

Low-intensity laser therapy improves tetanic contractions in mouse anterior tibialis muscle injected with *Bothrops jararaca* snake venom

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Abstract Introduction: Envenomation by *Bothrops* snakes can produce local pain, edema, hemorrhage and myonecrosis. However, standard antivenom therapy is generally ineffective in neutralizing these effects so that alternative methods of treatment have been investigated. In experimental animals, low-level laser therapy (LLLT) attenuates the local effects of *Bothrops* venoms, but the benefits of LLLT on muscle function after envenomation are unclear. In this study, we examined the influence of LLLT on the contractile activity of mouse skeletal muscle injected with venom from *Bothrops jararaca*, the principal cause of snakebite in southeastern Brazil. **Methods:** Twenty-seven male mice were used. Mice were injected with venom (40 µg in 50 µl) in the right anterior tibialis muscle, after which the muscle tendon was exposed, connected to an isometric transducer and subjected to a resting tension of 1 g. A bipolar electrode was attached to the tibial nerve for electrical stimulation. The mice were randomly allocated to five groups: A – Control (n = 3), B – Venom 3 h (n = 6), C – Venom 9 h (n = 6), D – Venom + Laser 3 h (n = 6), E – Venom + Laser 9 h (n = 6). **Results:** The two groups that received LLLT post-venom showed improved muscle contraction and contracture in relation to muscle treated with venom alone. **Conclusion:** These results indicate that LLLT can improve muscle function after damage induced by *B. jararaca* venom.

Keywords: *Bothrops jararaca*, Low-level laser therapy, Muscle activity, Muscle fatigue, Snake venom, Tetanus.

Introduction

Bothrops snakes account for 90% of venomous snakebites in Brazil, with *Bothrops jararaca* being responsible for most of these cases, especially in southeastern Brazil (Brasil, 2011; França and Málaque, 2009; Leobas et al., 2016). Envenomation by *Bothrops* species can result in local effects (pain, edema, inflammation, hemorrhage and necrosis) and systemic effects (coagulopathy, systemic hemorrhage, circulatory shock and renal damage) (França and Málaque, 2009; Warrell, 2004). In severe cases of envenoming, myonecrosis mediated predominantly by basic myotoxic phospholipases A₂ (PLA₂) in these venoms (Gutiérrez and Ownby, 2003; Montecucco et al., 2008) can result in important loss of muscle mass, with permanent damage to the bitten limb and loss of function. Part of this myotoxicity is attributable to ischemia caused by impaired circulation as a result of microvascular damage (Gutiérrez and Ownby, 2003; Queiróz et al., 1984).

The most effective treatment for envenomation by *Bothrops* species is antivenom therapy. However, despite its effectiveness in neutralizing systemic effects such as coagulopathy (França and Málaque, 2009;

Warrell, 2004), antivenom is generally ineffective in neutralizing local reactions (Barbosa et al., 2009; Guimarães-Souza et al., 2011; Nadur-Andrade et al., 2012; Pereira et al., 2009; Picolo et al., 2002). Indeed, a major problem in the treatment of *Bothrops* venom-induced myonecrosis is the rapid onset of this damage (within 15 min of venom injection) that makes treatment virtually impossible (Gutiérrez et al., 2007). Consequently, various alternatives have been investigated to reduce the local effects of myotoxicity caused by bothropic venoms, including the use of medicinal plants (Cavalcante et al., 2007; Collaço et al., 2012a; 2012b; Ferraz et al., 2012; 2014; Pereira et al., 2012; Tribuiani et al., 2014) and substances such as heparin and polyanions (Lomonte et al., 1994; Melo et al., 1993). In addition, low-intensity laser therapy (LLLT) has been investigated as a potential treatment for experimental envenomation (Barbosa et al., 2008; 2009; 2010; Doin-Silva et al., 2009; Dourado et al., 2003; Nadur-Andrade et al., 2012).

The effects of LLLT on biological tissues are very broad and induce trophic-regenerative, anti-inflammatory and analgesic actions, as well as

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enhanced local microcirculation, as demonstrated by studies *in vitro* and *in vivo* (Barbosa-Souza et al., 2011; Oliveira et al., 2013). This beneficial effect on the microcirculation is related to an indirect action of the laser on precapillary sphincters through the release of chemical mediators that maintain a functional local peripheral microcirculation (Maier et al., 1990; Miró et al., 1984). In spite of various studies have examined the potentially beneficial effects of LLLT in animal models of pain, edema, local inflammation, hemorrhage and myonecrosis (Medeiros et al., 2010; Oliveira et al., 2013), to date there has been no assessment of the influence of LLLT on the contractility of venom-injected skeletal muscle. In this study, we examined the effect of LLLT on the function of mouse tibial muscle injected with *B. jararaca* venom.

Methodology

Twenty-seven male Swiss mice (18-22 g), obtained from Animais de Laboratório (Anilab, Paulínia, SP), were housed six/cage in the Laboratory of Physiology and Pharmacodynamics of the Institute for Research and Development at the Universidade do Vale do Paraíba (UNIVAP). The mice were maintained on a 12 h light/dark cycle at 22-26 °C, with free access to food and water. Prior to use, the mice were randomly allocated to five groups: A – Control (n = 3), B – Venom 3 h (n = 6), C – Venom 9 h (n = 6), D – Venom + Laser 3 h (n = 6) and E – Venom + Laser 9 h (n = 6). The animal experiments approved by the institutional Committee for Ethics in Animal Use at UNIVAP (protocol no. CEUA/2012/A01). The experiments were done within the ethical guidelines of the Brazilian Society for Laboratory Animal Science (SBCAL).

Venom was extracted from adult specimens of *B. jararaca* maintained at the serpentarium at UNIVAP. The venom was extracted manually and stored at -80 °C until lyophilized. Storage was subsequently at 4 °C until use.

For the experiments, the right anterior tibialis muscle was used, as described by Giaretta et al. (2015). Mice were injected with *B. jararaca* venom (40 µg in 50 µl of 0.9% saline); this dose of venom was chosen based on previous publications by our group (Barbosa et al., 2010; Dourado et al., 2011). The tibialis muscle of control mice was injected with an equal volume of 0.9% saline. Mice treated with LLLT received irradiation (InGaAlP laser equipment, Twin Flex Evolution®, MMOptics) 30 min after venom injection, operated in direct contact mode (parameters described in Table 1).

At 3 h and 9 h post-injection, the mice were anesthetized (xylazine – 20 mg/kg, i.p.,

Table 1. Laser parameters.

Parameters	Values
Energy density (J/cm ²)	3
Energy (J)	0.6
Power (mW)	35
Irradiation time (s)	17
Spot (cm ²)	0.19
Wavelength (nm)	660 ± 5

ketamine – 100 mg/kg, i.p., atropine – 0.25 mg/kg, i.p., and diazepam – 5 mg/kg, i.p.), fixed on a surgical table and the tendon of the right anterior tibialis muscle and the tibial nerve were exposed. In the region of insertion close to the metatarsal plantar region, the muscle tendon was connected to an isometric transducer and the nerve was connected to a bipolar electrode.

The muscle was subjected to a constant tension of 1 g and stimulated indirectly by individual pulses from a Grass Technologies model S48® square pulse stimulator. Pulses (4-8 mV, 0.2 Hz, 2 ms) were applied for 3 min to determine the minimum and maximum voltages needed to induce muscle contraction with recruitment of all fibers (Doin-Silva et al., 2009; Lopes-Martins et al., 2006). Tetanic stimuli were applied by increasing the frequency to 50 Hz for 10 s and the tetanic response was recorded. Six tetanic stimuli were applied at 3 min intervals. During these intervals, muscle intactness and normal contractility were assessed using the stimulus parameters described above (4-8 mV, 0.2 Hz, 2 ms). The tetanic contractions were recorded via an isometric force transducer (model 7003, Ugo Basile®) coupled to a Gemini 7070 flat-bed recorder (Ugo Basile®, Varese, Italy). The tetanic responses were analyzed by measuring the amplitude of the first peak of contracture to the electrical stimulus.

At the end of the stimulation protocols, the anesthetized mice were killed with an overdose of 10% KCl administered intracardially (Barbosa et al., 2009; 2010; Barbosa-Souza et al., 2011; Santos et al., 2010). The muscles (control and venom-treated) were removed and fixed in 4% formaldehyde for 24 h, followed by routine processing for dehydration and embedding in paraffin or paraplast. Sections 5 µm thick were cut with a microtome, mounted on microscope slides and stained with hematoxylin-eosin (HE). The sections were examined with a Leica DM2500 microscope fitted with a Leica DFC425 camera. Aspects analyzed included the occurrence of myonecrosis, the intactness of the cell membrane, the position of the nuclei and the presence of cellular infiltration. Representative images were photographed and processed with Leica LASV3® software. In addition, the muscle fiber diameters (longitudinal section)

and area (cross-section) were quantified, using ImageJ 1.49t software. The changes in muscle fiber morphometry were assessed by measuring the fiber diameter in 6 non-overlapping fields per section in 10 non-consecutive sections for each muscle (total of 60 fibers/mouse). The fibers area was measured in 10 fibers/section/animal.

The results were expressed as the mean \pm standard error of the mean (SEM). Statistical comparisons between the control (saline), venom alone and venom + laser groups were done using ANOVA followed by the Tukey-Kramer post-test for multiple comparisons. Values of $p < 0.05$ were considered significant. All data analyses were done using Prism[®] v.5.0 (GraphPad, La Jolla, CA, USA).

Results

Figure 1 shows representative recordings of the tetanic responses of saline- (control) and venom-injected muscles. Control muscle showed consistent, reproducible responses to a series of six tetanic trains (Figure 1A), indicating that the muscle was not damaged by repeated stimulations. In contrast, 3 h and 9 h after venom injection the tibial muscle showed no responses to low voltage or tetanic stimuli

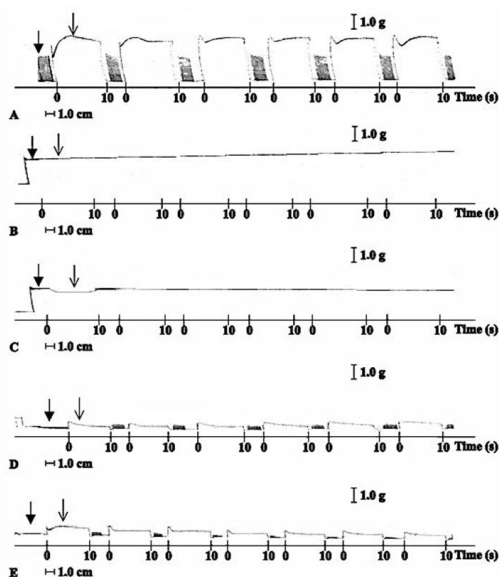


Figure 1. Myographic records of electrically-induced tetanic contractions in mouse tibialis muscle. (A) Control (saline; $n = 3$); (B) Venom 3 h ($n = 6$); (C) Venom 9 h ($n = 6$); (D) Venom + Laser 3 h ($n = 6$); and (E) Venom + Laser 9 h ($n = 6$). The baseline in control preparations remained stable throughout the experiment, indicating that the muscle did not go into contracture and was not injured during repeated stimulations. Venom-injected muscles treated with laser showed contractions and tetanus. In each record: first (dark) arrow – baseline and second (light) arrow – response to tetanic stimulus. The records are representative of the number of experiments indicated.

(Figure 1B, C), which suggested that the venom had caused important damage to the muscle contractile machinery. Treatment with LLLT 30 min after venom injection followed by assessment of muscle function 3 h and 9 h post-venom revealed small but significant recovery of the tetanic response (by 10% and 15%, respectively) compared to the responses to venom alone (Figures 1D, E, 2 and 3).

Histological analysis of saline-injected (control) muscle revealed myocytes with a normal appearance (intact cell membrane and muscle fibers, well-defined muscle fascicles, peripherally located nuclei and a normal interstitial space) (Figure 4A, B). In contrast to this normal appearance, 3 h after venom injection

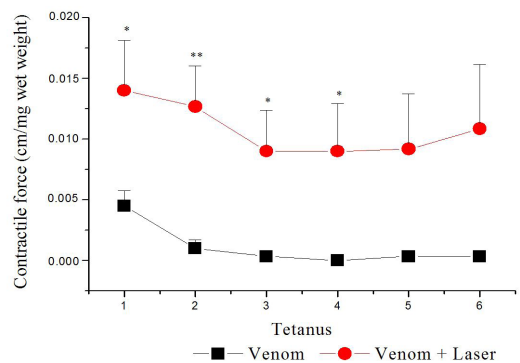


Figure 2. Contractile force generated by tetanic responses in mouse tibial muscle 3 h after injection of *B. jararaca* venom (40 μg) (■) and venom followed by treatment with LLLT (●). In contrast to venom alone, venom-injected muscle exposed to LLLT showed enhanced tetanic responses. The contractile force (muscle tension) was calculated from the initial peak of the tetanic response. The points are the mean \pm SEM. * $p < 0.05$ and ** $p < 0.01$ compared to venom alone.

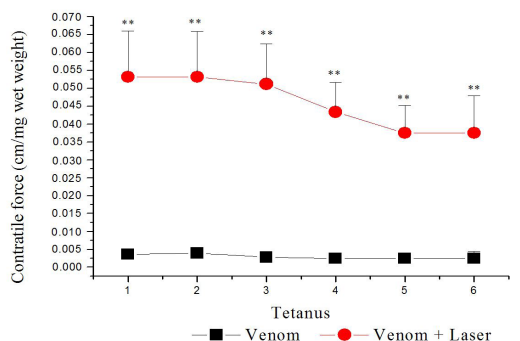


Figure 3. Contractile force generated by tetanic responses in mouse tibial muscle 9 h after injection of *B. jararaca* venom (40 μg) (■) and venom followed by treatment with LLLT (●). In contrast to venom alone, venom-injected muscle exposed to LLLT showed enhanced tetanic responses. The contractile force (muscle tension) was calculated from the initial peak of the tetanic response. The points are the mean \pm SEM. ** $p < 0.01$ compared to venom alone.

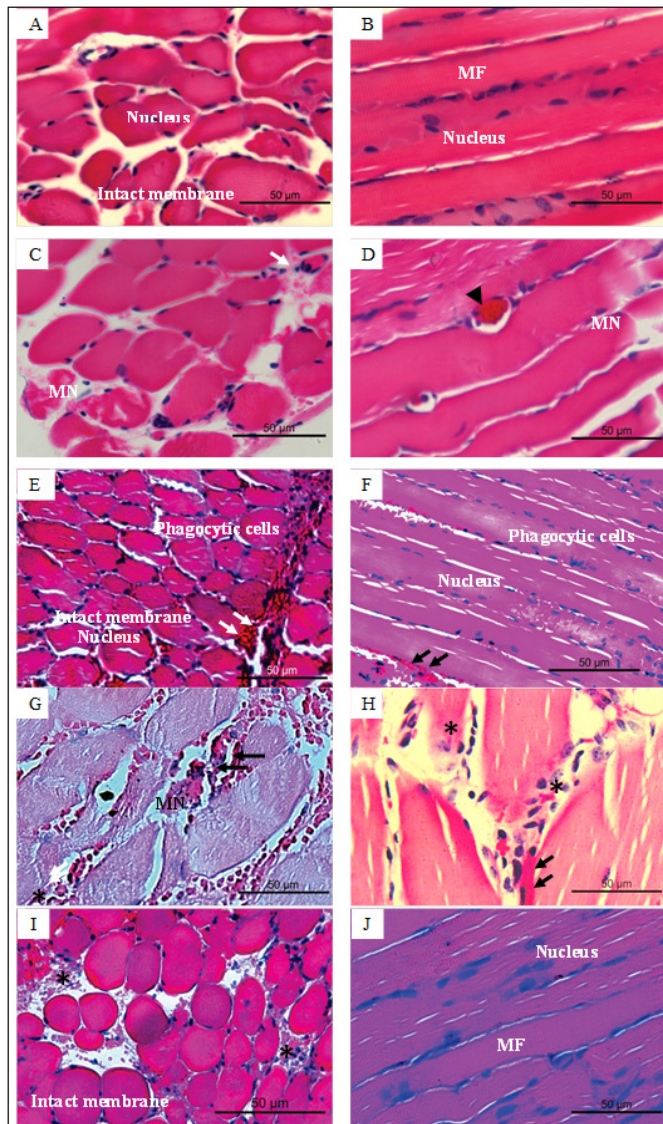


Figure 4. Histological sections of mouse tibial muscle injected with *B. jararaca* venom (40 μ g). (A, B): Control muscles 3 h after injection of 0.9% saline showing muscle fibers (MF) of uniform diameter with peripheral nuclei and an intact cell membrane. (C, D): Venom-treated muscle 3 h after venom injection. Note the myonecrosis (disruption of the cell membrane) and hemorrhage (red blood cells in the interstitial space). (E, F): Venom-treated muscle exposed to LLLT and analyzed 3 h after venom injection. Note the intact fibers, peripherally located nuclei, undamaged cell membrane and presence of inflammatory cells in the interstitial space. (G, H): Venom-treated muscle 9 h after venom injection. The histological changes were similar to those at 3 h, but there was a marked inflammatory infiltrate. (I, J): Venom-treated muscle exposed to LLLT and analyzed 9 h after venom injection. Note the unaltered cell membrane and the presence of hemorrhage and inflammatory cells. Treatment with LLLT attenuated the venom-induced myonecrosis but did not affect the hemorrhage or infiltration of inflammatory cells. Blood vessel – arrowhead; Hemorrhage – arrow; Inflammatory infiltrate – asterisks; Myonecrosis – MN. Scale bars = 50 μ m.

there was muscle fiber disorganization and degradation indicative of myonecrosis; there was also hemorrhage, cellular infiltration and an increase in fiber diameter suggestive of intracellular edema formation (Figures 4C, D, 5A, B). Similar changes occurred at 9 h post-venom, but were less marked (Figure 4G, H).

In venom-injected muscle treated with LLLT and analyzed 3 h post-venom, there was less myonecrosis but inflammatory (phagocytic) cells were observed (Figure 4E, F). At 9 h post-venom, LLLT-treated muscle showed an intact cell membrane (no myonecrosis), cellular infiltration and a small amount of hemorrhage

(Figure 4I, J). Overall, at both time intervals, there was attenuation of venom-induced myonecrosis. Histomorphometric analysis showed a significant decrease in the diameter and area of muscle fibers in mice treated with LLLT (3 h) compared to those treated with venom alone; this improvement persisted after 9 h, but was less marked than at 3 h, probably because of partial recovery in the venom-treated muscles, i.e., the diameter and area in venom-treated muscle were greater at 9 h compared to 3 h (Figure 5A, B).

Discussion

LLLT is a non-invasive, low-cost procedure that has been widely used in clinical practice to relieve pain and stimulate tissue regeneration (Buso, 2006; Dall-Agnol et al., 2009; Fikackova et al., 2006; Kato et al., 2006; Medeiros et al., 2010; Oliveira et al., 2013; Piva et al., 2011). Laser light interacts with biological tissues to produce significant beneficial therapeutic effects in various physiological systems (Leal et al., 2010; Piva et al., 2011). Lopes-Martins et al. (2006) found that GaAlAs laser at λ 630-680 nm protected against muscle damage, with a concomitant decrease in creatine kinase release, stimulated the release of cGMP to cause local vasodilatation and helped to

maintain muscle contractile strength in rats. More recently, Giaretta et al. (2015) observed that LLLT improved muscle function in mice, as assessed by the enhanced tetanic response and resistance to fatigue.

Based on studies of the beneficial effects of LLLT in a variety of models, various experimental investigations have shown that LLLT can reduce the myonecrosis, edema and inflammation induced by snake venoms (Barbosa et al., 2009; 2010; Barbosa-Souza et al., 2011; Doin-Silva et al., 2009; Oliveira et al., 2013). Dourado et al. (2003) examined the effects of laser irradiation with GaAs 4 J/cm² on the myonecrosis caused by *Bothrops moojeni* snake venom in mouse gastrocnemius muscle. Subsequently, Barbosa et al. (2008; 2010) observed that GaAs laser significantly decreased the myonecrosis caused by *Bothrops jararacussu* snake venom in mice. Although these studies reported the recovery of muscle cell structures, they did not assess possible improvements in muscle function. These beneficial effects of laser treatment on venom-induced damage are apparently not mediated by a direct action of laser light on venom activities since Barbosa et al. (2009) showed that prior irradiation of *B. jararacussu* venom did not adversely affect the myotoxicity of this venom.

As shown here, the intramuscular injection of *B. jararaca* venom adversely affected muscle contractility (electrically-induced contractions and contractures). Treatment with LLLT partially restored this muscle dysfunction 3 h and 9 h after venom injection, probably by reducing the muscle fatigue. This conclusion is supported by the findings of Leal et al. (2009), who reported that phototherapy (infrared radiation) attenuated the skeletal muscle fatigue in biceps muscles of professional male volleyball players. The possible cellular mechanisms involved in this enhanced resistance to fatigue have been investigated in recent studies (Giaretta et al., 2015; Kelencz et al., 2010; Maciel et al., 2013; Muñoz et al., 2013; Reis et al., 2015). Passarella and Karu (2014) reported that mitochondria respond to low intensity light and that this response increases ATP production and facilitates cell multiplication. Albuquerque-Pontes et al. (2015) also observed increased cytochrome c oxidase activity in response to a 660 nm laser with an output of 1 J. These authors noted a significant increase in the percentage of muscle fibers with >50% staining for cytochrome c oxidase within 5 min after LLLT; the enhanced cytochrome c oxidase activity probably facilitated the transfer of protons and electrons, thereby accelerating the rate of oxidative metabolism. This led to an increase in ATP that in turn enhanced cellular activity and muscle contractility.

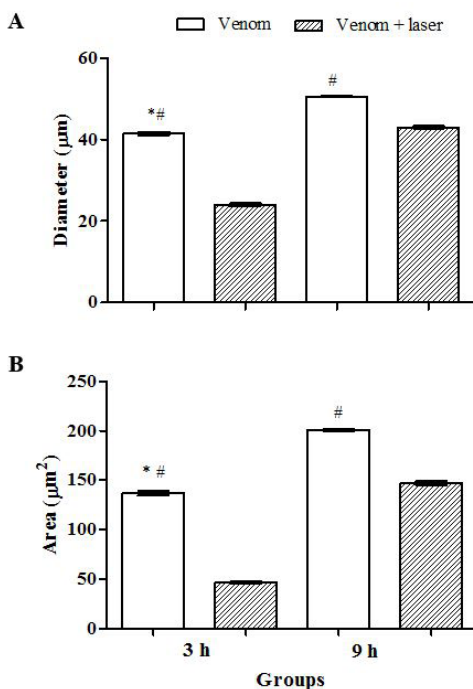


Figure 5. Histomorphometric analysis of venom and venom + laser treated tibial muscles 3 h and 9 h after LLLT. Muscle fiber diameters (A) and area (B) were quantified in longitudinal and cross-sections (n = 10 sections/mouse) and the columns represent the mean \pm SEM. $p < 0.0001$: * vs 9h; # vs laser.

Histological analysis of muscle injected with *B. jararaca* venom alone revealed myonecrosis, similar to but less marked than that caused by the venoms of *B. jararacussu* (Barbosa et al., 2009; Doin-Silva et al., 2009) and *B. moojeni* (Dourado et al., 2003). Treatment with LLLT attenuated this myonecrosis, as also observed by Doin-Silva et al. (2009) for *B. jararacussu* venom in rats. Dourado et al. (2003) reported that GaAs and HeNe laser therapy significantly reduced the inflammation and myonecrosis caused by *B. moojeni* venom, with GaAs laser being more effective than HeNe laser. Similarly, Doin-Silva et al. (2009) found that HeNe laser attenuated the damage to rat gastrocnemius muscle and associated nerve caused by *B. jararacussu* venom; these authors also noted an improvement in the contractility of irradiated muscle compared to muscle treated with venom alone. Barbosa et al. (2010) reported that GaAs laser reduced the edema, inflammation and myonecrosis caused by *B. jararacussu* venom in rat paw.

As shown here, LLLT reduced the diameter and area of venom-treated muscle fibers in relation to venom alone at 3 h, with the attenuation being less marked at 9 h. This attenuation by LLLT was accompanied by improved tetanic responses compared to venom alone. This finding suggests that LLLT had a beneficial effect on muscle contractility in the presence of venom.

Overall, the results of this study indicate that treatment with LLLT attenuated the myonecrosis caused by *B. jararaca* venom and partially but significantly reversed the venom-induced dysfunction in muscle contractility. The ability of LLLT to partially restore the function of envenomed muscle suggests that this intervention could be a potentially beneficial ancillary procedure for treating the myonecrosis and muscle dysfunction caused by *Bothrops* venoms.

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