Inhibition of *Tityus serrulatus* venom hyaluronidase affects venom biodistribution

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## Abstract

### Background

The hyaluronidase enzyme is generally known as a spreading factor in animal venoms. Although its activity has been demonstrated in several organisms, a deeper knowledge about hyaluronidase and the venom spreading process from the bite/sting site until its elimination from the victim’s body is still in need. Herein, we further pursued the goal of demonstrating the effects of inhibition of *T. serrulatus* venom (TsV) hyaluronidase on venom biodistribution.

### Methods and principal findings

We used technetium-99m radiolabeled *Tityus serrulatus* venom (⁹⁹mTc-TsV) to evaluate the venom distribution kinetics in mice. To understand the hyaluronidase’s role in the venom’s biodistribution, ⁹⁹mTc-TsV was immunoneutralized with specific anti-*T. serrulatus* hyaluronidase serum. Venom biodistribution was monitored by scintigraphic images of treated animals and by measuring radioactivity levels in tissues such as heart, liver, lungs, spleen, thyroid, and kidneys. In general, results revealed that hyaluronidase inhibition delays venom components distribution, when compared to the non-neutralized ⁹⁹mTc-TsV control group. Scintigraphic images showed that the majority of the immunoneutralized venom is retained at the injection site, whereas non-treated venom is quickly biodistributed throughout the animal’s body. At the first 30 min, concentration peaks are observed in the heart, liver, lungs, spleen, and thyroid, which gradually decreases over time. On the other hand, immunoneutralized ⁹⁹mTc-TsV takes 240 min to reach high concentrations in the organs. A higher concentration of immunoneutralized ⁹⁹mTc-TsV was observed in the kidneys in comparison with the non-treated venom. Further, *in situ* neutralization of ⁹⁹mTc-TsV by anti-*T. serrulatus* hyaluronidase serum at zero, ten, and 30 min post venom injection showed that late inhibition of
Hyaluronidases are known as the venom components responsible for disseminating toxins from the injection site to the victim's organism. Therefore, understanding how the venom distribution occurs and the role of hyaluronidases in this process is crucial in the field of toxinology. In this study, we inhibited *Tityus serrulatus* venom (TsV) hyaluronidase's action using specific anti-Ts-hyaluronidase antibodies. Labeling TsV with a radioactive compound enabled monitoring of its biodistribution in mice. Our results show that, upon hyaluronidase inhibition, TsV remains at the injection site for longer, and only a reduced amount of the venom reaches the bloodstream. Consequently, the venom arrives later at target organs like the heart, liver, lungs, spleen, and thyroid. Considering the possible application of hyaluronidase inhibition as a therapeutic resource in envenoming first-aid treatment, we performed the administration of hyaluronidase neutralizing antibodies at different times after TsV injection. We observed that TsV remains in the bloodstream and its arrival at tissues is delayed by 120 or 240 min after TsV injection, depending on anti-hyaluronidase administration times. Our data show that hyaluronidase plays a crucial role in TsV spreading from the injection site to the bloodstream and from the bloodstream to the organs, thus suggesting that its inhibition can help to improve envenoming's treatment.
pain, cramps, vomiting, hypotension, diarrhea, bradycardia, and dyspnea. Severe envenoming may present several potentially lethal complications, such as cardio-respiratory failure [7–11].

These symptoms are related to the synergic action of a variety of toxic components present in the venom. Ts venom (TsV) consists of a complex mixture of components such as mucus, lipids, amines, nucleotides, inorganic salts, hyaluronidases, serine proteases, metalloproteases, natriuretic peptides, bradykinin potentiating peptides, antimicrobial peptides, high molecular weight (Mw) proteins, and ion channel active neurotoxins, which are the major toxic components [12–24].

Hyaluronidases are extensively found in the venoms of various animals such as snakes, scorpions, spiders, and others [25]. Venom hyaluronidases are always referred to as "spreading factors" [26,27], as they hydrolyze the hyaluronic acid (HA) present in the interstitial matrix, thus helping the venom toxins to reach the victim’s bloodstream and invade its organism. Hyaluronidase’s enzymatic action increases membrane absorbency, reduces viscosity, and makes tissues more permeable to injected fluids (spreading effect). Therefore, hyaluronidase acts as a catalyst for systemic envenoming [25].

TsV hyaluronidase activity was first demonstrated by Possani’s group [28], and the enzyme was later isolated and partially characterized by Pessini and collaborators [14]. Horta et al. [16] further expanded these studies by performing extensive molecular, biological, and immunological characterization of TsV hyaluronidase. The authors described the sequence of two enzyme isoforms showing 83% identity, Ts-Hyal-1 and Ts-Hyal-2, by cDNA analysis of the venom gland. A purified native Ts hyaluronidase was used to produce anti-hyaluronidase serum in rabbits. Epitopes common to both isoforms were mapped, and it was shown that they include active site residues. Most importantly, it was demonstrated for the first time that in vivo neutralization assays with anti-hyaluronidase serum inhibited and delayed mouse death after injection of a lethal dose of TsV, thus confirming the influence of hyaluronidase in TsV lethality [16].

The active recombinant hyaluronidase Ts-Hyal-1 from TsV was produced and characterized. It is an important biotechnological tool for the attainment of sufficient amounts of the enzyme for structural and functional studies [29].

Herein, we further pursued the goal of demonstrating the effects of inhibition of TsV hyaluronidase on venom biodistribution. Our results show that inhibition of the hyaluronidase activity of TsV in mice hinders venom spreading from the injection site as well as its biodistribution to the tissues.

**Methods**

**Scorpions and venom extraction**

*T. serrulatus* scorpions were collected in Belo Horizonte, Minas Gerais, Brazil, with proper licensing from the competent authorities (IBAMA, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, protocol number 31800–1). Venom was obtained from female scorpions regularly milked twice a month by electrical stimulation of telson. After extraction, venom was solubilized in ultrapure water and centrifuged at 16,000g at 4˚C for 10 min. The supernatant was quantified using Bio-Rad "Protein DC assay" kit [30], and stored at -20˚C until use.

**Experimental animals**

Female Swiss CF1 mice (6–8 weeks old, 24–28 g) were obtained from the animal care facilities (CEBIO) of the Federal University of Minas Gerais (UFMG). Animals had free access to water and food and were kept under controlled environmental conditions.
Ethics statement

The Ethics Committee (Comissão de Ética no Uso de Animais, CEUA) of UFMG certifies that the procedures using animals in this work are in agreement with the Ethical Principals established by the Brazilian Council for the Control of Animal Experimentation (CONCEA). Protocol number 05/2016. Approved: March 8, 2016.

Anti-hyaluronidase serum

Rabbit anti-hyaluronidase and pre-immune sera used in this work were produced by Horta et al. [16].

Hyaluronidase activity: In vitro neutralization assay

Hyaluronidase activity was measured according to the turbidimetric method described by Pukrittayakamee et al. [31] with modifications [16]. The assay mixture contained 12.5 μg of HA (Sigma-Aldrich), acetate buffer (0.2 M sodium acetate-acetic acid pH 6.0, 0.15 M NaCl), and test (or control) sample in a final volume of 250 μl. Commercial hyaluronidase from bovine testis (12.5 μg; Apsen) was used as a positive control, and ultrapure water was used as a negative control. Assay mixtures were incubated for 15 min at 37˚C, and reactions were stopped by adding 500 μl of stop solution containing 2.5% (w/v) cetyltrimethylammonium bromide (CTAB) dissolved in 2% (w/v) NaOH.

Assays were monitored by absorbance at 400 nm against a blank of acetate buffer (250 μl) and stop solution (500 μl). Turbidity of the samples decreased proportionally to the enzymatic activity of hyaluronidase. Values were expressed as percentages of hyaluronidase activity relative to the negative (no addition of enzyme, 0% activity) and positive (addition of commercial enzyme, 100% activity) controls.

The tested samples were native hyaluronidase purified from TsV (0.5 μg, produced by Horta et al. [16]), TsV (2 μg), TsV neutralized with rabbit pre-immune serum (2 μg of TsV incubated for 1 h at 37˚C with 10 μl of pre-immune serum), TsV neutralized with anti-hyaluronidase serum (2 μg of TsV incubated for 1 h at 37˚C with 10 μl of anti-hyaluronidase serum).

Radiolabeling of T. serrulatus venom (TsV)

To label TsV with technetium-99m (99mTc; IPEN São Paulo), a sealed vial containing 200 μg of SnCl₂·H₂O solution in 0.25 mol/l HCl (2 mg/ml) and 50 μg of NaBH₄ solution in 0.1 mol/l NaOH (1 mg/ml) was prepared. The pH was adjusted to 7.4 using 1 mol/l NaOH. Next, 25 μl of TsV (5 g/l in saline 0.9% w/v) was added, and vacuum was applied to the vial, followed by addition of 0.1 ml of Na99mTcO₄⁻ (3.7 MBq). The solution was kept at room temperature for 15 min.

Radiochemical purity evaluation

Radiochemical purity was determined by thin layer chromatography (TLC-SG, Merck) using acetone as the mobile phase to quantify 99mTcO₄⁻. Strips radioactivity was determined by a gamma counter (Wallac Wizard 1470–020 Gamma Counter, PerkinElmer Inc.). 99mTcO₂ was removed from the preparation using a 0.45 μm syringe filter [32].

In vitro stability of 99mTc-TsV

Tests in saline 0.9% (w/v) and in mice plasma were performed to evaluate the stability of the radiolabeled complex 99mTc-TsV.
TLC-SG was used to evaluate the stability of the radiolabeled complex diluted in saline. The labeled solution was kept at room temperature, and aliquots were taken at 1, 2, 4, 6 and 24 h for determination of radiochemical purity. A volume of 90μl 99mTc-TsV solution was incubated with 1 ml of fresh mouse plasma at 37˚C under agitation. Radiochemical stability was determined by TLC-SG from samples taken at 1, 2, 4, 6 and 24 h after incubation.

**Blood clearance of 99mTc-TsV**

An amount equivalent to 3.7 MBq of 99mTc-TsV was diluted (10% v/v) in phosphate-buffered saline (PBS; control) or anti-hyaluronidase serum and incubated for 1 h at 37˚C. Then, 50 μl of the samples were intramuscularly injected into the right tight of healthy Swiss mice (6–8 weeks old, 24–28 g; n = 6 per group). Mice were anesthetized with a mixture of xylazine (15 mg/kg) and ketamine (80 mg/kg), and an incision was made in the animals' tails for blood collection in pre-weighed tubes at 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, and 1440 min after administration of the samples. The tubes were weighted, and their radioactivity determined by a gamma counter. These data were used to plot the percentage of dose injected per gram tissue (% ID/g) versus time.

**Scintigraphic images of mice injected with 99mTc-TsV**

Aliquots of 18 MBq of 99mTc-TsV in 10% (v/v) PBS (control) or anti-hyaluronidase serum (pre-incubated for 1 h at 37˚C) were intramuscularly injected (50 μl) into the right tight of healthy Swiss mice (6–8 weeks old, 24–28 g; n = 3 per group). Animals were anesthetized at 30, 60, and 120 min after sample administration with a mixture of ketamine (80 mg/kg) and xylazine (15 mg/kg) and placed horizontally under a gamma camera (Nuclide TM TH 22, Mediso). Images were collected with a Low Energy High Resolution (LEHR) collimator and 256x256x16 dimension matrices with a 300 s acquisition time, using a 20% symmetrical window with a fixed energy peak at 140 KeV.

**Biodistribution of 99mTc-TsV**

Aliquots of 3.7 MBq of 99mTc-TsV in 10% (v/v) PBS (control) or anti-hyaluronidase serum (pre-incubated for 1 h at 37˚C) were intramuscularly injected (50 μl) into the right tight of healthy Swiss mice (6–8 weeks old, 24–28 g; n = 6 per group). Mice were euthanized at 30, 60, 240, and 1440 min post-injection, and heart, liver, lungs, spleen, thyroid, and kidneys were dissected, dried with filter paper, and weighed. The radioactivity in each tissue was determined by a gamma counter. A standard dose containing the same injected amount of 99mTc-TsV was counted simultaneously in a separate tube, which was defined as 100% radioactivity. The results were expressed as the percentage of injected dose per gram of tissue (%ID/g).

**Evaluation of hyaluronidase neutralization as a first-aid treatment**

An amount equivalent to 3.7 MBq of 99mTc-TsV diluted in PBS was intramuscularly injected (25 μl) into the right tight of healthy Swiss mice (6–8 weeks old, 24–28 g; n = 6 per group). Next, anti-hyaluronidase serum was inoculated (25 μl, intramuscularly) into the same site of 99mTc-TsV injection at different time-points (0, 10, and 30 min post-injection of 99mTc-TsV). Mice were anesthetized with a mixture of xylazine (15 mg/kg) and ketamine (80 mg/kg), and an incision was made in the animals' tails for blood collection in pre-weighed tubes at 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, and 240 min after administration of 99mTc-TsV. The tubes were...
weighted, and their radioactivity determined by a gamma counter. Data were used to plot the percentage of dose injected per gram tissue (% ID/g) versus time.

**Statistical analyses**

Sample sizes were calculated using G Power version 3.1. To compare multiple means, the sample size was calculated considering alpha (α), power effect, effect size (f), and population size (n). To estimate number of mice needed in the $^{99m}$Tc-TsV biodistribution assays, parameters were set at $f = 0.7$, $\alpha = 0.05$, power $= 0.8$, and groups $= 4$. Data were expressed as mean $\pm$ S.E. M. Graphs were plotted using the software GraphPad PRISM version 5.00 (La Jolla, CA, USA).

All statistical tests were carried out on R version 3.4.4. Significance level was set at 0.05, and tests were performed two-sided. Effect of serum administration and time on the mean $^{99m}$Tc-TsV biodistribution was evaluated using two-way ANOVA. Normality and equal variance suppositions were assessed using Shapiro-Wilk and Levene’s tests, respectively. Effects of serum administration and time on $^{99m}$Tc-TsV mean blood clearance were analyzed using a linear mixed model (lme function on nlme package).

**Results**

**Anti-hyaluronidase serum neutralizes TsV hyaluronidase activity in vitro**

In the turbidimetric assays, commercial hyaluronidase from bovine testis exhibited high hyaluronidase activity (99.98 ± 0.01% activity), which was referred to as 100% activity (positive control). Ultrapure water had no enzyme activity (-0.003 ± 0.002% activity), which was referred to as 0% activity (negative control). TsV (99.57 ± 0.16% activity) and native hyaluronidase purified from TsV (99.73 ± 0.11% activity) presented high enzymatic activity, similar to that observed for the positive control (Fig 1). In the in vitro neutralization assay, pre-incubation of TsV with anti-hyaluronidase serum completely neutralized hyaluronidase activity (0.005 ± 0.003% activity). Rabbit pre-immune serum was used to test unspecific neutralization of the venom content and did not neutralize TsV enzymatic activity (99.39 ± 0.24% activity) (Fig 1). Western blot results for anti-hyaluronidase and pre-immune sera to TsV are shown in supporting information (S1 Methods, S1 Fig).

$^{99m}$Tc-TsV presents high radiochemical yields and radiolabeling stability

The radiochemical efficiency of the TsV labeling with technetium-99m was determined by TLC. The results indicated high radiochemical yield (95.2 ± 2.4%). Radiochemical yields higher than 90% are recommended for in vivo application of radiopharmaceuticals [33]. Therefore, our $^{99m}$Tc-TsV complex presented suitable radiochemical characteristics, which encouraged further in vivo studies.

The radiolabeling stability curve for $^{99m}$Tc-TsV is shown in Fig 2. Stability tests were performed after 1, 2, 4, 6 and 24 h of incubation of $^{99m}$Tc-TsV in saline 0.9% (w/v) at room temperature or in fresh mouse plasma at 37˚C. High stability was observed over long periods of time (>95%), thus indicating suitability for further biodistribution assays.

**Neutralization of hyaluronidase impairs TsV spreading**

Blood clearance of $^{99m}$Tc-TsV diluted in PBS or pre-neutralized with anti-hyaluronidase serum is shown in Fig 3. Following the injection into healthy Swiss mice, the $^{99m}$Tc-TsV complex showed quick absorption, reaching the highest bloodstream levels after 30 min. After this time point, $^{99m}$Tc-TsV concentration in the bloodstream decreases, which indicates biodistribution to the tissues.
Hyaluronidase is a spreading factor from scorpion venom

Fig 1. *In vitro* neutralization assay using rabbit anti-hyaluronidase serum. Hyaluronidase activity (%) was measured using a turbidimetric assay. Commercial hyaluronidase from bovine testis was used as a positive control, and ultrapure water was used as a negative control. Enzymatic activities of TsV (2 μg) and native hyaluronidase from TsV (0.5 μg) were tested. For the *in vitro* neutralization assay, TsV (2 μg) was incubated with pre-immune serum (10 μl) or anti-hyaluronidase serum (Anti-Hyal, 10 μl) for 1 h at 37˚C before testing. Anti-hyaluronidase serum neutralized the hyaluronidase activity in TsV. All values are expressed as the mean ± S.E.M. of duplicates from three independent experiments.

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Fig 2. *In vitro* stability of ⁹⁹ᵐTc-TsV. Stability of the complex ⁹⁹ᵐTc-TsV over time in the presence of saline 0.9% (w/v) at room temperature and in the presence of plasma at 37˚C. All values are presented as the mean ± S.E.M. of duplicates from three independent experiments.

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In contrast, the $^{99m}$Tc-TsV complex pre-neutralized with anti-hyaluronidase serum reaches lower levels in the bloodstream compared to the $^{99m}$Tc-TsV in PBS. This result shows that neutralization of TsV hyaluronidase activity significantly reduces TsV spreading from the injection site to the blood circulation.

Scintigraphic images of the mice corroborated the blood clearance results and showed that $^{99m}$Tc-TsV diluted in PBS quickly spreads from the injection site in the right tight muscle to the whole body between 30 and 120 min post-injection (Fig 4A). On the other hand, when $^{99m}$Tc-TsV was neutralized with anti-hyaluronidase serum, TsV spreading from the injection site was visibly reduced at all times evaluated. Noteworthy, the labeled neutralized TsV remained at the site of injection (right tight muscle) (Fig 4B).

Regarding the kinetics of TsV spreading, high levels of $^{99m}$Tc-TsV diluted in PBS were absorbed by the organs, particularly the kidneys and bladder (Fig 4A), while lower levels of $^{99m}$Tc-TsV neutralized with anti-hyaluronidase serum reached these tissues over time (Fig 4B).

Neutralization of hyaluronidase delays biodistribution of TsV to tissues

Tissues such as heart, liver, lungs, spleen, and thyroid displayed different uptake levels of $^{99m}$Tc-TsV diluted in PBS and $^{99m}$Tc-TsV neutralized with anti-hyaluronidase serum (Fig 5). Higher concentration of $^{99m}$Tc-TsV diluted in PBS was initially detected in these tissues (30 min), indicating a quick biodistribution of TsV from the injection site to the bloodstream and

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Fig 3. Blood clearance of $^{99m}$Tc-TsV. 3.7 MBq of $^{99m}$Tc-TsV diluted in PBS ($^{99m}$Tc-TsV + PBS) or neutralized with anti-hyaluronidase serum ($^{99m}$Tc-TsV + Anti-Hyal serum) was intramuscularly injected in Swiss mice (6–8 weeks old, 24–28 g; n = 6 per group). Radioactivity levels were measured in blood samples at 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, and 1440 min post-injection. Data are represented as the mean percentage of the injected dose of $^{99m}$Tc-TsV per gram of blood (% ID/g) ± S.E.M. of the mean. Values represent duplicates from two independent experiments. Statistical analyses were performed using a linear mixed model. Serum administration (p < 0.0001), time (p < 0.0001), and their interaction (p < 0.0001) had a statistically relevant effect on the mean $^{99m}$Tc-TsV blood clearance.

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subsequently to the organs. After reaching the organs, labeled TsV concentration decreased over time, thus suggesting the elimination of the radiolabeled complex from the mice’s body. In agreement, $^{99m}$Tc-TsV levels increased in the kidneys, pointing towards renal elimination.

In contrast, the neutralized $^{99m}$Tc-TsV was initially detected at lower concentrations in the heart, liver, lungs, spleen, and thyroid, only reaching high levels at 240 min post-injection, after which time levels begin to decrease again (Fig 5). Thus, hyaluronidase inhibition delays TsV incorporation into the bloodstream and organs. Moreover, lower concentrations of neutralized TsV reached the kidneys, when compared with $^{99m}$Tc-TsV diluted in PBS.

**TsV hyaluronidase neutralization as a first-aid treatment for scorpion sting**

A blood clearance test was performed to evaluate the efficiency of anti-hyaluronidase serum to neutralize previously injected TsV *in situ*, thus simulating a first-aid treatment for scorpion sting.

Animals injected with $^{99m}$Tc-TsV diluted in PBS (control group) presented a quick absorption of TsV from the injection site into the bloodstream, followed by a decrease in blood concentration (Fig 6), corroborating the results previously observed in the blood clearance assay (Fig 3). On the other hand, animals treated with anti-hyaluronidase serum injected in the right tight muscle at 0, 10, and 30 min post-injection of $^{99m}$Tc-TsV diluted in PBS showed higher concentrations of labeled TsV in the bloodstream over time at all times tested (Fig 6A–6C). The increased concentration of neutralized TsV in the bloodstream indicates hindered

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**Fig 4.** $^{99m}$Tc-TsV spreading in mice over time. Representative scintigraphic images of mice injected with 18 MBq $^{99m}$Tc-TsV diluted in PBS (A) or neutralized with anti-hyaluronidase serum (B). Samples were intravenously injected in Swiss mice (6–8 weeks old, 24–28 g; n = 3 per group). Radioactivity levels were measured 30, 60, and 120 min post-injection. Images show a quick and growing spread of $^{99m}$Tc-TsV diluted in PBS over time (A). On the other hand, TsV neutralized with anti-hyaluronidase serum remains at the injection site (right tight muscle) (B). Images are pseudocolored according to the color scale.

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Fig 5. Biodistribution of $^{99m}$Tc-TsV. $^{99m}$Tc-TsV (3.7 MBq) diluted in PBS ($^{99m}$Tc-TsV + PBS) or neutralized with anti-hyaluronidase serum ($^{99m}$Tc-TsV + Anti-Hyal serum) was intramuscularly injected in Swiss mice (6–8 weeks old, 24–28 g; n = 6 per group). Radioactivity levels were measured in the heart, liver, lungs, spleen, thyroid and kidneys at 30, 60, 240 and 1440 min post-injection. The results are expressed as the percentage of injected dose/g of tissue (%ID/g). All values are presented as the mean ± S.E.M. of two independent experiments. Statistical analysis was performed using two-way ANOVA (factors: serum administration and time). Anti-hyaluronidase serum significantly affected the mean distribution of TsV to the liver (p < 0.0001), spleen (p = 0.0115), and kidneys (p = 0.0009), while time was a significant factor for TsV distribution to the heart (p < 0.0001), liver (p < 0.0001), lungs (p = 0.0095), spleen (p = 0.0008), and kidneys (p < 0.0001). A significant interaction between serum administration and time was observed in the heart (p = 0.00003), liver (p < 0.0001), spleen (p = 0.0337), and thyroid (p < 0.0001).

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Fig 6. Hyaluronidase neutralization as a first-aid treatment for scorpion sting. 3.7 MBq of $^{99m}$Tc-TsV diluted in PBS was intramuscularly injected in Swiss mice (6–8 weeks old, 24–28 g; n = 6 per group). Subsequently, anti-hyaluronidase serum was injected in the same site of $^{99m}$Tc-TsV injection at different times (Anti-Hyal serum; 0, 10,
biodistribution to the tissues (Fig 6). Altogether, our data reveal the potential use of hyaluronidase inhibition as a novel first-aid strategy in envenoming.

Discussion

Hyaluronidases are involved in different processes such as inflammation, angiogenesis, embryogenesis, wound healing, tumor growth and progression, and systemic diffusion of venom toxins [34–38]. The role of hyaluronidases in venom spreading has been investigated, as this enzyme is a component vastly described both in vertebrate and invertebrate animal venoms. Specifically, for scorpions, a search in NCBI protein database reveals at least 20 hyaluronidase protein sequences already described for 15 different species [39]. However, no study so far has shown how hyaluronidase activity interferes with venom distribution [16,17,40–44].

Previous studies have demonstrated the role of hyaluronidase in venom dissemination [36,44], in enhancing the effects of hemorrhagic toxins from snake venoms [27,37], in triggering allergic reactions to bee and wasp venoms [45–47], and in increasing the effects of other toxins from spider and scorpion venoms [14,48]. Moreover, Horta and collaborators [16] have greatly advanced the understanding of hyaluronidase in *T. serrulatus* through characterization studies showing the role of this enzyme in venom lethality. However, evidence demonstrating the role of hyaluronidases in venom spreading and describing the steps from scorpion sting to venom elimination was still lacking. In the present study, we demonstrated how the inhibition of TsV hyaluronidase activity using anti-hyaluronidase serum affects venom biodistribution.

Our data show that TsV distribution kinetics is fast and efficient. TsV is distributed from the injection site to the bloodstream and organs in the first 30 min post-injection (Figs 3, 4A and 5). The biodistribution is not uniform for all tissues. After that time, the level of TsV decreases in the organs (Fig 5) and increases in the kidneys (Figs 4A and 5), thus indicating a renal route of elimination of the radiolabeled complex $^{99m}$Tc-TsV. This shows that the venom is quickly biodistributed from the injection site to target organs such as heart, liver, lungs, and spleen, where it activates receptors and other biological targets. The binding triggers signaling cascades that culminate in all the symptomatology of scorpion sting, including the potentially lethal cardiogenic shock and pulmonary oedema. Following biodistribution, renal excretion is an important route of elimination of TsV from the organism [49,50].

Previous studies have used a toxic fraction isolated from *T. serrulatus* venom radiolabeled with $^{99m}$Tc ($^{99m}$Tc-TsTx) for biodistribution assays in young rats [49]. In that study, it was observed that the isolated fraction is not regularly distributed, it is first detected at low levels in the organs and reaches its maximum concentration in the brain, heart, thyroid, lungs, spleen, liver, and blood after 60 to 180 min. In the kidneys, the highest concentration of $^{99m}$Tc-TsTx is detected 360 min after injection [49]. The biodistribution and elimination of TsTx are slower compared to that of the total venom observed in the present study and may be explained by the low molecular weight of the TsTx fraction (~7 kDa) that lacks hyaluronidase (~ 50 kDa) in its composition.

Studies of venom biodistribution and neutralization of circulating venom allow a better understanding of the pathophysiology of envenomation, especially through the determination of venom levels in tissues [49,51–55]. TsV is composed mainly by low molecular weight
neurotoxic peptides which modulate Na\(^+\), K\(^+\), Ca\(^{2+}\) and Cl\(^-\) channels in excitable membranes, thereby causing a massive release of neurotransmitters and stimulation of the autonomic nervous system. The synergism of various toxins from TsV is responsible for its deleterious effects [18, 50].

In the present work, we observed that the inhibition of hyaluronidase in TsV caused by anti-hyaluronidase serum delays the process of venom distribution. Higher levels of TsV are detected at the injection site, and reduced levels are detected in the organs at early times (30 min) when compared to the control group (Figs 4B and 5). The immunoneutralized venom is retained in the right tight muscle, and its spread from the injection site to the bloodstream is reduced (Fig 4B). Over time, the levels of TsV in the organs show delayed increase when compared to control. The highest concentrations of immunoneutralized venom are observed in the tissues 240 min after injection, which represents a 190-min delay in comparison with the untreated venom. Lower levels of immunoneutralized TsV were also observed in the kidneys, in relation to the control, indicating reduced renal clearance (Figs 4 and 5). The delay in the biodistribution of TsV caused by inhibition of hyaluronidase may compromise the synergistic action of the venom’s components, which are relevant in the envenoming process, and may culminate with the reduction of TsV lethality observed by Horta et al. [16].

Revelo et al. [51] demonstrated the effect of *T. serrulatus* antivenom on the biodistribution of TsV. High levels of venom were detected in mice serum and organs up to 8 h after subcutaneous injection of TsV. In contrast, when antivenom was applied intravenously at times 0 or 1 h after venom injection, venom levels detected in blood and tissues were significantly reduced [51]. These data show the effectiveness of antivenom in blocking venom biodistribution and indicate that anti-hyaluronidase antibodies may exist in total antivenom.

Due to the hyaluronidase action facilitating initial venom dissemination, we hypothesized that anti-hyaluronidase serum could complement anti-serum therapy as a first-aid treatment for envenomation. Some studies point to the use of hyaluronidase activity inhibition in envenoming processes as a first-aid action. As inhibitors of viper venom hyaluronidase have long been used for this purpose, neutralization of scorpion hyaluronidase could be a similar therapeutic approach to arrest the main effects of envenomation [56,57]. Here we proceeded with inhibition of hyaluronidase after venom injection at times 0, 10, and 30 min, and observed a higher concentration of labeled TsV in the bloodstream until 120 or 240 min after TsV injection, depending on anti-hyaluronidase administration time, when compared to the control group (Fig 6). These results corroborated the \(^{99m}\)Tc-TsV biodistribution assays previously neutralized with anti-hyaluronidase serum, which showed that the maximum concentration of TsV detected in tissues occurs 240 min after injection (Fig 5). Thus, aiming at using hyaluronidase neutralization as a first-aid treatment, our results were effective in showing a delay in the biodistribution of TsV to target organs and its accumulation in the bloodstream. In a real envenoming situation, delaying venom biodistribution may compensate for the time required for the sting victims to seek medical attention and treatment with antivenom serum, especially in remote locations with poor access to hospitals. This would represent a breakthrough in the treatment of systemic envenoming by venomous animals, which are considered neglected issues by the World Health Organization (WHO) due to the lack of adequate access to anti-venom therapy where they are needed [58].

In addition, the accumulation of TsV in the bloodstream as a result of hyaluronidase activity neutralization (Fig 6) suggests a correlation between hyaluronidase activity and venom flow from the bloodstream to the tissues. These results indicate that TsV hyaluronidase is important not only to allow venom access from the sting/bite site to the bloodstream (Figs 3 and 4) but is also involved in the biodistribution of TsV from the blood to the organs (Figs 5 and 6).
In endothelial cells, hyaluronic acid (HA) stimulates cell proliferation, migration, and neovascularization, and regulates endothelial barrier function [59]. As a key component of the glycocalyx in the vascular wall, HA is crucial for vascular integrity and maintenance of blood vessel continuity [60]. Especially in the glomerulus, HA is pivotal to the integrity of protein permeability barrier [61,62]. Our results suggest that TsV hyaluronidase is relevant to the cleavage of the HA present in the endothelial barrier and, therefore, promotes the biodistribution of TsV from the blood to the tissues.

Studies of this nature can contribute to the development of more effective envenomation therapies and help clarify the mechanisms of action of components from the TsV. Herein, hyaluronidase was shown as a key enzyme for the biodistribution of TsV from the venom injection site to the bloodstream and subsequently to the target tissues. This enzyme promotes the rapid distribution of TsV toxins through the victim’s body and is pivotal in the envenoming process. Since we have proved the critical role of hyaluronidase in scorpionism, our findings lead the way for new therapeutic strategies.

Supporting information
S1 Methods. Electrophoresis and immunoblotting analysis.

(S1 Fig. SDS-PAGE and immunoblotting analysis. A) 12% (w/v) SDS-PAGE analysis of T. serrulatus venom (15 μg; TsV). B) Immunoblotting of TsV probed with anti-hyaluronidase serum (1:5,000). C) Immunoblotting of TsV probed with pre-immune serum (1:5,000).)

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