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Research article

# Effects of tityustoxin on cerebral inflammatory response in young rats

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## HIGHLIGHTS

- TsTX injection can lead a cerebral inflammatory process.
- TsTX induces increase in cerebral microvascular leukocyte recruitment in vivo.
- Rats injected with TsTX present higher cerebral levels of TNF-α cytokine.

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## ABSTRACT

Accidents caused by scorpion stings, mainly affecting children, are considered an important cause of morbidity and mortality in tropical countries. Clinical studies demonstrate the relevant role of systemic inflammatory events in scorpion envenoming. However, remains poorly understood whether the major lethal component in *Tityus serrulatus* venom, tityustoxin (TsTX), is able to induce inflammatory responses in the cerebral microcirculation. In this study, we systematically examined leukocyte recruitment into the CNS in response to TsTX injection. Accordingly, developing rats were subjected to a subcutaneous (s.c.) injection of TsTX (0.75 mg/kg), and leukocyte recruitment (i.e., 4, 8 and 12 h after injection) and TNF-α levels were evaluated. Rats injected with TsTX presented a significant increase in leukocyte rolling and adhesion and higher levels of TNF-α at all time points studied, compared to the control group. Altogether, this work demonstrates the triggering of neuroimmunological mechanisms induced by TsTX injection in young rats.



## 1. Introduction

Scorpion stings are a common event in subtropical and tropical countries [1,2]. They are considered an important cause of morbidity and mortality, especially among children. In Brazil, more than 57,000 scorpion stings were reported in the last year,with the highest mortality rate occurring in children. *Tityus serrulatus* is the scorpion associated with the most severe cases of scorpion envenoming [1].

The composition of scorpion venom is a mixture of different toxic peptides that act upon voltage-gated ion channels (sodium, potassium, calcium, chloride) [3]. The toxin used in this study, TsTX, has been suggested as the major lethal component in *T. serrulatus* venom [4].

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The inflammatory response seems to play a significant role in the pathophysiology of scorpion envenoming, contributing to systemic manifestations [2,5–8]. There are several experimental studies addressing the systemic inflammatory action of *T. serrulatus* scorpion venom [7–9]. De Matos et al. have shown that the release of platelet-activating factor, leukotrienes and prostaglandins, may play a role in the induction of acute lung edema by scorpion venom in rats [10]. Furthermore, Fukuhara and co-workers have found a positive correlation between increased levels of cytokines and the severity of scorpion envenomation in humans [11,12]. It seems that the depolarization induced by the binding of scorpion toxin to voltage-gated sodium channels causes a cascade of inflammatory events [9]. Moreover, after mild, moderate and severe scorpion envenomation, there was a significant increase in leukocyte counts in child blood samples [13]. The association of leukocytosis in severe scorpion envenoming may be associated with the release of super oxide anion by activated leukocytes, leading to tissue hypoxia in the microcirculation and contributing to multiple organ failure[14].

Additionally, there are several studies addressing a high sensitivity of the central nervous system to TsTX [15–18]. Even small amounts of this specific toxin can have a lethal effect in adult rats when injected directly in the brain parenchyma [18,19]. Furthermore, studies with developing rats have shown very early vascular, metabolic and electroencephalographic alterations in the brainstem by systemic injection [16,20]. This effect strongly suggests that TsTX is crossing the blood brain barrier, as this barrier in young animals is not yet fully developed. In addition, it has already been shown that the pharmacokinetics of the venom is altered by age. In fact, young rats present a greater and faster distribution of TsTX, as well as a slower rate of elimination, when compared to adult animals [21]. Altogether, these factors can help to explain why the most severe symptoms of scorpion envenoming are found in children [2,22–24].

In summary, the venom's action on the central nervous system seems to play an important role in the pathophysiology of scorpion envenoming syndrome, involving peripheral immune cells and release of inflammatory mediators. Although there are previous data that demonstrate the occurrence of systemic inflammatory events after scorpion poisoning, little is known about a cerebral inflammatory response induce by TsTX. The aim of this work was to evaluate the inflammatory process in the brain of developing rats after a subcutaneous injection of a sub-lethal dose of TsTX.

## 2. Material and methods

### 2.1. Scorpion venom

TsTX was isolated from the venom of *T. serrulatus* according to the methodology described by Gomez and Diniz, and modified by Sampaio et al. [25,26]. The lyophilized toxin was solubilized in 500 μl of sterile saline. A known concentration of TsTX, as determined by the method of Hartree (serum bovine albumin as standard [27]), was used to determine the absorbance coefficient read at 280 nm: [Protein] (Ag/mL)/A280 = 279. Further determination of TsTX concentration was conducted by spectrophotometer reading (Hitachi spectrophotometer, model 2001, Japan).

### 2.2. Animals

Male Wistar rats ($n = 78$; 21 days-old) were injected s.c. with saline (control group) or a sub-lethal dose of TsTX (1/4DL50 = 0.75 mg/kg) [37]. The rats had free access to food and water. Animal protocols were approved by local Animal Care Committee(CETEA/UFMG; Protocol n°.005/09).

### 2.3. Intravital microscopy

Intravital microscopy of the cerebral microvasculature was performed as described elsewhere [28]. Fig. 1 shows a schematic image of the brain intravital microscopy protocol. Briefly, control and TsTX animals at 4, 8, 12 h and 7 days ($n = 48$; 6 rats/group) were anaesthetized by intraperitoneal (i.p.) injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). The parietal craniotomy was performed using a high-speed drill and the dura mater was removed to expose the underlying pial vasculature. The tail vein was cannulated for administration of rhodamine 6G (0.3 mg/kg; Sigma, USA) [29]. Leukocytes labeled with rhodamine 6G were visualized using a Zeiss Imager M.2 microscope with objective 20X-LD, fitted with a fluorescent light source (epi-illumination at 510–560 nm using a 590 nm emission filter) and a silicon-intensified camera (Optronics Engineering, Goleta, CA, USA). The microvessels (pial venules) were analyzed in a 100 μm long section. The diameter measured in each vessel ranged between 80 and 120 μm. Rolling leukocytes (cells/min) were defined as white cells moving at a velocity less than that of erythrocytes. Adherent leukocytes remained stationary for 30 s or longer on the venular endothelium (100 μm).

### 2.4. Determination of cerebral TNF-α levels

Control and TsTX animals were euthanized at 4, 8 and 12 h ($n = 30$; 5 rats/group). The whole brains were collected and homogenized (Ultra-Turrax) in extraction solution (100 mg of tissue/mL) (0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU aprotinin). The homogenates were centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatants were stored at −20 °C. The concentration of TNF-α was assayed by ELISA according to manufacturer (R&D Systems, USA).

### 2.5. Statistical analysis

Statistical analysis was conducted using the Mann–Whitney test for comparisons between different groups considering leukocyte-endothelium interactions and cerebral levels of TNF-α. These results are expressed as the median ± interquartile range. Statistical significance was established at $p < 0.05$.

## 3. Results

### 3.1. Adhesion of leukocytes in cerebral microvasculature

Intravital microscopy images from pial microcirculation from control animals injected with saline revealed no baseline rolling in any of the vessels at 4, 8 and 12 h and at 7 days. In contrast, s.c. injection of TsTX induced a significant increase in the number of rolling and adherent leukocytes at 4, 8 and 12 h (Fig. 2A and B, $p < 0.005$) and at 7 days (Fig. 2C and D; $p < 0.001$), when compared to the control group.

### 3.2. Effects of TsTXon TNF-α levels

Rats injected with TsTX exhibited a significant increase in cerebral TNF-α levels 4, 8 and 12 h after scorpion envenomation, compared to the control group ($p < 0.005$; Fig. 3).

## 4. Discussion

After the subcutaneous injection of TsTX (0.75 mg/kg), reactions such as lachrymation, piloerection and salivation were commonly

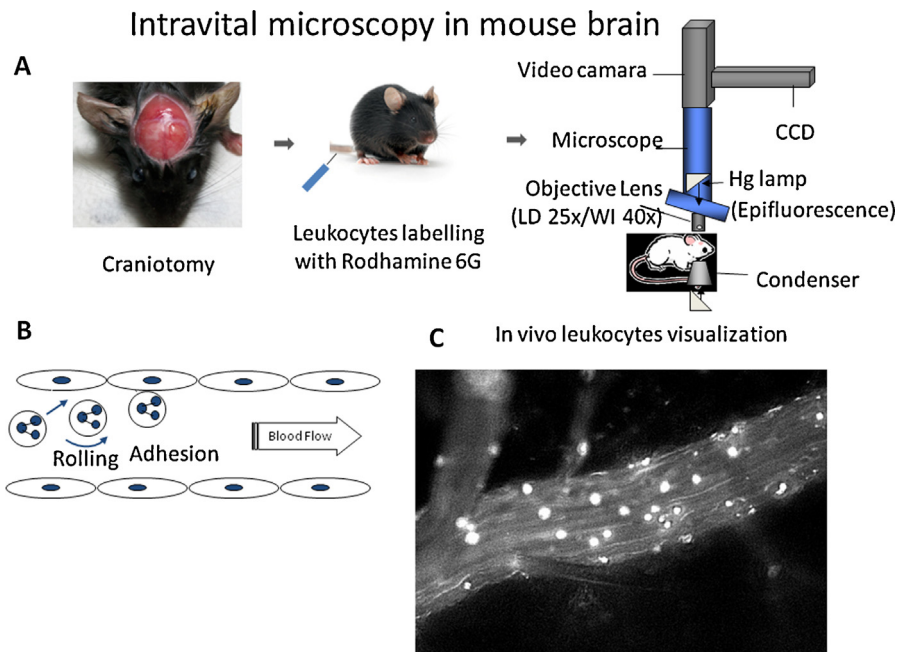


**Fig. 1.** Schematic of the brain intravital microscopy protocol: (A) In animals anesthetized with ketamine + xylazine, performed a craniotomy, followed by iv injection of rhodamine 6G to label the leukocytes and visualization of microcirculation of the pia mater in the fluorescence microscope. (B) The parameters evaluated were rolling and adhesion of leukocytes. (C) Representative image of an intravital microscopy experiment in which we observed a pia mater venule containing fluorescently labeled leukocytes.

visualized. The dose used in this work corresponds to ¼ DL50 for post-natal day 21 Wistar rats [21].

From accumulating basic and clinical evidence, the inflammatory response plays a major role in scorpion envenoming syndrome [2,7,8,12,30]. In fact, multiple organ dysfunction and death are associated with an increased production of pro-inflammatory cytokines in experimental models of severe scorpion envenoming [2,7,8,30]. Nevertheless, little is known about an inflammatory response involving the brain tissue after TsTX insult. In this context, our results have showed a significant increase in leukocyte rolling and adhesion in the brain microvasculature from 21-day-old rats at 4, 8 and 12 h after subcutaneous TsTX injection. This leukocyte

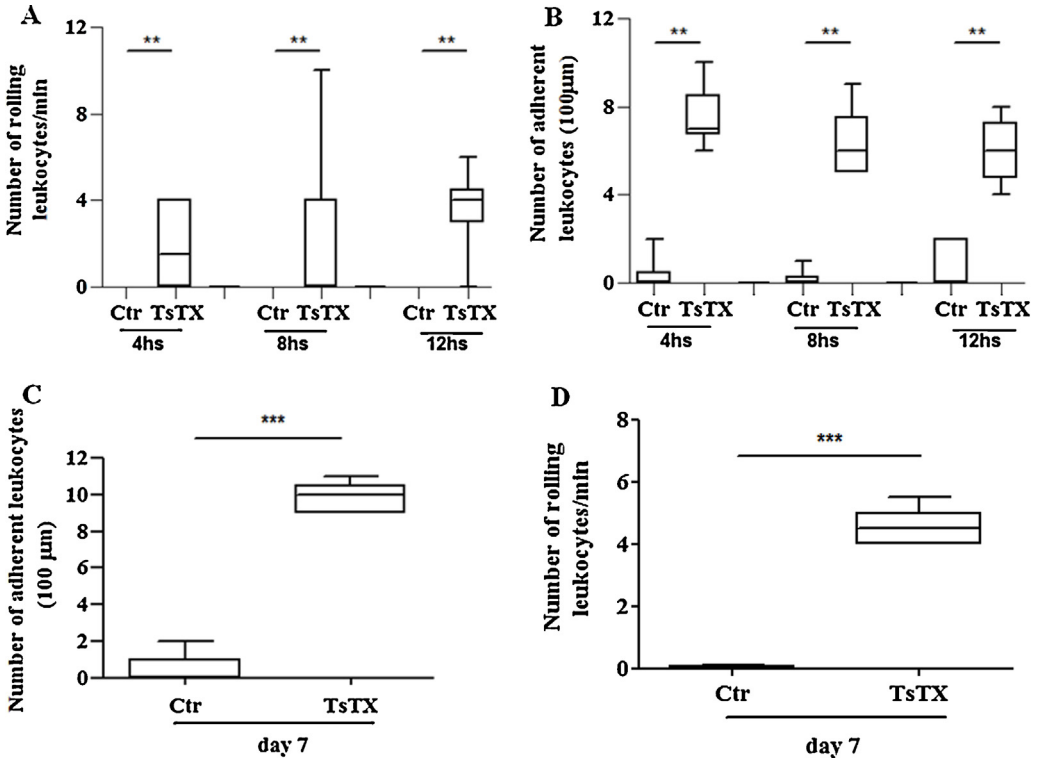


**Fig. 2.** Leukocyte-endothelial interactions in pial vasculature. Intravital microscopy was used to assess leukocyte rolling and adhesion at 4, 8 and 12 h (1A and B) and at 7 days (1C and D) after subcutaneous injection of vehicle or TsTX. The analyses were performed in 8–10 vessels/animal ($n = 6$ rats/group). The results are expressed as the median ± interquartile range. Significant difference: $^{**}p < 0.005$; $^{***}p < 0.001$ (Mann–Whitney test, for comparison between control and TsTX groups).

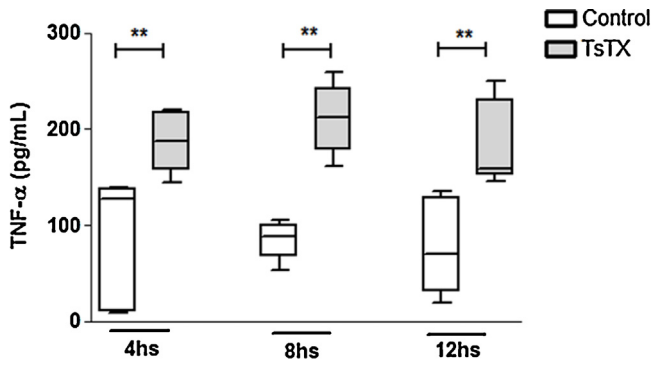


**Fig. 3.** Kinetics of cytokine production in the brain. There was an increase in TNF-α levels in TsTX rats, compared to the control group ($n = 5$ rats/group). The results are expressed as the median ± interquartile range. Significant difference: $^{**}p < 0.005$ (Mann–Whitney test, for comparison between control and TsTX groups).

recruitment was associated with increased brain levels of TNF-α in the TsTX group, compared to the control group. Indeed, leukocyte recruitment into the inflammatory site is essential for the development of an appropriate immune response [31].

Additionally, TNF-α is described as a pro-inflammatory cytokine that can be expressed and released after pathological brain insult [32]. TNF-α has a stimulatory role in glutamatergic excitotoxicity, inhibiting glial glutamate transporters on astrocytes and increasing the synaptic expression of AMPA receptors [33]. In this regard, the TsTX binds to site 03 of voltage-gated sodium channels, slowing their inactivation and increasing neurotransmitter release, including glutamate [9,34–36]. Additionally, the increased levels of TNF-α presented in the TsTX group can induce a vasoconstrictor effect, contributing to excitotoxic glutamatergic effects. Indeed, it was previously demonstrated that an intrastriatal injection of TNF-α in rats resulted in an acute, dose-dependent reduction of cerebral blood volume [37]. Moreover, TNF-α may contribute to an impaired microvascular perfusion, by increasing leukocyte recruitment obstructing cerebral vessels or via a direct vasoconstrictor effect [38]. Many studies have demonstrated increased TNF-α plasma levels in human or experimental models of *T. serrulatus* scorpion envenoming [11,13,39].

Altogether, our data strongly suggests the involvement of neuroimmunological mechanisms induced by injection of TsTX in developing rats. Further studies are crucial to assess the communication between immune cells and neurons as well as to determine the effect of this interaction on the severity of *T. serrulatus* envenomation.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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