Rev. Inst. Med. trop. São Paulo
26(5):247-253, *setembro outubro,* 1984

UDC 616-022.9

HISTOPATHOLOGICAL CHANGES CAUSED BY VENOM OF URUTU SNAKE (BOTHROPS ALTERNATUS) IN MOUSE SKELETAL MUSCLE (1)

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SUMMARY

Venom of urutu snake **(Bothrops alternatus)** injected into the Tibialis anterior muscle (Tib. ant.) of mice in a dose of 80 μg induced massive local haemorrhage within 10 min. Though muscle fibres appeared normal at this stage they later suffered necrosis in increasing numbers so that by 24 hr the whole muscle was necrotic. Arteries near the injection site often showed hyaline necrosis of the media and some were thrombosed. Phagocytosis of debris, which progressed from the periphery towards the centre of the necrotic area was usually complete by 2 weeks and was accompanied by muscle fibre regeneration. After 1 or 2 months several animals showed extensive recovery of the damaged muscle though a localized scar often remained. The regenerated muscle fibres showed central nuclei and varied in diameter, many appearing atrophic. In other mice, however, there was replacement of Tib. ant. by fibroadipose tissue with little or no muscle fibre regeneration. The results show that despite severe initial haemorrhage and necrosis, the affected muscles exhibit considerable capacity for regeneration. It is suggested that the poor regenerative response observed in some animals could result, at least to some extent, from permanent damage to the local blood vessels.

INTRODUCTION

Snakes of the genus **Bothrops**, responsible for some 90% of human snake bite accidents in São Paulo and environs [4,15] produce rapidly spreading oedema, haemorrhage and necrosis of the bitten extremity, often with disabling consequences [3]. In Brazil, the pathology of the local lesions of **Bothrops** envenomation has been studied mainly in the skin [5,16,18]. The effects of Brazilian snake venoms on skeletal muscle, presumably important for the pathogenesis of sequelae [7,10], have been the subject of little experimental work.

This paper reports the pathological changes caused in mice by intramuscular injection of venom of the "urutu" **(Bothrops alternatus,** Duméril, Bibron et Duméril, 1854), a snake widely distributed in Southern Brazil, whose bites are held popularly to be "crippling if not killing" [6]. Attention was given to the acute effects and to the regenerative capacity of skeletal muscle after challenge by venom.

MATERIAL AND METHODS

Desiccated whole venom of **Bothrops alternatus** purchased from Butantan Institute in São Paulo was donated by the Dept. of Biochemistry, UNICAMP. Venom crystals were dissolved just before use in sterile physiological saline to final concentrations of 50, 200 and 800 μg/ml.

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Adult male albino mice (25-30 g) were lightly anaesthetised with ether and 0.1 ml of venom solution was injected percutaneously into the upper two-thirds of the right Tibialis anterior muscle. After survival times ranging from 10 min to 2 months the mice were killed under deep ether anaesthesia by intracardiac perfusion of 40 ml of formol-calcium fixative (40% formaldehyde — 10 ml; distilled water — 90 ml; calcium acetate — 1 g). Usually 4 — 6 animals were used for each time and dose. The right hindlimbs were severed at the thigh, post-fixed overnight in the same fixative and then for 6 hr in FAM (40% formaldehyde — 10 ml; glacial acetic acid — 10ml; absolute methanol — 80 ml), decalcified for 2 days in formic-citrate solution (98% formic acid — 35 ml; 20% sodium citrate — 65 ml) and dehydrated for paraffin embedding. Serial transverse blocks of the whole limb were prepared by the method of BEESLEY & DANIEL[1] and 6 μm paraffin sections were stained with HE. In a few mice Tibialis anterior and Extensor digitorum longus were taken out as a single block immediately after perfusion fixation, post-fixed in FAM embedded flat in paraffin. Serial longitudinal sections were cut at 6 μm and stained with HE.

RESULTS

Control group

Mice injected with 0.1 ml saline in the Tibialis anterior muscle (Tib. ant.) and killed after 1 week showed leg muscles of normal histological appearance except for a small group of muscle fibres with central nuclei in the deep region of Tib. ant. These were interpreted as regenerated fibres after damage by the needle. There was no inflammatory infiltrate or fibrosis.

Mice injected with urutu venom

Clinical and macroscopic findings

A dose of **5 μg** of venom produced slight oedema of the limb in about 1 hr. Limb movements were preserved. The animals showed no general ill effects and fed normally. One hr after **20 μg** there was moderate local swelling and darkish discolouration of the injection site; toe movements were diminished and the mice moved around less than normally, but all eventually recovered. A dose of **80 μg** caused intense swelling and darkening of the injected limb in a few minutes. In mice injected under direct vision petechial haemorrhages were first seen at 4 — 5 min, and soon became confluent. Flexion and extension of the toes were abolished, the animals stayed quiet, eyes closed and refused food and water. A few hours later blood oozed from the needle puncture and remained uncoagulable. By 24 hr about 50% of the animals had died. Autopsy showed a layer of blood over the thigh and leg muscles of the injected limb, and widespread subcutaneous haemorrhages elsewhere, but no bleeding in serous cavities or viscera. Histological examination of lung, heart, liver, spleen and kidney was unremarkable. Surviving mice regained toe movements in a few days. Subcutaneous haemorrhage at the injection site was reabsorbed in about a week.

Microscopical observations

10 min to 24 hr

Only the dose of 80 μg was used in this group. At **10 min**, abundant extravasated red blood cells were found among muscle fibres in Tib. ant. (Fig. 1) and extended as far back as the superficial region of gastrocnemius, hamstrings and popliteal fossa. Muscle fibres looked normal except for a few ones in Tib. ant. which were pale, homogenous and had pyknotic nuclei. No myofibrils could be distinguished in these fibres, which were considered to be necrotic. At **30 min** the number of necrotic fibres had increased (Fig. 2). In longitudinal sections they were characterized by waxy hyaline appearance without cross striations. By **6 hr** very few surviving fibres remained in Tib. ant. (Fig. 3) and none were left at **24 hr.** Arteries in this muscle and its vicinity had necrotic appearance. There was little or no inflammatory infiltrate between the necrotic fibres, and only very few fibres at the periphery of the muscle were undergoing phagocytosis. The amount of haemorrhage remained relatively constant after 30 min.

All pictures show HE stained paraffin sections of mouse Tibialis anterior muscle injected with 80 μg of **B. alternatus** whole venom.

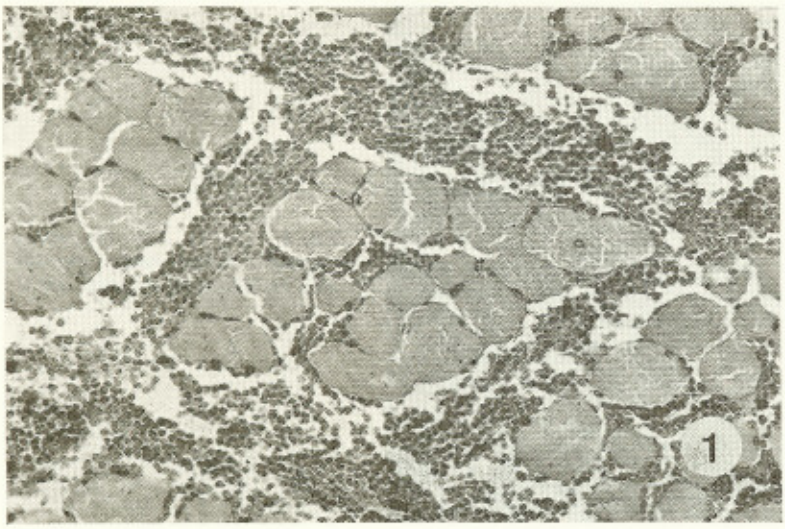

Fig. 1 — 10 min. Extensive haemorrhage between muscle fibres, which have normal appearance. 400 ×

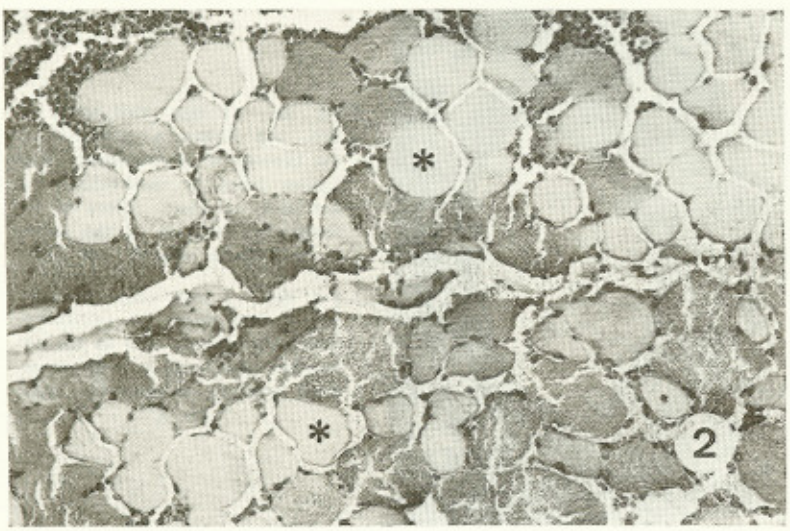

Fig. 2 — 30 min. Necrotic muscle fibres (*) stand out through their pale homogeneous appearance without distinct myofibrils. Note myofibrillar appearance of normal fibres. 400 ×

3 days to 1 week

From this stage on doses of 5, 20 and 80 μg were used. At **3 days** Tib. ant. showed an area of necrosis the size of which depended roughly on the venom dose. After 5 μg necrosis involved about one fourth to one half of the cross sectional area of Tib. ant., other muscles being spared. After 80 μg the whole of Tib. ant. and several neighbour muscles were necrotic. Phagocytosis of necrotic fibres advanced centripetally and **1 week** after doses of 5 or 20 μg the necrotic fibres had been removed, giving way to a punched out area of granulation tissue with neutrophils, lymphocytes, plasma cells, a few pre-existing muscle fibres and numerous small regenerating myotubes. With 80 μg a large proportion of the necrotic fibres, sometimes the whole Tib. ant. muscle had not been phagocytosed yet after 1 week. Breakdown of debris and formation of granulation tissue were observed only at the edge of the muscle, while the centre remained necrotic and acellular. Deep

arteries of the leg and those in Tib. ant. often showed hyaline necrosis of the media. A few arteries and veins were thrombosed or recanalized (Fig. 4a,b).

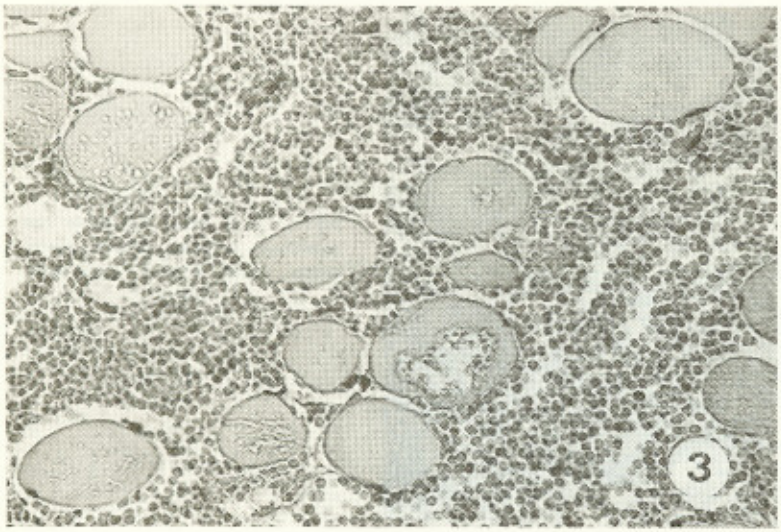

Fig. 3 — 6 hr. Tib. ant. shows extensive haemorrhagic necrosis with no inflammatory cells. 400 ×

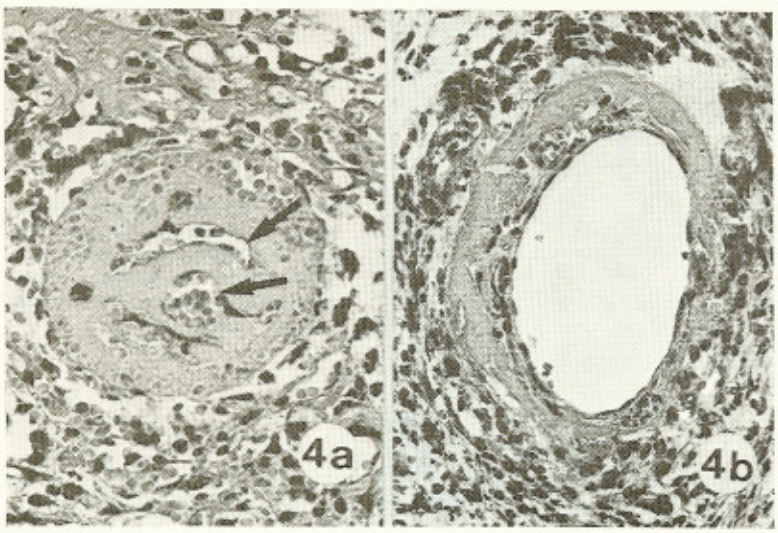

Fig. 4a — 4 days. This artery of Tib. ant. shows a thrombus in the process of recanalization. New lumina lined by endothelial cells are seen (arrows). There is inflammatory reaction in the necrotic muscle nearby. 400 ×

Fig. 4b — 1 week. Hyaline necrosis of the media of an artery, with inflammatory cells in the adventitia. The lumen is endothelium-lined and no thrombus is seen. 400 ×

2 weeks to 2 months

Two weeks after 5 or 20μg of venom many regenerated muscle fibres were present in Tib. ant. They were rounded or oval, had one or two central nuclei and were rich in myofibrils, but varied a great deal in size: most were one half to one third the diameter of preexisting fibres, others were minute. At **1 and 2 months** the injected muscles had recovered almost normal appearance, except for the persistence of central nuclei, some variation in fibre size and in some cases a localized area of fibrosis and/or newly formed fatty tissue in Tib. ant.

In most animals receiving 80 μg of venom, removal of the necrotic muscle fibres was complete by the end of the **2nd week.** A few mice injected with this dose (but none of lower doses or controls) developed subcutaneous or intramuscular abscesses at the injection site. In animals without abscesses regeneration of the muscle fibres in Tib. ant. took place to variable extent so that at **1 or 2 months** many mice had

recovered much of the original bulk of the muscle. However, there was marked variation in fibre size (Fig. 5) and an atrophic scar was often seen. In some animals Tib. ant. was replaced almost completely by fibroadipose tissue (Fig. 6). Regenerated muscle fibres were rare, atrophic and surrounded by collagen fibres.

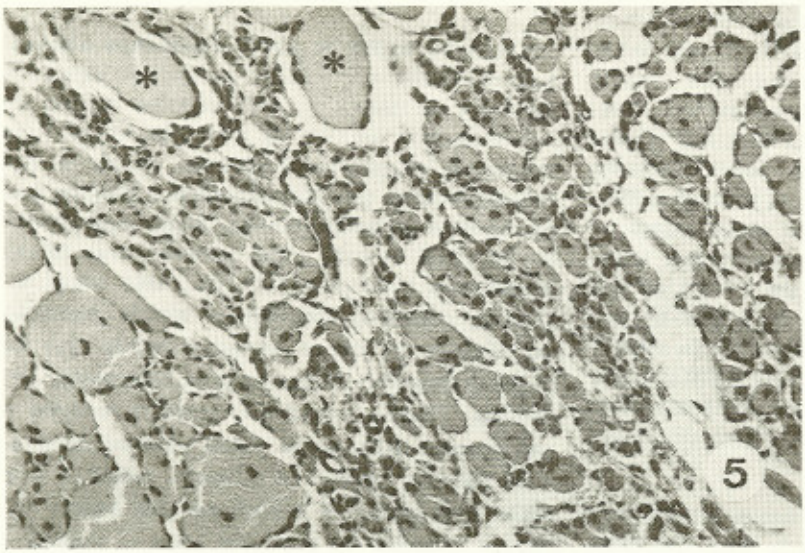

Fig. 5 — 2 weeks. Regenerated fibres in the injected muscle show variable diameter, some being very small, and contain central nuclei. Preexisting fibres (*) have peripheral nuclei. 400 ×

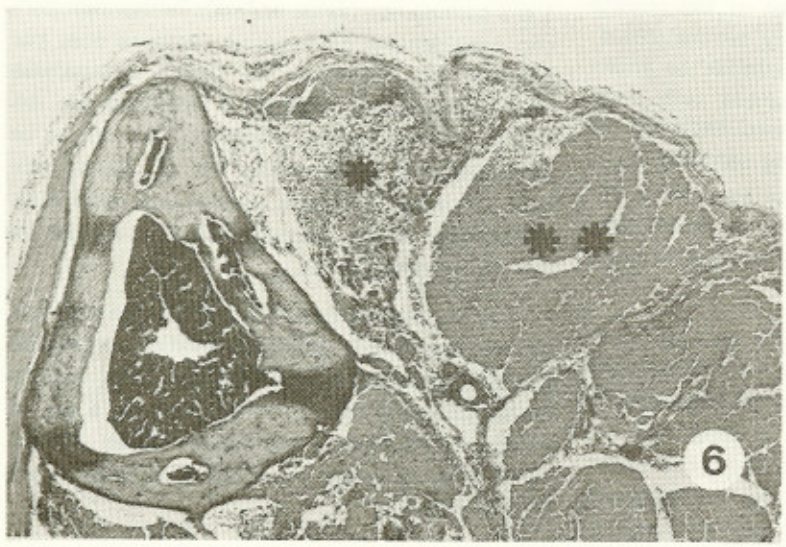

Fig. 6 — 2 months. In this animal the Tib. ant. muscle (*) has been replaced by fibrofatty tissue. The Extensor digitorum longus muscle (**) has normal size and appearance. 40 ×

DISCUSSION

The present results indicate that **B. alternatus** venom causes pronounced local haemorrhage within a few minutes of injection. The time lag was comparable to those observed after local injection of other haemorrhagic venoms (e. g. **Crotalus atrox**) [12] or purified fractions [9,11]. Necrosis of the muscle fibres set on only after haemorrhage. Whether myonecrosis is attributable to primary venom action, or is secondary to collapse of the microcirculation cannot be decided here. **In vitro** preparations of skeletal muscle exposed to venom could help settle the point.

The early effects of **B. alternatus** venom differ materially from those of **B. jararacussu** ve-

nom, which causes rapid muscle necrosis with little haemorrhage [14]. Necrotic muscle fibres after jararacussu venom break up into bands of hyaline myofibrillar material separated by empty-looking spaces ("myolytic type" of myonecrosis [8]). By contrast, necrotic fibres resulting from urutu envenomation were pale, waxy and homogeneous through considerable lengths ("coagulation type" of myonecrosis [8]). Reasons for such difference are unclear, but it may mean that the two venoms cause necrosis through diverse mechanisms, i.e., through differently acting components. The rapid onset of myonecrosis after **B. jararacussu** venom suggests a direct toxic action of the venom on the muscle fibres, an assumption supported by isolation of a fraction which is myolytic **in vitro** [17] and **in vivo** (QUEIROZ & SANTO NETO, unpublished). As far as we know no myotoxic components have yet been isolated from urutu venom.

In spite of the massive haemorrhagic necrosis of Tib. ant., particularly with the dose of 80 μg, many mice showed considerable muscle fibre regeneration, and most of the muscle in these animals was eventually reconstituted. The regenerated fibres were similar to those seen after necrosis by other agents (e.g. local anaesthetics) [2] but usually showed variation in diameter, many fibres remaining atrophic. These appearances resembled those seen after jararacussu venom and could perhaps be attributed to deficient reinnervation of the regenerated fibres.

Complete replacement of Tib. ant. by fibroadipose tissue observed in some animals could have resulted from permanent obliteration of the major blood vessels as a consequence of vascular necrosis and thrombosis in the early stage. Though definite conclusions on this point cannot be drawn from the present material, it has been shown experimentally as well as in patients that vascular lesions caused by venom of the Japanese crotalid snake "Habu" **(Trimeresurus flavoviridis)** may aggravate the effects of the venom and severely hinder muscle regeneration [7]. It is possible that gangrene and slough of whole extremities in humans following bites by urutu and other snakes [3,13] could at least partly be explained in this way.

RESUMO

Estudo histopatológico das lesões causadas pelo veneno de urutu (Bothrops alternatus) **em músculo esquelético de camundongos**

Veneno bruto de urutu **(Bothrops alternatus)** dissolvido em solução salina fisiológica foi injetado no músculo tibial anterior direito de camundongos adultos na dose de 80 μg. Os músculos foram examinados em cortes de parafina, corados por Hematoxilina e Eosina. Aos 10 minutos já havia intensa hemorragia difusa no M. tibial anterior, mas apenas raras fibras musculares estavam necróticas. Nas horas seguintes, contudo, observou-se rápido aumento do número de fibras afetadas, sendo que às 24 hs o músculo apresentava-se totalmente necrótico. Vasos sangüíneos intramusculares e nas proximidades do M. tibial anterior mostravam necrose hialina da camada média e por vezes trombose. A fagocitose dos restos celulares ocorreu da periferia para o centro e acompanhou-se de regeneração muscular. Após 1 a 2 meses, em vários animais houve recuperação considerável do músculo, embora com persistência de cicatriz. As fibras regeneradas possuiam núcleos centrais e variavam em diâmetro, estando muitas atróficas. Em outros camundongos a regeneração do M. tibial anterior foi muito precária, tendo este sido substituído por tecido fibroadiposo com apenas raras fibras musculares.

Os resultados mostram que, apesar da gravidade das lesões iniciais devidas ao veneno, ocorre regeneração muscular em grau variável de animal para animal. Sugere-se que a má regeneração observada em alguns casos poderia ser devida, ao menos em parte, a dano vascular permanente.

ACKNOWLEDGEMENTS

The Authors are grateful to Mr. J.C. Novello for the gift of venom, to Prof. J. Lopes de Faria for review of the manuscript, to Mr. Ismael Vêncio for the histological preparations, and to Messrs. Sebastião Henrique Militão and Joaquim Francisco do Prado who looked after the animals.

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Recebido para publicação em 30/11/1983.