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Brown spider (*Loxosceles* genus) venom toxins: Evaluation of biological conservation by immune cross-reactivity

Daniela Regina Buch [a], Fernanda Nunes Souza [a], Gabriel Otto Meissner [a], Adriano Marcelo Morgon [a], Luiza Helena Gremski [a, b], Valéria Pereira Ferrer [a], Dilza Trevisan-Silva [a], Fernando Hitomi Matsubara [a], Mariana Boia-Ferreira [a], Youssef Bacila Sade [a], Daniele Chaves-Moreira [a], Waldemiro Gremski [a, c], Silvio Sanches Veiga [a], Olga Meiri Chaim [a], Andrea Senff-Ribeiro [a, *]

[a] Department of Cell Biology, Federal University of Paraná, Curitiba, Paraná, Brazil
[b] Department of Clinical Pathology, Clinical Hospital, Federal University of Paraná, Curitiba, Paraná, Brazil
[c] Catholic University of Paraná, Health and Biological Sciences Institute, Curitiba, Paraná, Brazil

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ABSTRACT

Loxosceles spiders are responsible for serious human envenomations worldwide. The collection of symptoms found in victims after accidents is called loxoscelism and is characterized by two clinical conditions: cutaneous loxoscelism and systemic loxocelism. The only specific treatment is serum therapy, in which an antiserum produced with *Loxosceles* venom is administered to the victims after spider accidents. Our aim was to improve our knowledge, regarding the immunological relationship among toxins from the most epidemiologic important species in Brazil (*Loxosceles intermedia*, *Loxosceles gaucho* and *Loxosceles laeta*). Immunoassays using spider venoms and *L. intermedia* recombinant toxins were performed and their cross-reactivity assessed. The biological conservation of the main *Loxosceles* toxins (Phospholipases-D, Astacin-like metalloproteases, Hyaluronidase, ICK-insecticide peptide and TCTP-histamine releasing factor) were investigated. An *in silico* analysis of the putative epitopes was performed and is discussed on the basis of the experimental results. Our data is an immunological investigation in light of biological conservation throughout the *Loxosceles* genus. The results bring out new insights on brown spider venom toxins for study, diagnosis and treatment of loxoscelism and putative biotechnological applications concerning immune conserved features in the toxins.

1. Introduction

Loxosceles spiders, also called brown spiders, are responsible for human envenomations worldwide. Different *Loxosceles* species have been found and reported in South America, Central America, North America, Europe, Africa, Middle East, Oceania and Asia (Futrell, 1992; da Silva et al., 2004; Gremski et al., 2014). In South and Southeast Brazil, there are tree species of main medical importance, *Loxosceles intermedia*, *Loxosceles laeta* and *Loxosceles gaucho*; which are responsible for the most serious arachnid accident cases in the country (Barbaro et al., 1994; Dias-Lopes et al., 2010).

The spiders from the *Loxosceles* genus belong to the family *Loxoscelidae*, sub-order *Labidognatha*, order *Araneida*, class *Arachnida*, and phylo *Arthropoda* (Appel et al., 2005). *Loxosceles* spiders have a violin-shaped pattern on the dorsal surface of their cephalothorax and have six eyes arranged in non-touching pairs forming a U-shaped pattern (da Silva et al., 2004; Chaim et al., 2011a). The clinical manifestations following *Loxosceles* bites are called loxocelism and there are two clinical variants. Cutaneous loxoscelism is more common, occurs in approximately 83% of the cases, and is associated with necrotic skin lesions and gravitational spreading. Systemic loxoscelism includes renal failure; disseminated intravascular coagulation, intravascular hemolysis and may cause death in some victims (Futrell, 1992; da Silva et al., 2004; Hogan et al., 2004; Swanson and Vetter, 2006; Chaim et al.,

* Corresponding author. Centro Politécnico, Department of Cell Biology, Federal University of Paraná, UFPR, Jardim das Américas, CEP 81531-990, Curitiba, Paraná, Brazil.
E-mail address: senffribeiro@ufpr.br (A. Senff-Ribeiro).

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2011a; Gremski et al., 2014).

Studies in molecular biology have contributed to the identification of a great number of brown spider toxins. The cloning and expression of recombinant toxins have been a very useful tool for improving our knowledge of loxoscelism and the biological functions of toxins, as well as for biotechnological purposes (Senff-Ribeiro et al., 2008; Chaim et al., 2011a; Gremski et al., 2014). Toxins from different organisms could be used as reference molecules in designing and developing new drugs, for industrial applications or directly for therapeutic and diagnostic use (Senff-Ribeiro et al., 2008; Chaim et al., 2011a; Mendes et al., 2013).

The venom of *Loxosceles* spiders is a complex mixture of protein and peptide toxins with a molecular mass profile ranging from 3 to 40 kDa (da Silva et al., 2004). Over recent years, *Loxosceles* genus spider venoms have been studied by several scientific research groups worldwide, and many different toxins have been identified in the venoms. Although studies have shown that venom components act synergistically, the mechanisms by which the venom exerts its effects are still under investigation (da Silva et al., 2004; Appel et al., 2005; Gremski et al., 2014). Herein, biological conservation of 5 classes of *Loxosceles* toxins was studied: phospholipases-D, astacins (metalloproteases), hyaluronidase, ICK (insecticide peptides) and TCTP (histamine releasing factor). The dermonecrotic toxins, phospholipases-D, are the better characterized toxins in brown spider venoms. A recombinant form of this toxin by itself can exert most of the biological effects described for the whole venom: the cutaneous and systemic manifestations such as dermonecrotic lesions, hematological disorders, and renal failure (da Silva et al., 2004; Ribeiro et al., 2007; da Silveira et al., 2007a; Chaim et al., 2011b). Astacin-like metalloproteases degrade gelatin, fibronectin, fibrinogen and entactin, but the mechanism by which these toxins play a role in the noxious effects of the venom have not been fully determined (Feitosa et al., 1998; Veiga et al., 2000, 2001a, b; da Silveira et al., 2007a). However, they could putatively be involved in the gravitational spreading of dermonecrosis (a hallmark of loxoscelism), as well as in hemorrhagic disturbances which were also observed following some accidents and acting as systemic spreading factors (Futrell, 1992; da Silveira et al., 2002; da Silva et al., 2004; da Silveira et al., 2007a; Trevisan-Silva et al., 2010). The hyaluronidase from *L. intermedia* is an endo-β-N-acetyl-D-hexosaminidase and is suggested to act with astacins in the gravitational spreading and in edema (Futrell, 1992; Young and Pincus, 2001; Barbaro et al., 2005; da Silveira et al., 2007b; Ferrer et al., 2013). Hyaluronidases are known to be involved in physiological and pathological processes ranging from fertilization to aging (Girish and Kemparaju, 2007). The insecticide toxins, which are peptides similar to knottins (molecules that form an inhibitor cystin knot, called ICKs) are the most abundant toxins in *L. intermedia* transcriptome (de Castro et al., 2004; Gremski et al., 2010). The peptide studied herein, named U2-SCRTX-Li1b, was recently characterized (Matsubara et al., 2013). A translationally controlled tumor protein family member (TCTP) was also identified in the *L. intermedia* venom gland and studied. This protein is related with the histaminergic effects of the venom (Gremski et al., 2010; Chaim et al., 2011a; Sade et al., 2012).

Recent studies showed that there is a family of intra-species as well as inter-species toxins in the *Loxosceles* spiders (Machado et al., 2005; Ribeiro et al., 2007; Trevisan-Silva et al., 2010). The presence of isoforms of toxins found throughout the genus can be explored as putative immune biotools if their immunogenic epitopes are conserved among the species (Trevisan-Silva et al., 2013; Gremski et al., 2014).

An immunological investigation of the *Loxosceles* toxins was mainly performed while taking into consideration the phospholipases-D toxins, specifically using LiD1 (Kalapothakis et al., 2002; Araujo et al., 2003; Felicori et al., 2006, 2009; Dias-Lopes et al., 2010). The aim of the present study was to investigate the biological conservation of the main *Loxosceles* toxins (Phospholipases-D, Astacin-like metalloproteases, Hyaluronidase, ICK insecticide peptide and TCTP). The cross-reactivity between the venom of the most important epidemiologic species in Brazil (*L. intermedia*, *L. gaucho* and *L. laeta*) was assessed using immunoassays and recombinant toxins. A bioinformatics analysis of the putative epitopes is also discussed in relation to the experimental results. The data obtained took into account the biological conservation throughout the genus, and brings out putative biotechnological applications of the main brown spider venom toxins for study, diagnosis and the treatment of loxoscelism.

2. Material and methods

2.1. Reagents

SDS and Comassie blue were purchased from GibcoBRL (Grand Island, NY, USA). SDS-PAGE mass markers, BSA and Ponceau-S were purchased from Sigma (St. Louis, MI, USA). Alkaline phosphatase-conjugated anti-rabbit IgG were purchased from Promega (Madison, WI, USA). Secondary antibodies (anti-rabbit IgG) conjugated to horseradish peroxidase (Sigma) (St. Louis, MI, USA).

2.2. Loxosceles venoms and antibodies

Whole venom from *L. intermedia* obtained by electrostimulation (15 V) of the cephalothorax of spiders captured in the wild was solubilized in PBS and maintained frozen until used, as described by Feitosa et al., 1998. Polyclonal antibodies against *L. intermedia* whole venom were produced as previously described (Luciano et al., 2004). The *L. laeta* and *L. gaucho* whole venoms used in this work were a kind gift from CPPI (Center of Production and Research on Immunobiologicals-CPPI, Curitiba, Paraná, Brazil). These venoms were used to produce the antibodies against *L. laeta* and *L. gaucho* venoms in rabbits following the same protocol established for *L. intermedia* venom (Luciano et al., 2004). Antibodies integrity and reactivity were assayed by titration before the experimental procedures (data not shown).

2.3. L. intermedia recombinant toxins and antibodies

L. intermedia recombinant toxins were previously produced in procariotic expression systems as described (LiRecDT1 – Chaim et al., 2006; LiRecDT2 – da Silveira et al., 2006; LiRecDT3 – da Silveira et al., 2006; LiRecDT5 – da Silveira et al., 2007a; LiRecDT6 – Appel et al., 2008; LALP – Trevisan-Silva et al., 2010; TCTP – Sade et al., 2012). *L. intermedia* recombinant hyaluronidase (Ferrer et al., 2013). *L. intermedia* recombinant ICK peptide (Matsubara et al., 2013). Before their use, integrity, purity and concentration of recombinant toxins were assessed. Protein was quantified by Coomassie Blue method (BioRad, Hercules, USA). Polyclonal hiperimmune sera against each recombinant toxin were previously produced in rabbits by our group (anti-LiRecDT1- Chaim et al., 2006; anti-LALP – Trevisan-Silva et al., 2010; anti-TCTP – Sade et al., 2012). Anti-hyaluronidase and anti-ICK polyclonal sera were obtained as previously described (Ferrer et al., 2013 and Matsubara et al., 2013; respectively).

2.4. Electrophoresis and western blotting

The venom or recombinant protein concentrations were