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Effect of perimuscular injection of *Bothrops jararacussu* venom on plasma creatine kinase levels in mice: influence of dose and volume

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Abstract

The effect of dose and volume of a perimuscular injection of *Bothrops jararacussu* venom on myonecrosis of skeletal muscle was studied in mice. An increase of the venom dose (0.25 to 2.0 µg/g) at a given volume (50 µl) resulted in an increase in plasma creatine kinase (CK) levels 2 h after injection. Plasma CK activity increased from the basal level of 129.27 ± 11.83 (N = 20) to 2392.80 ± 709.43 IU/l (N = 4) for the 1.0 µg/g dose. Histological analysis of extensor digitorum longus muscle 4 h after injection showed lesion of peripheral muscle fibers, disorganization of the bundles or the complete degeneration of muscle fibers. These lesions were more extensive when higher doses were injected. Furthermore, an increase in volume (12.5 to 100 µl) by dilution of a given dose (0.5 µg/g) also increased plasma CK levels from 482.31 ± 122.79 to 919.07 ± 133.33 IU/l (N = 4), respectively. These results indicate that care should be taken to standardize volumes and sites of venom injections.

Key words
- *Bothrops* venoms
- Myotoxicity
- Skeletal muscle
- Plasma creatine kinase

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Local myonecrosis is a serious manifestation of snake bites causing prolonged and sometimes permanent disability. A number of snake venoms induce histological alterations when injected into or close to muscle tissue (1-5). The myotoxic activity of snake venoms can be monitored by the increase of plasma creatine kinase (CK) activity and morphological analysis. The increase in plasma CK levels results from sarcolemmal damage due to myotoxic components of the venom (1-3,6-10).

In the present study, by using a different method to inject the crude venom over the muscle tissue rather than directly into it, we investigated whether not only the dose, but also the volume of the venom injected can affect plasma CK activity. Adult Swiss mice (20-25 g) were lightly anesthetized with diethyl ether and injected with four different doses of *Bothrops jararacussu* crude venom (0.25-2.0 µg/g) in a final volume of 50 µl. The solution was positioned over the extensor digitorum longus (EDL) muscle. The venom was not injected directly into the muscle as previously described (7-10), but was inoculated under the tibial anterior muscles and the tibia, close to the external

surface of the EDL muscle. Thus, the venom solution should have surrounded the whole muscle, bathing the epimysium and the gutter where the muscle works. In addition, the venom was applied at the single dose of 0.5 µg/g in four volumes, 12.5, 25.0, 50.0 and 100.0 µl. Venom was diluted in physiological saline (NaCl). Myotoxicity is reported as an increase in plasma CK activity.

Blood samples were collected before and 2 h after venom injection. The serum was separated and stored at 4ºC for subsequent determination of plasma CK activity using a diagnostic kit (Diagnostica Merck, São Paulo, SP, Brazil). Enzyme activity is reported as IU/l, where 1 IU is the amount that catalyzes the transformation of 1 µmol of substrate (NADH) per min at 25ºC (2). Basal plasma CK activity was that obtained for the blood samples from each group collected before the injection of either venom or NaCl.

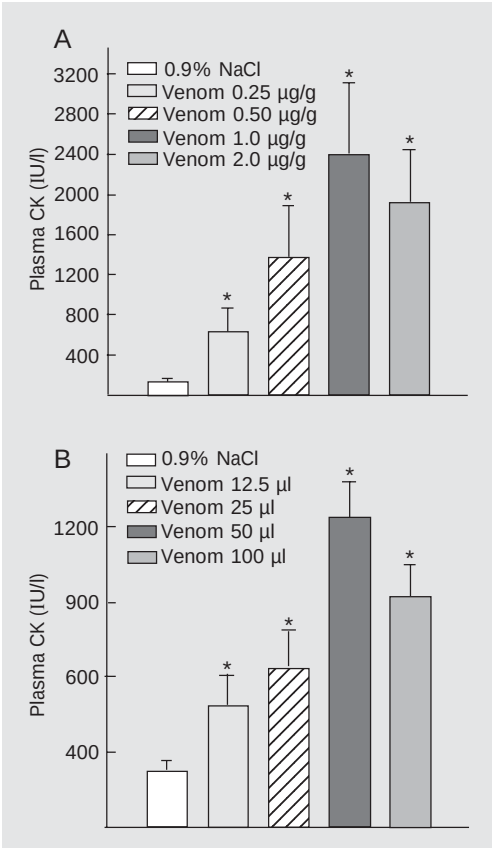

Figure 1. Effects of dose and volume on *in vivo* myotoxicity of *Bothrops jararacussu* venom in mice. *A*, The venom protein dose was increased from 0.5 to 2.0 µg/g in the same volume (50 µl). Plasma creatine kinase (CK) levels were measured 2 h after venom injection. *B*, Effect of injection volume. Venom, 0.5 µg/g was injected in increasing volumes, 12.5 to 100 µl, and plasma CK levels were measured 2 h after injection. In both protocols the control group received 50 µl of 0.9% NaCl. Data are reported as means ± SEM (N = 4). *P<0.05 compared to the control group (ANOVA).

Histological analysis was performed 4 h after venom injection. The mice were killed under ether anesthesia and the right posterior EDL muscles were dissected. Fragments of this muscle were immersed in fixative solution (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 2 to 4 h. The fragments were then washed in buffer and fixed again for 1 h in 1% aqueous OsO_4. The tissue was dehydrated in acetone and embedded in Polybed 812 resin. Semi-thin sections (500 nm) were obtained for light microscopy and stained with 1% toluidine blue (1,3,5).

Figure 1A shows the effect of increasing the concentration of the dose of the *B. jararacussu* venom on plasma CK levels. CK activity increased from the basal level of 129.27 ± 11.83 (N = 20) to 618.30 ± 242.98 IU/l (N = 4) for the 0.25 µg/g dose and up to 2392.80 ± 709.43 IU/l (N = 4) for the 1.0 µg/g dose. Doses higher than 1.0 µg/g induced the maximum effect (data not shown). These results indicate that plasma CK levels depend on the doses of venom. It is interesting to note that plasma CK activity was lower (1917.90 ± 530.47 IU/l, N = 4) at 2.0 µg/g, but not significantly different from the activity observed at the dose of 1.0 µg/g (2932.50 ± 709.43 IU/l, N = 4) of *B. jararacussu* venom. One possible explanation for this plateau of plasma CK activity is the interplay of local venom effects. Although the amount of CK released from the damaged muscle was greater, the local hemorrhage, edema and local stasis caused by the venom did not allow the washout of CK to the capillaries.

Figure 1B shows the effect of a single dose, 0.5 µg/g of *B. jararacussu* venom injected in four different volumes, 12.5, 25.0, 50.0, and 100.0 µl on plasma CK levels. The injection of 0.5 µg/g of the venom dissolved in 12.5 µl NaCl increased basal plasma CK activity from 128.53 ± 19.65 IU/l (N = 20) to 482.31 ± 122.79 IU/l (N = 4), while the injection of the same dose of the venom dissolved in 50 µl NaCl increased CK activ-

ity to 1239.95 ± 144.85 IU/l (N = 4), showing an increase in plasma CK activity in a volume-dependent fashion. A plausible mechanism for the increased lesion occurring with increasing injection volumes is that the venom comes into contact with a larger area surrounding the muscle, and is thus able to reach and affect more muscle fibers. However, we cannot explain why 100 µl did not result in the highest CK activity.

Figure 2A-C demonstrates the effects of increasing venom doses on the histology of muscle fibers. The figure shows a panoramic view of transverse sections of EDL muscle 4 h after receiving 50 µl injections of NaCl (Figure 2A), 0.25 µg/g of venom (Figure 2B), and 2.0 µg/g of venom (Figure 2C). These data suggest that increasing concentrations of venom result in increasing muscle lesions, and further demonstrate that increasing volumes of the injection can also increase these lesions and plasma CK levels. These results indicate that care must be taken in standardizing and reporting injection doses and volumes in future studies to ensure consistency and reliability of the experimental conditions.

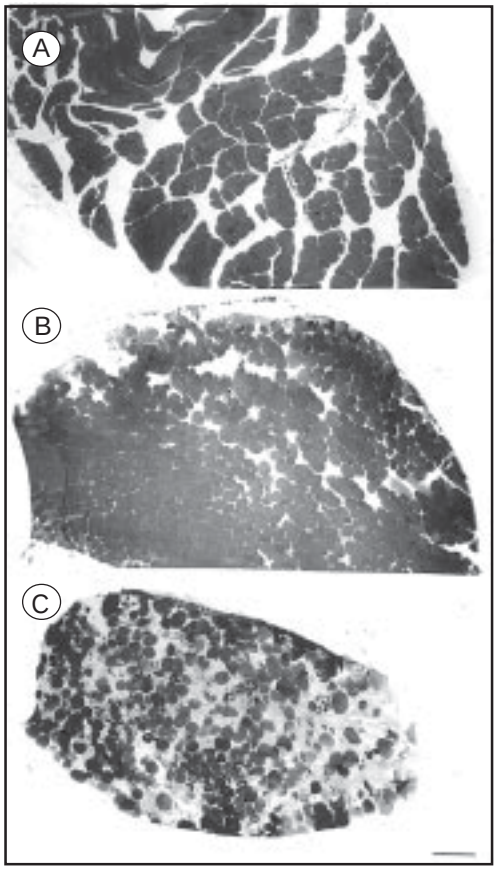

Figure 2. Transverse sections of extensor digitorum longus muscle 4 h after receiving *A*: 50 µl NaCl injections, *B*: 0.25 µg/g of venom, and *C*: 2.0 µg/g of venom. *A*, Panoramic view of control muscle showing normal cells and organization. In *B* note the lesion of peripheral muscle fibers and the disorganization of the bundles. In *C* note the complete degeneration of muscle fibers (bar = 110 µm).

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