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Stinging caterpillars from the genera *Podalia*, *Leucanella* and *Lonomia* in Misiones, Argentina: A preliminary comparative approach to understand their toxicity

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ABSTRACT

Dermal contact with Lepidoptera specimens at their larval stage (caterpillar) may cause systemic and/or local envenomation. There are multiple venomous species of them in Argentina, but their overall venom composition is poorly known. Lately, several cases of envenomation have been reported in the Misiones province, Northeastern Argentina. Thus, this work aimed to compare the protein composition, and the enzymatic properties of bristle extracts from caterpillars belonging to the families Megalopygidae (*Podalia* ca. *fuscescens*) and Saturniidae (*Leucanella memusae* and *Lonomia obliqua*) - the most common causative agents of accidents in Misiones -, and additionally to test their cross-reactivity with the *L. obliqua* antivenom produced in Brazil. Saturniidae venoms exhibited striking similarity in both their electrophoretic protein profile, and antigenic cross-reactivity. All venoms degraded azocasein – with the highest proteolytic activity observed in the *P.* ca. *fuscescens* bristle extract –, and hyaluronic acid, but the latter at low levels. *Lonomia obliqua* venom exhibited the highest level of phospholipase A_2 activity. Bristle extracts from *P.* ca. *fuscescens* and *L. obliqua* both degraded human fibrin(ogen) and shortened the clotting time triggered by calcium, while *L. memusae* venom inhibited plasma coagulation. Proteins related to the coagulation disturbance were identified by mass spectrometry in all samples. Altogether, our findings show for the first time a comparative biotoxinological analysis of three genera of caterpillars with medical relevance. Moreover, this study provides relevant information about the pathophysiological mechanisms whereby these caterpillar bristle extracts can induce toxicity on human beings, and gives insight into future directions for research on them.

1. Introduction

The order Lepidoptera comprises approximately 160.000 species of moths and butterflies worldwide (Gullan and Cranston, 2008), particularly in temperate and tropical climate zones (French and Brillhart, 2015). At their larval stage, when they are also called caterpillars, some of them display stinging or hairy bristles, which are chitinous evaginations of the cuticule. Usually, accidents with caterpillars occur when the victim inadvertently leans against the bristles, penetrating into the subcutaneous tissue and allowing the release of toxins – e.g. proteolytic enzymes, histamine and other pro-inflammatory substances – from the caterpillar body into the skin (Haddad and Lastoria, 2014).

Cutaneous reactions, such as excruciating pain, edema and erythema are frequent local manifestations of caterpillar envenomation. These reactions are typically mild and self-limited, but the contact with *Lonomia* caterpillars can cause a potentially fatal hemorrhagic syndrome (Hossler, 2010). Species of medical interest in Argentina are those related to Erebidae, Notodontidae, Limacodidae, Megalopygidae and Saturniidae families (de Roodt et al., 2000; Specht et al., 2008). Caterpillars from the last two families are the most common causative

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agents of accidents in the Misiones province, Northeastern Argentina, and *Lonomia obliqua* (Saturniidae) induces the most severe cases of envenomation (Sánchez et al., 2015). Caterpillars belonging to both families can be easily differentiated by the shape of their bristles: Megalopygidae caterpillars exhibit fine setae throughout the body, whereas Saturniidae ones show setae in small pine tree format (Haddad and Lastoria, 2014).

With the exception of detailed studies of the bristle extract from Brazilian *Lonomia obliqua* (Chudzinski-Tavassi et al., 2013), other bristle extracts from common caterpillars have not been investigated yet, and therefore their properties and overall venom composition are poorly known. For this reason, we initiated a comparative study of bristle extracts of caterpillars belonging to the families Megalopygidae (*Podalia* ca. *fuscescens*[1]) and Saturniidae (*Leucanella memusae* and *Lonomia obliqua*), which have caused several cases of human envenomation in Misiones. Besides shedding light on the pathophysiological mechanisms following envenomation, we aimed to determine the extent to which individual toxin families occur in common among the venoms of these caterpillars. Furthermore, in order to support the use of a specific treatment in case of *Lonomia* envenomation in Argentina, we investigated whether the specific antivenom produced by Instituto Butantan in Brazil recognizes toxic components present in *L. obliqua* bristle extract from Argentina. On the whole, this study aims to provide relevant information concerning the venom composition and enzymatic activity of bristle extracts of caterpillars from Argentina and nearby countries, and their possible neutralization by the sole antivenom available worldwide for caterpillar envenomation, and to give insight into future directions for research on these venoms.

2. Material and methods

Living larvae of *Lonomia obliqua*, *Leucanella memusae* and *Podalia* ca. *fuscescens* caterpillars were collected in the Misiones province (authorized by the Ministry of Ecology and Natural Renewable Resources of this province, authorization number 046), transported and maintained in the Insectarium of the Instituto Nacional de Medicina Tropical (INMeT) (Argentina).

Specimens of *L. obliqua* from the Southern region in Brazil were collected and sent to Instituto Butantan to obtain the antigen for antivenom production and quality control. From each batch of *Lonomia* venom, protein content and *in vitro* biological activity are determined to establish a reference standard for the bristle extract. As part of this characterization, one sample from Santa Catarina state (produced on February 2015) was used herein to compare the *Lonomia* venom from Argentina with the one used for the production of the *Lonomia* antivenom produced by Instituto Butantan.

For this study, samples were named as: Lo A: *Lonomia obliqua* from Argentina; Lo B: *Lonomia obliqua* from Brazil; Lm: *Leucanella memusae*; and Pf: *Podalia* ca. *fuscescens*. *Lonomia* heterologous antivenom (batch 1304060, expiration date: 03-2016) was kindly donated from the Instituto Butantan.

2.1. Morphology of the scoli and spines

Caterpillar scoli and spines were studied by scanning electron microscopy (SEM). The median dorsal region of caterpillar bodies were dissected and isolated, cleaned, critical-point dried and coated with a thin layer of gold (Denton Vacuum Desk II). Preparations were examined using a JEOL 5800LV scanning electron microscope at an acceleration voltage of 15 kV.

[1] This is equivalent to *Podalia* sp. (near *P. fuscescens*).

2.2. Caterpillar venoms

Preparation of bristle extracts (venoms) was carried out by manually removing the bristles, homogenizing them in cold phosphate-buffered saline (PBS), pH 7.4, and then the suspension was centrifuged and filtered to remove insoluble material (Da Silva et al., 1996). The protein content of bristle extracts was determined by fluorometry using the Qubit 2.0 (Life Technologies, USA) and/or the bicinchoninic acid (BCA) assay (Pierce, USA), and thereafter aliquots were stored at − 20 °C until use.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In order to evaluate and compare the protein profile of caterpillar bristle extracts, samples were electrophoresed on 12% polyacrylamide slab gels (Laemmli, 1970) under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions, and then silver stained (Blum et al., 1987). Each lane was loaded with 4 μg of protein.

2.4. Cross-reaction among caterpillar components

The presence of components reacting with the *Lonomia* antivenom produced by Instituto Butantan was tested by Western Blotting. Proteins separated by one-dimensional electrophoresis (see above) were transferred to 0.2-μm nitrocellulose membranes in a tank transfer system (Hoeffer mini VE, Amersham Biosciences) at 25 V for 1.5 h. Membranes were then blocked with 5% nonfat dry milk, and incubated with the *Lonomia* antivenom diluted 1:500, and subsequently with 1:10,000 peroxidase-conjugated anti-horse IgG (Sigma A9292). The reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma D5637) as reported elsewhere (Antunes et al., 2010).

In order to determine antibody titers against the *L. obliqua* bristle extracts from Argentina and Brazil, an ELISA assay was carried out as described previously (Antunes et al., 2010), except that microplates were incubated at 37 °C for 1 h with the *Lonomia* antivenom diluted 1:4,000, and subsequently with 1:10,000 peroxidase-conjugated anti-horse IgG (Sigma A9292).

2.5. Proteomic assay

Proteomic analyses were performed using bristle extracts in solution. Briefly, samples were treated with 8 M urea for 15 min at 80 °C, 100 mM dithiothreitol (DTT) for 30 min at 60 °C and then 200 mM iodoacetamide (IAA) for 30 min at room temperature, for destruction of 3D structures and reduction and alkylation of disulfide bridges. Then, the samples were hydrolyzed with 20 ng/mL ultrapure trypsin solution (Sigma T8658) diluted in 50 mM ammonium bicarbonate. The reaction was carried out at 37 °C overnight and stopped with 5% formic (FA) acid. The peptides were dried and then dissolved into 0.5% FA for LC-MS/MS analysis, in an IT-TOF mass spectrometer system (Shimadzu, Japan). Sample aliquots were injected in a C18 column (Phenomenex 2.1 × 50 mm, 300 Å) and eluted with a linear gradient of B over A, from 5 to 40% in 25 min, under a constant flow rate of 0.2 mL min^{-1}. The solvents were A = 0.5% FA in ultrapure water and B = 90% acetonitrile containing 0.5% FA in ultrapure water. Instrument control and data acquisition were performed by the LCMS Solution (Shimadzu, Japan). The MGF-converted MS2 profiles were analyzed by MS/MS ion search algorithms by PEAKS studio 7.0 for matches with known proteins sequences deposited on the public UniProt database. The MS and MS/MS tolerances were fixed as 0.1 Da (Zhang et al., 2012).

2.6. Quantitative enzyme assays

Caseinolytic activity was determined as reported previously