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A new venomous scorpion responsible for severe envenomation in Argentina: *Tityus confluens*

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ABSTRACT

In Argentina the scorpions of medical importance belong to the genus *Tityus* (*T.*), particularly the species *T. trivittatus*, the only scorpion whose sting is recognized to be associated with severe human envenoming and death. This genus is distributed from the north of the Patagonian region to the center and some provinces in the north of the country. During the period 2003–2006 four children died following scorpion stings, of which one was certainly and three were probably by *T. confluens*. In 2006, in the province of Tucumán, a girl died by scorpion envenoming and the scorpion responsible for the death, found in her shoe, was *T. confluens*. We thus studied the toxicity of venom gland homogenates from *T. confluens* from the provinces of Jujuy and Catamarca, and of crude venom from specimens from Catamarca and the province of La Rioja. The lethal potencies of the telson homogenates were 7.0 and 18.6 μg/g for Jujuy and Catamarca, respectively, while the lethal potency of the crude venom was 0.7 μg/g. Injected mice showed generalized congestion and hepatic lesions. Pancreatic damage was observed in some animals. Lungs showed congestion and foci of hemorrhage and mild edema. The heart showed injury in the muscular fibers. The venom showed high reactivity against anti-*T. trivittatus* antivenom and against two anti-*T. serrulatus* antivenoms. The anti-*T. trivittatus* antivenom neutralized the lethal activity of *T. confluens* venom. In addition, the venom reacted very slightly against an anti-*Centruroides* antivenom. Therefore, the stings of this scorpion must be considered of risk for humans to the same degree as the stings of *T. trivittatus*.

1. Introduction

The scorpions responsible for severe human envenomation in the Americas belong to the Buthidae Family (Buchler, 1971). In Argentina there are three Buthidae genera: *Ananteris*, *Zabius* and *Tityus*. The latter includes at least six different species: *T. argentinus*, *T. uruguayensis*, *T. paraguayensis*, *T. bahiensis*, *T. confluens* and *T. trivittatus*,

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not all related to human envenoming (Ojanguren-Affilastro, 2005).

The venom of *Tityus* scorpions is a secretion rich in neurotoxic peptides, especially those that modulate the Na^+ channels or block the K^+ channels (Becerril et al., 1997), and can thus alter the functionality of the transmission of the nerve impulse, which are the most conspicuous alterations observed in the autonomous nervous system (Cupo et al., 2003). At the nerve terminals, these toxins cause alterations in the Na^+ channels delaying their inactivation, impairing its activation, and/or blocking the K^+ channels (Becerril et al., 1997). Then, the scorpion envenoming syndrome is the result of the alterations caused by the scorpion toxins on the polarization and repolarization processes on excitable tissues. Thus, the injection of *Tityus* venoms results in the disorganized release of acetylcholine, catecholamines and other neurotransmitters (Correa et al., 1997; Vasconcelos et al., 2005).

In Argentina, envenomation by scorpions is a public health problem with increasing importance in more recent years (Salomón and de Roodt, 2001; de Roodt et al., 2003, 2006, de Roodt, 2007; Ministerio de Salud 2004, 2005, 2008; Saracco et al., 2006). The species associated with severe human envenomation are mainly *T. trivittatus*, which can be found from the north of the Patagonian region to the north of the country, and *T. bahiensis*, only found in the province of Misiones in the North East of the country (Ojanguren-Affilastro, 2005).

During the period 1993–1999, three fatal cases were reported (de Roodt et al., 2003). However, deaths by scorpionism have drastically increased from 1999 to date. Many severe cases, including 10 fatalities in which *T. trivittatus* was identified as the species responsible for the accident, have been recorded.

In 2003, in the province of Jujuy in the north of the country, two children died after a scorpion sting. The search for scorpions in the house resulted in the finding of several specimens of *T. confluens*, a scorpion previously described in this province (Neder de Román et al., 2007), and of some other scorpions, but not of *T. trivittatus*. In 2005, some fatal cases of children showing typical signs of scorpion sting were recorded in the province of Catamarca in the north west of Argentina. The only scorpion species found both in and near the house of one of those children was *T. confluens*. In 2006, in the province of Tucumán, a girl died after a sting from a specimen of *T. confluens* found by her relatives in the girl's shoe immediately after the scorpion sting (de Roodt et al., 2006). Although there are reports indicating *T. confluens* as a dangerous species of scorpion (Acosta, 2005; Ojanguren-Affilastro, 2005), there are no studies on its venom or cases reported about envenomation due to this species, and, to our knowledge, there are no reports available concerning its venom and lethality in experimental animals.

In the present work, we studied the toxicity of venomous gland homogenates of *T. confluens* from the provinces of Jujuy and Catamarca, and the toxicity of venom milked from specimens from Catamarca and La Rioja. The lethal potencies were assayed in mice and the course of the envenomation and the histopathological lesions produced by the venom were observed and recorded. The immunological reactivity of the milked venom and the venom gland homogenates was tested against four anti-scorpion antivenoms.

2. Materials and methods

2.1. Venom

The venom was obtained from telson homogenates of scorpions from Jujuy ($n = 20$) and Catamarca ($n = 17$) by conventional methods. Telsons were homogenized in 0.15 M NaCl. The homogenates were then centrifuged at $5000 \times g$ for 15 min in a refrigerated centrifuge at 4 °C. In addition, crude venom was obtained by milking scorpions from Catamarca and La Rioja. Briefly, scorpions were sedated with CO_2, after which they were electrically stimulated in the articulation of the telsons at 12 V using a commercial battery. The venom was diluted in 0.15 M NaCl and stored at −20 °C until use. The protein concentration was determined by the Bradford method (1976) using the Protein Assay Kit (Bio-Rad).

2.2. Experimental animals

NIH mice (18–22 g), provided by the Insituto Nacional de Producción de Biológicos – A.N.L.I.S. "Dr. Carlos G. Malbrán", Buenos Aires, Argentina (henceforth INPB), were used for all the experiments. The animals were kept under a controlled environment and received food and filtered water *ad libitum*. The institutional ethical approval regarding the handling of animals was provided by the I.N.P.B., in agreement with the recommendations of the National Research Council (2002).

2.3. Determination of the lethal potency

Lethal potency was studied in groups of six mice (NIH, 18–20 g) applying different doses of venom by the intraperitoneal route. After 24 h, the deaths were recorded and the plot of number of surviving animals as a function of the dose was analyzed by non-linear regression. The lethal potency was expressed as median lethal dose (LD_{50}), which represents the amount of venom (in μg) necessary to kill half of the challenged mice 24 h after venom injection.

2.4. Evolution of the experimental envenomation

The mice injected with the venom used for lethal potency determination were observed and the evolution of their signs was recorded from the moment of the injection until their death or resolution.

2.5. Histopathology

Animals inoculated were necropsied immediately after death and gross pathological examination was conducted in all the mice. Samples of lungs, heart, pancreas, liver and other selected organs were fixed in 10% formaldehyde in 0.15 M NaCl, 1.0 M phosphate buffer, pH 7.4. Paraffin-embedded tissues were sliced and stained for

light microscopic examination (Bancroft and Stevens, 1990).

2.6. Immunological studies

The immunochemical reactivity of the milked venom and the telson homogenates was tested against four available therapeutic antivenoms. All the antivenoms were constituted by $F(ab')_2$ fragments of equine immunoglobulins. The antivenoms used were: (1) "Antiveneno escorpiónico" (henceforth A-*Tt*) of the INPB (this antivenom is presented in 2 -ml ampoules), batch 907, expiry date 07/2003, developed with telson homogenate from *T. trivittatus*; (2) "Soro Antiaracnídico" (henceforth AA) of the Instituto Butantan of Sao Paulo, Brazil (presented in 5 -ml ampoules), produced with *T. serrulatus*, *Loxosceles gaucho* and *Phoneutria* sp. venoms, batch 950a d 921, expiry date 11/15/1998; (3) "Soro antiescorpión" (henceforth A-*Ts*) from the Instituto Butantan of Sao Paulo (presented in 5 -ml ampoules), produced with milked venom of *T. serrulatus*, batch 0204040/E, expiry date 20/03/2005; and (4) an anti-*Centruroides* antivenom (henceforth A-*Cn*), Alacramyn NR, from the Instituto Bioclón, México DF (lyophilized presentation, to be reconstituted in a volume of 5 ml), produced with telson homogenates from *Centruroides* sp., batch [B-8J-04] B-7E-06, expiry date 06/2011.

The double immunoprecipitation was carried out by conventional methods (Margni, 1990). Briefly, 10 μl of each antivenom were confronted against 10 μl of a telson homogenate or milked venom in a concentration of 0.8 mg/ml in 0.15 M NaCl. The gels were incubated for 48 h at room temperature, washed in 0.15 M NaCl for 48 h, dried and stained with Amido Schwartz.

We also studied the neutralization of the venom. Because of the lack of venom, the only test we were able to perform was the study of the protection conferred by a tested A-*Tt* antivenom over *T. confluens* venom in a single level of dose. Briefly, groups of six CF-1 mice were injected either with 2.5 i.p. LD_{50} of *T. confluens* venom or with the same dose of venom preincubated for 30 min at 37 °C with 1.5 ED_{50} of A-*Tt* (ED_{50}: dose of antivenom that protects 50% of challenged mice against 2.5 LD_{50} of *T. trivittatus* venom) in a final volume of 0.5 ml/mouse. The deaths were recorded 48 h after the injections.

3. Results

3.1. Protein content

The protein content per telson was 0.336 mg for specimens from Jujuy and 0.161 mg for specimens from Catamarca. The mean protein content by specimen obtained by milking was of 68.5 μg.

3.2. Lethal potency

The LD_{50}s found in the telson homogenates from Jujuy and Catamarca were 143 (118–174) and 372 (342–405) μg/mouse, respectively. The LD_{50} of the milked venom was 14.0 (9.0–21.0) μg/mouse. The potencies were 7.0 (4.5–1.05) and 18.6 (17.1–18.6) mg/kg for those of Jujuy and Catamarca, respectively, and 0.7 (0.45–1.05) mg/kg for the milked venom.

3.3. Evolution of the experimental envenomation after inoculation

At 1 min, mice showed restlessness. At 2 min, mice reduced the movements and showed tachypnea. At 3 min, some mice showed piloerection on their backs. At 4 min, mice showed high excitation to external stimulation. At 10 min, we observed salivation and lacrimation. At 12 min, we observed extension and crossing of the forelimbs, extension movements of the neck and difficulty in walking. At 20 min, we observed deep dyspnea, staggering and muscular contractions. At 25–40 min, some mice injected with the amount of venom equivalent to one or more LD_{50}s showed prostration and laid laterally. Mice died with marked dyspnea and cyanotic mucosa. Despite the initial signs of envenomation, mice surviving after 48 h did not show abnormalities.

3.4. Pathological studies

On necropsy, the gross pathology showed a generalized congestion. In the microscopic study, marked congestion, infiltration of mononuclear cells, thickening of the alveolar septa with rupture of the alveolar structure and foci with intraalveolar edema and hemorrhage were observed in all samples (Fig. 1a,b). Intrafibrillar edema and focal fragmentation of myocardial fibers were observed in the hearts and several samples showed isolated and cytoplasmic condensation and eosinophilia (Fig. 2a,b). Diffuse cytoplasmic vacuolization and sinusoidal congestion was observed in the liver (Fig. 3a,b). Pancreatic damage consisting of peripancreatic hemorrhage and signs of severe epithelial injury of acini was observed in 40% of mice (Fig. 4).

3.5. Immunological studies

The venom showed reactivity against all the antivenoms tested. The strongest reactivity was observed against the A-*Tt* antivenom and the lowest reactivity against the A-*Cn*. The reactivity against the AA antivenom was higher than that observed against the A-*Ts* antivenom both against the telson homogenates and the milked venom. The neutralization assay showed that 1.5 ED_{50} of A-*Tt* protected 100% of challenged mice, which survived without any sign of envenomation for more than 48 h, and that no mice from the control group survived for more than 45 min after of the venom injection.

4. Discussion

The clinical signs observed after the inoculation of *T. confluens* venom were very similar to those described in mice experimentally envenomed with *T. trivittatus* venom (de Roodt et al., 2000), and were consistent with the clinical signs observed in the envenomation by *T. trivittatus* (de Roodt et al., 2003) and other South American scorpions (Ministerio de Saúde 1999; Mazzei de Davila et al., 2002;

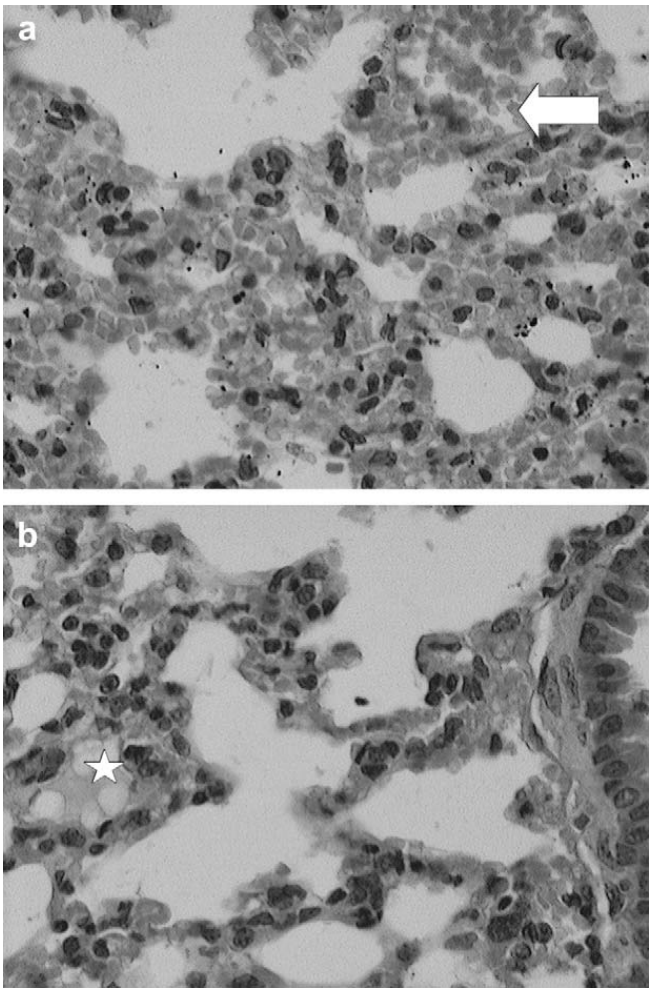

Fig. 1. (a) Histology of the lungs showing marked congestion and infiltration of mononuclear cells. Observe the thickening of the alveolar septa and edema. In the superior right corner is clearly observed intra-alveolar hemorrhage (arrow) (H&E, 200). (b) Observe in addition to the thickening of septa and the cellular infiltration, the rupture of the alveolar structure. On the left, congestion and focal intra-alveolar edema can be observed (asterisk) (H&E, 200).

Cupo et al., 2003). Clinical signs of experimental envenomation in mice injected with 1 or more i.p. LD_{50} of venom gland homogenates or milked venom were short-lived since the envenoming syndrome progressed to death in 40 min. These mice showed sialorrhea and lacrimation probably due to stimulation of cholinergic receptors (Andrade et al., 1981) and tachypnea, slight piloerection, slight sudoration and tremors probably of adrenergic origin (Cupo et al., 2003). However, injected mice did not show the rather profuse sudoration (adrenergic origin) and/or the diarrhea (cholinergic stimulation) observed in the experimental envenomation by *T. trivittatus* (de Roodt et al., 2000).

Pulmonary edema, respiratory distress and cardiac failure are considered among the frequent causes of death in human victims stung by *Tityus* scorpions (Cupo et al., 1994; Freire-Maia et al., 1994; Freire-Maia, 1990) and in animal models injected with these venoms (Freire-Maia, 1990; ÓSuze et al., 1999, 2003; de Roodt et al., 2000; Cupo et al., 2003). The histopathological lesions observed in lungs from mice injected with *T. confluens* venom showed edema, rupture of alveolar septa and hemorrhagic foci (Fig. 1a,b), but did not show the important intraalveolar deposit of hyaline material observed in mice injected with *T. trivittatus* venom (de Roodt et al., 2000). The pathogenesis of pulmonary edema induced by the venom of a scorpion sting is complex (Amaral et al., 1994: Amaral and Rezende, 1997; D'Suze et al., 1999) and involves both left ventricular failure due to acute arterial hypertension and sinus tachycardia (Cupo et al., 1994; Freire-Maia 1990) and increased pulmonary vascular permeability due to the release of bradykinin (Rotschild and Castania, 1976), prostaglandins (Amaral and Rezende, 1997), histamine (Cuhna-Melo et al., 1987) and interleukins (Magalhães et al., 1999; Andrade et al., 2006). The high levels of IL-1α, IFNγ, nitric oxide and GM-CSF observed in severe envenomations are similar to those observed in the systemic inflammatory response (Magalhães et al., 1999; D'Suze et al., 2003, 2004). Increasing plasma (D'Suze et al., 2003) and tissue (Andrade et al., 2006) levels of cytokines, which can be responsible for the exacerbation and maintenance of the inflammatory response of lungs, have been observed in the pulmonary edema or distress respiratory syndrome in *Tityus* envenoming. The edema, thickening of the alveolar septa, hemorrhage and cellular infiltration experimentally observed in mice injected with *T. confluens* were similar to those observed in the experimental envenoming by other *Tityus* venoms.

In this work, we observed lesions in cardiac muscle, represented by early ischemic changes consisting of interfibrillar edema, cytoplasmic condensation and eosinophilic like apoptotic bodies (Fig. 2). The lesions in cardiac muscle in *Tityus* envenomation were attributed to a direct action of the venom and to an indirect action by the autonomic alterations caused by the envenomation (Amaral and Rezende, 1997), with a toxic and an ischemic origin. The release of catecholamines, acetylcholine and other neurotransmitters leads to pronounced and long-lasting hemodynamic alterations, such as acute hypertension and sinus tachycardia (Freire-Maia 1990; Cupo et al., 1994). Catecholamines seem to play a very important role in the myocardial lesions (Nouira et al., 2005; Ouanes-Besbes et al., 2005). However, the ischemic myocardial lesions may not be caused only by catecholamines but by the cytokines and/or the neuropeptide Y released in the envenoming by its constrictor action on coronary vessels and the high levels of glucose that occur in this envenoming (Bahloul et al., 2005).

The involvement of kinins in scorpion envenomations is consistent with the observation of pancreatic lesions in some of the animals injected with *T. confluens* venoms (Fig. 4), as described previously in other experimental *Tityus* envenoming (Correa et al., 1997; de Roodt et al., 2000; D'Suze et al., 1995, 2004; Fletcher et al., 1994). Several animals showed hemorrhage, inflammation and lesions in the acini (Fig. 4). The release of substances from the acini can begin a sequence of systemic effects which can evolve until death. The pancreatic lesions are caused at least partially by the activation of muscarinic receptors (Possani et al., 1991; Fletcher et al., 1994; Fletcher et al., 1996; D'Suze et al., 1995) and can evolve into a systemic inflammatory response (Norman, 1998; Steer and

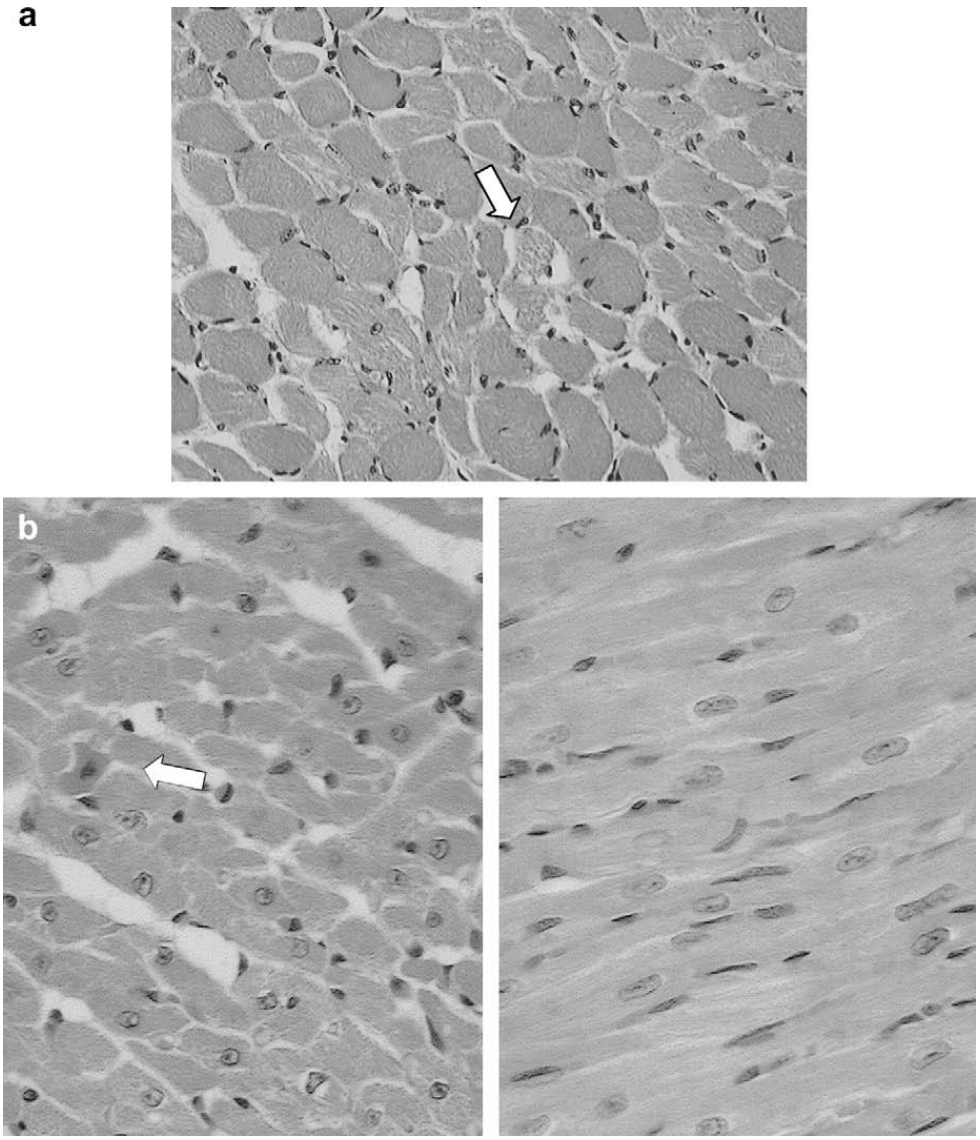

Fig. 2. (1) The hearts showed ischemic changes consisting of intrafibrillar edema with focal fragmentation of the myocardial fibers (arrow) (H&E, 200). (b) Details of the intrafibrillar edema, focal fragmentation of fibers and necrosis. On the right cytoplasmic condensation and eosinophilia-like apoptotic bodies can be observed (arrow) (H&E, 200).

Meldolesi, 1988; Frossard et al., 2001, 2002; Frossard and Pastor, 2002). In pancreatitis, the release of pro-inflammatory factors and other deleterious components contribute to the lesions in other organs. In this way, the acute lung injury is the most frequent complication occurring in the course of acute pancreatitis (Ranson et al., 1973; Steer, 1989). This mechanism has been suggested in the envenomation by *Tityus* venoms (D'Suze et al., 2004). The kallikrein-bradykinin system seems to play a role in scorpion envenoming (Fukuhara et al., 2004) and its activity has been related to the hyperthermia observed in the envenomation by *Tityus* (Pessini et al., 2006). The cardiovascular manifestations and the accumulation of vasodilator substances (kinins and/or prostaglandins and some interleukins) may contribute to the terminal shock refractory to the hypertensive action of noradrenaline (Ismail, 1995).

The lethal potency of the milked venom (0.70 mg/kg) was very high when compared with the venom from other *Tityus* venoms, ranging from 0.77 to 1.59 mg/kg for the venoms of the Brazilian *Tityus*, of highest sanitary importance (Nishikawa et al., 1994), and from 0.50 to 1.75 mg/kg for *T. trivittatus* milked venom (unpublished results). The lethal potency of telson homogenates was around those found for *T. trivittatus* venom gland homogenates from regions of Argentina where humans deaths were recorded, ranging from 100 to 500 μg (de Roodt et al., 2005).

The venom and telson homogenates showed immunochemical reactivity against all the antivenoms, the A-*Tt* antivenom being the most reactive (Fig. 5). Precipitation bands with the heterologous A-*Cn* antivenom were also observed. When the crude venom was confronted with the antivenoms, the strongest recognition was observed again with A-*Tt* antivenom, followed by AA and A-*Ts*, showing the A-*Cn* the lowest reactivity (Fig. 5). This indicates important immunochemical cross-reactivity between *T. confluens*, *T. trivittatus* and *T. serrulatus*

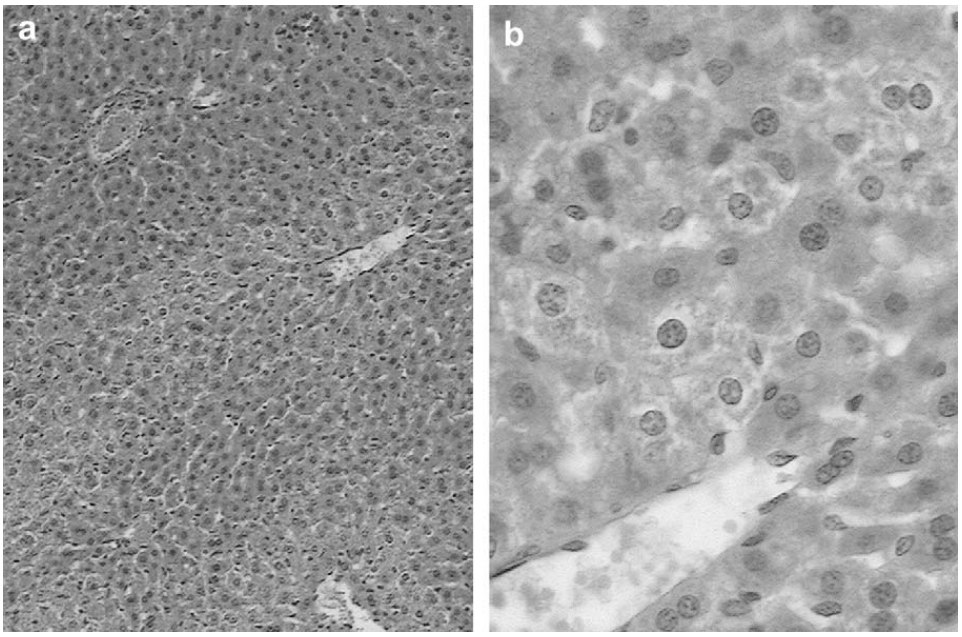

Fig. 3. (a) Sinusoidal congestion in the liver of mice injected with *T. confluens* venom (H&E, 100). (b) Hepatocytes with focal granular cytoplasm, and vacuolization. Vascular congestion (H&E, 400).

venoms, in agreement with the cross-reactivity observed between *Tityus* venoms (Nishikawa et al., 1994). The low reactivity observed with the A-*Cn* antivenom is in agreement with the low reactivity found between some species of *Tityus* and *Centruroides* venoms (D'Suze et al., 2007). It has been suggested that a monovalent antivenom could be useful to neutralize the venom from Brazilian scorpions (Nishikawa et al., 1994); this could be the case among the Argentinean *Tityus*. In a single experiment we observed that the A-*Tt* antivenom totally protected mice against 2.5 LD_{50} of *T. confluens* telson homogenate. At present, we are working in order to obtain *T. confluens* specimens to evaluate the neutralization of the antivenoms used to treat scorpion bites, a difficult work due the characteristics of this scorpion.

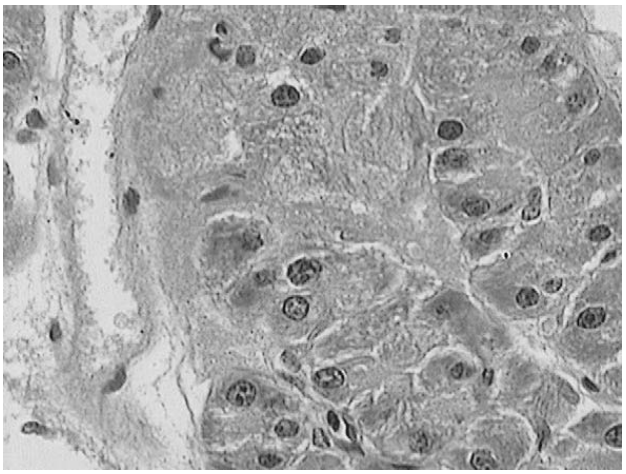

Fig. 4. Pancreatic lesions. The damage consisted of peripancreatic hemorrhages and signs of severe epithelial injury of acini (H&E, 400).

Although some of the available literature reports *T. confluens* as responsible for the deaths (Ojanguren-Affilastro, 2005), there is no local registry on envenomation by this species or on the toxicity of its venom. However, from the Public Health point of view, after this report of the toxicological properties of the *T. confluens* venom, and the deaths associated with this species, two points should be remarked. First, the neutralizing power of the heterologous antivenoms deserves further investigation. Second, the fatal cases of scorpionism due to this species happen in synanthropic domestic scenarios, different from those described in its typical disrupted geographical range in the Chaco dry formation. In addition, a trend to sub-speciation has been reported (Bolivia, Paraguay, Salta-Argentina, and Mato Grosso do Sul-Brazil) (Lourenço et al., 2004; Acosta 2005; Bertani et al., 2005; Lourenço and Da Silva 2006, 2007), similar to that observed in the past with *T. trivittatus*.

Therefore, envenomation by scorpion stings in Argentina is an increasing public health problem, and in addition to the already known *T. trivittatus*, we must include *T. confluens* as a potential scorpion responsible for severe envenomation and death in humans. The range of toxicity in mice, the course of the experimental envenomation, and the histopathological lesions indicate its toxicity, which showed characteristics similar to those described for other species of *Tityus* in South America. Although the venom reacted with the antivenoms commonly used in Argentina and it was neutralized by A-*Tt* antivenom, neutralization experiments should be carried out to test the usefulness of these antivenoms on the venom of *T. confluens*.

To our knowledge, this is the first work on the toxicity of the venom of *T. confluens*, the course of the experimental envenomation, the histopathological lesions caused by the venom and its immunochemical characteristics.

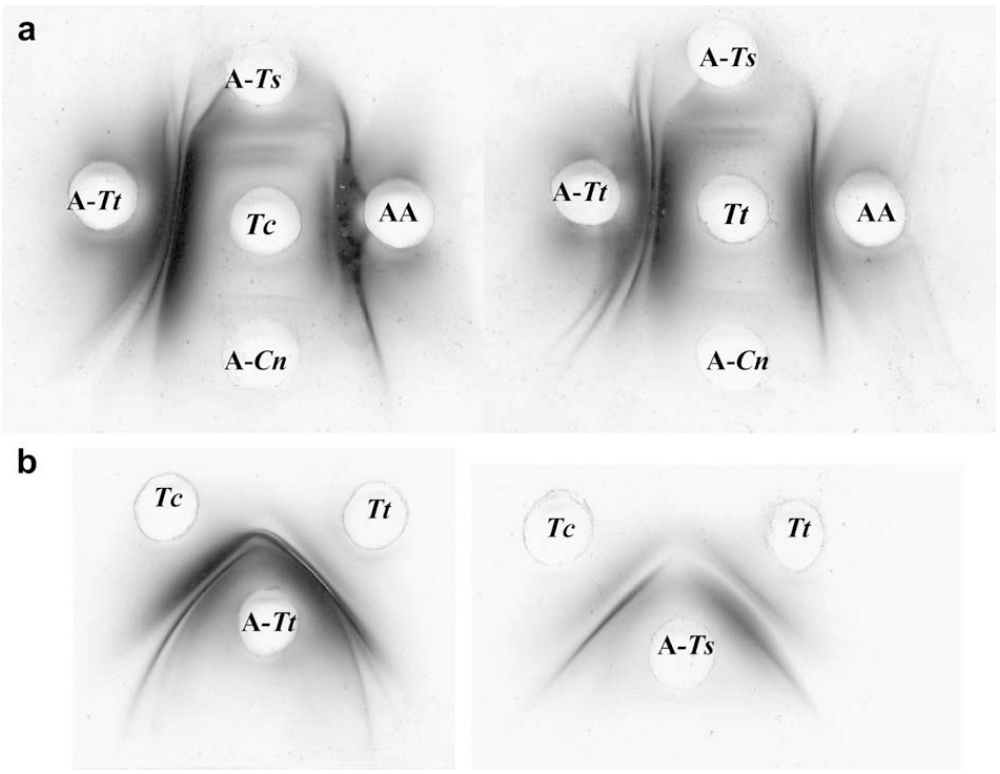

Fig. 5. (a) Double immuno diffusion of the venom and antivenoms. In the left are shown the double diffusion of four antivenoms (10 μl each one) against 10 μg of *Tityus confluens* venom *(Tc)*. In the right, the same test using the same antivenoms against 10 μg of *Tityus trivittatus (Tt)* venom. A-*Ts*: anti *T. serrulatus antivenom*; A-*Tt*: Anti *T. trivittatus* antivenom; AA: Anti *T. serrulatus*, Anti *Loxosceles gaucho* and anti *Phoneutria venoms, A-Cn*: Anti *Centruroides* spp. venom. (b) The Anti *T. trivitattus* (left) and Anti *T. serrulatus* (right) were confronted with venom of *T. confluens (Tc) and T. trivittatus (Tt)*.

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Conflict of interest

There are no conflicts of interest in the content of this work.

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