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Cutaneous loxoscelism caused by *Loxosceles similis* venom and neutralization capacity of its specific antivenom

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ABSTRACT

Members of the spider genus *Loxosceles* pose a marked health risk to humans because of the seriousness of the necrotic and systemic effects of their bite, known as loxoscelism. The recent confirmation of *Loxosceles similis* in residences of Belo Horizonte in Minas Gerais Province, Brazil increases the local potential risk of loxoscelism at higher levels. The first characterization of the venom from this species showed that its main biological effects had a similar intensity as other species (e.g. *Loxosceles intermedia*, *Loxosceles laeta*, and *Loxosceles gaucho*). Therefore, we wished to further analyse the biological activity of the *L. similis* venom as well as the capacity of anti-*L. similis*-venom serum to reduce dermonecrotic effects to rabbit skin. Histological analysis of rabbit skin 2, 4 and 8 h after intradermal injection of *L. similis* venom demonstrated a dense inflammatory infiltrate, edema, degeneration and necrosis of the skin muscle, dissociation of collagen fibers, and disruption of reticular fibers. Importantly, pre-incubation of the venom with anti-*L. similis*-venom serum significantly decreased all of these effects. Anti-*L. similis* antivenom generated antibodies that were strongly reactive to *L. similis* venom and capable of neutralizing the dermonecrotic effects in rabbits caused by this venom. Moreover, the antivenom significantly reduced the sphingomyelinase activity of *L. similis* crude venom. Venoms produced by male and female spiders were equally reactive towards anti-*L. similis* and anti-*L. intermedia* antivenoms, but female venom induced larger lesions on rabbits. In contrast, female venom acted as an immunization enhancer and protected animals from *L. similis* envenomation to a greater degree than male venom. In conclusion, the results shown in this study for *L. similis* antivenom merits a more in depth study of its properties, which may become a valuable tool against loxoscelism.

1. Introduction

The spider genus *Loxosceles* (Araneae, Sicariidae) is comprised of 101 species worldwide located in the temperate and tropical zones of North, Central, and South America as well as Europe, Asia, Africa, and Australia (Platnick, 2011). Several members of the genus have attracted the scientific interest of researchers, including *Loxosceles reclusa* (Gertsch and Mulaik, 1940), *Loxosceles gaucho* (Gertsch, 1967), *Loxosceles laeta* (Nicolet, 1849), and *Loxosceles intermedia* (Mello-Leitão, 1934), mainly due to the health risk to humans from the necrotic and systemic effects of their bite (loxoscelism). The three latter species are prominent in most of the southern

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provinces/states of Brazil, and *L. laeta* is also found in the state of Bahia. In addition, *L. similis* (Moenkhaus, 1898) has been found in the state of Minas Gerais, Brazil (Machado et al., 2005).

Extensive studies have been conducted on this genus in recent years and have revealed the biological effects of the venom (Barbaro et al., 2005; De Oliveira et al., 2005; Gomez et al., 2001; Silvestre et al., 2005) or of specific, isolated fractions of the protein components (Chaim et al., 2006; Guilherme et al., 2001; Tambourgi et al., 1995, 1998), the mechanism of action (Dias-Lopes et al., 2010b; Gomes et al., 2011), and the particular involvement of these proteins towards the production of broadly used and effective antivenoms (De Oliveira et al., 2005; Dias-Lopes et al., 2010a; Pauli et al., 2006; Olvera et al., 2006; Tambourgi et al., 2004).

Molecular cloning of the genes that code for these proteins and their particular biological effects on mammals has also been the focus of several studies in this scientific area (Castro et al., 2004; Kalapothakis et al., 2002; Silvestre et al., 2005; Tambourgi et al., 2004). Kalapothakis et al. (2007) described several new proteins from the most lethal family of toxins expressed in the venom gland of *Loxosceles* spiders, known as Loxtox, and also described important characteristics of this group. The highly conserved antigenic profile from the *Loxosceles* species has been shown by both amino acid sequence similarities and by high cross-reactivity between antivenoms and crude or purified fractions of individual species (Barbaro et al., 1994, 1996, 2005; Olvera et al., 2006; Silvestre et al., 2005; Tambourgi et al., 2004; Toro et al., 2006).

Several *Loxosceles* species are synanthropic and presumably have a strong ability to colonize urban areas due to their high dispersal competence and ecological features, such as tolerance to starvation and lack of water, great longevity, avoidance of extreme temperatures, and preference for relative aridity (Fischer and Vasconcellos-Neto, 2005a,b,c). This ecological profile, in combination with the increasingly high numbers of envenomations reported annually by the Brazilian Ministry of Health (Ministério da Saúde, Governo Federal), calls for more detailed research not only on known species, but also on other species that may prove to be a threat to human health in the future.

In line with this approach, *L. similis* (Moenkhaus, 1898) has been the focus of some recent biological studies (Machado et al., 2005; Silvestre et al., 2005). This species is one of the three reported in the state of Minas Gerais, Brazil, together with *L. laeta* and *L. anomala* (Mello-Leitão, 1917). Based on morphology, this species belongs to the *gaucho* group, together with *L. gaucho*, *L. adelaida*, and *L. variegata* (Gertsch, 1967). Until recently, it was thought to be mainly a cave-dwelling spider that frequented the areas of Pará, Bahia, Minas Gerais, Mato Grosso do Sul, and São Paulo (Andrade et al., 2001; Ferreira et al., 2000, 2005; Trajano and Gnaspini, 1990). However, Machado et al. (2005) reported its presence inside residences of Belo Horizonte in Minas Gerais Province, which added another species to the list of synanthropic members of this genus and increased the potential risk of loxoscelism at higher levels. Because of this, and because of an ongoing interest in speleology and touristic activities around the caves of Minas Gerais, Silvestre et al. (2005) conducted the first characterization of the *L. similis* venom and identified its main biological effects. *L. similis* venom is capable of inducing haemolysis of human erythrocytes, dermonecrotic lesions in rabbits, and lethality in mice at a relatively low LD_{50} (0.32 mg/kg). Importantly, these biological effects are of similar intensity to those of other species, such as *L. intermedia*, *L. laeta*, and *L. gaucho*.

Recently, the number of incidents of loxoscelism caused by *L. similis* has markedly increased in one of the biggest cities of Brazil, Belo Horizonte. This increase in occurrence has justified additional investigation of the *L. similis* venom, sex-linked variation of its potency, and the neutralization effect of anti-*L. similis*-venom on rabbit skin.

2. Materials and methods

2.1. Spiders, venoms, and animals

L. similis spiders (350 individuals) were collected in a country house in the area of Sabará (Minas Gerais, Brazil) and identified using the method described by Gertsch (1967). Venom glands were removed, macerated, and centrifuged, and the cleaned supernatant was stored at −80 °C before use. Protein quantification of venom was performed using the Bradford technique (Bradford, 1976). Bovine serum albumin (BSA) was used as a protein standard. Absorbance was measured at 600 nm with a Spectra MAX 340 microplate spectrophotometer system (Molecular Devices, CA, USA). Adult female New Zealand white rabbits (2.0–2.5 kg) were used in this study and received water and food under controlled environmental conditions throughout the experimental study. Ethical approval regarding the management of the animals used in this study was obtained by the Internal Ethics Committee for Animal Experimentation (CETEA) from the Federal University of Minas Gerais.

2.2. Antivenom production and cross reactivity

After collection of pre-immune serum, rabbits were initially injected subcutaneously with 30 μg of *L. similis* crude venom emulsified in complete Freund's adjuvant at four different points. Four consecutive boosters, each containing 50 μg venom, were then emulsified in incomplete Freund's adjuvant and administered at fifteen day intervals to each rabbit. Immunizations were performed using samples of venom that originated from male or female spiders separately or pooled. One week after each booster, blood samples were taken from the ears of the rabbits, and serum was extracted and stored at −20 °C before use. One week after the last injection, blood was withdrawn and a serum titration was performed by ELISA as described in Chavez-Olortegui et al. (1997). In parallel, the titration of anti-*L. intermedia*-venom serum was evaluated as a comparative control.

2.3. Neutralization assays

2.3.1. In vivo *neutralization assay*

Rabbits immunized with *L. similis* venom extracted from male or female spiders were challenged 7 days after the last immunization by injecting 10 μg of *L. similis* venom diluted into 100 μl of phosphate-buffered saline (PBS) intradermally