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Low-level laser therapy decreases local effects induced by myotoxins isolated from *Bothrops jararacussu* snake venom

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Abstract: The prominent myotoxic effects induced by *Bothrops jararacussu* crude venom are due, in part, to its polycationic myotoxins, BthTX-I and BthTX-II. Both myotoxins have a phospholipase A2 structure: BthTX-II is an active enzyme Asp-49 PLA2, while BthTX-I is a Lys-49 PLA2 devoid of enzymatic activity. In this study, the effect of low-level laser therapy (LLLT), 685 nm laser at a dose of 4.2 J/cm2 on edema formation, leukocyte influx and myonecrosis caused by BthTX-I and BthTX-II, isolated from *Bothrops jararacussu* snake venom, was analyzed. BthTX-I and BthTX-II caused a significant edema formation, a prominent leukocyte infiltrate composed predominantly by neutrophils and myonecrosis in envenomed gastrocnemius muscle. LLLT significantly reduced the edema formation, neutrophil accumulation and myonecrosis induced by both myotoxins 24 hours after the injection. LLLT reduced the myonecrosis caused by BthTX-I and BthTX-II, respectively, by 60 and 43%; the edema formation, by 41 and 60.7%; and the leukocyte influx, by 57.5 and 51.6%. In conclusion, LLLT significantly reduced the effect of these snake toxins on the inflammatory response and myonecrosis. These results suggest that LLLT should be considered a potential therapeutic approach for treatment of local effects of *Bothrops* species venom.

Key words: *Bothrops jararacussu*, myotoxins, inflammation, myonecrosis, low-level laser therapy.

INTRODUCTION

Venom phospholipases A_2 (PLA_2, EC 3.1.1.4) catalyze the hydrolysis of the *sn*-2-acyl bond of glycerophospholipids in a calcium-dependent fashion to release free fatty acids and lysophospholipids. These reaction products may display direct biological activities or may be transformed into other active compounds with hemostatic, cardiotoxic, convulsant, hemolytic, hypotensive, hepatotoxic, myotoxic and neurotoxic activities (1-4).

Numerous experimental studies have shown that *Bothrops* PLA_2s are involved in venom-induced inflammatory responses such as edema, pain, leukocyte migration and necrosis (5-8). *Bothrops* PLA_2s exist as monomers of ~14 kDa or as homodimers of ~28 kDa, and may be classified as Asp49 or Lys49 PLA_2, depending on the residue at position 49 in the amino acid sequence (9, 10). PLA_2s with Asp49 are enzymatically active whereas Lys49 PLA_2s show little or no enzyme activity, although both types are biologically active (10). Two myotoxins had been isolated from *Bothrops jararacussu* venom, bothropstoxin I (BthTX-I) – a basic Lys 49 major – and bothropstoxin II (BthTX-II) – a basic Asp 49 (11, 12). These proteins play a relevant role in the pathogenesis of local tissue damage induced by *Bothrops jararacussu* venom, causing myotoxic and edema-forming effects. Moreover, a conspicuous inflammatory cell infiltrate has been described in muscle that had been affected by those PLA_2s (13).

Myotoxic PLA_2 homologues can be inhibited by polyclonal or monoclonal antibodies, as well as by heparin, plant extracts and serum/plasma factors (14-18). A thorough understanding of the local action of *Bothrops* snake venoms is required for the successful development of alternative therapeutic strategies. The low-level laser therapy (LLLT) has been clinically utilized to promote anti-inflammatory effects, pain relief and to accelerate the regeneration of the damaged tissue (19, 20). Furthermore, laser therapy has shown positive effects on the reduction of edema, pain and migration of inflammatory cells (21-23). We previously reported that laser therapy significantly reduces the edema formation, leukocyte influx and myonecrosis induced by *B. jararacussu* snake venom in gastrocnemius muscle when the muscle was irradiated with a dose of 4.2 J/cm^2 immediately after the venom injection (24, 25). However, the effect of laser therapy on the reduction of edematogenic reaction, leukocyte migration and myonecrosis induced by snake myotoxins has not been yet determined. The aim of this work was to investigate the ability of low-level laser therapy to reduce the local inflammation and myonecrosis after injection of bothropstoxin-I and bothropstoxin-II in the gastrocnemius muscle to assess the involvement of these toxins in the myonecrosis and inflammatory reaction induced by *B. jaracussu* crude venom and their neutralization by laser therapy.

MATERIALS AND METHODS

Myotoxins

Myotoxins bothropstoxin-I (BthTX-I) and bothropstoxin-II (BthTX-II) were supplied by Dr. Andreimar M. Soares, from the University of São Paulo, USP, Ribeirão Preto, SP, Brazil. BthTX-I and II were isolated and purified as previously described (12, 26).

Animals

All animal tests were in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL/COBEA) and were approved by the Ethics Committee on Animal Research of UNIVAP (protocol number A020/2006/CEP). Male Swiss mice weighing between 22 and 25 g were employed and randomly divided into groups of five animals each. Animals were kept in plastic cages, offered water and food *ad libitum*, maintained under controlled temperature (26°C) and lighting (12-hour light-dark cycle).

Laser Device

A low-level semiconductor GaAs (gallium arsenide) laser (Thera Lase®, DMC Equipamentos, Brazil) operating continuously at 685 nm (red) was employed to experimentally irradiate the animals. The parameters that corresponded to a laser dose of 4.2 J/cm^2 were: 29 mW of power, 29 s of irradiation time and an irradiated area of 0.2 cm^2. Mice were irradiated at the same site where myotoxins were injected, from a distance of 15 mm. The optical laser power was determined by a Newport 1835-C Multi Function Optical Power Meter® (Newport Corp., USA). Laser dose was low enough to avoid any thermal effect and chosen based on studies that had shown a beneficial effect of low-level laser therapy on the inflammatory process and myonecrotic effect (24, 27-29).

Surgical Procedure and Laser Irradiation

BthTX-I and BthTX-II were prepared by diluting 2 mg/kg (animal weight) into 50 µL of a sterile saline solution (SS). Shaving and antiseptic preparation were performed on the skin located directly over the gastrocnemius muscle for the BthTX-I or BthTX-II injection.

The animals received intramuscular (IM) injections of myotoxins in the central part of the right gastrocnemius muscle, whereas the contralateral muscle received the same volume of an apyrogenic saline solution. Animals were manually immobilized while the laser was applied to both muscles (right and contralateral), at the same site of myotoxin or saline solution injection. Mice were irradiated immediately and at 1, 3 and 12 hours after the injection.

Morphological Studies

Twenty-four hours after myotoxin injection mice were euthanized by intraperitoneal injections of 10 mg/kg of xylazine and 100 mg/kg of ketamine, followed by intracardiac administration of 10% potassium chloride. Then, the gastrocnemius muscle was collected for histological processing. Briefly, after rinsing with phosphate buffered saline (PBS) the samples were fixed in 10% buffered formalin for 24 hours, rinsed again, dehydrated in graded ethanol series,

and embedded in paraffin. Histological cross-sections of 5 mm were stained with hematoxylin and eosin (HE) and Masson's trichrome.

Myotoxic Activity (Creatine Kinase)

The myotoxic activity was assessed by measuring creatine kinase (CK) in the gastrocnemius muscle after injection of BthTX-I or BthTX-II. In brief, gastrocnemius muscles were dissected out and homogenized in 4 mL of PBS, pH 7.2, for 10 seconds in a homogenizer (Brinkmann, USA). Then, 1 mL of PBS containing 0.5% Triton X-100 was added. Homogenates were centrifuged at 5,000 x *g* for five minutes, and the supernatant was diluted to 1:35 with PBS for the quantification of CK activity. Muscle CK level was used as a quantitative index of muscle activity whereas CK activity was determined by a diagnostic kit (CK-NAC®, Labtest Diadgnóstica SA, Brazil) (5, 30). CK activity was expressed as U/L; one unit was defined as the amount of enzyme that produces 1 µmoL of NADH per minute under the conditions of the assay.

Quantification of Edema

To measure the muscle edema, mice were injected in the right gastrocnemius muscle with 50 µL of BthTX-I or BthTX-II, at the same time that the contralateral muscle received the same volume of a sterile saline solution, as previously described. After mice were euthanized (24 hours after myotoxin injection) their gastrocnemius muscles were dissected out for subsequent analysis. Both muscles were weighted and the edema was expressed as the percentage of the increase in the weight of the myotoxin-injected muscle compared to the corresponding contralateral muscle (5).

Quantification of Inflammatory Infiltrate in Muscle

To quantify the inflammatory infiltrate, after the injection of myotoxins, mice were euthanized and their gastrocnemius muscles were dissected out and chopped with a blade into very small pieces before the addition of 2 mL of PBS. The suspension was incubated for 30 minutes at 4°C. Then, it was filtered through gauze that was subsequently washed with an additional 1 mL of saline solution. After that, a fraction of the filtered solution was diluted in Türk's solution (1:20) to count total leukocytes in a Neubauer chamber. Suspensions were centrifuged for six minutes at 800 rpm and the pellet was ressuspended in 100 µL of PBS. Differential leukocytes were stained with Instant-Prov® (Newprov Produtos para Laboratório Ltda., Brazil) (5).

Statistical Analysis

Mean and standard deviation were calculated for each group. To establish whether the difference between the mean values of two experimental groups was significant the Student's t-test was performed, using a statistical significance level of $p < 0.05$. When more than two groups were compared a two-way analysis of variance was applied, followed by the Tukey-Kramer test.

RESULTS

Edema Formation Induced by BthTX-I or BthTX-II and Treatment by LLLT

Intramuscular injection of 2 mg/kg of BthTX-I or BthTX-II caused a prominent weight increase of treated gastrocnemius muscle, at 24 hours after injection, as compared to control muscle (Figure 1). BthTX-II caused the most pronounced effect ($p < 0.05$ for BthTX-II *versus* BthTX-I, Figure 1). The LLLT significantly reduced edema formation by 41 and 60.7% respectively for BthTX-I and BthTX-II.

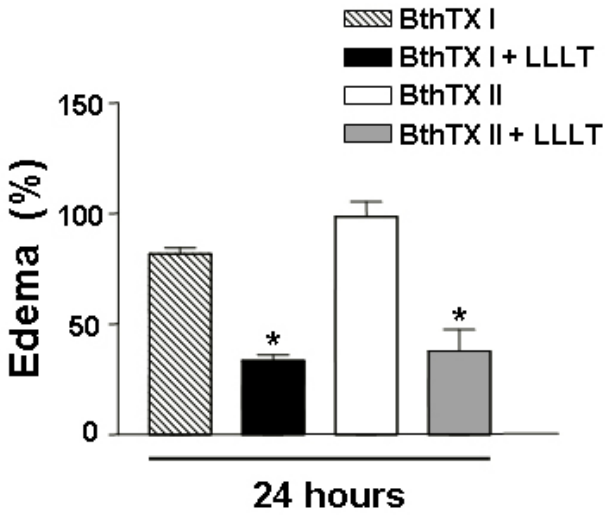

Figure 1. Edema induced by BthTX-I (diagonally striped bar), BthTX-II (empty bar), and treatment with LLLT (black and gray bars).
*$p < 0.05$ *versus* the corresponding myotoxin group; #$p < 0.05$ BthTX-II *versus* BthTX-I.

Inflammatory Infiltrate in Gastrocnemius Muscle Induced by BthTX-I or BthTX-II and Treatment by LLLT

The total number of leukocytes in gastrocnemius muscle was determined 24 hours after intramuscular injection of 2 mg/kg of BthTX-I or BthTX-II. Both myotoxins had induced an inflammatory infiltrate at 24 hours after inoculation (BthTX-I: 330 ± 56 x 10^5 cells/mL; BthTX-II: 310 ± 74 x 10^5 cells/mL) as shown in Figure 2 – A, which also shows statistically significant respective reductions in the leukocyte number produced by LLLT of 57.5 and 51.6% for BthTX-I and BthTX-II groups.

Differential counts showed that gastrocnemius muscle cells were predominantly polymorphonuclear leukocytes, mainly neutrophils. Figure 2 – B shows the number of polymorphonuclear leukocytes in the two groups, BthTX-I (261 ± 75 x 10^5 cells/mL) and BthTX-II (231 ± 79 x 10^5 cells/mL). It was observed that laser treatment induces a statistically significant reduction in the number of polymorphonuclear leukocytes (BthTX-I: 6.4 ± 5 x 10^5 cells/mL and BthTX-II: 45 ± 14 x 10^5 cells/mL), a decrease that is more remarkable when compared to the total leukocyte number. On the other hand, mononuclear cells (BthTX-I: 43 ± 17 x 10^5 cells/mL and BthTX-II: 31 ± 13 x 10^5 cells/mL) significantly increased in laser-irradiated animals (BthTX-I: 123 ± 47 x 10^5 cells/mL and BthTX-II: 97 ± 27 x 10^5 cells/mL), as shown in Figure 2 – C.

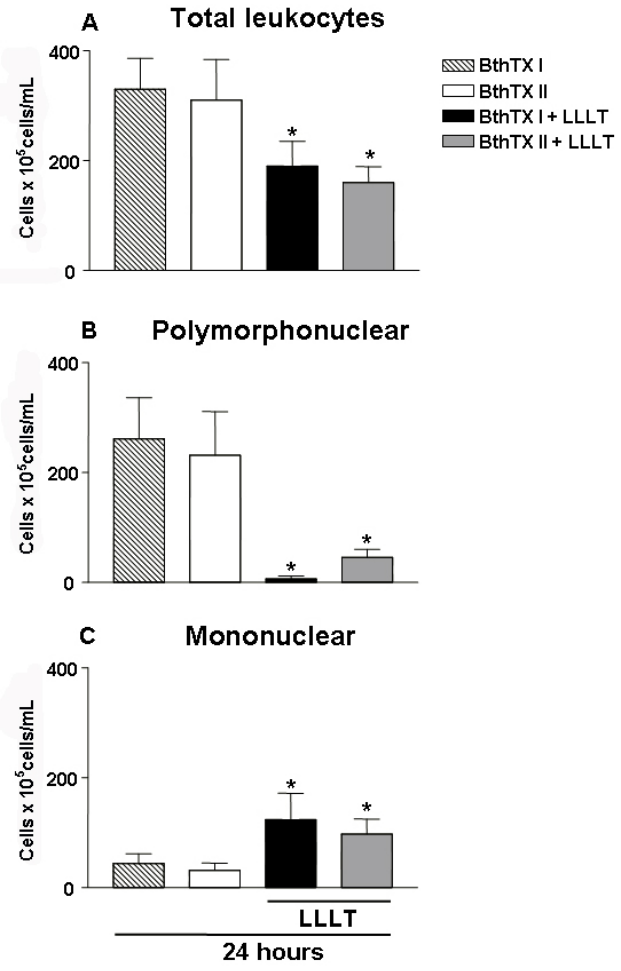

Figure 2. Leukocyte influx into the gastrocnemius muscle induced by intramuscular injection of 2 mg/kg of BthTX-I (black bar) or BthTX-II (gray bar). (**A**) Total leukocytes, (**B**) polymorphonuclear cells, and (**C**) mononuclear cells.
*$p < 0.05$ *versus* the corresponding myotoxin group.

Effect of LLLT on Myonecrotic Activity in Gastrocnemius Muscle Induced by BthTX-I or BthTX-II

The myonecrotic effect on gastrocnemius muscle was determined 24 hours after intramuscular injection of 2 mg/kg of BthTX-I or BthTX-II, as displayed in Figure 3. As shown in this same figure, both myotoxins were able to drop muscle CK content at 24 hours post-injection, compared to controls (control: 2,274 ± 78 U/L; BthTX-I: 1,060 ± 67 U/L and BthTX-II: 1,419 ± 218 U/L). LLLT produced a statistically significant increase in muscle CK content by 60 and 43%, respectively against BthTX-I or BthTX-II envenomation, at 24 hours.

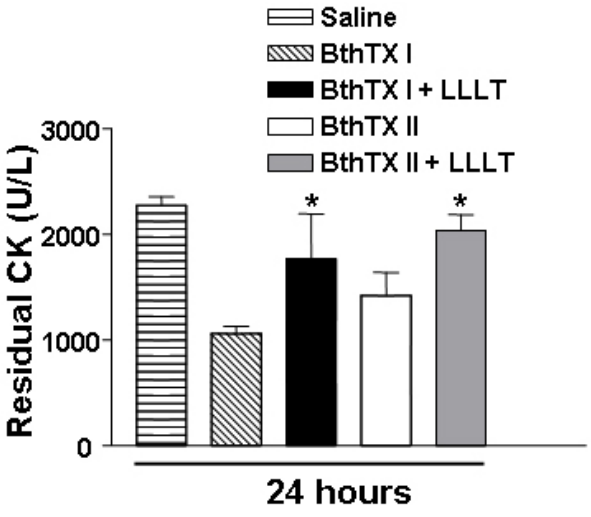

Figure 3. Myonecrosis in gastrocnemius muscle induced by BthTX-I or BthTX-II and LLLT treatment. Saline solution in contralateral muscle (horizontal striped bar), BthTX-I (gray bar), BthTX-I + LLLT (empty bar), BthTX-II (black bar) and BthTX-II + LLLT (diagonally striped bar).
*$p < 0.05$ *versus* the corresponding myotoxin group.

Histopathological Analysis

The acute local pathological alterations induced by intramuscular injection of BthTX-I or BthTX-II are illustrated in Figure 4. The degenerative phase included the appearance of necrotic areas in muscle tissue 24 hours after the inoculation. Muscle from the control group showed normal cell structure with regular fibers, defined muscular fascicles, and unbroken membranes (Figure 4 – A). Light micrograph sections showed considerable changes in mouse gastrocnemius muscle 24 hours after BthTX-I or BthTX-II inoculation, which included vascular congestion, edema, loss of muscular fascicle definition and infiltration of inflammatory cells (Figure 4 – B and C). At 24 hours post-injection, LLLT treatment reduced the number of damaged fibers compared with muscle injected with BthTX-I or BthTX-II (Figure 4 – D and E).

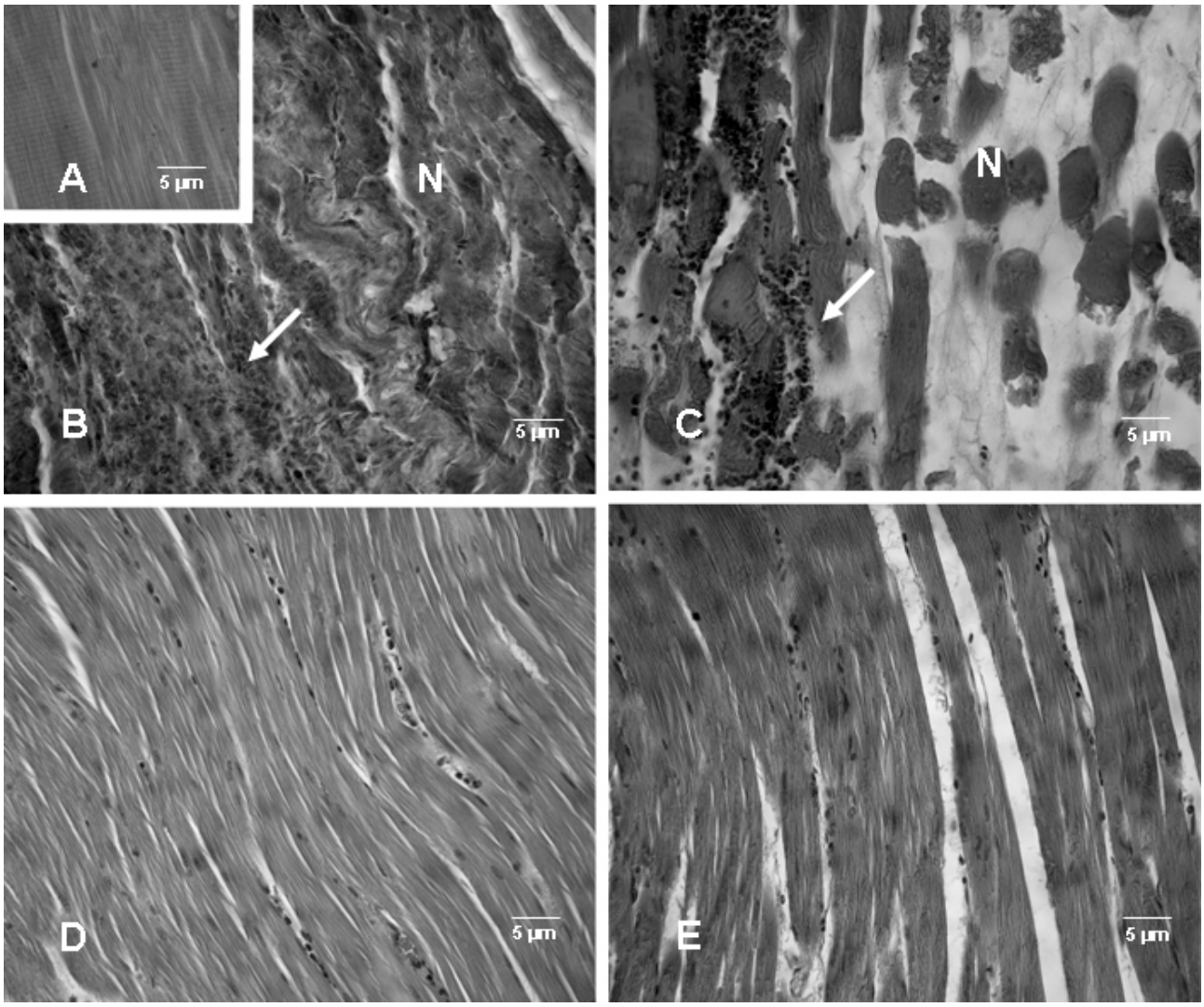

Figure 4. Light micrographs of sections of mouse gastrocnemius muscle 24 hours after intramuscular inoculation of BthTX-I or BthTX-II. (**A**) Control gastrocnemius muscle injected with a saline solution presents normal fibers; (**B**) BthTX-I and (**C**) BthTX-II inoculations show muscles with typical alterations due to myonecrosis (N) and infiltration of leukocytes (arrows). Alterations of the gastrocnemius muscle after inoculation of BthTX-I and BthTX-II with LLLT are shown in (**D**) and (**E**), respectively. It is observed that LLLT is effective in decreasing the number of destroyed fibers. Bar corresponds to 5 µm in all panels.

DISCUSSION

Venom PLA_2s are proven to induce inflammatory responses, such as edema formation and inflammatory cell infiltrates (13, 31-33). Two myotoxic PLA_2s were isolated from *Bothrops jararacussu* snake venom and characterized as bothropstoxin I (BthTX-I) and bothropstoxin II (BthTX-II) (11, 12). These proteins can be classified into two categories: Asp49 PLA_2s, catalytically active, and Lys49 PLA_2s, devoid of significant catalytic activity upon artificial substrate (2, 10, 18, 34). In the present work, the local inflammatory process and myonecrosis induced by BthTX-I (Lys49 PLA_2) and BthTX-II (Asp49 PLA_2), and their possible blockade by laser treatment, were investigated.

Both BthTX-I and BthTX-II induced a prominent edema in gastrocnemius muscle, which corroborates previous observations of the edema-forming activity of venom PLA_2 (33, 35). Our results demonstrated that the catalytically active BthTX-II (Asp 49 PLA_2) is more potent in promoting edema formation than BthTX-I (Lys 49 PLA_2) ($p < 0.05$ BthTX-II *versus* BthTX-I, Figure 1). Various enzymatically inactive Lys-49 PLA_2s have been shown to induce edema, clearly indicating the existence of molecular regions, different from the catalytic site in these PLA_2s homologues, which are responsible for mast cell degranulation and edema formation (13, 36, 37).

A prominent leukocyte infiltrate, composed predominantly of neutrophils, was observed after injection of BthTX-I or BthTX-II in the present study. This finding corroborates previous studies on mouse skeletal muscle after injection of BthTX-II myotoxins from *B. jararacussu* venom (38). Other authors have also documented polymorphonuclear and mononuclear cellular infiltrates in mouse skeletal muscle after injection of myotoxic PLA_2s from the venoms of *B. asper* and *B. nummifer* (13, 39). Furthermore, Castro *et al.* (32) showed that BthTX-I and BthTX-II are able to recruit leucocytes into the rat pleural cavity as a consequence of the generation of chemoattractant mediators (leukotriene B4 and platelet-activating factor) by the action of these proteins that stimulate cytosolic PLA_2. In our model, both myotoxins revealed the same ability to promote leukocyte influx into gastrocnemius muscle. In contrast to our results, Castro *et al.* (32) reported that BthTX-II was a more potent leukocyte attractant (particularly of neutrophils) than BthTX-I in the rat pleural cavity. The mechanisms underlying these differences are still unclear. However, we may speculate that such discrepancies might be due to the animal model studied and/or the site of myotoxin injection.

BthTX-I and BthTX-II induced myonecrosis in gastrocnemius muscle 24 hours after injection, as measured by the residual muscle CK levels. The decrease in CK activity in muscle indicates the presence of myonecrosis (5, 30). Histological results confirm CK results, which are in agreement with those reported by Silva *et al.* (40). Likewise, our findings on the myonecrotic and edema-inducing effects are also similar to those of Soares *et al.* (41).

Envenomation by snakes is often treated by intravenous administration of antiophidian serum. During serum therapy, the toxic systemic effect is usually counteracted by the antivenom, but reversal of local tissue damage usually does not occur (42, 43). Neutralization of snake venoms and isolated toxins by plant extracts has been extensively explored as an alternative treatment to serum therapy (17, 18, 44). Natural and synthetic compounds such as heparin, suramin, fucoidan and animal serum factors have also been studied (45-51).

Various studies have tested the efficacy of low-level laser irradiation in promoting inflammatory and tissue repair processes (22, 52, 53). In the present study, we investigated the effect of LLLT on the myotoxic and local inflammatory process induced by BthTX-I or BthTX-II. Treatment with LLLT was capable of diminishing by 41 and 60% the edemathogenic activity, and by 57 and 51% the leukocyte influx induced respectively by BthTX-I and BthTX-II. In the literature is reported that LLLT acts by reducing the inflammation process and accelerating wound healing in rats (29, 54). Several authors showed that laser irradiation caused inhibition of PGE_2 through reduction of COX-2 mRNA levels (22, 55-57).

In addition, low-level laser irradiation significantly inhibited the gene expression of IL-1β and IFN-γ (55, 58). IL-1β, PGE_2, and IFN-γ are involved in different immune responses and in the acute phase of inflammatory processes (56, 59). Also, IFN-γ is an important macrophage activator and plays an important role in the inflammatory process (59). IL-1β, TNF-α, and IFN-γ are key mediators of inflammatory

processes and, therefore, laser irradiation may control inflammation via decreased production of these mediators. In view of this fact, one may speculate that reduction in the inflammatory process induced by myotoxins isolated from *Bothrops jararacussu* snake venom can be due to the inhibited expression of IL- β and PGE_2 in LLLT-treated mice. This hypothesis is supported by other authors who observed that venom PLA_2 increases cytokines, such as IL-1, IL-6 and TNF-α (33). In our model, the number of mononuclear cells increased after LLLT, which agrees with the literature and shows that laser irradiation stimulates macrophages and lymphocytes (27, 60).

LLLT significantly reduced the cell damage caused by BthTX-I or BthTX-II 24 hours after injection, as evidenced by the increase in muscle CK content. Moreover, histological observation showed that LLLT diminished the number of destroyed fibers when compared to muscle injected only with myotoxins without laser treatment. Recently, we demonstrated that myonecrosis induced by *B. jararacussu* crude venom was diminished by laser treatment (25). Similar results were found by Dourado *et al*. (27, 28) when studying myonecrosis provoked by *Bothrops moojeni* and *Bothrops neuwiedi* venoms and treated with LLLT. They suggested that laser treatment is able to block the ability of venom to disrupt the plasma membrane integrity. There are no data from the literature concerning LLLT treatment after injection of isolated myotoxins. Evidence in the literature suggests that at the cellular level, photo-irradiation at low power causes significant biological effects including cellular proliferation, collagen synthesis and release of growth factors from cells (61).

In conclusion, this work indicates that LLLT is capable of inhibiting inflammatory and myonecrotic processes caused by myotoxins isolated from *Bothrops jararacussu* snake venom. The observation that LLLT acts at the same intensity to reduce the inflammatory and myonecrosis processes for both BthTX-I and BthTX-II suggests that enzymatic activity is not relevant for laser treatment. Furthermore, our findings indicate that LLLT should be considered a potential therapeutic approach for treatment of local effects of *Bothrops* snakebite, as well as an interesting tool for the study of the mechanisms underlying the inflammatory process and myonecrotic activity induced by those venoms.

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CONFLICTS OF INTEREST

There is no conflict.

ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee on Animal Research of the Vale do Paraíba University, UNIVAP (protocol number A020/2006/CEP).

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