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# Dose-dependent effect of carbamazepine on weanling rats submitted to subcutaneous injection of *tityustoxin*

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**Abstract**

The scorpion envenoming syndrome is a serious public health matter in Brazil. The most severe cases occur during childhood and elderly. Previous results from our laboratory suggest that the effects of scorpion toxins on the central nervous system play a major role on the lethality induced by scorpion envenoming. The aim of this work is to evaluate the therapeutic potential of carbamazepine (CBZ) injected i.p. 90 min before s.c. tityustoxin (TsTX) injection in weanling rats. Rats were divided into six experimental groups according to s.c. injection (saline or TsTX) and i.p. treatment (vehicle or CBZ 12, 50 and 100 mg/kg): Sal/Veh group ($n=4$); Sal/CBZ100 ($n=4$); TsTX/CBZ12 ($n=6$); TsTX/CBZ50 ($n=8$); TsTX/CBZ100 ($n=8$) and, at last, TsTX/Veh ($n=8$). The dose of TsTX was the same for all groups: 6.0 mg/kg, twice the DL50 for weanling rats. Video images were recorded until death or for a maximum period of 240 min. Lungs were excised and weighed to evaluate edema. The results showed that CBZ (12, 50 and 100 mg/kg) was able to increase the survival rate and latency-to-death of the rats. Only the group treated with 100 mg/kg of CBZ had a decrease in the pulmonary edema. The known effect of CBZ reducing neuronal excitability most likely protected the neural substrates targeted by TsTX. Although treatment was performed before TsTX inoculation, the results are promising regarding CBZ as a therapeutic coadjuvant in the treatment of scorpion poisoning. The pharmacokinetics of CBZ can be very much improved by either changing the form of administration or encapsulating the drug in order to enhance solubility.


*Keywords:* Tityustoxin; Carbamazepine; Neuroprotection; Scorpion envenoming

The scorpion envenoming syndrome is a serious public health matter with high prevalence in tropical countries. In Brazil, there are 8000 accidents notified per year, with an incidence of three cases for each 100,000 inhabitants. *Tityus serrulatus* is responsible for the most serious accidents [12]. Clinical symptoms of the envenoming depend on the type of accident, body region of venom inoculation and the age of the patient. The consequences can vary from mild or moderate local pain and paresthesia (90% from envenoming accidents) to serious systemic dysfunctions such as cardiac arrhythmias, gastrointestinal alterations, lung edema, pancreatitis, convulsions, neurological lesions, coma and even death [30,10]. The most serious envenoming cases occur in children and in senior subjects [19]. Scorpion antivenom is indicated in 10% of the cases [12], considered as severe scorpion envenoming. However, even with treatment, there is a general lethality of 0.6% prevailing in children under 6 years old.

*Tityus serrulatus* scorpion venom consists of a complex mixture of toxic and non-toxic peptides. Two types of toxins within the venom have been proposed as responsible for its toxic effects: toxin gamma (a β-type toxin) and tityustoxin (TsTX, an α-type toxin), both with specific affinity to the voltage-gated sodium channels (VGSCs) [2]. The toxin used in this study, TsTX, has

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been suggested as the major lethal component in the *Tityus serrulatus* venom [20]. TsTX binds to the site 3 of the voltage-gated sodium channel, slowing its inactivation and has higher affinity to the channel in its open state [2,22,21].

Nunan et al. had s.c. injected radio-labeled TsTX and reported that the distribution of TsTX in the brain of young animals was close to three-fold that of adult rats. Furthermore, the presence of labeled TsTX in the brain of young rats indicates that it can cross the blood brain barrier and, together with its presence in the heart, may explain the association of cardiovascular and central nervous system (CNS) symptoms observed frequently in children after scorpion accidents [28,27]. In another study, Nunan et al found that weanling male Wistar rats showed around 3.5 times more toxic sensitivity than adult male rats [26]. In clinical practice, children are more vulnerable than adults to death after the inoculation of the venom [34,18,3]. Clot-Faybesse et al. (2000) showed a small, but significant, amount of labeled toxin recovered from the brains of young mice 2 h after the subcutaneous injection [36]. Intracerebroventricular administration of the toxin is able to generate similar systemic effects, nevertheless more potentiated, than toxins injected subcutaneously [18,25]. Altogether these data suggest that, especially in infants where the blood brain barrier is not fully developed, the direct effect of the venom on the CNS cannot be discarded.

Pulmonary edema is a frequent complication after the scorpion envenoming in humans, especially children, stung by scorpions of several species [11,18]. Thus, there are many studies throughout the literature trying to explain the pathophysiology of this specific symptom, which source is controversial between the authors. Santana et al. studying the effect of *T. serrulatus* crude venom (5 μg/100 g, s.c.) in anesthetized rats, reported finding unilateral lung edema without the presence of cardiac dysfunction, which would corroborate to the theory of a central effect of the venom [33]. On the other hand, De Matos et al. showed that the release of vascular permeability factors, leukotrienes and prostaglandins may play a role in the induction of acute lung edema by scorpion venom in rats [5]. In 2001, De Matos et al. suggested an important role of mast cells in formation of this edema [6]. Furthermore, D'Suze et al. suggested that neutrophil activation, inflammation and coagulation are correlated in the genesis of scorpionism acute lung injury [7]. Fatani et al. showed that lignocaine, which blocks voltage-gated sodium channels, was efficient in attenuating symptoms after the scorpion envenoming in rabbits, decreasing the lethality [9]. In addition, rats pre-treated with Phenobarbital presented decrease in pulmonary edema after TsTX inoculation [24].

Thus, in the present work, we tested the known anticonvulsant and mood stabilizer carbamazepine (CBZ), a drug that reduces neural excitability by modulating the activity of voltage-gated sodium channels, against the symptoms observed after the s.c. inoculation of the venom in Wistar weanling rats. The clinical relevance of this work is based on the assumption that death by scorpion envenomation is primarily a consequence of the venom's action on the CNS rather than a direct action on peripheral organs.

Tityustoxin (TsTX) was isolated from the venom of *Tityus serrulatus*, in agreement with the methodology described by Gomez [13], and modified by Sampaio et al. [32]. The lyophilized toxin was solubilized in 500 μl of sterile saline. A known concentration of TsTX, as determined by Hartree [17] (serum bovine albumin as standard), was used to determine the absorbance coefficient read at 280 nm: [Protein] (mg/ml)/A280 = 279. Further determination of TsTX concentration was done by the direct reading of samples in the spectrophotometer (Hitachi spectrophotometer, model 2001, Japan). After determining the concentration of protein (4.76 μg/μl), the initial pool was stored in 200 μl Eppendorf tubes, in volumes of 10 μl each, and stored at −20 °C until the time of the experiments. All experiments used the same initial pool of TsTX.

The TsTX was injected subcutaneously (dorsum s.c. injection) in a fixed dose of 6.0 mg/kg; which corresponds to twice the DL50 for post-natal day 21 female rats [26]. Due to its highly hydrophobic characteristic, CBZ (Galena Chemistry and Pharmaceuticals LTDA) was dissolved in physiological saline (NaCl 0.9%) and propyleneglycol in a ratio of 4:6. The correct dose of 12, 50 or 100 mg/kg of CBZ was determined, after weighting the animal, and the volume was completed to 0.2 ml using propyleneglycol and saline. Thus, a fixed volume of 0.2 ml was injected either for vehicle or the various doses of CBZ.

The CeBIO-ICB-UFMG vivarium supplied post-natal day 21 female Wistar rats ($n = 38$; weighting $40.13 \pm 5.69$) used in this study. The animals were submitted to an artificial lighting system (lights on at 7:00 and lights off at 19:00), room temperature at 22° and with free access to food and water. Efforts were made to avoid any unnecessary distress to the animals, in accordance to the University Federal of Minas Gerais Guidelines for Animal Experimentation. This experiment was approved by the University Committee for Animal Experimentation – (CETEA-UFMG protocol 055/05).

Rats were divided into six experimental groups according to s.c. injection (saline or TsTX) and i.p. treatment (vehicle or CBZ 12, 50 and 100 mg/kg): saline injected and vehicle treated ($n = 4$; Sal/Veh group); saline injected and CBZ treated ($n = 4$; Sal/CBZ100 group; 100 mg/kg); TsTX injected and CBZ 12 mg/kg treated ($n = 6$; TsTX/CBZ12 group); TsTX injected and CBZ 50 mg/kg treated ($n = 8$; TsTX/CBZ50 group); TsTX injected and CBZ 100 mg/kg treated ($n = 8$; TsTX/CBZ100 group) and, at last, TsTX injected and vehicle treated ($n = 8$; TsTX/Veh group).

CBZ or vehicle was injected intraperitoneally (i.p.) 90 min before the subcutaneous (s.c.) inoculation of the TsTX (6 mg/kg). According to Graumlich et al. [14], the CBZ reaches a maximum cerebral and plasmatic concentration, in rats, 90 min after its i.p. injection.

After the subcutaneous (s.c.) injection of the TsTX, the rats were recorded in individual acrylic boxes for 240 min or until their death. If the death did not occur within a period of 240 min after s.c. injection, animals were sacrificed under sodium thiopental (80 mg/kg, i.p.) overdose. The lungs were harvested immediately after death and the pulmonary index (lung/body weight × 100) was calculated for each animal.

The statistical analysis was done using the Product Limit Method of Kaplan and Meier with curve comparison through

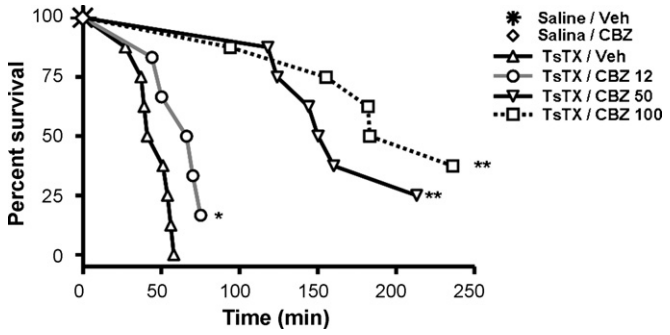


Fig. 1. Survival curves after the s.c. injection of TsTX (6 mg/kg) or saline in post-natal day 21 Wistar rats treated with either CBZ (12, 50 and 100 mg/kg) or vehicle. Experimental groups were divided according to s.c. injection (saline or TsTX) and i.p. treatment (vehicle or CBZ 12, 50 and 100 mg/kg), respectively: Sal/Veh, Sal/CBZ100, TsTX/Veh, TsTX/CBZ12, TsTX/CBZ50 and TsTX/CBZ100. Each data entry in the figure corresponds to one obit: plotted at a specific time and percentage of remaining animals alive. *$p < 0.01$; **$p < 0.0001$ – product limit method of Kaplan and Meier with curve comparison through Logrank test.

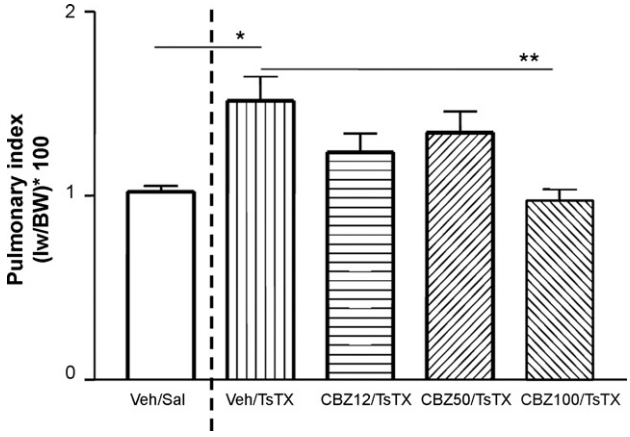


Fig. 2. Pulmonary index (lung weight/body weight × 100). Experimental groups were divided according to treatment (vehicle or CBZ i.p. injection) and to s.c. injection (saline or TsTX), respectively: Sal/Veh, TsTX/Veh, TsTX/CBZ12, TsTX/CBZ50 and TsTX/CBZ100. *$p < 0{,}05$ – unpaired *t*-test; **$p < 0.01$ – ANOVA, with Dunnett's multiple comparison test. LW – lung weight; BW – body weight. Results are expressed as mean ± S.E.M.

Logrank, and ANOVA with Dunnett's multiple comparison test to evaluate lung edema. Results are expressed as mean ± S.E.M.

Within few minutes after the inoculation of TsTX, rats presented piloerection, tremor, salivation, immobility, and sometimes vocalization. Fig. 1 shows the survival curves of rats pre-treated with vehicle or with different doses of CBZ (12, 50 and 100 mg/kg). There was an increase in the latency-to-death in rats pre-treated with 12 ($p < 0.01$), 50 and 100 mg/kg ($p < 0.0001$) of CBZ, when compared with rats pre-treated only with vehicle. Two and three rats pre-treated with respectively 50 and 100 mg/kg of CBZ did not die within the period of observation (240 min). The median survival for the different groups was: 46 min to TsTX/Veh group, 68 min to TsTX/CBZ12 group, 155 min to TsTX/CBZ50 group and 209.5 min to TsTX/CBZ100 group. These results are consistent with other toxicological reports, which used neurotropic agents that targeted voltage-gated sodium channels (VGSCs). Al-Shanawani et al. showed that mice pre-treated with lignocaine survived longer after the inoculation of the *Leiurus quinquestriatus* venom than animals that received only vehicle [1]. The best results were obtained when the mice were treated minutes before the venom inoculation. Fatani et al. obtained similar results with this same VGSCs blocker [9]. It seems reasonable to assume that drugs targeting the same proteins as does TsTX may be potential candidate agents for attenuating the scorpion envenoming symptoms. Nevertheless, CBZ is also known to have a variety of other actions, including inhibiting the reuptake of norepinephrine, modulating the hippocampal serotoninergic system and blocking adenosine and *N*-methyl-D-aspartate (NMDA) receptors [29,35]. Moreover, is described that CBZ affects neutrophil function through an action on peripheral benzodiazepinic receptors, but it seems occur only in chronic treatments [4]. Thus, the specific mechanism by which CBZ protects the brain against envenomation still needs to be investigated.

On the other hand, the pulmonary edema was blocked only in the group pre-treated with 100 mg/kg of CBZ (Fig. 2), when compared with the control group (TsTX/Veh group). This datum suggests that this edema was not the primary cause of death, as the animals pre-treated with 50 mg/kg of CBZ survived despite this effect. Although unable to prevent death by envenomation, Mesquita et al. blocked the pulmonary edema, which followed an i.c.v. injection of TsTX in adult Wistar rats by pre-treating animals with Phenobarbital [24]. In contrast, Guidine et al. [15] used CBZ to block pulmonary edema and prevent death in similar experimental conditions as used by Mesquita et al. [24]. In both studies, it seems that the pulmonary edema induced by envenomation is neurogenic in nature and, although may contribute, it is not the major cause of death. Nevertheless, several reports throughout literature have suggested that the edema is caused by a direct action of the scorpion venom in the lungs [5–7]. Although CBZ has been described as a drug able to affects the neutrophil function in chronic treatment [4], the recruitment of neutrophils does not seem to play a major role in the development of acute lung oedema following i.v. injections of scorpion venom in rats [23].

The current therapeutic view regarding scorpion envenoming syndrome does not emphasize central effects of the venom. Instead, treatment has focused on the use of antivenom therapy and alternative/adjunct therapy, which includes analgesics, anti-inflammatory drugs, antiemetics and vasodilators [16]. Despite the standard therapeutical approaches, the scorpion envenoming syndrome is still responsible for many deaths in tropical countries [8,31]. Although the experimental conditions in this work are not ideal for treatment, considering that CBZ was injected 90 min before TsTX inoculation, they are ideal for testing the hypothesis relative to the major role of the CNS in severe scorpion accidents: two-fold the DL50 of TsTX was used to guarantee 100% death in controls and CBZ pharmacokinetics are consistent with maximum drug distribution in the brain parenchyma at the time of inoculation. Thus, the perspective of others drugs that treat successfully scorpion envenoming syndrome is crucial to decrease the general lethality after these accidents. Although the studies with a neurotropic agent like

CBZ are promising, more investigations are necessary to use these agents to increase the efficacy of the scorpion envenoming treatment. Moreover, the pharmacokinetics of CBZ can be very much improved by either changing the form of administration or encapsulating the drug in order to enhance solubility.

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