

We investigated morphological alterations induced by s.c. injection of 2.5 µg of *Bothrops jararaca* venom in rats. Intense disorganisation of collagen fibres was observed 1 min after the venom injection, particularly at regions near vessels and nerves. Mast cells were degranulated, and erythrocytes were seen leaving venules throughout the endothelial junctions. At this time, damaged endothelial cells were not observed. In rats envenomed as above, but immediately after cardiorespiratory failure induced by deep ether anaesthesia, alterations in the connective tissue structures, as previously described, were not observed. The mediation of this haemorrhage was investigated by injecting the venom into the foot pad of mice and compared to the mediation of oedema. Local haemorrhage was significantly reduced in mice pre-treated with capsaicin or guanethidine or submitted to a surgical section of sciatic and saphenous nerves. In these animals, oedema was not affected. Groups treated with methysergide or morphine showed both haemorrhage and oedema significantly reduced. Indomethacin or dexamethasone pre-treatments significantly reduced the oedema, but not the haemorrhage. Moreover, in animals treated with promethazine or mepyramine, oedema and haemorrhage were not affected. These data suggest that local haemorrhage induced by *Bothrops jararaca* venom is partially controlled by serotonin and neurohumoral mediators. Furthermore, results indicate that haemorrhage and oedema are mediated by different pharmacological systems.

**Key words:** Snake venom, *Bothrops jararaca*, Local haemorrhage, Oedema, Neurogenic inflammation

## Local haemorrhage induced by *Bothrops jararaca* venom: relationship to neurogenic inflammation

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## Introduction

Haemorrhage and oedema, besides blood coagulation disturbances, are the main signs of envenoming by *Bothrops* snake venoms.<sup>1,2</sup> Despite serum therapy being an effective treatment for the control of systemic symptoms in this type of envenoming, it does not control the oedema and local haemorrhagic and necrotic lesions which develop at the site of venom inoculation.<sup>1,3–5</sup>

Among the toxins responsible for these local effects, haemorrhagic factors were isolated from various viperid venoms.<sup>6</sup> Even though some of these haemorrhagic factors are devoid of proteolytic activities,<sup>7–11</sup> they are characterised as metalloproteinases.<sup>6</sup> Some of these factors digest proteins from the extracellular matrix, and this property has been related to the pathogeny of haemorrhagic lesions.<sup>6,12–17</sup>

On the other hand, efforts have also been made to characterise the pharmacological mediators involved in oedema formation,<sup>18–23</sup> pain<sup>24</sup> and other para-

eters of the inflammatory response<sup>25–29</sup> induced by viperid venoms. Nevertheless, all this information is not sufficient to draw a comprehensive mechanism to explain the local lesions observed in this type of envenoming.

In the present study it is shown that oedema and haemorrhage induced by *B. jararaca* venom have distinct pharmacological mediation and our results suggest that haemorrhage is partially controlled by serotonin and neurogenic mediators.

## Methods

### Venoms

A pool of lyophilised venom obtained from adult specimens of *B. jararaca* snakes, supplied by the Laboratory of Herpetology, Instituto Butantan, was used throughout this investigation. The venom was kept at –20°C, and venom solutions were prepared (w/v) with sterile saline just before use.

## Animals

Male Wistar rats (220–250 g) or male Swiss mice (18–22 g) were used. The animals were maintained with water and food *ad libitum* in appropriate environment conditions and used under ethical conditions, according to guidelines of the International Society of Toxicology.<sup>30</sup>

## Morphology of venom-induced lesions

Rats, maintained under ether anaesthesia, were injected into the subcutaneous tissue of the scrotal bag with 2.5 µg of venom (50 µl) in saline. Control rats received the same volume of saline. One minute after the injection, animals were killed and samples of tissue from injected areas were obtained. In order to evaluate the role played by blood circulation in the evolution of the venom-induced lesions, the same procedure as above was performed in rats whose heartbeats had just stopped due to prolonged ether anaesthesia. Tissue samples from the sites of venom injection were collected 1 min after the injection and fixed in 2.5% buffered glutaraldehyde. Routine double fixation, embedding in Araldite and staining with uranyl acetate and lead citrate were employed. The processed samples were analysed in a Phillips (EM-201) electron microscope.

## Evaluation of haemorrhage and oedema

Local haemorrhage and oedema were simultaneously evaluated using a modification of the methods described by Ownby *et al.*<sup>31</sup> and by Yamakawa *et al.*<sup>32</sup> Mice were injected s.c. in the right foot pad with 50 µl of the venom solution. The contra-lateral foot pad received the same volume of sterile saline. Three hours later (time determined by a time-course experiment for haemorrhage and oedema), the animals were sacrificed in an ether chamber. The paws were removed at the level of the tibio-tarsic junction, weighted, fragmented and put in tubes containing 4 ml of von Kampen–Zijlstra reagent modified by Matsubara *et al.*<sup>33</sup> (200 mg K<sub>3</sub>[Fe(CN)<sub>6</sub>], 50 mg KCN, 120 mg KH<sub>2</sub>PO<sub>4</sub>, 50 mg NaCl and 1 ml non-ionic detergent per litre). The tissue was homogenised and the tubes were centrifuged at 3000 g for 30 min. The supernatant was filtered in Millipore membranes (0.8 µ pores), and the absorbance at 540 nm was determined in a spectrophotometer (Micronal B382, Brazil).

The haemoglobin concentration was determined as follows:

$$[\text{Hb}] = \frac{A_{540 \text{ nm}} \times 64.458}{44 \times d \times 1000}$$

where: [Hb] = haemoglobin concentration; A<sub>540nm</sub> = absorbance obtained; 64.458 = molecular weight of

haemoglobin; 44 = 44 mmol<sup>-1</sup>/cm = molecular absorption of haemoglobin; d = 1 = cuvette thickness; 1000 = converting factor (litre to millilitre); 4 = volume of von Kampen–Zijlstra reagent.

In order to eliminate the influence of oedema, the concentration of haemoglobin obtained in the paw injected with venom was divided by the weight of the paw injected with saline. The difference between haemoglobin content of the paw injected with venom and of that injected with saline was the estimated haemorrhage, expressed as mg Hb/g of tissue. The minimal haemorrhagic dose (MHD) was defined as the minimal concentration of venom able to induce an increase of three times the haemoglobin concentration in relation to that of a control tissue. Finally, oedema, expressed in mg, was evaluated by the difference in weight between one paw injected with venom and the other injected with saline.<sup>32</sup>

## Treatment of animals

Groups of 6 to 10 mice were injected s.c. into the foot pad with 5 MHDs of the venom (5 µg in 50 µl) after the following treatments: (1) dexamethasone (corticosteroid, phospholipase A<sub>2</sub> inhibitor: Decadron®, Promade, Brazil), 1 and 0.4 mg/kg, i.p., 24 and 1 h before the venom; (2) indomethacin (inhibitor of the prostaglandin-forming cyclooxygenase: Sigma), 30 mg/kg, s.c., 30 min before the venom; (3) methysergide (5HT receptor antagonist: Sigma), 0.8 mg/kg, s.c., 30 min before the venom; (4) promethazine (H<sub>1</sub> receptor antagonist: Rhodia Pharma, Brazil), 10 mg/kg, i.p., 30 min before the venom; (5) mepyramine (H<sub>1</sub> receptor antagonist: Sigma), 5 mg/kg, i.p., 30 min before the venom; (6) morphine (opioid: Merck), 30 mg/kg, s.c., 30 min before the venom; (7) guanethidine (peripheral post-ganglionic adrenergic neurone inhibitor; sympathetic blockade: Ciba Geigy), three doses of 30 mg/kg, s.c., with 24 h intervals, before the venom injection; (8) chronic denervation: mice were anaesthetised with a Ketalar® (Park–Davis) – Rumpum® (Bayer) solution (1:2 v/v, diluted 1:4 v/v in saline), i.p., 0.1 ml/10 g body weight, and the sciatic and saphenous nerves of the right leg were surgically sectioned and the venom injected into the right foot pad 7 days later; (9) capsaicin (substance P releaser from sensory afferent neurones: Merck), 50 mg/kg, s.c., on the second day after birth (in neonate animals, capsaicin treatment causes degeneration of sensory afferent neurones) (animals were used when they achieved the appropriate weight); (10) respective control groups received saline by the same route. In the case of the chronic denervation, control animals were sham operated, and in the case of the capsaicin, control group received the capsaicin solvent.

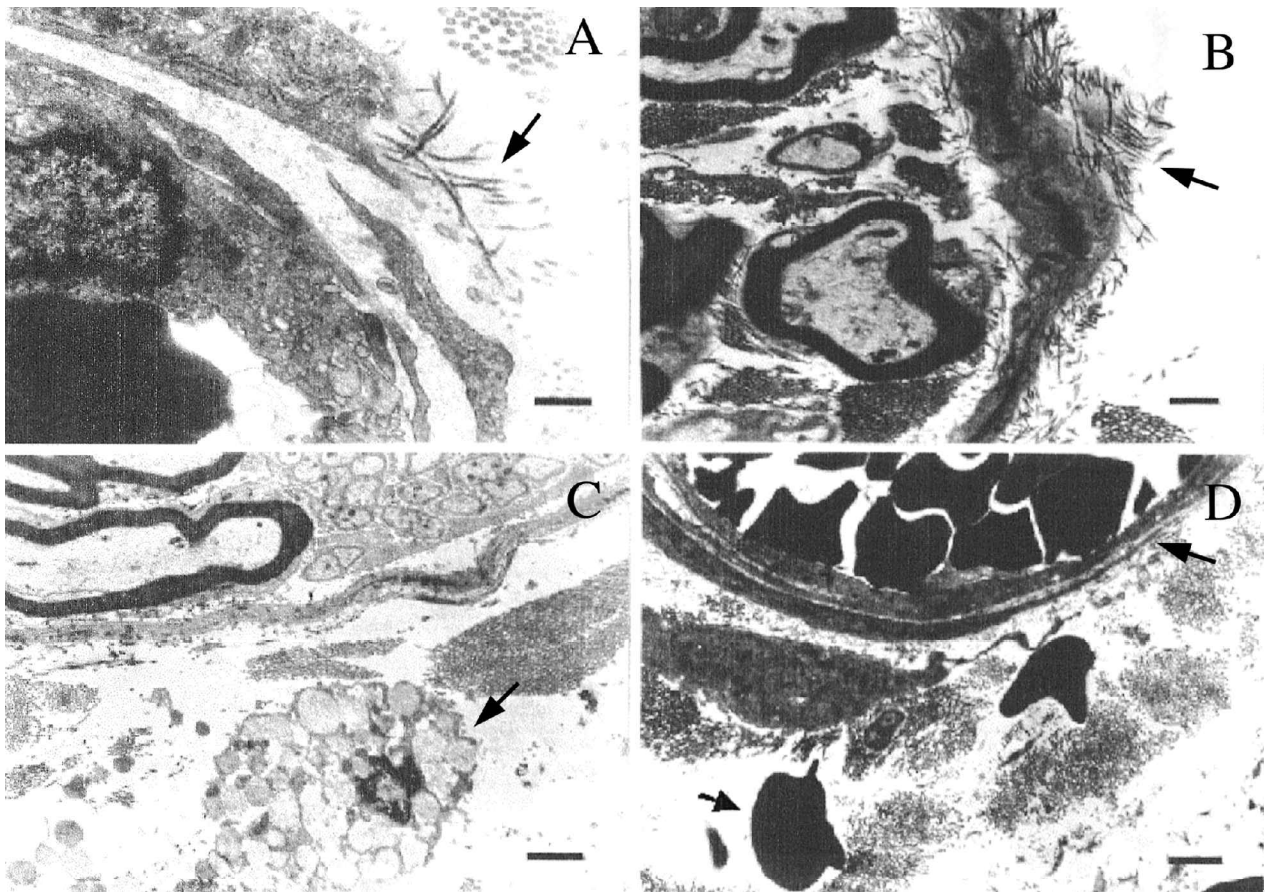


FIG. 1. Electron micrographs of the connective tissue of scrotal bag of a rat 1 min after injection of 2.5  $\mu\text{g}$  of *B. jararaca* venom. Arrows indicate: (A) disorganisation of perivascular collagen fibres (bar = 0.25  $\mu\text{m}$ ); (B) disorganisation of perineural collagen fibres (bar = 0.5  $\mu\text{m}$ ); (C) mast cell degranulation near a nerve bundle (bar = 1  $\mu\text{m}$ ); and (D) vascular congestion and haemorrhage (bar = 1  $\mu\text{m}$ ).

## Statistics

Results were analysed by Student's *t*-test and considered significantly different when  $p < 0.05$ .

## Results

### Morphology of the envenomed connective tissue

Disorganisation of collagen fibres, particularly near blood vessels and nerve bundles (Fig. 1A and 1B, respectively), mast cell degranulation (Fig. 1C) and intense vascular congestion (Fig. 1D) were observed 1 min after venom injection into the subcutaneous tissue of the scrotal bag of rats. Morphological alterations in venular endothelial cells were not clearly detected, although red blood cells were seen squeezing out through open endothelial cell junctions, characterising a *per diapedesis* type of haemorrhage (Fig. 2). Alterations such as disorganisation of collagen fibres and mast cell degranulation were not observed when the venom was injected into the s.c. tissue of clinically dead animals.

### Time-course of haemorrhage and oedema induced by the venom

Haemorrhage induced by 1.25  $\mu\text{g}$  of venom showed a peak at the 3rd hour after injection, persisting up to 6 h. Oedema was maximal from 30 min up to 3 h, decreasing after 6 h of venom injection. As both haemorrhage and oedema were maximal 3 h after venom injection, this time was selected to evaluate some parameters of the pharmacological mediation of these processes.

The haemorrhagic activity was dose-dependent, and the MHD obtained was 0.95  $\mu\text{g}$ . A dose of 5  $\mu\text{g}$  of venom was used for the study of the pharmacological mediation of the lesions.

### Mediation of venom-induced haemorrhage and oedema

With the dose of venom used, only local events were evaluated, as it was considered that envenomed mice do not present such systemic effects as blood incoagulability or thrombocytopenia (data not shown). Dexamethasone and indomethacin treat-

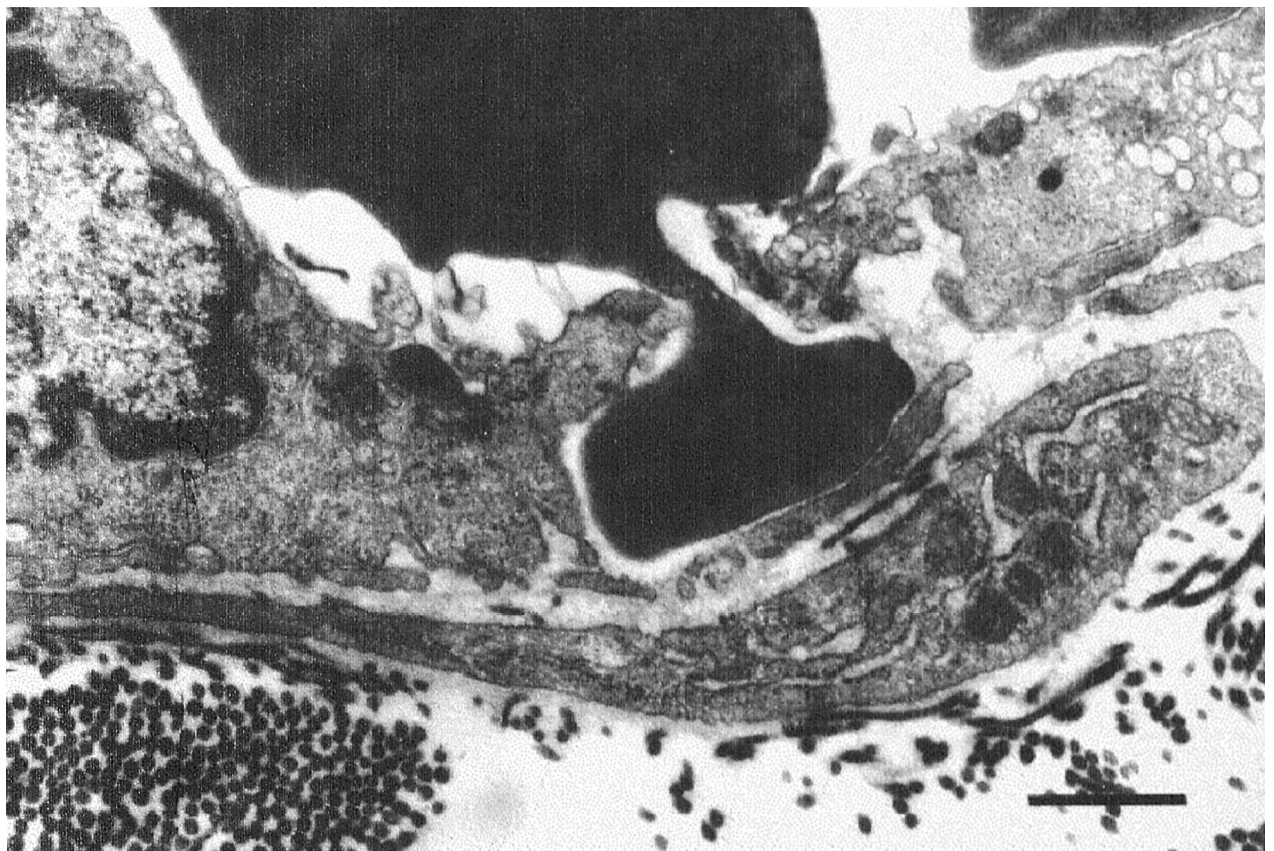


FIG. 2. Electron micrograph of a venule 1 min after injection of 2.5 µg of *B. jararaca* venom showing an erythrocyte escaping through an endothelial junction (bar=0.5 µm).

ments did not alter the haemorrhagic pattern induced by the venom, but significantly inhibited oedema (Fig. 3). Pre-treatment with morphine or methysergide inhibited both haemorrhage and oedema (Fig. 4), and guanethidine, surgical denervation and capsaicin treatments significantly inhibited haemorrhage, but not oedema induced by the venom (Fig. 5). In promethazine or mepyramine treated animals, oedema and haemorrhage were not affected.

## Discussion

Local haemorrhage induced by snake venoms is credited to metalloproteinases present in these venoms.<sup>6</sup> This effect has been associated with the activity of these toxins on proteins of the extracellular matrix.<sup>6,12</sup>

In the present study, the injection of a low dose of *B. jararaca* venom into the connective tissue of the scrotal bag of rats induced prompt and significant alterations to the morphology of this tissue. The premature manifestation of these alterations contrast with the long time (hours) needed for the *in vitro* degradation of proteins of extracellular matrix by haemorrhagic factors isolated from viperid ven-

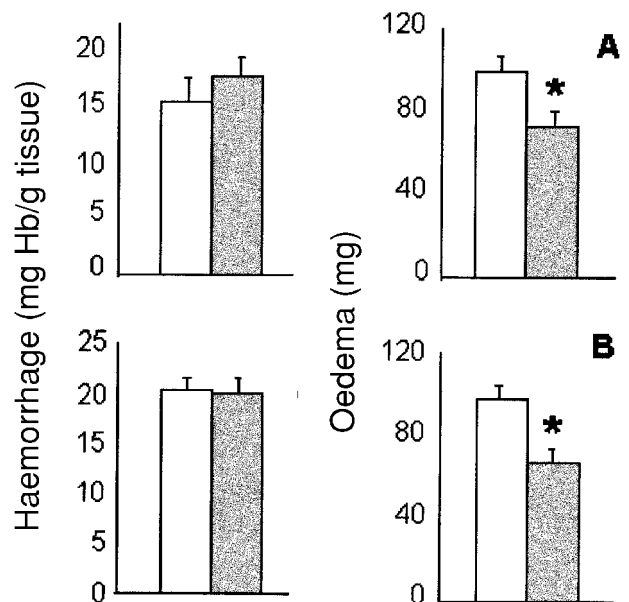


FIG. 3. Effect of treatments with (A) dexamethasone and (B) indomethacin on *B. jararaca*-induced haemorrhage and oedema. Mice treated with (A) dexamethasone (1 and 0.4 mg/kg, i.p., 24 and 1 h before the venom) or (B) indomethacin (30 mg/kg, s.c., 30 min before the venom) received 5 µg of *B. jararaca* venom into the foot pad. Haemorrhage and oedema in treated animals (dark bars) were compared with results obtained in saline treated control groups (white bars). Mean ± SE. \* $p < 0.05$ .

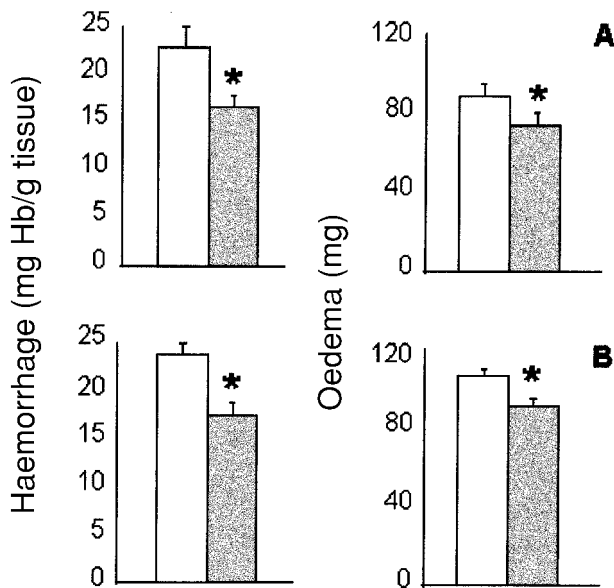


FIG. 4. Effect of treatments with (A) methysergide and (B) morphine on *B. jararaca*-induced haemorrhage and oedema. Mice were treated with (A) methysergide (0.8 mg/kg, s.c.) or (B) morphine (30 mg/kg, s.c.), 30 min before *B. jararaca* venom injection (5  $\mu$ g) into the foot pad. Haemorrhage and oedema in treated animals (dark bars) were compared with results obtained in saline treated control groups (white bars). Mean  $\pm$  SE. \* $p$ <0.05.

oms.<sup>13-17</sup> Moreover, the absence of alterations such as disorganisation of collagen fibres and degranulation of mast cells, when the venom was injected into the connective tissue of rats immediately after ceasing heartbeat, suggest that such an effect depends on the mediation of some factor(s) liberated from injected tissue, and possibly from the active blood circulation.

The possible participation of mediators from the damaged tissue in the pathogenesis of local haemorrhage induced by the venom was tested in paw of mice submitted to pharmacological or surgical treatments and compared with the mediation of the oedema induced by the venom in the same paw. As shown in the Results section, the major mediators of oedema induced by this venom were derivatives of arachidonic acid, since treatments with dexamethasone (phospholipase A<sub>2</sub> inhibitor) or indomethacin (cyclooxygenase inhibitor) were effective in inhibiting this effect. These results are in agreement with data from Trebien and Calixto<sup>19</sup> and Perales *et al.*<sup>21</sup> The participation of these mediators in the process was also demonstrated for other viperid venom-induced oedema.<sup>18,23</sup> While histamine seems to play a minor role in oedema induced by *B. jararaca* venom in rats,<sup>19</sup> in mice this vasoactive amine does not participate at all in the process, confirming previous observations made by Perales *et al.*<sup>21</sup> By contrast to the observations of these authors, we found that serotonin partially mediates venom-induced oedema,

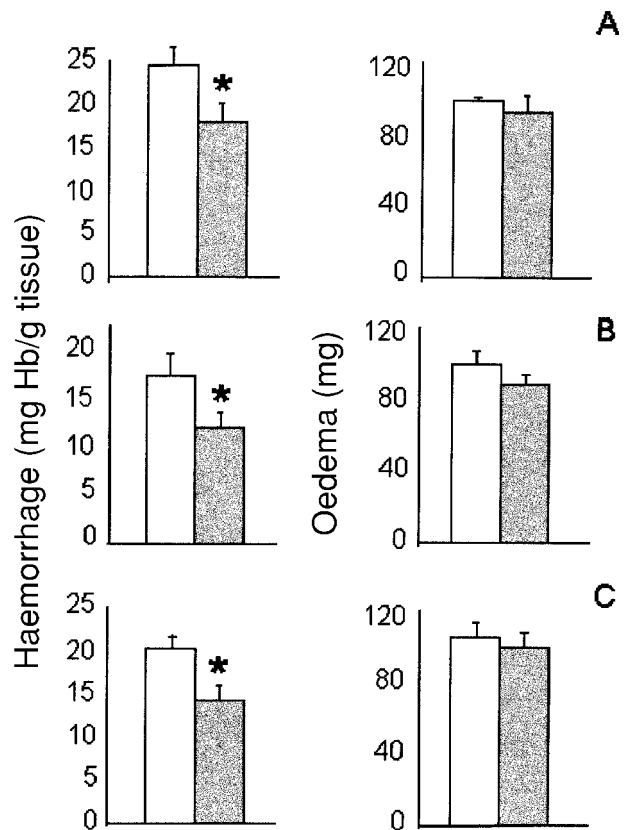


FIG. 5. Effect of treatments with (A) capsaicin, (B) guanethidine and (C) surgical denervation on *B. jararaca*-induced haemorrhage and oedema. Mice were treated with (A) capsaicin (50 mg/kg, s.c., on the 2nd day after the birth), (B) guanethidine (3 doses of 30 mg/kg, with 24 h intervals, lasting 24 h before the venom) or (C) surgical denervation by sciatic and saphenous nerve section, 7 days before the venom injection. Animals received 5  $\mu$ g of *B. jararaca* venom into the foot pad. Haemorrhage and oedema in treated animals (dark bars) were compared with results obtained in saline treated control groups (white bars). Mean  $\pm$  SE. \* $p$ <0.05.

considering that pre-treatment of the animals with methysergide decreased the intensity of the process.

The oedema induced by *B. jararaca* venom seems to be also mediated by opioid receptors, considering that treatment of the animals with morphine inhibits the process. This inhibitory effect of morphine was also demonstrated for oedema caused by other viperid venom (*Trimeresurus flavoviridis*)<sup>20</sup>, as well as that caused by other flogistic agents such as carrageenan.<sup>34</sup> Nevertheless, the mechanisms which underlie the anti-oedematogenic effect of morphine are not yet understood.

Our results indicate that the mediation of oedema formation differs from that involved in the induction of haemorrhage. Based on results obtained in mice pre-treated with dexamethasone or indomethacin, arachidonic acid derivatives seems not to participate in the local haemorrhage induced by *B. jararaca* venom, confirming previous observations by Perales *et al.*<sup>21</sup> Conversely, in mice pre-treated with guanethi-

dine, capsaicin or surgical denervation, local haemorrhage but not oedema was attenuated. On the other hand, methysergide or morphine significantly diminished both oedema and haemorrhage, indicating that, at least in mice, serotonin and opioid receptors are partially implicated in both effects. The fact that partial inhibition was obtained with these treatments suggests that haemorrhage and oedema are indeed multimediated phenomena.

The participation of opioid receptors in inflammatory events, as well as the relationship between antidromic stimulation of sensitive nerve fibres and vascular permeability disturbance, have been demonstrated.<sup>20,34-43</sup> It is known that neuropeptides, particularly substance P, can induce a direct vascular disturbance or may act indirectly by inducing liberation of histamine from mast cells.<sup>39</sup> Added to this hypothesis is the fact that the blockade of opioid receptors (e.g. treatment with morphine) can prevent the liberation of substance P.<sup>44,45</sup> These results, and the inhibition of haemorrhage observed in animals treated with capsaicin or which are chronically denervated, strongly indicate that haemorrhage induced by the venom could be classified as a neurogenic type of haemorrhagic inflammatory response. Reinforcing this interpretation is the fact that neurogenic vascular disturbances can be inhibited by sectioning nerve routes or by chemical depletion of neuropeptides after capsaicin treatment.<sup>36</sup>

Taken together, these data might explain the explosive alterations in the connective tissue of rats 1 min after venom injection. Malucelli and Mariano<sup>40</sup> also observed a similar phenomenon of instantaneous haemorrhagic response on the diaphragm of guinea pigs after electric or chemical stimulation of the phrenic nerve. These authors described a *per diapedesis* haemorrhage in diaphragmatic vessels. Furthermore, this type of haemorrhage was also observed after *T. flavoviridis* venom injection.<sup>46</sup> Conversely, after intramuscular injection of various viperid crude venoms or isolated haemorrhagic factors, the usual feature is a *per rhexis* haemorrhage observed in capillaries.<sup>47-49</sup> Whether these differences are due to the dose of venom injected, the route of injection or other peculiar characteristics of the microvasculature in these tissues remains to be investigated. It must be considered that *per diapedesis* haemorrhage is mainly observed in venules while *per rhexis* haemorrhage is reported to occur mainly in capillaries. Nevertheless, only vessels greater than 20 µm in diameter, like venules, are innervated.<sup>50</sup> This fact and results here described indicate that *per diapedesis* haemorrhage induced by *B. jararaca* venom can be controlled by neurogenic factors. Further studies should be made in order to identify the specific neurogenic factor(s) involved in this process.

In conclusion, our results suggest that local haemorrhage induced by *B. jararaca* venom is partially mediated by serotonin and by neurogenic factors. Furthermore, they show that oedema and local haemorrhage induced by this venom have distinct chemical mediation.

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