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Pro-inflammatory response and hemostatic disorder induced by venom of the coral snake *Micrurus tener tener* IN C57BL/6 mice

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Abstract

Micrurus venoms are known to induce mainly neurotoxicity in victims. However, other manifestations, including hemorrhage, edema, myotoxicity, complement activation, and hemostatic activity have been reported. In order to develop a more complete pharmacological profile of these venoms, inflammatory responses and hemostasis were evaluated in C57BL/6 mice treated with a sub-lethal dose of *M. t. tener* (*Mtt*) venom (8 µg/mouse), inoculated intraperitoneally. The venom induced moderate bleeding into the abdominal cavity and lungs, as well as infiltration of leukocytes into the liver. After 30 min, the release of pro-inflammatory mediators (TNF-α, IL-6, and NO) were observed, being most evident at 4 h. There was a decrease in hemoglobin and hematocrit levels at 72 h, a prolongation in coagulation times (PT and aPTT), a decrease in the fibrinogen concentration and an increase in fibrinolytic activity. In this animal model, it was proposed that *Mtt* venom induces inflammation with the release of mediators such as TNF-α, in response to the toxins. These mediators may activate hemostatic mechanisms, producing systemic fibrinolysis and hemorrhage. These findings suggest alternative treatments in *Micrurus* envenomations in which neurotoxic manifestations do not predominate.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

Ethical statement

All experimental procedures in this study involving animals were performed in accordance with ethical standards of the Ethics Committees of the Instituto Venezolano de Investigaciones Científicas (IVIC) and the Institute of Anatomy of the Universidad Central de Venezuela, and respecting guidelines for the care and use of laboratory animals, published by the Institute of Animal Laboratory Resources (2011).

Keywords

Inflammation; *Micrurus tener tener*; Hemostasis; Edema; Snake venom

1. Introduction

Snake envenomation is a major public health problem in rural areas of tropical and subtropical countries in Africa, Asia, Oceania and the Americas (WHO, 2007). Clinical signs and symptoms of envenomation vary according to the species of snake involved, composition of the venom, the amount of venom injected and the geographic origin of the offending snake (Aguilar et al., 2007; Rey-Suárez et al., 2011).

Clinical manifestations in snake bites are complex due to the numerous toxic components that act individually or synergistically, and due to the physiological responses of victims. It has been widely observed that *Micrurus* envenomation produces neurotoxic effects, due to the action of pre- and post-synaptic neurotoxins, which may block neuromuscular transmission (Rosenfeld, 1971; Bucarechi et al., 2016).

In addition to neurotoxicity, *Micrurus* venom produces other effects, including hemolysis, hemorrhage and edema (Barros et al., 1994), cardiotoxicity (Ramsey et al., 1972), myotoxicity, nephrotoxicity or renal failure, as a consequence of muscle damage (de Roodt et al., 2012), and activation of complement (Tanaka et al., 2012).

In previous *in vitro* studies conducted in our laboratory, we found that venoms of *M. t. tener* and *M. f. fulvius*, from the USA, and *M. isozonus* from different regions of Venezuela, showed hemostatic activities, including anti-platelet aggregation with ADP as an agonist, anti-coagulant (anti-FXa activity), thrombin-like (formation of an unstable fibrin gel), fibrinolytic, and anti-plasmin activities (Salazar et al., 2011).

The interaction between hemostasis and inflammation has been poorly studied in snake envenomation, where both systems can potentiate alterations triggered by the toxins in the victims (Josepha et al., 2003). This led us to evaluate alterations in the inflammatory response and hemostasis, induced by a sub-lethal dose of *M. t. tener* venom in C57BL/6 mice, to better understand clinical manifestations, to open doors to alternative treatments, as well as to enable production of more specific and effective anti-venoms.

2. Material and methods

2.1. Reagents

Cadmium granules, potassium nitrate, bovine thrombin, acetic acid, trisodium citrate, hippuryl arginine substrate, calcium chloride, cyanuric chloride, potato carboxypeptidase inhibitor (CPI), o-methyl-hippuric acid, hippuric acid, lipopolysaccharide, urea, hematoxylin and eosin and other reagents were obtained from Sigma Aldrich, USA. Avidin peroxidase and streptavidin peroxidase were obtained from Santa Cruz Biotechnology, USA. 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from Scytech, USA. Chromogenic substrates were obtained from Chromogenix AB, Italy. Human fibrinogen, FXa and double chain t-PA

standards (tcu-PA) were obtained from Sekisui, USA. Mouse IL-6 minikit and mouse TNF- α minikit were supplied by Thermo Scientific, USA. PT and aPTT reagents were obtained from Stago, USA.

2.2. Venoms

A pool of *Micrurus tener tener* venom (*Mtt*) was purchased from the National Natural Toxin Research Centre (NNTRC) -Texas A & M University Kingsville, Kingsville, Texas, USA. *Bothrops isabellae* (*Bi*) and *Crotalus durissus cumanensis* (*Cdc*) venoms, used as a positive control for these *in vivo* studies, was donated by the Tropical Medicine Institute Serpentarium, Universidad Central de Venezuela (UCV).

2.3. Experimental animals

Male C57BL/6 mice, weighing between 18 and 20 g, were supplied by the animal facility at Instituto Venezolano de Investigaciones Científicas (IVIC). Mice were acclimated for at least 1 week before each experiment, and received water and food *ad libitum*. The Bioethics Committee of IVIC approved this study. Standards and protocols for animal use and management were obtained from the Institute of Animal Laboratory Resources (2011).

2.4. Lethality

Venom lethality was determined by intraperitoneal (i.p.) injection of different doses of *Mtt* venom (7.1–36.0 μ g/mouse) in groups of 4 mice/dose. The LD₅₀ was calculated after 48 h of observation, according to the method of Spearman-Kärber (1964).

2.5. Treatment of mice with a sub-lethal dose of *Mtt* venom

Based on the LD₅₀ results, a sub-lethal dose of *Mtt* venom was injected in experimental animals to evaluate effects that could be masked by neurotoxicity. Mice were injected i.p. with a dose of 0.4 mg/kg, equivalent to 8 μ g of venom/20 g mouse weight, dissolved in 200 μ L sterile saline solution. Sterile saline solution was used for the negative control group.

To evaluate the effect of *Mtt* venom on secretion of inflammatory mediators and alterations of hemostatic and hematological parameters, blood samples (0.7–1.2 mL) were obtained at indicated times from each animal by cardiac puncture under ether anesthesia. Mice were then sacrificed in a CO₂ chamber. Lipopolysaccharide (LPS, from *Escherichia coli* serotype O26:B6) (8 μ g/animal) was used as a positive control for the inflammatory response.

2.6. Histopathological studies

At 72 h, mice were sacrificed in a CO₂ chamber and the lungs, liver, and kidneys were removed from each animal, fixed in 30% buffered formaldehyde, and embedded in paraffin in an automatic processor (Leica Microsystems TP 1020, Germany). Five micron paraffin tissue sections of experimental and control groups were stained with hematoxylin and eosin in an automatic stainer (Leica Autostainer XL, Germany). The average number of infiltrating inflammatory cells in each organ was calculated from 3 different fields at 400 $\times$ magnification.

2.7. Necrotizing activity

Necrotizing activity was measured by the method of Theakston and Reid (1983). Groups of 4 mice were inoculated i.d. on the right dorsal side with 8 µg of *Mtt* venom dissolved in 100 µL sterile saline solution. *Bothrops isabelae* venom at same dose and sterile saline solution (100 µL) were used as positive and negative controls respectively. At 72 h, mice were sacrificed in a CO₂ chamber and the dorsal skin was removed. Areas of lesions were measured (mm²) on the inner surface of the skin with background illumination.

2.8. Edema-forming activity

Edema-forming activity was measured using a modification of the method of Yamakawa et al. (1976). Briefly, groups of 4 mice were injected in the right hind pad with 8 µg of *Mtt* venom dissolved in 30 µL of sterile saline solution. Sterile saline solution was injected into the left footpad as a negative control. At different times, animals were sacrificed in a CO₂ chamber and the hind limbs were removed and weighed. Results were expressed as a percentage of edema.

2.9. Inflammatory mediators

At 30, 60, 120, 240, and 480 min, blood samples were obtained from each animal, centrifuged at 385 RCF for 15 min at 4 °C to obtain serum, and then stored at -70 °C until use. Serum pro-inflammatory cytokines (IL-6 and TNF-α) were determined using ELISA kits. Concentrations were calculated employing a standard curve obtained with recombinant TNF-α and IL-6 (Thermo Scientific, USA) and expressed in pg/mL. The sensitivity of this assay was approximately 6000 and 2000 pg/mL, for TNF-α and IL-6, respectively.

NO concentrations in mouse serum were evaluated indirectly using the Griess reaction after reduction of NO₃⁻ to NO₂⁻ with cadmium granules (Cortas and Wakid, 1990). Nitrite concentrations were determined using a standard curve obtained with nitrates (KNO₃) and expressed in µM. The sensitivity of this assay was approximately 1 µM nitrite.

2.10. Hematological and hemostatic parameters

In the same groups of mice in which histopathologic studies were performed at 72 h, blood samples were collected to determine hemostatic and hematological parameters. For hemostatic parameters, 0.7 mL of blood was treated with 3.8% sodium citrate (9:1 v/v) to inhibit coagulation and Platelet Poor Plasma (PPP) was obtained by centrifugation at 385 RCF for 10 min at 4 °C. The remaining blood was treated with 1% EDTA, as an anti-coagulant to determine hematological variables. Samples were processed within an hour after blood extraction.

Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) were determined according to the method of Speer et al. (2013) and clotting time was recorded manually. Qualitative determination of factor XIII was performed using the clot dissolution test in 5 M urea. Fibrinogen was determined using the gravimetric method (Ingram, 1952). Endogenous fibrinolytic activity was measured according the method of Barrios et al. (2009) using the plasma euglobulin fraction. TAFIa was measured using a modification of the method described by Schatteman et al. (1999).

Hemoglobin was measured spectrophotometrically using the cyanomethaemoglobin method (Van Kampen and Zijlstra, 1965). Hematocrit measurements employed the micro-hematocrit method. Leukocyte differential was determined on blood smears with Giemsa staining. Leukocyte counts were made with Turk solution, and platelets were counted by the method of Brecher and Cronkite (1950). Cell counts were performed in a Neubauer chamber.

2.11. Statistical analysis

Data were expressed as arithmetic means $\pm$ the standard deviation (SD) of 3 independent experiments. Student's t-test was employed to evaluate possible differences between control and experimental groups, using GraphPad PRISM® 6.01. Values of $p < 0.05$ and 0.01 were accepted as statistically significant for all experiments.

3. Results

3.1. Lethality

Table 1 indicates deaths for doses used to calculate the LD₅₀. An LD₅₀ of 0.99 mg/kg was observed for this pool of *Mtt* venom in C57BL/6 mice. Commencing approximately 2 h after i.p. injection, experimental mice exhibited evident malaise. The most remarkable activities were “face-washing” episodes, equilibrium alterations, and involuntary movements. Experimental animals did not worsen significantly until after 72 h, when a decrease in fibrinogen and a prolongation of coagulation times were also observed.

3.2. *Mtt* venom sub-lethal dose effects in C57BL/6 mice

Initially, the most remarkable macroscopic effects of the venom were observed at 72 h post-inoculation. Dissection of experimental mice showed moderate bleeding in the abdominal cavity, as well as the presence of clots in the lungs, with no such changes observed in the negative control group (results not shown). Histopathological studies of the lungs showed moderate bleeding with a large number of erythrocytes in areas close to the alveoli compared with the negative control (Fig. 1 A and C). In hepatic tissue, there was no hemorrhage although leukocyte infiltrate with a predominance of mononuclear cells was observed in areas adjacent to blood vessels (Fig. 1 B and D). The number of infiltrating mononuclear leukocytes in this tissue was 77 ± 5 cells/field compared to 9 ± 1 cells/field in control tissue ($p < 0.001$) (Fig. 1 E). No changes were seen in the kidneys (results not shown).

3.3. Necrotizing activity

In animals treated with a sub-lethal dose of *Mtt* venom (8 μ g/ mouse), minimal vasodilatation was detected in the inoculation area, in contrast to *Bi* venom (positive control) where a 49 mm² necrotic lesion was observed (results not shown).

3.4. Edema-forming activity

Mtt venom induced mild edema after 30 min ($22.4 \pm 4.7\%$), with the major effect at 240 min ($40.7 \pm 4.4\%$). In contrast, *Bi* venom produced more edema after 30 min ($37.6 \pm 7.7\%$) and the most notable effect was detected at 120 min ($70.5 \pm 17.3\%$) (Fig. 2 A).

Additionally, edematogenic activity was compared to that observed with venoms from the most common venomous snakes in Venezuela, *Crotalus durissus cumanensis* (*Cdc*) and *Bothrops isabellae* (*Bi*). The highest effect was seen with *Bi* venom. *Mtt* venom induced mild edema at the doses employed, while *Cdc* venom produced a lesser effect (Fig. 2 B). Eight and 20 µg of venom/mouse of *Bi* venom induced 47.9 ± 1.1 and $59.9 \pm 4.8\%$ of edema respectively. *Mtt* induced 42.3 ± 2.0 and $43.4 \pm 1.1\%$ of edema with 8 and 20 µg per mouse, respectively, while the values for *Cdc* venom were 23.4 ± 2.0 and $30.0 \pm 8.6\%$ of edema, respectively.

3.5. Effect of *Mtt* venom on release of pro-inflammatory cytokines and NO in C57BL/6 mice

The edema observed in experimental mice treated with *Mtt* venom, led us to evaluate serum levels of pro-inflammatory mediators, TNF-α, IL-6 and NO, which are actively involved in the innate immune response.

Mice inoculated with *Mtt* venom and LPS (positive control) produced the highest concentrations of TNF-α after 30 min (1688.7 ± 294.4 and 1714.0 ± 918.7 pg/mL, respectively) ($p < 0.01$), compared to 179.5 ± 209.2 pg/mL in the negative control group (Fig. 3A). At 240 min, this cytokine decreased to undetectable levels in the LPS and negative control groups, while in the case of animals treated with *Mtt* venom, significant levels of TNF-α persisted at 240 min (190.5 ± 121.5 pg/mL), returning to baseline at 480 min.

Serum levels of IL-6 increased to 569.0 ± 141.6 pg/mL at 30 min in mice injected with *Mtt* venom (Fig. 3B), reaching the highest levels at 240 min post-injection (1008.9 ± 175.7 pg/mL), which were statistically significant compared to the negative control (380.1 ± 178.23 pg/mL; $p < 0.05$). In mice treated with LPS, a similar increase was observed at 30 min (598.9 ± 122.3 pg/mL). However, the highest concentration in this group was detected at 120 min (1028.0 ± 18.0 pg/mL), being higher than the negative control (147.6 ± 5.6 pg/mL) and the experimental group inoculated with *Mtt* venom (426.6 ± 21.4 pg/mL) ($p < 0.05$).

Serum NO rose to 4.1 ± 1.3 pM (*Mtt* venom) and 3.6 ± 0.9 (LPS) at 30 min, levels significantly higher than observed in the negative control group (2.0 ± 0.6 pM) ($p < 0.05$) (Fig. 3 C). Maximum levels were detected at 240 min in mice inoculated with *Mtt* venom (7.3 ± 0.6 pM), a value similar to that observed at 120 min in the LPS group (7.5 ± 1.1 pM) ($p < 0.05$), and higher than the value shown by the negative control group (5.6 ± 0.5 pM).

Serum NO persisted at high concentrations up to 480 min, in contrast to the return to baseline levels observed with TNF-α and IL-6.

3.6. Effect of *Mtt* venom on hematological and hemostatic parameters in C57BL/6 mice

The hemorrhage observed in peritoneal cavities of mice treated with *Mtt* venom, as well as the vasodilation and localized inflammation in the inoculation area, led us to evaluate hematological and hemostatic parameters. Samples were collected 72 h after i.p administration of 8 µg/mouse of this venom, due to the earlier pro-inflammatory effect of

Mtt venom that might promote a pro-coagulant state in experimental mice, inducing delayed alterations in the hemostatic system.

The results showed that some hematological parameters decreased in mice treated with *Mtt* venom, compared to controls (Table 2), notably with statistically significant differences in hemoglobin ($p < 0.01$) and hematocrit ($p < 0.01$).

Mice treated with *Mtt* venom did not show alterations in platelet count (Table 3). However, this venom prolonged PT and aPTT times in experimental mice compared to the negative control group ($p < 0.05$). A decrease in plasma fibrinogen concentration was also observed in treated mice, compared to the negative control group ($p < 0.01$). FXIII, evaluated by the technique of clot solubility in urea (which detects alterations when the concentration of this factor is less than 2%), was not altered in any of the groups.

General performance of the fibrinolytic system, which was assessed by evaluating euglobulin lysis area on fibrin plates, showed higher fibrinolytic activity in samples of animals treated with *Mtt* venom at 24 h, compared to the negative control group ($p < 0.01$). Increased TAFI activity was observed in venom-treated mice compared to negative controls, although the difference was not statistically significant.

4. Discussion

Studies of the effects of coral snake venoms in experimental models are essential to understand clinical symptoms other than neurotoxicity, thus providing valuable information for production of more effective anti-venoms (Gutiérrez, 2011). *Micrurus* envenomation is mainly characterized by neurotoxicity. However, activation of inflammatory responses and the hemostatic system have been clinically and experimentally described (Barros et al., 1994; Manock et al., 2008; Salazar et al., 2011; Tanaka et al., 2010).

To study inflammatory, hematological, and hemostatic parameters that might be altered by *Mtt* venom, we used the i.p. route because it allows lower diffusion of toxins than the i.v. route, favoring evaluation of systemic effects other than neurotoxicity. We chose C57BL/6 male mice as our experimental model, because this strain is susceptible to venoms from snakes, scorpions, spiders and caterpillars, responding similarly to human victims of envenomation (Lima et al., 1991; Barrios et al., 2009).

Micrurus venoms are highly toxic (Barros et al., 1994; Manock et al., 2008; Rey-Suárez et al., 2011). Results from this study showed a LD₅₀, following i.p. injection (0.99 mg/kg), comparable to those reported by Sánchez et al. (2008), Salazar et al. (2011) and Bénard-Valle et al. (2014), 0.78, 0.79 and 1.2 mg/kg, respectively, in mice injected i.v. These findings demonstrate the extreme lethality of the venom, which could be related to the high proportion of phospholipase A₂ (PLA₂) (46%) and 3FTxs (38%) already described in these venoms (Bénard-Valle et al., 2014).

C57BL/6 mice showed moderate bleeding in the abdominal cavity and clots in the lungs, 72 h after inoculation with a sub-lethal dose of *Mtt* venom. This result may be associated with a loss of vascular wall integrity caused by PLA₂s and proteolytic enzymes. In previous studies

with *Mtt* venom, SVMP and PLA₂ have been identified, as well as fibrino (geno)lytic, fibronectinolytic, gelatinolytic and hyaluronidase activities (Salazar et al., 2011). The latter has also been reported in other *Micrurus* venoms (Tanaka et al., 2010). These activities may degrade subendothelial membranes and increase toxin influx, causing capillary rupture, endothelial dysfunction and hemorrhage in organs such as the lungs (Gutiérrez et al., 2005).

In envenomation by elapids from Asia and Oceania, necrotic lesions and myotoxicity have been reported in the bite area (Heller et al., 2007). In addition, in victims bitten by *M. laticollaris*, *M. fulvius* and *M. lemniscatus*, muscle lesions were observed associated with PLA₂ (de Roodt et al., 2012). In the current study, mice inoculated with *Mtt* venom showed no necrosis, but displayed capillary vasodilation, which may be associated with the low proportion of proteolytic enzymes, such as SVMP, as reported in *M. mipartitus* (3.6%) (Rey-Suárez et al., 2011), *M. corallinus* (2.1%) (Aird et al., 2017) and *M. clarkii* (1.6%) (Lomonte et al., 2016).

In addition to hemorrhage, an accumulation of mononuclear leukocytes in the liver was observed, which may be associated with diffusion of proteolytic enzymes that increase vascular permeability and induce histamine release (Teixeira et al., 2003). The influx of toxins could induce an inflammatory response leading to recruitment of immune cells to the liver, first neutrophils, followed at 24–48 h by mononuclear cells (macrophages and T cells) to prevent tissue damage (Scheller et al., 2011).

Edema formation was comparable using doses close to the LD₅₀ (20 µg/mouse) and the sub-lethal dose of 8 µg/mouse. A similar response was reported with *M. lemniscatus* and *M. spixii* venoms (Barros et al., 1994; Cecchini et al., 2005; Terra et al., 2015). PLA₂ induces edema by degrading cell membrane phospholipids, releasing arachidonic acid, a substrate for the biosynthesis of several lipid pro-inflammatory mediators (Teixeira et al., 2003). In addition, edema can be associated with SVMP, as has been described in *Micrurus*, promoting complement activation and release of anaphylatoxins, C3a, C4a and C5a, which in turn stimulate histamine release, increasing vascular permeability and promoting vasodilation (Tanaka et al., 2012).

When the edema induced by *Mtt* venom was compared with those produced by *Bi* and *Cdc* venoms at equivalent doses, *Mtt* venom produced a much more moderate response, probably due to the lower metalloprotease content (Rey-Suárez et al., 2011; Sanz et al., 2016), while the greatest effect was found with *Bi* venom, which was probably due to the greater concentration of pro-inflammatory SVMPs (Teixeira et al., 2009). In contrast, *Cdc* venom produced the least edema, which may be attributed to the anti-inflammatory effect that this venom can induce (Nunes et al., 2010).

Since we observed infiltration of leukocytes into liver and edema in experimental mice, it was important to evaluate the release of pro-inflammatory mediators. In mice treated with *Mtt* venom, high concentrations of serum pro-inflammatory mediators were measured, following kinetics similar to those described for LPS (DeForge and Remick, 1991). The highest concentration was recorded at 30 min for TNF-α and at 240 min for IL-6 and NO. Different pathological mechanisms, such as infection, trauma, and toxins, induce the release

of TNF- α , a molecule of great importance in the acute inflammatory response, because it activates the nuclear transcription factor κ B (NF- κ B) and activating protein 1 (AP-1), which in turn activate gene transcription and stimulate the release of other pro-inflammatory mediators (Aird, 2002; Zelova and Hošek, 2013).

IL-6 binds to its specific receptor, IL-6R, which also exists in soluble form (IL-6Rs) forming complexes (IL-6/IL-6R or IL-6/IL-6Rs) that can bind gp130. Even in cells lacking the surface receptor, such as endothelial cells, adhesion molecule expression and chemokine release are induced, favoring mononuclear cells recruitment to the site of inflammation (Jones and Rose-John, 2002). Furthermore, NO is a vasodilator that participates in pathogenesis of snake bites, by inducing formation of peroxynitrite (ONOO⁻), reacting with locally produced superoxide anions and contributing to the hypotension described in these envenomations (Zamuner et al., 2001; Aird, 2002; Zelova and Hošek, 2013).

The release of pro-inflammatory mediators, the edematizing effect, and histopathological studies, suggest that *Mtt* venom triggers an acute inflammatory response in C57BL/6 mice. In this sense, venom components may stimulate the release of pro-inflammatory and vasoactive mediators by tissue-resident cells, directly or indirectly, which increase vascular permeability and activate the endothelium. Activated endothelial cells in turn release pro-inflammatory cytokines, including IL-6 and TNF- α , as well as NO, which activate leukocytes and recruit them to the site of the lesion. This response may also lead to systemic inflammation, with leukocyte infiltration in remote organs, and may activate the coagulation system through Tissue Factor (TF) expression (Zelova and Hošek, 2013).

In elapid venoms, pro-coagulant proteins (FV, FVIII, FX, prothrombin and fibrinogen activators), and anti-coagulant proteins (FX, thrombin and platelet aggregation inhibitors) have been described. These provoke hemostatic disorders including coagulopathies (Lu et al., 2005; White, 2005; Salazar et al., 2011). For this reason, monitoring the hemostatic system in victims would be helpful when evaluating the severity of envenomation and would enable more appropriate co-adjuvant treatments. This may be particularly important in *Micrurus* envenomation, where other clinical manifestations beside neurotoxicity appear, as in *M. lemniscatus* envenomation, which causes early alterations in coagulation times (PT and aPTT) (Manock et al., 2008).

In dogs bitten by the banded cobra, *Naja annulifera*, with a mainly neurotoxic venom, a significant increase in acute phase proteins including fibrinogen was observed in the first 24 h post envenomation. This promoted a hypercoagulable state, which, in turn, potentiated thrombosis and pulmonary thromboembolism (Nagel et al., 2014). In our *in vivo* model, alterations in coagulation were not seen 24 and 48 h after *Mtt* venom inoculation (data not shown). Thrombin-like activity was identified in *Mtt* crude venom that, in addition to its pro-coagulant activity, may stimulate earlier release of pro-inflammatory mediators (Salazar et al., 2011). In turn, TNF- α can positively regulate TF expression in endothelial cells and monocytes, promoting generation of thrombin, which stimulates the generation of fibrin and intensifies the inflammatory response via PAR receptors (Petäjä, 2011; Zelova and Hošek, 2013). In addition, IL-6 up-regulates the release of acute phase proteins, including fibrinogen, which might promote a pro-coagulant state (Medcalf, 2007).

However, a decrease in fibrinogen and a prolongation of coagulation times (PT and aPTT) were observed after 72 h in the mice. These findings may be associated with an early activation of coagulation, which consumes hemostatic factors, leading to later alteration of coagulation tests, as has been described in snake envenomation by Australian elapid snakes (Berling and Isbister, 2015). In addition, these observations may be associated with inhibitors of coagulation factors, such as anti-FXa, described in *in vitro* experiments with *Mtt* venom (Salazar et al., 2011). This anti-FXa activity might interfere with the interaction between FXa and FVa or prevent the assembly of the prothrombinase complex, thus consuming hemostatic factors and promoting an anticoagulated status (Kini, 2011). Furthermore, these results may be due to fibrino(geno)lytic activity associated with SVMPs, also reported in this venom, which is capable of cleaving fibrinogen A α and B β chains and, generating fibrinogen degradation products (FDP) (Salazar et al., 2011). FDP may contribute to the anti-coagulant state, the prolongation of coagulation times, and may result in the hemorrhage seen in experimental mice (Swenson and Marckland, 2005; Hamza et al., 2010; Nagel et al., 2014). However, these effects could be delayed due to the low proportion of proteases in this venom, such as SVMPs and serine proteases, which are widely known for their hemostatic activity (Rey-Suárez et al., 2011; Lomonte et al., 2016; Aird et al., 2017).

Our results showed increased endogenous fibrinolytic activity, which may be related to primary fibrinolysis induced by toxins from *Mtt* venom (Salazar et al., 2011), through fibrino(geno)lytic activity previously described in the venom of the caterpillar *Lonomia achelous* (Arocha-Piñango et al., 2000). Moreover, secondary fibrinolysis may occur because of early coagulopathy induced by the venom itself or by the inflammatory response. *Mtt* venom may also induce fibrinolysis indirectly, through cellular activation (endothelial cells, monocytes, macrophages), with synthesis and secretion of fibrinolytic enzymes that enhance inflammation. Plasmin thus generated would amplify the inflammatory response by promoting release of pro-inflammatory mediators such as IL-1 β , growth factors including TGF- β , and activating matrix-specific metalloproteases (MMPs) (Schuliga, 2015). Therefore, the high level of fibrinolytic activity without upregulation of one of the main regulators, TAFIa, may also modulate hemorrhage and recruitment of leukocytes seen at 72 h in our experimental model.

In summary, in mice treated with *Mtt* venom, diffusion into tissues of enzymatic toxins such as hyaluronidases, PLA₂s, and SVMP, as well as 3FTxs, may induce tissue injury, triggering an acute systemic inflammatory response within a few hours of inoculation, characterized by release of pro-inflammatory mediators. These mediators could generate a pro-coagulant state, associated with expression of TF in inflammatory cells and endothelium, with an initial activation of coagulation. Indeed, this state later promotes an important release of FDP and an activation of the fibrinolytic system. Subsequently, endogenous fibrinolysis, as well as the fibrino(geno)lytic activities of *Mtt* venom, could lead to anti-coagulation, with reduced fibrinogen, prolonged coagulation tests and hemorrhage.

It is evident that effects induced by the complex mixture of toxins in *Mtt* venom, including activation of inflammatory and hemostatic systems in this mouse model, are the result of interactions between the two systems, both of which should be evaluated in victims in which neurotoxic symptoms are not predominant, to aid in the selection of alternative treatments

for complications that may lead to morbidity. Further experiments will address the role of the inflammatory response in hemostatic alterations presented in this study.

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Abbreviations:

Mtt	Micrurus tener tener
LD₅₀	lethal dose 50
i.p.	intra peritoneal
i.d.	intra dermal
NO	nitric oxide
TNF-α	tumor necrosis factor alpha
PT	Prothrombin Time
aPTT	Activated Partial Thromboplastin Time
FXIII	factor XIII
t-PA	tissue plasminogen activator
PAI-1	plasminogen activator inhibitor type 1
u-PA	urokinase plasminogen activator
IL-6	Interleukin 6
Cdc	Crotalus durissus cumanensis
Bi	Bothrops isabelae

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Fig. 1. Hemorrhage and cell infiltrates in lungs and livers of mice inoculated with *Mtt* venom. C57BL/6 male mice inoculated i.p. with sterile saline solution (Control) or *Mtt* (8 μ g/mouse) (Experimental). Lung (A–B) and liver (C–D) samples obtained 72 h post-inoculation, were fixed in 30% formaldehyde, stained with hematoxylin and eosin and observed under light microscopy at 400 $\times$ magnification. Sections: **A–C:** Control; **B–D:** Experimental; **E:** Infiltration of mononuclear cells in hepatic tissue. Results represent 3 independent experiments and are expressed as the number of infiltrating cells/field (mean $\pm$ SD from 3

different zones in 10 mice). * $p < 0.001$ vs control. E: Erythrocytes. MC: Infiltrating mononuclear cells.

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Fig. 2. Edematogenic effect of *Mtt* venom in C57BL/6 mouse paws.

A) Mice were inoculated i.d. in the hind foot pad, with 8 or 50 µg of *Mtt* or *Bi* venom/mouse, respectively, or sterile saline solution (control), and then sacrificed at different times (0–480 min). Percentage of edema = weight venom-injected limb/weight control limb. Results represent 3 independent experiments in triplicate (mean ± SD). **B)** Mice were inoculated i.d. in the posterior limb foot pad with 2 different doses of venom (*Mtt*, *Cdc* or *Bi*), or sterile saline (control), then sacrificed at 240 min. Percentage of edema = weight venom-injected limb/weight control limb. Results represent 3 independent experiments in

triplicate (mean $\pm$ SD). * $p < 0.05$ *Cdc* vs. *Mtt* (8 μ g) and *Cdc* vs *Bi* (20 μ g). *Mtt*: *Micrurus tener tener* venom; *Bi*: *Bothrops isabelae* venom; *Cdc*: *Crotalus durissus cumanensis* venom.

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Fig. 3. Pro-inflammatory mediators in serum from mice inoculated with *Mtt* venom.

C57BL/6 male mice were inoculated i.p. with 200 μ L sterile saline solution (Negative Control), 10 μ g LPS/200 μ L sterile saline solution (Positive Control) or 8 μ g *Mtt*/200 μ L sterile saline solution (Experimental). After 30, 60, 120, 240 and 480 min, serum TNF- α (A) and IL-6 (B) were determined by ELISA, and serum NO (C) by the Griess method. Results represent 3 independent experiments in triplicate (mean $\pm$ SD). * $p < 0.05$ vs. negative control; $^{\circ}$ $p < 0.001$ vs. negative control.

Table 1

Death and survival rate of C57BL/6 mice up to 48 h after i.p. injection, to determine LD₅₀ of *Mtt* venom.

Dose (µg/mice)	Death (mice)			Survival (mice)	Death rate (%)
	4h	24 h	48h		
7.1	0	0	0	5	0
10.6	0	0	0	5	0
16.0	0	1	1	3	29
24.0	1	2	0	2	71
36.0	5	0	0	0	100

n = 5.

Table 2Effects of *Mtt* venom (8 µg/mouse) on hematological parameters in C57BL/6 mice, 72 h post-inoculation i.p.

Parameters		Controls	Experimentals
Hb (g/dL)		13.47 ± 1.00	12.00 ± 1.22 **
Hto (%)		39 ± 2	32 ± 5 **
Leukocytes (x 10 ³ /mm ³)		5.0 ± 4.4	3.4 ± 1.8
Leukocyte Differential formula (%)	Neutrophils	21 ± 10	17 ± 9
	Lymphocytes	75 ± 9	80 ± 11
	Eosinophils	2 ± 1	2 ± 3
	Basophils	0	0
	Monocytes	2 ± 2	1 ± 1

n = 10; bold = statistically significant difference compared to control group;

**
p < 0.01.

Table 3

Effect of *Mtt* venom (8 µg/mouse) on hemostatic parameters of C57BL/6 mice, 72 h post-inoculation i.p.

Parameters	Controls	Experimentals
Platelets (x 10 ⁵ /L)	9.5 ± 2.1	8.5 ± 2.6
PT (s)	15.0 ± 0.4	18.3 ± 1.5 *
aPTT (s)	29.5 ± 4.5	37.5 ± 6.6 *
Fibrinogen (mg/dL)	305 ± 51	205 ± 69 **
FXIII (Urea 5 M/24 h)	Insoluble	Insoluble
Euglobulin Lysis (mm ²)	47.2 ± 56.0	143.7 ± 54.0 **
TAFIa (µg/mL)	14.37 ± 3.79	17.55 ± 9.02

n = 10; bold = statistically significant difference compared to control group;

*
p < 0.05

**
p < 0.01.