capillary endothelium (triangle) was seen, showing disappearance of fenestrae, and caveoles (empty square) (×20,000). (B) Pleomorphic mitochondria with different electron density (Mit) and lost of cristae (arrowhead) were seen. Intense edema and capillary fenestra lost (triangle) were also seen. Intercellular spaces without defined membranes and nucleus capsule disappearance in some places were also noticed (circle). Nucleoli (Nu) with differences in electron density were observed. Plasmatic membrane separation by edema (rhombus) was observed; however, smooth endoplasmic reticulum seemed to be normal (×26,000).
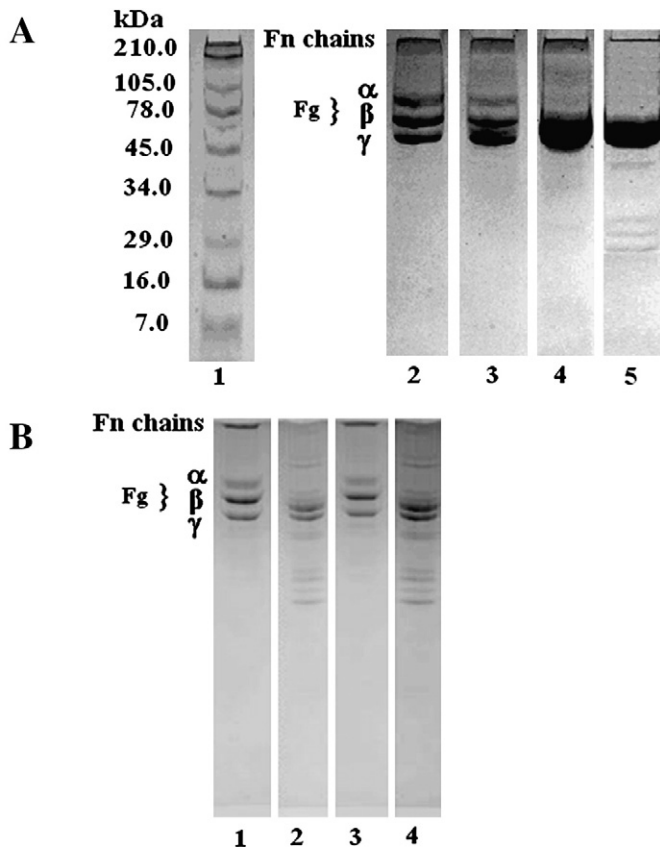
Fig. 6. Fibrinogenolytic activity of *L. geometricus* venom. Fibrinogen treated with BrWSV at 5 μg BrWSV/100 μg Fg ratio for 37 °C, and at different incubation times. Samples (30 μg) were electrophoresed under reducing conditions in the presence of SDS on 10% gel and stained with Coomassie blue. (A) Lanes: 1) high molecular weight markers; 2) Fg control in the presence of fibronectin (Fn) as contaminant; 3–5) Fg + BrWSV at 30 min, 2 h and 24 h, respectively. (B) Fibrinogenolytic effect at 5 μg BrWSV/100 μg Fg ratio for 37 °C, and at 24 h incubation, in the presence of protease inhibitors. Lanes: 1) Fg control in the presence of fibronectin (Fn) as contaminant; 2) Fg + BrWSV; 3) Fg + BrWSV in the presence of MPI; 4) Fg + BrWSV in the presence of SPI.
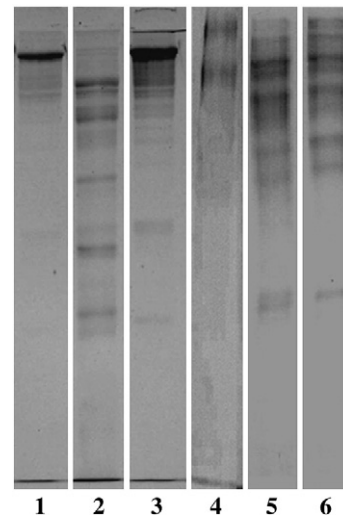

Fig. 7. The effects of *L. geometricus* venom on ECM proteins. BrWSV was incubated for 2 and 24 h at 37 °C with purified fibronectin (FN), laminin (LM) at a 10 μg BrWSV/100 μg adhesive protein ratio. Samples (20 μg) were electrophoresed under reducing conditions in the presence of SDS on 4 to 8% gel and stained with Coomassie blue. Lanes: 1) FN control; 2) FN + BrWSV at 24 h; 3) FN + BrWSV at 2 h; 4) LM control; 5) LM + BrWSV at 2 h; 6) LM + BrWSV at 24 h.

abolished the proteolytic effect in fibrinogen chains. In addition to the fibrinogenolytic effect, this figure also shows fibronectin degradation. Fibronectin is a contaminant adhesive protein of the purified fibrinogen used in the test, which was demonstrated using an immunoblotting assay (data not shown). At 24 h, total degradation of the fibronectin was observed, and metalloproteinase inhibitors also abolished this degradation activity.

Furthermore, the crude VGH did not present significant fibrinolytic activity on fibrin plate or amidolytic-like activities to hemostatic enzymes such as thrombin, factor Xa, plasmin, urokinase, kallikrein, or on t-PA likes or on proteolytic enzymes such as chymotrypsin-like. This VGH had no effect on ristocethin-induced platelet aggregation. However, it showed an inhibitory effect on collagen and/or ADP-induced platelet aggregation (between 15 and 20% in the presence of 40 μg VGH/3.0×10^5 platelets), results not shown. The VGH at a dose up to 50 μg did not show gelatinolytic activity on a gelatin zymogram.

To determine the activity of VGH on ECM proteins, the VGH with purified fibronectin, laminin and collagen type IV was tested. As shown in Fig. 7, under the conditions assayed (2 and 24 h of incubation time at 37 °C, and at a 10 μg venom/100 μg protein ratio), fibronectin and laminin were degraded. In the SDS-PAGE, under reduced conditions, control fibronectin appeared as a broad band positioned at the top of the gel. After incubation with VGH, fibronectin degradation was evident as early as 2 h, in which this large band disappeared and many degradation products with lower molecular masses were observed. After 24 h, fibronectin was cleaved into several fragments that ranged in sizes from 105 to 16 kDa. Laminin degradation was manifested as early as 2 h, at this incubation time several fragments were observed. Furthermore, the collagen type IV molecule was lightly changed in the presence of VGH (data not shown). The proteolytic activity on ECM proteins was abolished only by metalloprotease inhibitors (results not shown).

3.4.2. Venom gland homogenate fractions

The VGH fractions were tested for fibrinogenolytic activity on fibrinogen (Fg) (100 μg Fg: 10 μg fraction) for 24 h at 37 °C (Fig. 8). The SDS-PAGE under reduced conditions demonstrated that F1 and F2 fractions were active, degrading both αA and βB chains, as well as fibronectin chains which appear as high molecular weight bands. In contrast, F6, F14 and F15 fractions presented activity only on αA. Additionally, these fractions were not active on the fibronectin chains.

Gelatinolytic activity was observed only in fraction F11. The zymogram-gelatin gel showed three well-defined areas of lysis that correspond to enzymes with molecular masses of 50–55 kDa; ~45 kDa; and ≥29 kDa, respectively (Fig. 9). This proteolytic activity on fibrinogen and fibronectin chains and gelatinolytic activity present in the chromatographic fractions was abolished by metalloprotease inhibitors (results not shown).

4. Discussion

L. geometricus is frequently known as the brown widow or geometric button spider. The brown widow is described in South America, United States, South Africa, and Australia. They are responsible for a number of bites and envenomations around the world (Jelinek, 1997). Latrodectism also represents a problem of health in some highly populated communities. For instance, in America they constitute a true medical emergency contributing to the rapid evolution of aracnoidism syndrome. *L. geometricus* VGH contains neurotoxins that cause alterations on striated muscle, causing several symptoms including paralysis, especially thoracic paralysis (Müller, 1993; Kiriakos et al., 2008; Goddard et al., 2008; Reyes-Lugo et al., 2009).

A comprehensive examination of the venomous proteins from spiders is not easily obtainable since it is very difficult to get enough quantities of spider venoms by extraction (even the milked venom may contain regurgitated material). This has been a challenge for analytical and identifying procedures, which require methodologies that
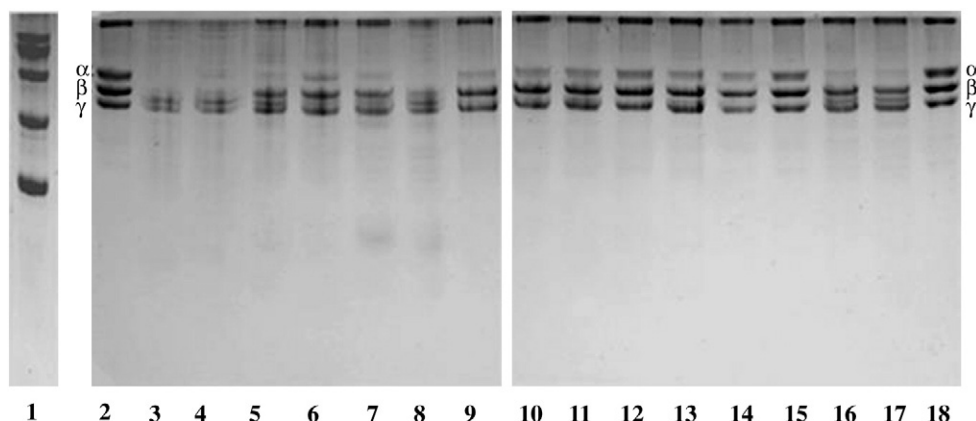
Fig. 8. Fibrinogenolytic effect of *L. geometricus* venom size exclusion fractions. Fibrinogen (Fg) and fractions were incubated at a 10 µg fraction/100 µg Fg ratio, for 24 h at 37 °C. Samples (30 µg) were electrophoresed under reducing conditions on a 10% gel SDS-PAGE and stained with Coomassie blue. Lanes: 1) high molecular weight markers; 2) Fg control in the presence of fibronectin (Fn) as contaminant; 3–17) Fg + chromatographic fractions: F1 to F15, respectively; and 18) Fg control.

successfully detach and recognize a multifaceted combination of toxins, peptides and proteins with different physicochemical characteristics. For these reasons it was compulsory to use venom gland homogenates inevitably contaminated by other gland cellular constituents instead of pure venom (Kristensen, 2005). The intraperitoneal lethal dose fifty value found for *L. tredecimguttatus* venom whole-gland homogenate was 0.59 mg/kg (McCrone, 1964); which was the same as that reported by others (Wang et al., 2007), but more toxic when injected subcutaneously at 0.33 mg/kg. The LD_{50} of *L. mactans* venom subcutaneously injected was 1.20 mg/kg (Daly et al., 2001). In the present experiment, BrWSVGH was extracted from dissected venom gland homogenate and injected intraperitoneally resulting in an LD_{50} of 0.225 mg/kg. According to these results, the BrWS appears to have one of the most toxic venoms of the *Latrodectus* genus.

Adrenotoxic activities in this current study, which was suspected due to clinical signs observed in the experimental mice, indicated that the mice adrenal glands were particularly vulnerable to BrWSVGH. Mice became irritable and aggressive immediately following injections and then they began to vomit, show fatigue evident by slow, sluggish and lethargic movement, followed by profound weakness. They also showed signs of unusual and excessive sweating, tachypnea and symptoms interpreted as shaking chills. The animals turned into adynamic and atonic states and their incapacity to continue moving preceded a coma and lifeless conduct. Finally, respiratory paralysis occurred, due in part to neurotoxic activity, but this does not exclude the severe acute adrenocortical insufficiency, which could also be contributed to the animals' death. Evidence of acute adrenal damage is manifested by circulatory collapse, which is characterized by a rapid

thready pulse, laboured respiration, an alarming drop in blood pressure, and coma. Other features may include vomiting and diarrhoea, occasionally anuria. Death usually occurred after a few hours, adrenal insufficiency being the immediate cause (Waterhouse, 1911). As it is known, primary adrenal insufficiency can be a result of adrenal gland destruction by either direct or indirect actions of infectious agents caused by hemorrhage or coagulation disorders (e.g., human Waterhouse–Friderichsen syndrome), or by the activities of natural toxins such as those found in bee (*Apis mellifera*) (Rodriguez-Acosta et al., 2003; Rodriguez-Acosta et al., 2004) and scorpion (*Tityus discrepans*) venoms (Rodriguez-Acosta et al., 2000).

The profound adrenal cortex damages by BrWSVGH observed from 3 to 24 h on endothelial capillaries (a formation of great significance for vessel stability), and mitochondrial membranes (if these membranes are injured, a lesser amount of energy will be formed and consequently the performance of the organ will then be distressed), intracellular membranes, edema of rough endoplasmic reticulum cisterns and the destruction of the nucleus capsule and plasmatic membrane could explain the acute clinical picture presented by the experimental animals. The lesions observed in mitochondria and endoplasmic reticulum might be sufficient to cause impairment of steroid synthesis. These results suggest that different degrees of adrenal dysfunction could be present in animals displaying the clinical manifestation of envenoming described above. Picotti et al. (1982), by intraarterial injections of α-latrotoxin (alpha LTx) into rats, provoked rapid and clear rises in plasma adrenaline and noradrenaline concentrations, indicating that the toxin stimulates catecholamine release from the adrenal medulla and other catecholamine tissue secretors. The catecholamine release from the adrenal medulla was possibly mediated by preganglionic release of acetylcholine. In primary adrenocortical insufficiency, glucocorticoid and mineralcorticoid properties are lost. Although more than 50 steroids are produced within the adrenal cortex, cortisol and aldosterone are by far the most abundant and physiologically active. Strongly affected animals may present a life-threatening adrenocortical crisis (Harvey et al., 2007).

Several compounds with activities similar to or against components of hemostatic systems have been identified in invertebrate venoms. Approximately three types of enzymes from animal venoms have been described affecting hemostatic proteins: 1) thrombin-like enzymes; 2) plasminogen activators; and 3) fibrinolytic enzymes (Zhang et al., 1995; Markland, 1998a,b; Arocha-Piñango et al., 1999; Tatematsu et al., 2000; Champagne 2004; Swenson and Markland, 2005). BrWSVGH did not present significant fibrinolytic activity or thrombin, factor Xa, plasmin, urokinase, kallikrein, or t-PA-like activities. However, the VGH did show an inhibitory effect on collagen or ADP-induced platelet aggregation (between 15 and 20% in the
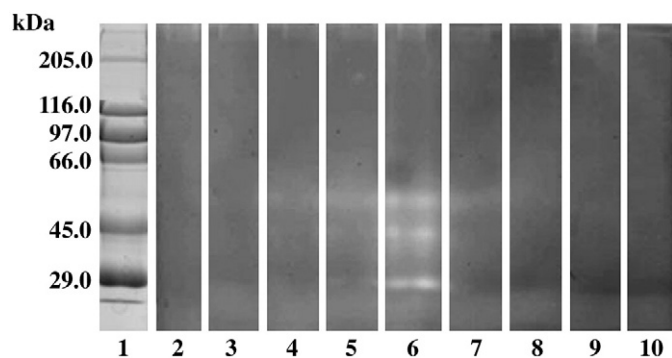

Fig. 9. Zymographic-gelatin-of *L. geometricus* venom size exclusion fractions. Twenty-five micrograms of each fraction was run on a 10% SDS-PAGE gel in the presence of 1 mg/mL gelatin. Lanes: 1) high molecular weight markers; BrWSV fractions: 2) F1; 3) F2; 4) F9; 5) F10; 6) F11; 7) F12; 8) F13; 9) F14; and 10) F15. The gelatinolytic activity is in the 60–30 kDa area.

presence of 40 μg venom/3.0×10^5 platelets), which could be explained by the fibrinogenolytic activity described above. Studies on platelet functions in the presence of *L. geometricus* venom fractions are currently being performed in our laboratory.

Several extracellular matrix molecules (ECM) such as fibronectin, fibrinogen, laminin, vitronectin, entactin, and heparan sulfate proteoglycan have been described as targets for degradation evoked by proteases present in other arthropod venoms such as those found in spiders or caterpillars (Paludo et al., 2006; Lucena et al., 2006, 2008). BrWSVGH demonstrated other proteolytic activities and could also degrade fibronectin, laminin and collagen. To our knowledge, no enzymes acting on extracellular matrix proteins and those with activities on hemostatic system have been described in *L. geometricus* venom until now.

Our results demonstrated that the BrWSVGH contains fibrinogenolytic activity since the Aα and Bβ chains of fibrinogen were degraded, while the γ chain was unaffected. The Aα and Bβ chains of fibrinogen were very susceptible to BrWSVGH, because they were completely degraded within 2 h. The α/β-fibrinogenase and α fibrinogenase activities have been also described in venoms of *Loxosceles* spiders (Veiga et al., 2000, 2001; Zanetti et al., 2002; Da Silva et al., 2004).

The molecular exclusion fractionation of crude venom showed 15 fractions, of which F1 and F2 presented α/β-fibrinogenase activity, also presenting action on fibronectin. Fractions F6, F14 and F15 showed only α-fibrinogenase activity, and had no activity on fibronectin chains. Additionally, a zymograph gelatin was also carried out. In fraction F11, gelatinolytic activity is evident in the 55–30 kDa area (Fig. 9).

The fibrinogenolytic activities observed in BrWSVGH, as well as in chromatographic fractions were abolished by EDTA–EGTA and 1,10-phenanthroline, metal chelators suggesting that these enzymes belong to the family of metalloproteinases. Metalloproteinases from natural toxins producing local or systemic hemorrhage have been separated from snake and spider venoms (Huang et al., 1995; Feitosa et al., 1998; Da Silveira et al., 2002), and according to our present results and the literature data, metalloproteinase activities have been identified for the first time in the VGH of *L. geometricus* venom.

In addition to gelatinolytic and fibrinogenolytic activities, BrWSVGH demonstrated other proteolytic activities and could also degrade fibronectin, laminin and collagen. Fibronectin plays important functions in the adhesive and migratory performance of cells associated to basic processes such as embryogenesis, malignancy, hemostasis, wound healing, host defense and preservation of tissue integrity. Plasma fibronectin was first revealed as a contaminant of purified plasma fibrinogen (Cho and Mosher, 2006). Fibronectin proteolysis by BrWSVGH could damage its functions, such as those of cross-linking to fibrin and its cellular adhesion. Laminin is the major non-collagenous complex glycoprotein, which plays a part of the architectural of basement membranes (Paulsson, 1992). We evaluated the relative proteolytic susceptibility of the two laminin subunits by BrWSVGH and our results showed that the venom degraded the laminin chains generating fragments with molecular masses from 105 to 16 kDa.

Some of the pathogenic activities of *Latrodectus* VGH detected in the current work were probably imputable to the presence of proteolytic enzymes such as hyaluronidase, described in other spiders, which is an enzyme that breaks down intercellular reinforcements and basement membrane molecules (da Silveira et al., 2007; Young and Pincus, 2001) allowing other venom components to spread to other areas of the body. In spiders, electrostimulated venom and venom gland extract demonstrated very similar hyaluronidase activity, signifying that hyaluronidases are self-components of spider venom instead of oral digest contamination (da Silveira et al., 2007). These proteolytic actions could contribute to the dissemination of venom across the vessel walls. These proteinases probably have numerous synergistic functions within different organs (adrenal gland, nervous system, muscles, etc.).

The fibronectin, fibrinogen and laminin proteolysis may play a role in the production of degradation fragments that may assist the stimulation of inflammatory processes, which indirectly injures other new tissue. The identity of the metalloproteinase(s) present in *Latrodectus* venom and the role in the pathology of *Latrodectism* remains to be established. Even though the clinical signals of latrodectism have been well acknowledged in the past years, data regarding the hemorrhagic syndromes induced by *L. geometricus* venom have not been described and the effects of the venom on hemostasis and platelet aggregation are unclear. However, VGH actions on platelet aggregation and proteolytic effects on extracellular matrix components, including fibrinogen chains, evidenced in the current work, could be related to the potential hemorrhagic syndromes that may be produced when encountered with this spider venom. Additional studies to identify these factors could shed light on human hemostasis alterations after envenomation.

To our knowledge, these results represent the first ultrastructural description of severe adrenal gland damage triggered in mice by BrWSVGH, in addition to its interrelations with cell-matrix adhesion proteins and hemostatic activities.

References

Arocha-Piñango, C.L., Marchi, R., Guerrero, B., 1999. Inventory of exogenous hemostatic factors derived from arthropods. Registry of Exogenous Hemostatic Factors of the Scientific and Standardization Subcommittee of the International Society on Thrombosis and Haemostasis. Thromb. Haemost. 81, 647–656.

Champagne, D.E., 2004. Antihemostatic strategies of blood-feeding arthropods. Curr. Drug. Targets. Cardiovasc. Haematol. Disord. 4, 375–396.

Cho, J., Mosher, D.F., 2006. Role of fibronectin assembly in platelet thrombus formation. J. Thromb. Haemost. 4, 1461–1469.

Da Silva, P.H., da Silveira, R.B., Appel, M.H., Mangili, O.C., Gremski, W., Veiga, S.S., 2004. Brown spiders and loxoscelism. Toxicon 44, 693–709.

Da Silveira, R.B., dos Santos Filho, J.F., Mangili, O.C., Veiga, S.S., Gremski, W., Nader, H.B., von Dietrich, C.P., 2002. Identification of proteases in the extract of venom glands from brown spiders. Toxicon 40, 815–822.

da Silveira, R.B., Chaim, O.M., Mangili, O.C., Gremski, W., Dietrich, C.P., Nader, H.B., Veiga, S.S., 2007. Hyaluronidases in *Loxosceles intermedia* (Brown spider) venom are endo-beta-N-acetyl-D-hexosaminidases hydrolases. Toxicon 49, 758–768.

Daly, F.F., Hill, R.E., Bogdan, G.M., Dart, R.C., 2001. Neutralization of *Latrodectus mactans* and *L. hesperus* venom by redback spider (*L. hasseltii*) antivenom. J. Toxicol. Clin. Toxicol. 39, 119–123.

Feitosa, L., Gremski, W., Veiga, S.S., Elias, M.C., Graner, E., Mangili, O.C., Brentani, R.R., 1998. Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in brown spider (*Loxosceles intermedia*) venom. Toxicon 36, 1039–1051.

Girón, M.E., Salazar, A.M., Aguilar, I., Pérez, J.C., Sánchez, E.E., Arocha-Piñango, C.L., Rodríguez-Acosta, A., Guerrero, B., 2008. Hemorrhagic, coagulant and fibrino(geno)lytic activities of crude venom and fractions from mapanare (*Bothrops colombiensis*) snakes. Comp. Biochem. Physiol., C. Toxicol. Pharmacol. 147, 113–121.

Goddard, J., Upshaw, S., Held, D., Johnnson, K., 2008. Severe reaction from envenomation by the brown widow spider, *Latrodectus geometricus* (Araneae: Theridiidae). South. Med. J. 101, 1269–1270.

Guerrero, B., Arocha-Piñango, C.L., 1992. Activation of human prothrombin by the venom of *Lonomia achelous* (Cramer) caterpillars. Thromb. Res. 66, 169–177.

Harvey, P.W., Everett, D.J., Springall, C.J., 2007. Adrenal toxicology: a strategy for assessment of functional toxicity to the adrenal cortex and steroidogenesis. J. Appl. Toxicol. 27, 103–115.

Huang, K.F., Hung, C.C., Pan, F.M., Chow, L.P., Tsugita, A., Chiou, S.H., 1995. Characterization of multiple metalloproteinases with fibrinogenolytic activity from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*): protein microsequencing coupled with cDNA sequence analysis. Biochem. Biophys. Res. Commun. 216, 223–233.

Jelinek, G.A., 1997. Widow spider envenomation (latrodectism): a worldwide problem. Wilderness Environ. Med. 8, 226–231.

Kiriakos, D., Núñez, P., Parababire, Y., García, M., Medina, J., Sousa, L.D., 2008. First case of human latrodectism in Venezuela. Rev. Soc. Bras. Med. Trop. 41, 202–204.

Kristensen, C., 2005. Comments on the natural expression and artificial extraction of venom gland components from spiders. Toxin Rev. 24, 257–270.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lucena, S., Guerrero, B., Salazar, A., Gil, A., Arocha-Piñango, C.L., 2006. Degradation of extracellular matrix proteins (fibronectin, vitronectin and laminin) by serine-proteases from *Lonomia achelous* caterpillar haemolymp. Blood Coagul. Fibrinolysis 17, 427–435.

Lucena, S., Salazar, A., Gil, A., Arocha-Piñango, C.L., Guerrero, B., 2008. Effect of Lonomin V, a protein isolated from *Lonomia achelous* caterpillar on some biological activities of fibronectin. Thromb. Res. 121, 653–661.

Markland, F.S., 1998a. Snake venom and the hemostatic system. Toxicon 36, 1749–1800.

Markland, FS., 1998b. Snake venom fibrinogenolytic and fibrinolytic enzymes: an updated inventory. Registry of Exogenous Hemostatic Factors of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb. Haemost. 79, 668–674.

Marsh, N.A., Arocha-Pinango, C.L., 1972. Evaluation of the fibrin plate method for estimating plasminogen activators. Thromb. Diath. Haemorrh. 28, 75–88.

Mccrone, J.D., 1964. Comparative lethality of several latrodectus venoms. Toxicon 69, 201–203.

Müller, G.J., 1993. Black and brown widow spider bites in South Africa. A series of 45 cases. S. Afr. Med. J. 83, 399–405.

NIH, 1985. Principles of laboratory animal care. National Institute of Health of United States of America, Pub. 85-23, Maryland, pp. 1–112.

Orlova, E.V., Rahman, M.A., Gowen, B., Volynski, K.E., Ashton, A.C., Manser, C., van Heel, M., Ushkaryov, Y.A., 2000. Structure of alpha-latrotoxin oligomers reveals that divalent cation-dependent tetramers form membrane pores. Nat. Struct. Biol. 7, 48–53.

Paludo, K.S., Gremski, L.H., Veiga, S.S., Chaim, O.M., Gremski, W., de Freitas Buchi, D., Nader, H.B., Dietrich, CP., Franco, C.R., 2006. The effect of brown spider venom on endothelial cell morphology and adhesive structures. Toxicon 47, 844–853.

Paulsson, M., 1992. Basement membrane proteins: structure, assembly, and cellular interactions. Crit. Rev. Biochem. Mol. Biol. 27, 93–127.

Picotti, G.B., Bondiolotti, G.P., Meldolesi, J., 1982. Peripheral catecholamine release by alpha-latrotoxin in the rat. Naunyn-Schmiedeberg's Arch. Pharmacol. 320, 224–229.

Reyes-Lugo, M., Sánchez, T., Finol, H.J., Sánchez, E.E., Suárez, J.A., Guerrero, B., Rodríguez-Acosta, A., 2009. Neurotoxic activity and ultrastructural changes in muscles caused by the brown widow spider *Latrodectus geometricus* venom. Rev. Inst. Med. trop. S. Paulo 51, 95–101.

Rodríguez-Acosta, A., Strauss, M., Pulido-Mendez, M., Finol, H.J., 2000. Adrenal gland ultrastructural changes in mice inoculated with *Tityus discrepans* (Buthidae) venom. Rev. Cient. FCV-LUZ. 10, 480–485.

Rodriguez-Acosta, A., Vega, J.L., Finol, H.J., Pulido-Mendez, M., 2003. Ultrastructural alterations in cortex of adrenal gland caused by the toxic effect of bee (*Apis mellifera*) venom. J. Submicrosc. Cytol. Pathol. 36, 91–96.

Rodriguez-Acosta, A., Peña, L., Finol, H.J., Pulido-Mendez, M., 2004. Cellular and subcellular changes in muscle, neuromuscular junctions and nerves caused by bee (*Apis mellifera*) venom. J. Submicrosc. Cytol. Pathol. 35, 309–314.

Salazar, A.M., Rodrıguez-Acosta, A., Giron, M.E., Aguilar, I., Guerrero, B., 2007. A comparative analysis of the clotting and fibrinolytic activities of the mapanare (*Bothrops atrox*) snake venom from different geographical areas in Venezuela. Thromb. Res. 120, 95–104.

Schägger, H., von Jagow, G., 1987. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 66, 368–379.

Shukla, S., Broome, V.G., 2007. First report of the brown widow spider, *Latrodectus geometricus* C. L. Koch (Araneae: Theridiidae) from India. Curr. Sci. 93, 775–777.

Stoscheck, C.M., 1990. Quantitation of protein. Meths Enzymol. 182, 50–68.

Swenson, S., Markland Jr., F.S., 2005. Snake venom fibrin(ogen)olytic enzymes. Toxicon 45, 1021–1039.

Tatematsu, R., Komori, Y., Nikai, T., 2000. A new thrombin-like enzyme, flavoviridiobin from the venom of *Trimeresurus flavoviridis* (habu). J. Nat. Toxins 9, 327–339.

Ushkaryov, Y.A., Rohou, A., Sugita, S., 2008. Alpha-latrotoxin and its receptors. Handb. Exp. Pharmacol. 184, 171–206.

Veiga, S.S., Feitosa, L., dos Santos, V.L., de Souza, G.A., Ribeiro, A.S., Mangili, O.C., Porcionatto, M.A., Nader, H.B., Dietrich, C.P., Brentani, R.R., Gremski, W., 2000. Effect of brown spider venom on basement membrane structures. Histochem. J. 32, 397–408.

Veiga, S.S., Zanetti, V.C., Braz, A., Mangili, O.C., Gremski, W., 2001. Extracellular matrix molecules as targets for brown spider venom toxins. Braz. J. Med. Biol. Res. 34, 843–850.

Wang, X.C., Duan, Z.G., Yang, J., Yan, X.J., Zhou, H., He, X.Z., Liang, S.P., 2007. Physiological and biochemical analysis of *L. tredecimguttatus* venom collected by electrical stimulation. J. Physiol. Biochem. 63, 221–230.

Waterhouse, R.A., 1911. Case of suprarenal apoplexy. Lancet 1, 577–578.

Young, A.R., Pincus, S.J., 2001. Comparison of enzymatic activity from three species of necrotising arachnids in Australia: *Loxosceles rufescens*, *Badumna insignis* and *Lampona cylindrata*. Toxicon 39, 391–400.

Zanetti, V.C., da Silveira, R.B., Dreyfuss, J.L., Haoach, J., Mangili, O.C., Veiga, S.S., Gremski, W., 2002. Morphological and biochemical evidence of blood vessel damage and fibrinogenolysis. triggered by brown spider venom. Blood Coagul. Fibrinolysis 13, 135–148.

Zhang, Y., Wisner, A., Xiong, Y., Bon, C., 1995. A novel plasminogen activator from snake venom. Purification, characterization, and molecular cloning. J. Biol. Chem. 270, 10246–10255.