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Role of nitric oxide in the local and systemic pathophysiological effects induced by *Bothrops asper* snake venom in mice

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Abstract. *Objective:* To assess the role of nitric oxide in the most relevant local and systemic manifestations in mice injected with the venom of the snake *Bothrops asper*. Mice were pretreated with nitric oxide synthase inhibitors, and the modifications of the pathological effects induced by the venom were tested.
Results: Inhibition of NO synthesis did not affect acute local myonecrosis and hemorrhage in muscle tissue upon intramuscular injection of venom. Local footpad edema was reduced in mice pretreated with the NO synthase inhibitor L-NAME, and a reduction in the extent of inflammatory infiltrate in muscle tissue was observed after envenomation in mice pretreated with L-NAME and aminoguanidine. The most pronounced effect of NOS inhibition by L-NAME was an increment in the lethal activity of the venom, when injected by the intraperitoneal route.
Conclusion: Nitric oxide does not seem to play a significant role in the local acute pathological alterations (hemorrhage and myonecrosis) induced by *B. asper* venom in mice, although it contributes to edema and inflammatory infiltrate. Nitric oxide exerts a protective role in the systemic pathophysiological manifestations leading to lethality.

Key words: NO synthase – NO – L-NAME – *Bothrops asper* venom – myonecrosis – hemorrhage – lethality

Introduction

Envenomations by pit vipers (family Viperidae, subfamily Crotalinae) constitute a public health hazard in many regions of the world [1, 2]. In Central America, most snakebite envenomations are inflicted by *Bothrops asper* [3], a large and widely distributed species in tropical rainforests and in altered areas devoted to agriculture and cattle raising [4]. Venoms of *Bothrops* sp snakes in general, and of *B. asper* in particular, induce envenomations of complex pathophysiology associated with prominent tissue alterations (hemorrhage, edema, necrosis, blistering) at the site of venom injection and, in moderate and severe cases, with systemic perturbations (hemodynamic alterations, defibrination and other manifestations of coagulopathy and renal effects) [2, 3].

The pathogenesis of local and systemic effects induced by *B. asper* venom has been partially investigated. Various locally-acting toxins, mostly metalloproteinases and phospholipases A_2, are responsible for the acute local pathological effects [5–7]. In addition, a variety of venom components, such as metalloproteinases, serine proteinases and proteins of the C-type lectin family, induce defibrination, systemic bleeding and thrombocytopenia, therefore promoting profuse bleeding and coagulopathy [8–12]. Furthermore, experimental and clinical evidence indicates that *B. asper* envenomations also concur with a conspicuous inflammatory response, both local and systemic, which might contribute to the pathophysiology of envenomation [13–18]. However, despite their well demonstrated occurrence, the precise role of inflammatory mediators as contributors to local and systemic tissue damage in these envenomations remains obscure.

Nitric oxide (NO) is a short-living mediator implicated in an amazing variety of tissue and cellular processes [19]. Increments in NO synthesis have been demonstrated in experimental animals injected with *B. asper* venom [15, 20]. Owing to the role that NO plays in various models of cardiovascular shock, and as precursor of tissue-damaging peroxynitrites [19], the possibility has been raised that increments in NO production in *Bothrops* sp envenomations may contribute to the complex pathophysiology characteristic of this pathology [15, 20]. Inhibition of NO synthesis drastically reduced the extent of myonecrosis induced by crotoxin, a myotoxic and neurotoxic phospholipase A_2 complex present in the venom of the rattlesnake *Crotalus durissus terrificus* [21]. Therefore, the elucidation of the role of NO in the pathogenesis of tissue damage inflicted by snake venoms is a relevant task. The present investigation assessed the involvement of NO in the local and systemic effects induced by *B. asper* venom in a murine experimental model.

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Materials and methods

Venom and drugs

B. asper venom was obtained from more than 40 adult specimens collected in the Pacific region of Costa Rica and kept at the serpentarium of Instituto Clodomiro Picado. Upon collection, venom was immediately lyophilized and stored at $-20\,°C$. Immediately before its use, venom was dissolved in apyrogenic and sterile saline solution (150 mM NaCl) (SS) and filtered through $0.22\,\mu m$ membranes. The following drugs were used: N^g-nitro-L-arginine methyl ester (L-NAME), N^g-nitro-D-arginine methyl ester (D-NAME), aminoguanidine, nitroprusside and L-arginine, all from Sigma-Aldrich (St. Louis, MO, USA). Drugs were dissolved in SS immediately before their use.

Protocols used for administration of drugs

Swiss mice (18–20 g), obtained from the Animal Colony of Instituto Butantan (Sao Paulo, Brazil), were used. Throughout the study, mice were maintained on a 12 h: 12 h light: dark cycle and received food and water *ad libitum*. L-NAME and D-NAME were injected intraperitoneally (i. p.), at a dose of 50 mg/kg, 24 h and 30 min before venom injection [20]. Aminoguanidine, an inhibitor of iNOS [22], was administered i. p. 24 h and 30 min before venom injection, at doses of 50 mg/kg and 100 mg/kg, respectively [20]. Sodium nitroprusside was used in experiments dealing with edema and myonecrosis, and was injected subcutaneously (s.c.) (for edema) and intramuscularly (i. m.) (for myonecrosis), at a dose of 5 mg/kg. L-arginine was administered i. p. 24 h and 1 h before venom injection at a dose of 300 mg/kg. Some animals were pretreated with SS alone. After these drug pretreatments, mice were injected with *B. asper* venom or SS to assess the extent of local and systemic alterations, as described below. Drug pretreatments and venom administration protocols were approved by the Committee for the Care and Use of Animals (CICUA) of the University of Costa Rica.

Quantification of NO in envenomated muscle

Groups of 5 mice received an i. m. injection, in the right gastrocnemius muscle, of $50\,\mu g$ of *B. asper* venom, dissolved in $100\,\mu L$ of SS. Control mice received $100\,\mu L$ of SS alone. At various time intervals (1 h, 3 h, 6 h, 24 h, 48 h) animals were killed and the injected gastrocnemius muscles were dissected and homogenized on ice with a 50 mM Tris, 0.1 mM ethylene diamine tetracetic acid (EDTA), 0.1 mM ethylene glycol bis (2-aminoethyl ether)-N, N, N′ N′-tetracetic acid (EGTA), 12 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.4 (Sigma-Aldrich), using a Polytron PT MR 3000 homogenizer (Brinkmann, Westbury, NY). Then, samples were centrifuged 7 min at 13,000 g. Nitrate was extracted according Wu and Yen [23]. Nitrate was then converted to NO by the addition of a reducing agent (0.8 % VCl$_3$ in 1N HCl). The sample was then applied to a Sievers Nitric Oxide analyser 280 NOA (Sievers, Boulder, CO, USA). Nitrate concentrations were calculated by comparison with standard solutions of NaNO$_3$ (Sigma). In another experiment, the increments in muscle NO were studied in groups of mice pretreated with either saline solution, L-NAME or aminoguanidine, as described before. Then, 1 h after venom injection, mice were killed and muscle NO concentration was determined as described.

Inhibition of venom-induced local effects

Inhibition of hemorrhagic activity. Mice (18–20 g) were pretreated as described, and then injected intradermally (i. d.), in the ventral abdominal region, with $20\,\mu g$ of *B. asper* venom, dissolved in $100\,\mu L$ of SS. This dose corresponds approximately to 10 minimum hemorrhagic doses, i. e. ten times the amount of venom that induces a hemorrhagic halo of 10 mm diameter [24]. Two hr after envenomation, mice were killed by an overdose of CO_2, and the diameters of the hemorrhagic halos in the inner side of the skin were measured. In another type of experiment, pretreated

mice received an i. m. injection, in the right gastrocnemius muscle, of $50\,\mu g$ venom, dissolved in $100\,\mu L$ SS. Controls were injected with SS alone. One hr after envenomation, mice were killed by CO_2 overdose, and the injected gastrocnemius muscles were dissected out and cut into small pieces to quantify the amount of hemoglobin [25]. Tissue was placed in 2.0 mL of Drabkin solution and left at $4\,°C$ for 24 h. After centrifugation at $600 \times g$, the supernatant was collected and its absorbance at 540 nm was recorded as a quantitative estimation of the amount of hemoglobin present in the tissue as a consequence of hemorrhage.

Inhibition of myotoxic activity. Mice pretreated as described were injected i. m., in the right gastrocnemius muscle, with $50\,\mu g$ of *B. asper* venom, dissolved in $100\,\mu L$ of saline solution. Control mice received SS alone. Three hr after envenomation, mice were anesthetized with CO_2 and bled from the tail, blood being collected into heparinized capillary tubes. The plasma creatine kinase (CK) activity was determined using the Sigma kit No. 47-UV. In some experiments, mice were killed by an overdose of CO_2 24 h after envenomation, and both envenomated and contralateral gastrocnemius muscles were dissected out and homogenized, as previously described [26]. After centrifugation at $5000 \times g$ for 5 min, the supernatants were diluted 1:35 with saline solution, and CK activity of the supernatants was determined as described. Residual CK activity in envenomated muscle was expressed as percentage, taking as 100 % the CK activity of non-envenomated, contralateral gastrocnemius.

Inhibition of edema-forming activity. Mice pretreated as described received a s. c. injection of $2.5\,\mu g$ *B. asper* venom, dissolved in $50\,\mu L$ SS. Other groups of pretreated mice were then injected s. c. with $50\,\mu L$ of SS without venom. This dose was selected because it induces evident edema without hemorrhage [14]. A group that received venom was injected simultaneously with sodium nitroprusside. The thickness of injected footpads was measured at various time intervals with a low pressure spring caliper (H.C. Kröplin, Germany) [13], and edema was expressed as the percentage increment in thickness of envenomated footpad as compared with the footpad injected with SS only.

Effects on inflammatory cell infiltrate. Mice pretreated as described were injected i. m. in the right gastrocnemius muscle with either $50\,\mu g$ of *B. asper* venom, dissolved in $100\,\mu L$ of SS or with $100\,\mu L$ of SS alone. Then, at 24, 48 and 72 h, animals were killed and the injected gastrocnemius muscles were dissected out and chopped with a blade into very small pieces before addition of 2 mL of SS containing trypsin. The rest of the procedure was performed as previously described [26]; briefly, the suspension was incubated for 15 min at $4\,°C$ and then for 20 min at $37\,°C$, with mild agitation every 5 min. Then, it was filtered through gauze, and the gauze was washed with an additional 1 ml of SS. Filtered suspensions were centrifuged and the pellet was resuspended in $200\,\mu L$ of SS. Total leucocyte counts in these suspensions were performed in a Neubauer chamber.

Inhibition of systemic effects

Inhibition of venom-induced lethality. Groups of 5 mice each were pretreated as described above. Then mice were injected i. p. with various amounts of venom, dissolved in $200\,\mu L$ of SS. Deaths were recorded during 48 h and LD$_{50}$ was estimated by using the probits method. In another type of experiment, mice receiving the same pretreatments were challenged with 2 LD$_{50}$s of venom by the i. p. route. Animals were observed and the time of death recorded. Controls received saline solution instead of venom.

Inhibition of defibrinating effect. Groups of 5 mice each were pretreated as described above. Then, various amounts of venom (1.5 μg, 3.0 μg and 6.0 μg, dissolved in $200\,\mu L$ of SS) were administered intravenously (i. v.) in the tail. Control mice were injected with $200\,\mu L$ of SS alone. One hr later, and under CO_2 anesthesia, animals were bled by cardiac puncture, blood was placed in clean, dry glass tubes, and clotting times were recorded.

Changes in blood leucocyte and platelet counts. Groups of 6 mice each were pretreated as described above. Then, they were injected i. p. with

50 µg venom, dissolved in 200 µL SS; this corresponds to a venom dose slightly lower than LD_{50} [27]. Control mice were injected with 200 µL of SS alone. One and three hr after injection, mice were bled by cardiac puncture, under CO_2 anesthesia. Total and differential leucocyte counts, as well as platelet counts, were performed in an automated hematological analyzer (Micros 60, ABX Hematologie, France).

Statistical analyses. The Student's *t* test was used to determine the significance of the differences between two pairs of means. When more than two means were compared, Analysis of Variance was used, followed by Tukey-Kramer test to compare pairs of means. A P value of 0.05 was selected to establish significance.

Results

Increment of NO in envenomated muscle

A significant increment in the NO content of gastrocnemius muscle was observed in mice injected with SS and with 50 µg *B. asper* venom, when compared with non-injected muscle. Increments were significantly higher in envenomated mice than in SS-injected mice at 1, 3, 6 and 24 h (Fig. 1A). When mice were pretreated with either L-NAME or aminoguanidine, the venom-induced increments in NO were abrogated (Fig. 1B).

Effects of inhibition of NO synthesis in local tissue damage

*Hemorrhagic activity.* When using the mouse intradermal test, pre-treatment with L-NAME or aminoguanidine result-ed in a significantly larger hemorrhagic lesion in mice when compared with animals pretreated with SS or D-NAME (Fig. 2A). However, when hemorrhagic activity was assessed in muscle tissue, mice pretreated with either L-NAME or aminoguanidine did not differ in the extent of hemorrhage, assessed by the amount of hemoglobin present in the tissue, when compared with control mice pretreated with SS or D-NAME (Fig. 2B).

Myotoxic activity. Mice injected i. m. with SS alone showed plasma CK activity of 200 ± 25 U/L 3 h after injection. Plasma CK activity increased drastically in mice pretreated with SS and then receiving an i. m. injection of *B. asper* venom, reflecting a prominent myonecrotic process (Fig. 3A), as has been previously described [28]. No significant variations in the plasma CK activity was observed in mice pretreated with either L-NAME, D-NAME or aminoguanidine, despite a trend of increment in myonecrosis in mice receiving L-NAME (Fig. 3A). Moreover, when myonecrosis was quantitated by assessing the reduction in muscle CK activity at 24 h, there were no differences between the various experimental groups (Fig. 3B). Pretreatment of animals with the NO-enhancing compounds L-arginine and nitroprusside did not affect the extent of myonecrosis (results not shown).

Edema-forming activity. Mice injected s. c. in the footpad with *B. asper* venom developed an edema of rapid onset which peaked 1 h after injection (Fig. 4). When mice were pretreated with D-NAME, no significant changes were observed in the curve of edema, whereas pre-treatment with L-NAME significantly reduced the extent of this effect
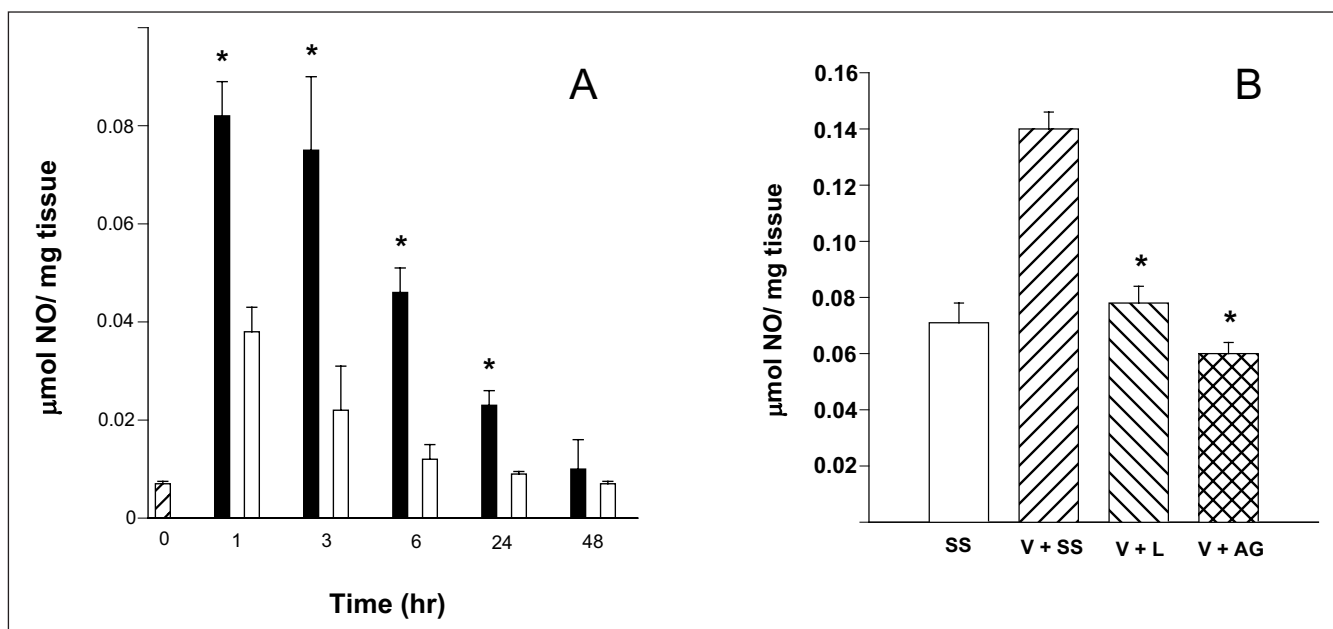

Fig. 1. (A) NO concentration in mouse gastrocnemius muscle after i. m. injection of either saline solution (white bars) or venom (filled bars). As control, NO from muscle of mice that did not receive saline solution or venom is included (shaded bar). Saline (100 µL) or venom (50 µg/100 µL) were injected i. m. in the right gastrocnemius muscle. At various time intervals, mice were sacrificed and injected muscles were dissected out and homogenized. Nitrate was extracted from tissues and converted to NO in order to determine its concentration in an analyzer, as described in Materials and methods. (B) Effect of pre-treatment with various drugs on NO synthesis in gastrocnemius muscle. Animals were pretreated with either saline solution (SS), L-NAME (L) or aminoguanidine (AG); then, they were injected i. m. with either saline solution (SS) or venom (V). One hour after venom or SS injection, mice were sacrificed and muscle NO quantified as described. Results are presented as mean ± SEM (n = 5). In (A) * P < 0.05 when comparing saline solution group versus venom group. In (B) * P < 0.05 when compared with mice pretreated with SS and then injected with venom.
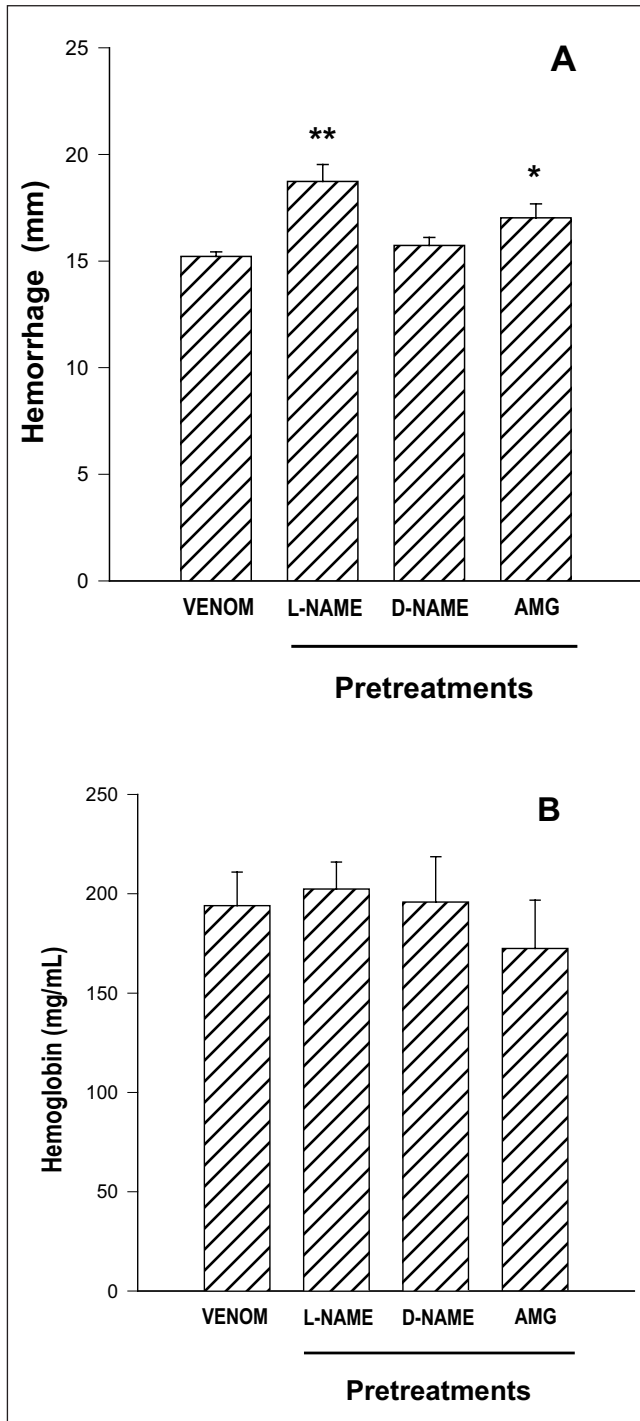
Fig. 2. (A) Effects of pre-treatment with NOS inhibitors in skin and muscle hemorrhage induced by *B. asper* venom. Mice were pretreated with either saline solution, L-NAME, D-NAME or aminoguanidine (AMG), as described in Materials and methods. Then, venom (20 µg/100 µL) was injected intradermally in the abdominal region. Two hr later, mice were sacrificed, their skin dissected and the diameter of the hemorrhagic lesion in the inner part of the skin measured. (B) Other groups of mice, pretreated as described, were injected i.m. in the right gastrocnemius muscle, with 50 µg venom/100 µL. One hr after envenomation, mice were sacrificed and injected muscles were dissected out and homogenized. After centrifugation, the haemoglobin concentration of the supernatant was determined. Results are presented as mean ± SEM (n = 5). * P < 0.05, ** P < 0.01 when compared with the group pretreated with saline solution and then injected with venom.
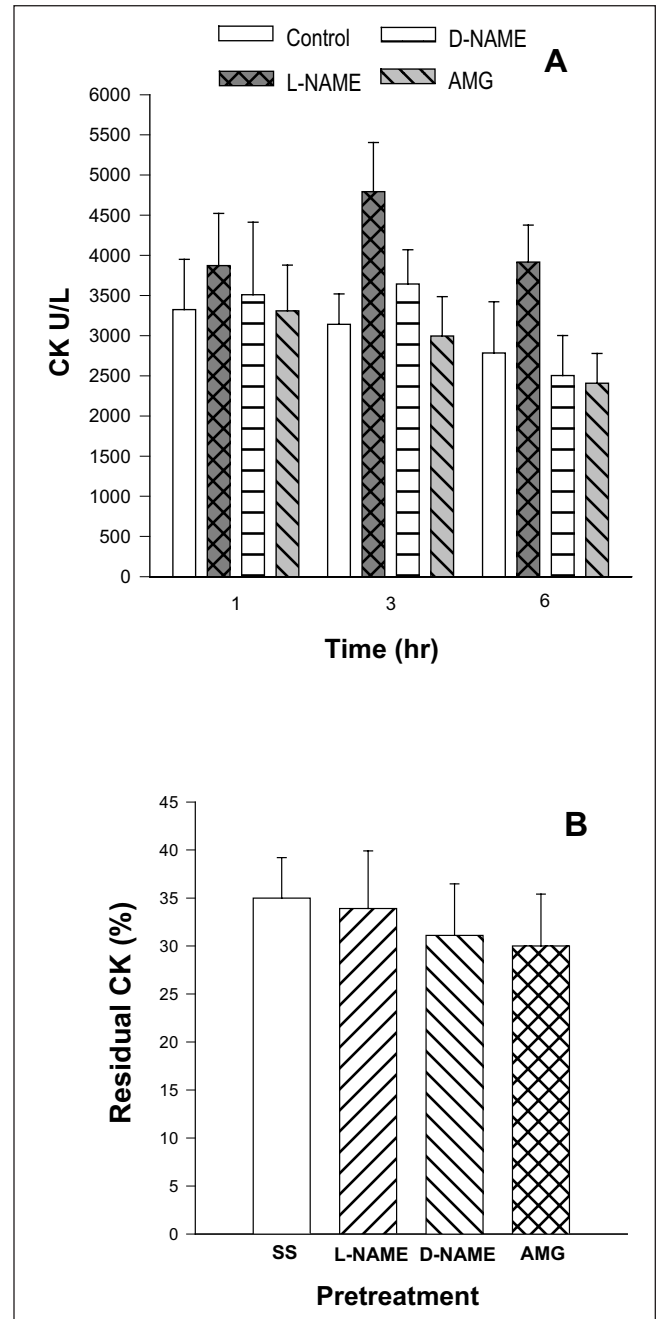
Fig. 3. Effects of pre-treatment with NOS inhibitors in myotoxicity induced by *B. asper* venom. Mice were pretreated with either saline solution, L-NAME, D-NAME or aminoguanidine (AMG), as described in Materials and methods. Then, venom (50 µg/100 µL) was injected i.m. in the right gastrocnemius muscle. At various time intervals after envenomation, plasma CK activity was determined (A). Then, 24 h after envenomation, mice were sacrificed and both right and left gastrocnemius muscles were dissected out and homogenized, followed by centrifugation. CK activity of the supernatant was determined and residual CK in envenomated muscle was expressed as percentage, taking as 100% the CK activity of the left, non-envenomated muscle (B). Results are presented as mean ± SEM (n = 5). No significant differences were observed between any of the experimental groups (P > 0.05).
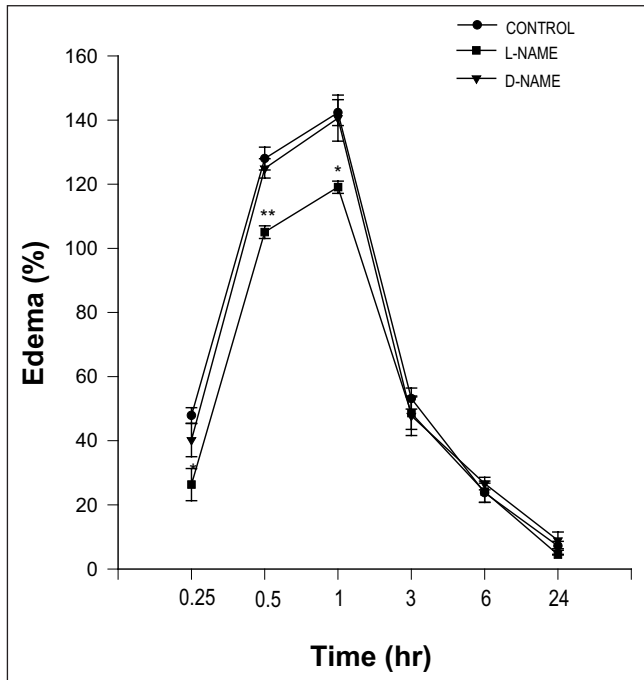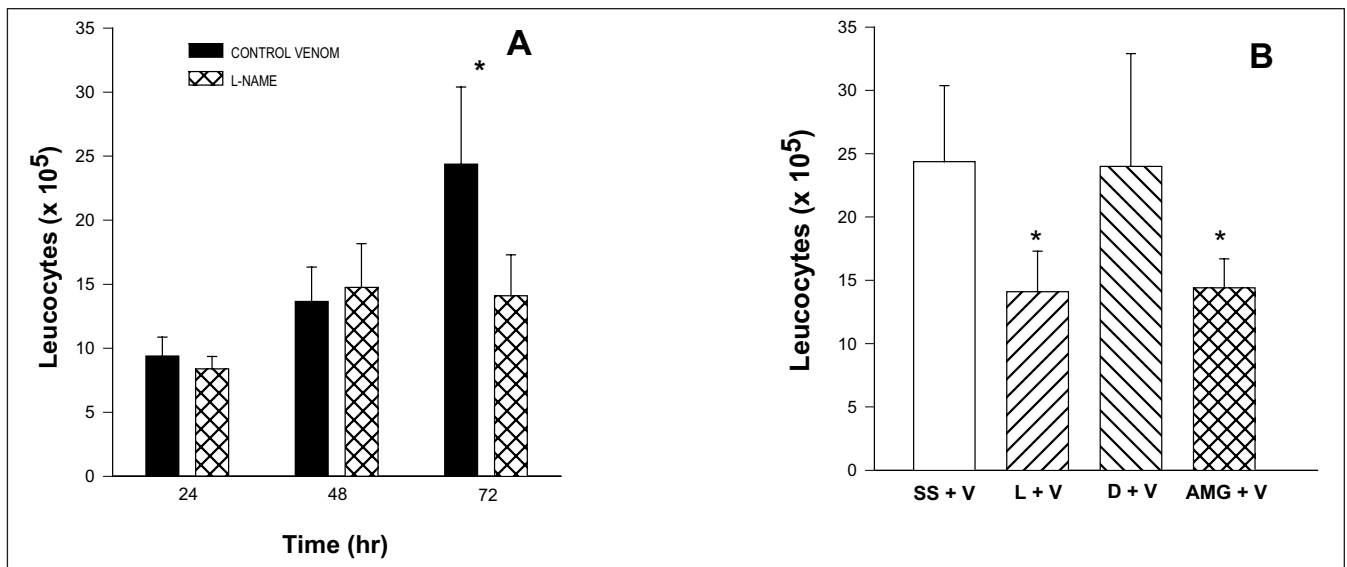
Fig. 4. Effects of pre-treatment with NOS inhibitors in edema induced by *B. asper* venom. Mice were pretreated with either saline solution, L-NAME or D-NAME, as described in Materials and methods. Then, mice were injected with either venom (2.5 μg/50 μL) or with saline solution (50 μL) subcutaneously in the foot pad. At various time intervals after venom injection, the footpad thickness was determined by plethysmography. Edema was expressed as percentage increase of thickness, when compared with the foot pads of mice injected with saline solution. Results are presented as mean ± SEM (n = 5). *P < 0.05, **P < 0.01 when compared with mice pretreated with saline solution and then injected with venom.

(Fig. 4). In order to assess if this effect was due to iNOS, mice were pretreated with aminoguanidine; however, there was not a reduction in the extent of edema when compared with mice pretreated with SS in these circumstances (results not shown).

Leucocyte infiltrate in muscle. A prominent inflammatory infiltrate was observed in gastrocnemius muscle injected with *B. asper* venom, as previously described [26, 29]. Such infiltrate increased with time, reaching highest numbers by 72 h (Fig. 5A). Pretreatment with L-NAME caused a significant reduction in the infiltrate at 72 h, but not at 24 or 48 h (Fig. 5A). Pretreatment with aminoguanidine also promoted a reduction in the infiltrate at 72 h. In contrast, no differences were observed at any time interval between mice pretreated with SS and those pretreated with D-NAME (Fig. 5B).

Effects of inhibition of NO synthesis in systemic alterations

Lethality. LD_{50} in mice pretreated with SS and then injected i. p. with venom was 63.5 μg in 16–18 g mice (95 % confidence limits: 53.7–73.2 μg), a value similar to one previously reported for this venom [27]. Pretreatment with L-NAME significantly reduced the value of LD_{50}, reflecting an increment in the toxicity of the venom in these conditions (LD_{50}: 43.3 μg; 95 % confidence limits: 36.1–47.0 μg). In contrast, no significant variation in the value of LD_{50} was observed in mice pretreated with D-NAME (LD_{50}: 64.4 μg; 95 % confidence limits: 54.8–104.4 μg), aminoguanidine (LD_{50}: 57.7 μg; 95 % confidence limits: 51.4–67.1 μg) or L-arginine (LD_{50}: 63.4 μg; 95 % confidence limits: 48.7–84.9 μg). When a venom dose corresponding to 2 LD_{50}s was injected i. p. in mice pretreated with either SS or L-NAME, there was a sig-
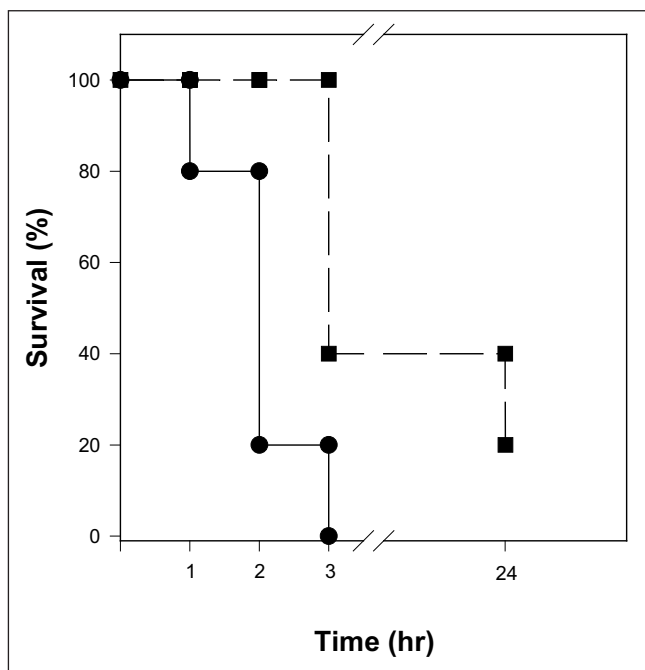

Fig. 5. Effects of pre-treatment with NOS inhibitors in the inflammatory infiltrate induced by *B. asper* venom. (A) Mice were pretreated with either saline solution or L-NAME, as described. Then, they were injected i. m. in the right gastrocnemius muscle with 50 μg/100 μL venom. At various time intervals, mice were sacrificed, the injected muscle dissected out and the leucocytes present in the tissue quantified as described in Materials and methods. (B) Mice were pretreated with either saline solution, L-NAME (L), D-NAME (D) or aminoguanidine (AMG), and were then injected with *B. asper* venom (V). Inflammatory infiltrate was quantified at 72 h. Results are presented as mean ± SEM (n = 5). *P < 0.05 when compared with infiltrate in mice pretreated with saline solution and then injected with venom.

nificant difference in the time of death, since mice pretreated with L-NAME died at earlier time intervals than those pretreated with SS (Fig. 6).

Defibrination. Mice injected i. v. with SS only had a whole blood clotting time of 121 ± 56 sec, whereas mice receiving *B. asper* venom developed a characteristic dose-dependent defibrination syndrome, as previously described [30]. Doses of 1.5 and 3.0 µg did not prolong the clotting time when compared with mice receiving SS alone (112 ± 34 sec and 88 ± 14 sec, respectively; $P > 0.05$). Mice pretreated with L-NAME and then receiving SS presented clotting time of 137 ± 40 sec ($P > 0.05$ when compared with control mice injected with SS only). In contrast, mice receiving 6.0 µg venom were defibrinated, i. e. had unclottable blood, in both L-NAME and SS-pretreated groups. Thus, NOS inhibition did not modify venom-induced defibrination.

Leucocyte and platelet counts. Mice pretreated with SS and then injected with venom by the i. p. route did not show significant variations in the total leucocyte and platelet counts when compared with control mice injected with SS instead of venom (Table 1). However, there was a significant change in the percentage of polymorphonuclear leucocytes (at 1 h) and monocytes (at 1 and 3 h). Envenomated mice had less polymorphonuclear leucocytes and more monocytes than control mice (Table 1). The same changes were observed in envenomated mice that had been pretreated with either L-NAME or D-NAME (Table 1).

Fig. 6. Effect of pre-treatment with the NOS inhibitor L-NAME in the time course of lethality in mice injected with *B. asper* venom. Groups of 5 mice were pretreated with either saline solution (discontinuous line) or L-NAME (continuous line). Then, they were injected with 2 LD$_{50}$ of venom, dissolved in 200 µL, by the intraperitoneal route. The time of death was recorded and survival was expressed as percentage of surviving mice at a given time point.

Discussion

An increment in NO levels was demonstrated in skeletal muscle rapidly after i. m. injection of *B. asper* venom. Such a rapid elevation may depend on the activity of constitutively-expressed NOS, known to be present in skeletal muscle [31]. However, the ability of pre-treatment with aminoguanidine to abrogate such NO increment strongly suggests that it depends on inducible forms of NOS associated with resident macrophages or with inflammatory cells reaching affected tissue after the insult [26, 29]. An increment in the expression of iNOS, together with an increase in protein nitrosylation, was previously demonstrated in peritoneal macrophages 48 h after ip injection of this venom [20]. Animals injected with SS alone had muscle NO levels higher than non-injected control mice, probably indicating that the mechanical damage induced by SS injection induces a small extent of NO synthesis.

NO has been implicated in acute muscle damage in various experimental models in vivo and in cell culture. Muscle cells cocultured with both neutrophils and macrophages developed a NO-dependent cytotoxic process [32], and inhibition of NOS by L-NAME decreased the extent of muscle cell damage in an experimental model of modified muscle use [33], as well as in models of muscle crush injury [34]. Furthermore, knockout mice for iNOS develop less muscle injury in a model of ischemia-reperfusion [35]. Regarding snake venoms, pre-treatment with L-NAME drastically abrogated the extent of myonecrosis induced by crotoxin [21], a highly potent neurotoxic and myotoxic phospholipase A$_2$ responsible of the paralysis and systemic myotoxicity characteristic of envenomations by the South American rattlesnake *Crotalus durissus terrificus* [36, 37]. On these grounds, it was of interest to assess if NO plays a role in *B. asper* venom-induced local myonecrosis. Our observations clearly argue against this hypothesis, since no significant differences in the release of CK from damaged muscle were observed between envenomated mice pretreated with SS, L-NAME, D-NAME and aminoguanidine. Previous studies had shown that other participants of the inflammatory reaction, i. e. neutrophils, matrix metalloproteinases and the cytokines TNF-α, IL-1β and IL-6, are not directly involved in acute myonecrosis in a similar experimental model of *B. asper* envenomation [14, 18, 26]. Hence, local myonecrosis induced by this venom very likely depends on the direct toxic effects of myotoxic phospholipases A$_2$ [5, 28] and hemorrhagic metalloproteinases [38, 39]. The former inflict muscle damage by directly affecting the integrity of skeletal muscle cell plasma membrane [7, 28], whereas myonecrosis induced by hemorrhagic proteinases is likely to be secondary to the ischemia resultant in the tissue from the deleterious effects on blood supply [39]. It is necessary to carry out a more comprehensive assessment on the role of NO in myotoxicity induced by a variety of myotoxic proteins present in other snake and arthropod venoms.

Inhibition of NO synthesis influenced the extent of skin hemorrhage induced by *B. asper* venom, since pre-treatment with L-NAME and aminoguanidine promoted an increment in the diameter of the local skin hemorrhagic lesion. However, when hemorrhage was assessed by quantifying the amount of hemoglobin in muscle tissue, no differences were

Table 1. Changes in leucocyte and platelet counts in peripheral blood in mice pretreated with saline solution (SS), L-NAME or D-NAME and then injected i. p. with *B. asper* venom[a]

1 h after venom injection

Parameter	Control (SS)	SS + venom	L-NAME + venom	D-NAME + venom
Total leucocytes (per µL)	3160 ± 251	3960 ± 739	3750 ± 804	3910 ± 439
Polymorphonuclear (%)	26 ± 1.6	13 ± 2.4*	8 ± 1*	13 ± 2.0*
Lymphocytes (%)	61 ± 3.2	62 ± 2.9	67 ± 3.6	58 ± 3.0
Monocytes (%)	13 ± 1.8	25 ± 1.1*	25 ± 2.7*	29 ± 1.6*
Platelets (per µL)	745,160 ± 58,000	696,000 ± 111,000	545,710 ± 85,160	599,028 ± 87,750

3 h after venom injection

Parameter	Control (SS)	SS + venom	L-NAME + venom	D-NAME + venom
Total leucocytes (per µL)	3160 ± 251	4660 ± 486	3950 ± 769	5130 ± 791
Polymorphonuclear (%)	26 ± 1.6	19 ± 2.2	18 ± 2.8	22 ± 2.2
Lymphocytes (%)	61 ± 3.2	58 ± 2.4	61 ± 2.6	57 ± 2.6
Monocytes (%)	13 ± 1.8	23 ± 2.2*	21 ± 1.1*	21 ± 2.5*
Platelets (per µL)	745,160 ± 58,000	581,400 ± 60,410	543,500 ± 116,500	545,500 ± 46,300

[a] Control mice received 200 µL of SS alone, by the i. p. route. The other groups of mice were pretreated by the same route with either SS, L-NAME or D-NAME; then, they were injected i. p. with 50 µg *B. asper* venom, dissolved in 200 µL SS. Blood samples were collected 1 h and 3 h after envenomation and cell counts were determined in an automatic analyzer. Data of controls injected with only SS correspond to samples collected 1 h after injection. Results are presented as mean ± SEM (n = 6).

* $P < 0.05$ when compared with values in control mice injected with SS alone.

observed between the various experimental groups. This discrepancy may be related to the marked heterogeneity of the action of NO in the microvasculature of various organs [40]. It is suggested that the effect in skin may be secondary to the alterations resultant from NO synthase inhibition in hemodynamic parameters. NOS inhibition promotes an increase in blood pressure which, in turn, may contribute to capillary wall rupture after degradation of basement membrane components by venom hemorrhagic metalloproteinases. Accordingly, it has been proposed that biophysical forces operating in the microvasculature in vivo are likely to participate in the mechanism of venom metalloproteinase-induced hemorrhage [41]. Differences in the effect of NO on the microvasculature of skin and skeletal muscle may explain the differences observed in the two experimental systems. Nevertheless, even in the case of skin, the differences in the extent of hemorrhage between the different groups were not impressive. It can be therefore concluded that NO does not seem to play a prominent role in the pathogenesis of *B. asper* venom-induced local hemorrhage. Similar observations were performed by Laing et al. [42] in the case of the P-III hemorrhagic metalloproteinase jararhagin, from the venom of *B. jararaca*, using knock-out mice for iNOS.

A significant reduction of venom-induced foot pad edema occurred in mice pretreated with L-NAME, thus suggesting that NO participates in this phenomenon. Moreover, the fact that L-NAME, but not aminoguanidine, reduced venom-induced edema, strongly suggests that constitutive eNOS, but not iNOS, plays a key role in this phenomenon. Edema induced by *Bothrops* sp venoms is multifactorial, since a number of inflammatory mediators are involved [14,

43]. NO participates in the edematogenic effect induced by carrageenin in rats [44]. In this case, it was suggested that such effect was due to a decrease in local blood flow, secondary to NO synthase inhibition, and not to an effect in vascular permeability [44]. Pain is another effect induced by *B. asper* venom, and previous results evidenced a role for neuronal NOS-dependent spinal NO synthesis in hyperalgesia and allodynia caused by intraplantar injections of myotoxic phospholipases A_2 present in this venom [45]. In addition, pretreatment with L-NAME reduced the extent of inflammatory infiltrate in muscle tissue 72 h after envenomation, but not at 24 and 48 h. Also, a similar inhibition was observed at 72 h when mice were pretreated with aminoguanidine, strongly suggesting the involvement of iNOS in the development of inflammatory infiltrate in muscle injected with *B. asper* venom. A reduction in inflammatory infiltrate, associated with reduced L-selectin expression, was described as a consequence of L-NAME treatment in a model of *B. jararaca* venom-induced inflammation in rats [46]. Hence, despite conflicting findings on the role of NO in inflammatory phenomena (see for example [47] and [48]), which may depend on the use of different NOS inhibition protocols and differences in the microvasculature of various tissues, our findings and those of others suggest that NO promotes various aspects of inflammation in models involving *Bothrops* sp venom injection. The lack of effect of NOS inhibition in the inflammatory infiltrate at 24 and 48 h suggests that inflammatory mediators other than NO play a predominant role in leucocyte recruitment at these earlier time intervals.

The most notorious consequence of NO synthase inhibition in our experimental model was a significant increment

in the lethal effect, and a shortening in the time of death, when venom was administered i. p. Such effect is not due to alterations in venom-induced coagulopathy nor in variations in the numbers of circulating leucocytes or platelets, which are conspicuous manifestations in *B. asper* envenomations [3]. It is likely that this effect depends on alterations in hemodynamic and tissue perfusion parameters. Observations with various inhibitors strongly suggest that a constitutive form of NOS, probably eNOS, is responsible for this effect, since pre-treatment with L-NAME, but not with aminoguanidine, increased lethality. NO generated by eNOS has been shown to play a critical role in vascular function, and therapeutic interventions aimed at increasing eNOS activity have been protective in various models of cerebral ischemia [49]. After its synthesis in vascular endothelial cells, NO exerts cytoprotective effect and contributes to vascular homeostasis [49, 50]. Hence, since systemic envenomation by crotaline snake venoms is associated with prominent hemodynamic disturbances [2, 51], inhibition of eNOS by L-NAME may play a deleterious role by jeopardizing the constitutively local vascular production of NO by eNOS. The role of NO in ischemia is complex and multifactorial, since NO generated by iNOS present in inflammatory cells exerts deleterious roles contributing to late-phase damage [52]. Nevertheless, our data suggest that the overall effect of NO inhibition in this model of *B. asper* venom-induced lethality is deleterious instead of beneficial. The mechanisms operating behind such protective role of NO require further investigation.

In conclusion, NO does not seem to play a critical role in the acute local pathological alterations (hemorrhage and myonecrosis) induced by *B. asper* venom, with the exception of a mild protective role on hemorrhagic activity in the skin. In contrast, NO contributes to local edema and to late phases of inflammatory infiltrate. Inhibition of NO synthesis does not affect venom-induced coagulopathy. However, inhibition of NOS increases the lethal effect of *B. asper* venom, when tested by the i. p. route, thus suggesting that NO has a protective role in this model of envenomation, probably associated with its participation in vascular homeostasis.

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