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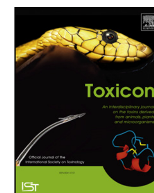

Light emitting diode (LED) therapy reduces local pathological changes induced by *Bothrops asper* snake venom

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ABSTRACT

The therapeutic effect of the Light Emitting Diode (LED) treatment in two wavelengths (635 or 945 nm) was evaluated in the local pathological alterations induced by *Bothrops asper* snake venom. Mice received irradiation of infrared LED (120 mW, 945 nm) or red LED (110 mW, 635 nm) applied immediately, 1 and 2 h after venom injection. LED treatment reduced edema formation in the plantar region and gastrocnemius muscle and significantly reduced neutrophil migration and hyperalgesia after the venom injection. Also, both infrared LED and red LED treatment significantly reduced myonecrosis, as revealed by muscle CK and plasma CK levels. Histological analysis corroborated the reduction in the extent of venom-induced myonecrosis. In conclusion, our data demonstrates that PBM with LED light in both red and infrared wavelengths, when applied after envenomation in mice, reduces the extent of myotoxicity, edema, inflammatory infiltrate and hyperalgesia, suggesting that photobiomodulation is a potential therapeutic approach that should be further investigated for the treatment of local effects of *Bothrops* snakebite.

1. Introduction

The majority of snakebites in Latin America are inflicted by species of the genus *Bothrops*. The snake *Bothrops asper* is responsible for 50–80% of snakebite accidents and 60–90% of deaths secondary to snakebites in Central America and in some regions of Mexico, Ecuador, Venezuela and Colombia (Gutiérrez, 2010; Herrera et al., 2016; Otero-Patiño, 2009; Saldarriaga-Córdoba et al., 2017). In untreated cases, or when antivenom administration is delayed, local necrosis frequently occurs and may lead to permanent tissue damage and, in some cases, leads to amputation (Jorge et al., 1999; Saborío et al., 1998). Additionally, victims of *Bothrops asper* snakebite suffer systemic effects of envenomation, which are responsible for fatalities in severe envenomations (Otero-Patiño, 2009).

The local effects caused by *B. asper* snakebites are characterized by intense reactions that include edema, pain, local hemorrhage, blisters, dermonecrosis and myonecrosis (Chacur et al., 2001; Gutiérrez et al., 2009a; Zamuner et al., 2001). In addition, systemic manifestations in severe cases are associated with thrombocytopenia, platelet hypoaggregation, disseminated intravascular coagulation, cardiovascular shock and kidney injury (Rucavado et al., 2005; Chugh et al., 1975; Gutiérrez et al., 2009b). The mainstay in the treatment of *Bothrops* sp. snakebite envenomation is the parenteral administration of antivenom therapy, which is highly efficient in neutralizing the systemic effects, but has been shown to be partially ineffective in the neutralization of local pathological and inflammatory reactions (Camey et al., 2002; Zamunér et al., 2004; Gutiérrez et al., 1998). In order to circumvent the limitations of antivenom in the neutralization of local effects, various therapeutic alternatives have been explored at the experimental level, such as the use of natural and synthetic inhibitors of venom metalloproteinases (SVMPs) and phospholipases A_2 (PLA_2s) (Rucavado et al., 2000, Lizano et al., 2003, Rostelato-Ferreira et al., 2010, Tribuiani et al., 2017). In addition, photobiomodulation therapy (PBMT) has been explored for it is potential to inhibit local pathological effects of

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venoms (Barbosa et al., 2008; Doin-Silva et al., 2009; Dourado et al., 2003; Nadur-Andrade et al., 2012).

PBMT is a medical technique in which exposures to low level lasers (LLL) or light emitting diode (LED) stimulate cellular function leading to beneficial clinical effects. Several studies have suggested that PBMT could be an effective device for wound healing, anti-inflammation and pain-killing as well as for promoting the processes of regeneration and angiogenesis in many clinical disorders (de Oliveira Melo et al., 2016; Magri et al., 2017, Ruaro et al., 2014). Thus, PBMT may be a significant therapeutic tool in reducing the local effects caused by *Bothrops* sp venom.

Previous studies have demonstrated that LLL treatment decreased considerably the extent of myonecrosis induced by *Bothrops moojeni* (Dourado et al., 2003) and *B. jararacussu* venoms (Barbosa et al., 2009). In addition, LLL treatment also prevented edema formation and leukocyte influx in muscle and paw of mice injected with *Bothrops* venom (Aranha de Sousa et al., 2013, Barbosa et al., 2008, Nadur-Andrade et al., 2012, Souza et al., 2011). Moreover, the anti-edematogenic effect caused by LLL treatment, after *B. jararacussu* venom injection in gastrocnemius muscle, is enhanced when antivenom and LLL were used in association (Barbosa et al., 2009). Furthermore, LLL treatment was effective in reducing the hemorrhage and hyperalgesia induced by *B. moojeni* venom (Aranha de Sousa et al., 2013; Nadur-Andrade et al., 2012, Souza et al., 2011). Additionally, Nadur-Andrade et al., (2014a,b) showed that LED treatment was effective in the reduction of edema, hyperalgesia and hemorrhage induced by *B. moojeni* venom.

Owing to the medical relevance of envenomations by *B. asper* in Central America and parts of Mexico and South America, a comprehensive analysis of the effect of LED treatment on the outcome of *B. asper* venom-induced local pathological reaction was performed. In addition, we compared the near infrared LED and red LED to identify which is the best for envenomation treatment.

2. Materials and methods

2.1. Animal

All animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation and was approved by the Committee for Ethics in Animal Research of the University of the Vale do Paraíba (UNIVAP) under number A118/2007/CEP. Experiments were performed using 45-day-old male Swiss mice (22–25 g), randomly divided into groups of five animals each. Animals were kept in plastic cages with water and food ad libitum, maintained under controlled temperatures (20–22 °C) and on a 12 h light/dark cycle.

2.2. Venom

Lyophilized crude *Bothrops asper* venom (Bav) was supplied by Instituto Clodomiro Picado, San José, Costa Rica. It is a pool obtained from more than 40 adult specimens collected in the Pacific versant of this country. The venoms were dissolved in 0.9% saline solution only at the moment of its use.

2.3. LED irradiation

A 635 nm red and 945 nm infrared LED devices (Super Bright LEDs, Inc., St. Louis, MO, USA) were employed to irradiate animals. The red LED parameters were 110 mW of power, 44 s irradiation time, and 1.2 cm^2 irradiated area, which corresponded to an irradiation dose of 4 J/cm^2. The infrared parameters were 120 mW of power, 40 s irradiation time, and 1.2 cm^2 irradiated area, which corresponded to an irradiation dose of 4 J/cm^2. Animals were irradiated at 0, 1, and 2 h after Bav injection. The LED irradiation angle was kept perpendicular to the skin surface so that only the point of venom injection was irradiated. The LED dose, low enough to avoid any thermal effect, was chosen on the basis of studies reported in the literature that had shown a beneficial effect of the LED therapy on venom-induced local effects (Nadur-Andrade et al., 2012, 2014a; 2014b).

2.4. Irradiated Bothrops asper venom (iBav)

To verify whether the LED irradiation could change venom toxicity, the lyophilized venom of Bav was diluted in saline solution and irradiated immediately before the injection of the animals, using the same parameters used to irradiate animals. The rationale of this experiment was to elucidate if the LED light can modify the biological activities of the venom.

2.5. Evaluation of paw edema

The ability of the LED to reduce paw edema was studied in mice. To that end, 50 μL of sterile saline 0.9% (w/v) containing Bav or iBav (2.5 μg/paw) were injected in the subplantar region of the right hind paw. The left paw received an equal volume of sterile saline alone and served as control. The volumes of both hind paws were measured plethysmographically (model 7140 plethysmometer, Ugo Basile, Italy) before and at 30 min, 1, 2, 4 and 6 h after venom administration. The edemathogenic effect was calculated as the difference between both paws and expressed as % increase in the volume of venom-injected paw.

2.6. Evaluation of muscle edema

To measure edema in muscle, animals received an i.m. injection of Bav or iBav (50 μg/50 μL) in the central part of the right gastrocnemius muscle and the same volume of apyrogenic saline solution in the contralateral muscle. After 3 and 24 h mice were euthanized and their gastrocnemius muscles were dissected out. Both muscles were weighed, and the edema was expressed as the percentage of the weight increase of the venom injected muscle as compared to the contralateral muscle (Barbosa et al., 2009).

2.7. Leukocyte harvesting and counting

The leukocyte migration into the peritoneal cavity was evaluated 6 h after injection of Bav or iBav (5 μg/cavity dissolved in a volume of 500 μL apyrogenic saline solution) [8]. Leukocytes were harvested 6 h after venom injection by washing the cavity with 2 mL of saline containing heparin (10 U/mL). Aliquots of the washes were collected and used to determine the total cell counts in a Neubauer chamber after dilution (1:20 v/v) in Turk solution (0.2% crystal violet staining in 3% acetic acid). For differential cell counts, cytospin preparations were stained with Instant Prov stain. Differential cell counts were performed by counting at least 100 cells, which were classified as either polymorphonuclear (PMN) or mononuclear (MN) cells, based on conventional morphological criteria.

2.8. Mechanical hyperalgesia

Testing for mechanical sensitivity (von Frey filaments Touch-Test® Sensory Evaluators -North Coast Medical) was based on the method of Chaplan et al. (1994). Mice were placed individually in plastic cages with a wire bottom, which were in contact with the paws. Filaments were applied to the plantar region of the left hind paw of each animal for 5 s. To reduce stress, mice were habituated to the experimental environment one day before the first measurement. Animals were injected with 2.5 μg of crude Bav or iBav diluted in 50 μL of sterile saline into the subplantar surface of the right hind paw. Control group animals received the same volume of sterile saline. The contralateral paw was not injected. The pain threshold was measured at 1, 3, 6 and 24 h after venom or saline injection.

2.9. Determination of myotoxic activity

2.9.1. Quantification of CK activity

The myotoxic effect on gastrocnemius muscle was evaluated by intramuscular injection of 50 µg of Bav and the extent of myonecrosis was assessed by measurement of CK activity both in plasma and in muscle (residual CK). Groups of mice (18–20 g), received an intramuscular injection, in the right gastrocnemius muscle, of either saline solution (control), Bav or iBav (50 µg) dissolved in 50 µL of saline solution. For the assessment of plasma CK activity, mice were bled from the tail at 3 h, blood was collected into heparinized capillary tubes, and plasma was obtained by centrifugation. Then, at 24 h, mice were sacrificed and the gastrocnemius muscles injected with venom were homogenized in 4 mL of PBS. Homogenization was performed in a homogenizer (Polytron, Lucerne, Switzerland) for 10 s. Then, 1 mL of PBS containing 0.5% Triton X-100 was added. Homogenates were centrifuged at 5000 g for 5 min, and the supernatant was diluted 1:35 (v/v) with PBS for the quantification of CK activity. The CK activity was determined in plasma and in gastrocnemius muscle using a diagnostic kit (LABTEST CK-NAC, Labtest Diagnóstica, Lagoa Santa, MG, Brazil) using a spectrophotometer (Spectra MAX; l¼190–340 nm, MDS, Toronto, Canada). The CK activity was expressed in units per liter (U/L), where one unit is defined as the amount of enzyme that produces 1 µmol of NADH per minute under the conditions of the assay (Barbosa et al., 2009).

2.9.2. Histological assessment of myonecrosis

Twenty-four hours after venom injection in gastrocnemius muscle, mice were euthanized with lethal dose of 200 mg/kg of sodium pentobarbital via intraperitoneal injection, and the injected muscle was excised, sectioned in the middle of the muscle mass, and placed in Bouin fixative solution (formaldehyde, picric acid, acetic acid). After routine tissue processing, samples were embedded in paraffin sections were prepared and stained with haematoxylin–eosin.

2.10. 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 *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 a Tukey–Kramer test.

3. Results

3.1. Effect of red LED or infrared LED treatment on paw edema induced by Bav

Intraplantar injection of Bav (2.5 µg/paw) caused a time-dependent increase in paw volume (edema). The peak increase in hind paw swelling occurred at 1 h and decreased gradually until 6 h, but the edema was still significantly higher than in saline injected (control) mice (Fig. 1). Irradiated venom (red or infrared LED) had a similar edematogenic activity as non-irradiated venom. Both LED (635 and 945 nm) irradiations on mice paw significantly attenuated edema formation from 1 to 6 h. In the peak of hind paw swelling (1 h) the reduction was 70% and 52% by red and infrared LED, respectively, compared to the response with Bav alone (Fig. 1).

3.2. Effect of red LED or infrared LED treatment on muscle edema induced by Bav

Intramuscular injection of 50 µg of Bav caused a prominent increment in muscle weight of envenomated gastrocnemius muscle at 3 h as well as at 24 h post-injection compared to the control muscle (Fig. 2).

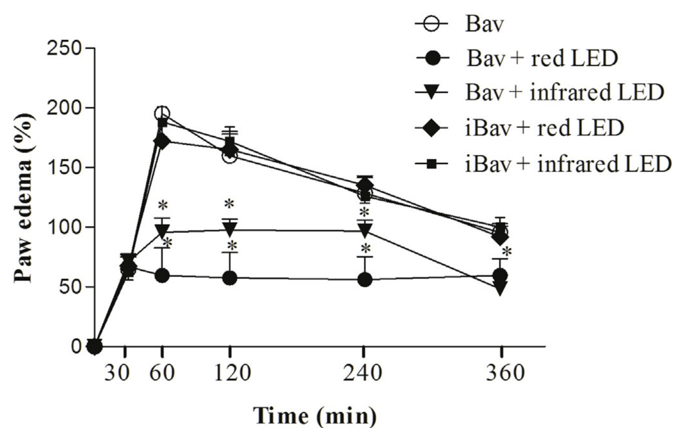

Fig. 1. Effect of red LED (635 nm) or infrared LED (945 nm) on paw edema induced by Bav or iBav. Animals were injected in the subplantar region with 2.5 µg per paw of Bav or iBav. LED irradiation was performed immediately, 1 h and 2 h after venom injection. The increase of the paws was evaluated by plethysmometer in the following times: 0, 30, 60, 120, 240 and 360 min after venom injection. Data are expressed as % of change as compared to control paws injected with saline solution. Data correspond to the mean ± S.E.M. of 5 animals per group. *p < 0.05 compared to Bav. Error bars indicate SEM.

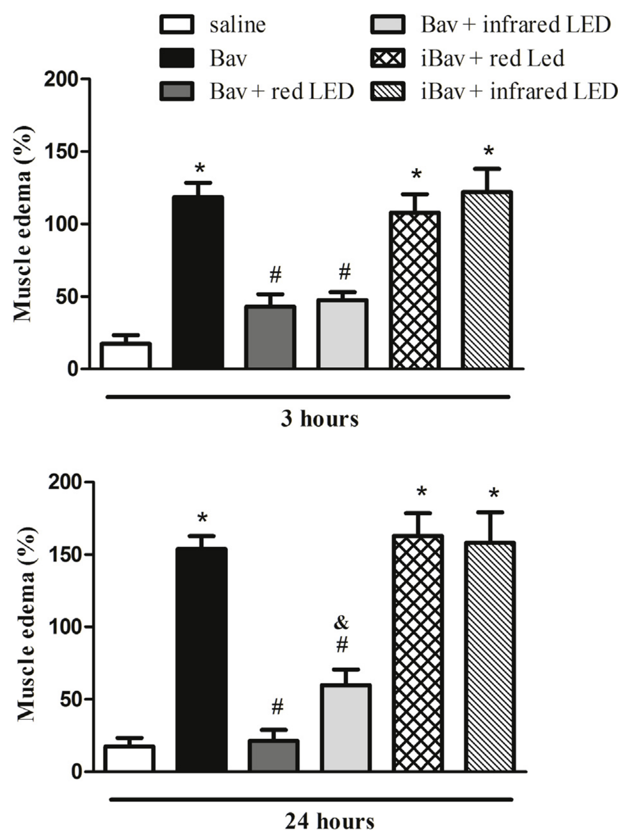

Fig. 2. Effect of red LED (635 nm) or infrared LED (945 nm) on muscle edema induced by Bav or iBav. Bav or iBav was injected in the right gastrocnemius muscle (50 µg per muscle). The contralateral muscle received the same volume of saline solution. LED irradiation was performed immediately, 1 h and 2 h after venom injection and edema was evaluated at 3 h (A) and 24 h (B). Edema was expressed as the percentage of the weight increase of the venom injected muscle, compared to the contralateral muscle (control). Data correspond to the mean ± S.E.M. of 5 animals per group. *p < 0.05 compared to control; #p < 0.05 compared to Bav; &p < 0.05 compared to Bav + red LED. Error bars indicate SEM.

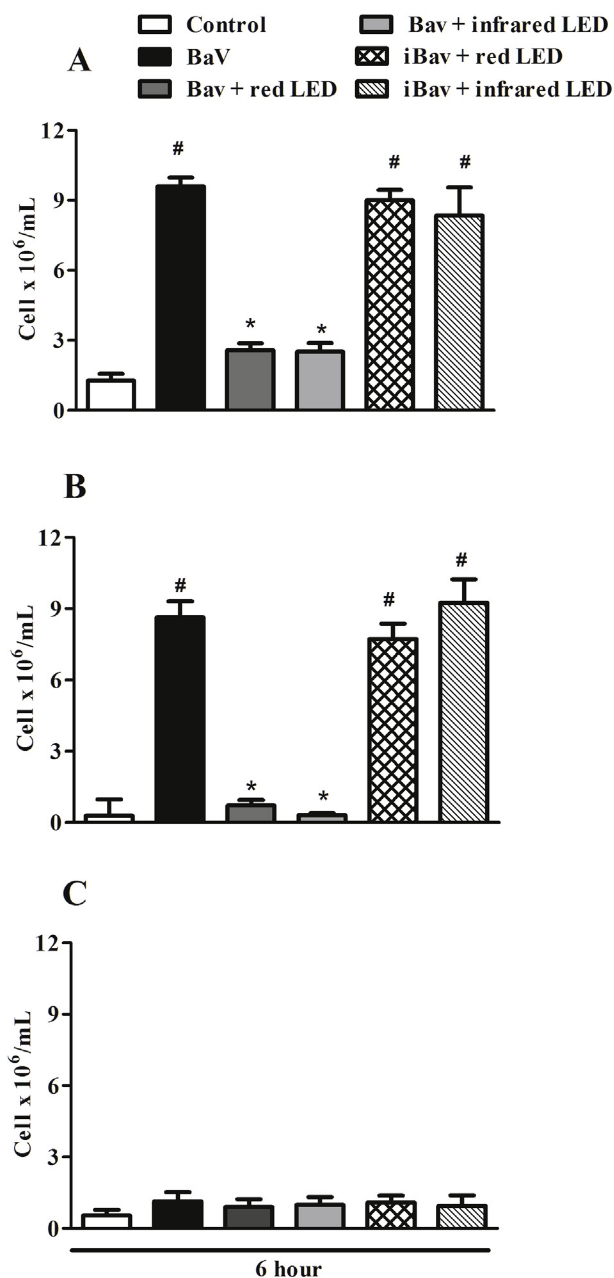

Fig. 3. Effect of red LED (635 nm) or infrared LED (945 nm) on leukocyte influx induced by Bav or iBav. (A) Total leukocytes, (B) polymorphonuclears cells, (C) mononuclear cells. Mice were injected with 5 μg of Bav or iBav in the peritoneal cavity. The LED device was applied immediately, 1 h e 2 h by contact in the same local of the venom injection. Animals of the control group received 50 μL of sterile saline solution. Six hours after venom injections, groups of animals were sacrificed and the inflammatory exudates were removed after washing the peritoneal cavity. Data correspond to the mean ± S.E.M. of 5 animals per group. *p < 0.05 compared to control; #p < 0.05 compared to Bav. Error bars indicate SEM.

Irradiated venom (red or infrared LED) did not show differences, as compared to non-irradiated venom in its edematogenic effect in muscle. Three hours after venom injection infrared LED treatment significantly reduced the muscle edema formation by 64% whereas red LED treatment reduced it by 60% (Fig. 2A). At 24 h after the Bav injection, the reduction in the muscle edema was 86% and 61% by infrared LED and red LED, respectively (Fig. 2B). The effect of infrared LED on muscle edema exposed to Bav at 24 h was still significantly different from that of saline-treated (control) mice.

3.3. Effect of red LED or infrared LED treatment on leukocyte influx into the peritoneal cavity

Fig. 3 shows the number of leukocytes in the peritoneal cavity of mice 6 h after i.p. injection of Bav (5 μg/cavity). Bav stimulated a migration of leukocytes that was significantly higher than that of saline-treated (control) mice (Bav: 9.59 ± 0.38 × 10^6 cells/mL; control: 1.25 ± 0.28 × 10^6 cells/mL). Irradiated venom by red or infrared LED exerted a similar effect on leukocyte migration as non-irradiated venom. Fig. 3A shows that both LED treatments reduced total leukocyte migration caused by Bav (red LED: 2.52 ± 0.36 × 10^6 cells/mL, Infrared LED 2.59 ± 0.29 × 10^6 cells/mL). The differential leukocyte counts indicated that most of the Bav-induced increase in cell migration was attributable to PMN rather than MN cells (Fig. 4 B, C). The infrared LED and red LED treatment significantly reduced PMN influx induced by Bav (Bav: 0.86 ± 6.75 × 10^6 cells/mL; red LED: 0.71 ± 0.23 × 10^6 cells/mL; Infrared LED: 0.31 ± 0.07 × 10^6 cells/mL). The number of MN was not modified in any of the treatments used (Fig. 3C).

3.4. Effect of red LED or infrared LED treatment on hyperalgesia induced by Bav

Intraplantar injection of Bav (2.5 μg) induced a significant decrease in nociceptive threshold (hyperalgesia) from 1 to 6 h (Fig. 4). When animals were treated with red LED the hyperalgesic response was significantly reduced from 6 to 24 h. The infrared LED treatment promoted a reduction of the threshold from 3 to 24 h after venom injection.

3.5. Effects of red LED or infrared LED treatment on myotoxicity induced by Bav

3.5.1. CK activity

Bav caused an increase of CK levels in plasma of animals (2510 ± 290 U/L). As shown in Fig. 5A, there was a significant reduction in plasma CK levels, with Red LED (670 ± 520 U/L) and infrared LED treatment (360 ± 110 U/L) as compared to the venom group. Furthermore, Bav caused a decrease of residual CK levels of the gastrocnemius muscle 24 h after venom injection (553 ± 131 U/L), compared to control muscle (2660 ± 156 U/L). LED treatment significantly increased residual CK content by infrared LED (1642 ± 255 U/L) and red LED (1415 ± 82 U/L) (Fig. 5B).

3.5.2. Histological assessment of muscle damage

Sections of gastrocnemius muscles of mice that received Bav, iBav or saline injection are illustrated in Fig. 6. The photomicrography illustrating the control gastrocnemius muscle was histologically normal in aspect with a typical structural organization of skeletal muscle (Fig. 6A). Fig. 6B shows significant changes in gastrocnemius muscle after 24 h of Bav inoculation, including the appearance of necrotic areas of muscle tissue. It included edema, represented by intersticial enlargement, loss of myofibrils, hypercontracted fibers and a massive infiltration of inflammatory cells. Histological alterations induced by iBav (red or infrared LED) were similar to those induced by non-irradiated venom (Fig. 6C and D). The venom group of mice treated with red or infrared LED showed some differences in the necrotic picture in relation to non-irradiated envenomed mice. The extension of damaged area in irradiated mice showed fewer destroyed fibers and a decrease in disorganization of myofibrils. Foci of more preserved fibers were intermingled with injury fibers. In addition, the extent of inflammatory cell infiltrate was clearly reduced in comparison to non-irradiated ones (Fig. 6E and F). There was no difference between red and infrared LED treatments.

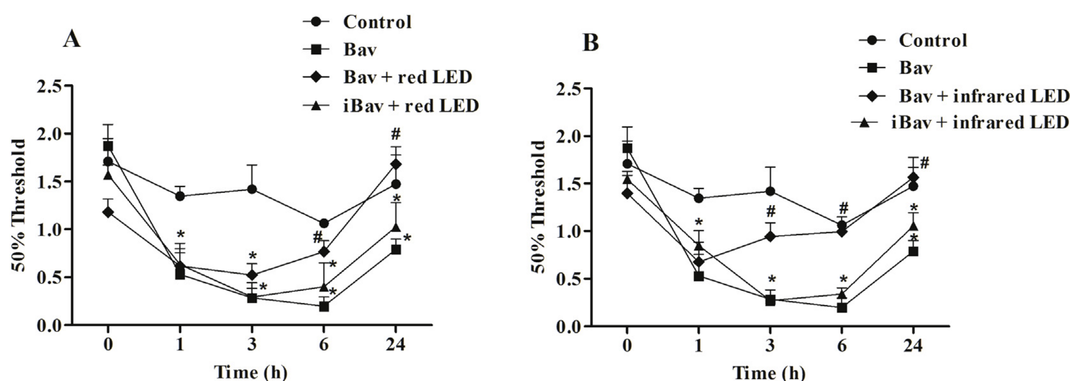

Fig. 4. Effect of red LED (635 nm) or infrared LED (945 nm) on mechanical hyperalgesia induced by Bav or iBav. Bav or iBav were injected into the plantar surface of the hind paw of mice. Mechanical hyperalgesia was measured by von Frey filaments before, 1, 3 and 6 h after venom injection. Red LED (A) or infrared LED (B) were applied directly on the site of injection. Results are presented as mean ± S.E.M. (n = 5). *$p < 0.05$ compared to control; #$p < 0.05$ in comparison to Bav. Error bars indicate SEM.

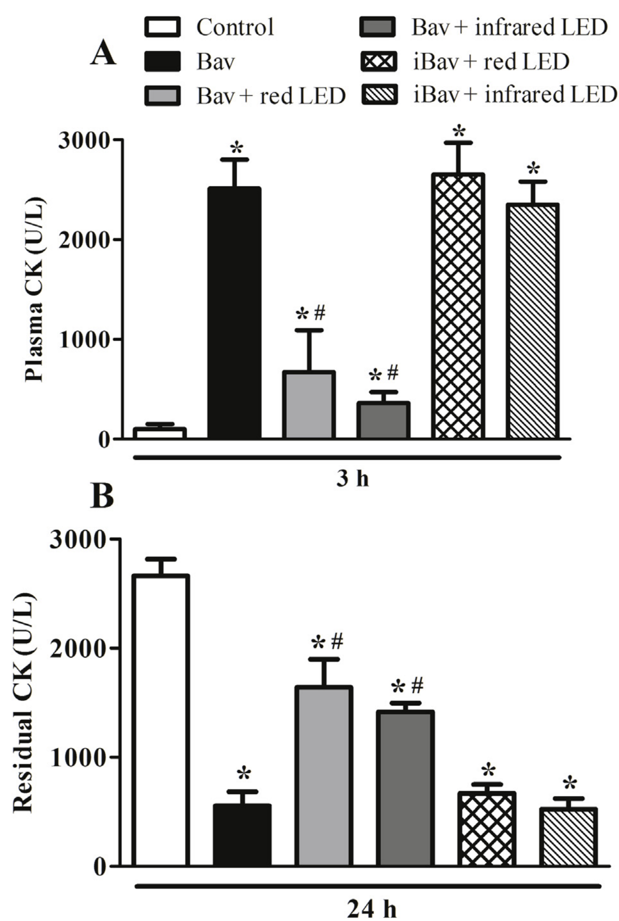

Fig. 5. Effect of red LED (635 nm) or infrared LED (945 nm) on creatine kinase (CK) release induced by Bav or iBav. Mice were injected in right gastrocnemius muscle with 50 μg of Bav or iBav, dissolved in 50 μL of saline solution. The groups received irradiation immediately and again 1 h and 2 h after the inoculation of the venom. Three hours after venom injection, mice were bled from the tail, and the plasma CK activity was determined (A). The animals were killed 24 h after administration of venom, both right and left gastrocnemius muscles were dissected and homogenized, and the residual CK activity was determined (B). Results are expressed in units (U/L), where a unit corresponding to the phosphorylation of 1 μmol of NADH, per min at 25 °C. *$p < 0.05$ compared to control; #$p < 0.05$ in comparison to Bav. Error bars indicate SEM.

4. Discussion

Antivenoms are the main therapeutic approach used to treat snakebite envenomations. Although antivenom has proven highly effective in the neutralization of systemic effects, they are only partially effective in the neutralization of the local effects induced by *Bothrops* sp. (Gutiérrez et al., 1986, 1998; Zamunér et al., 2004; Picolo et al., 2002). For this reason, researchers have increasingly sought alternatives for the treatment of venom-induced local effects. Here, we used the LED treatment to counteract the main manifestation of local tissue damage induced by the venom of *B. asper*. LED is a non-invasive, low-cost procedure that has been used in clinical practice to promote wound healing, stimulate tissue regeneration, and relieve pain, inflammation and edema (Yeh et al., 2010).

In order to verify whether the LED irradiation would be capable of altering the venom components and thus decrease their ability to cause tissue damage and inflammation, venom solutions were irradiated before injection in mice. Results demonstrated that venom subjected to prior irradiation showed the same toxicity profile observed with non-irradiated venom, demonstrating that the beneficial effects of LED treatment on venom-induced damage are not mediated by a direct inactivation of venom toxins by LED light, which is consistent with observations from our group using LLL irradiation (Barbosa et al., 2009). In this previous study, the irradiated lyophilized *B. jararacussu* venom did not affect myotoxic effect. Hence, the beneficial effects observed in our experiments are more likely due to changes in susceptibility of tissue to the action of venom.

Wavelength is considered a relevant parameter for beneficial outcomes of PBMT and determines the ability of a light to penetrate tissue. Studies have shown that shorter wavelengths in the rage of 600–700 nm penetrate more superficially, and longer wavelengths in the range of 780–950 nm, penetrates deeper in tissues (Chung et al., 2012). In this study we used the wavelength of 635 and 945 nm, and the results showed that both wavelengths produced positive results in all parameters analyzed, with no significant difference between them, with the exception of muscular edema, in which the red LED showed a better response. Dourado et al. (2011) carried out a study using 632.8 and 904 nm and reported that 632.8 nm laser produced a better angiogenic effect while 904 nm laser light improved fiber regeneration of muscle fascicles in gastrocnemius muscle exposed to snake venom. Another study, evaluating snake-envenomated gastrocnemius muscle of mice using enzyme biomarkers found that irradiation with 904 nm was superior to treatment with 632.8 nm laser light (Dourado et al., 2017). Moreover, Nadur-Andrade et al. (2014a) reported best results with 635 nm LED irradiation when compared to 945 nm LED light assessed venom-induced hyperalgesia. Although studies using red or infrared wavelengths showed some differences, both red and infrared

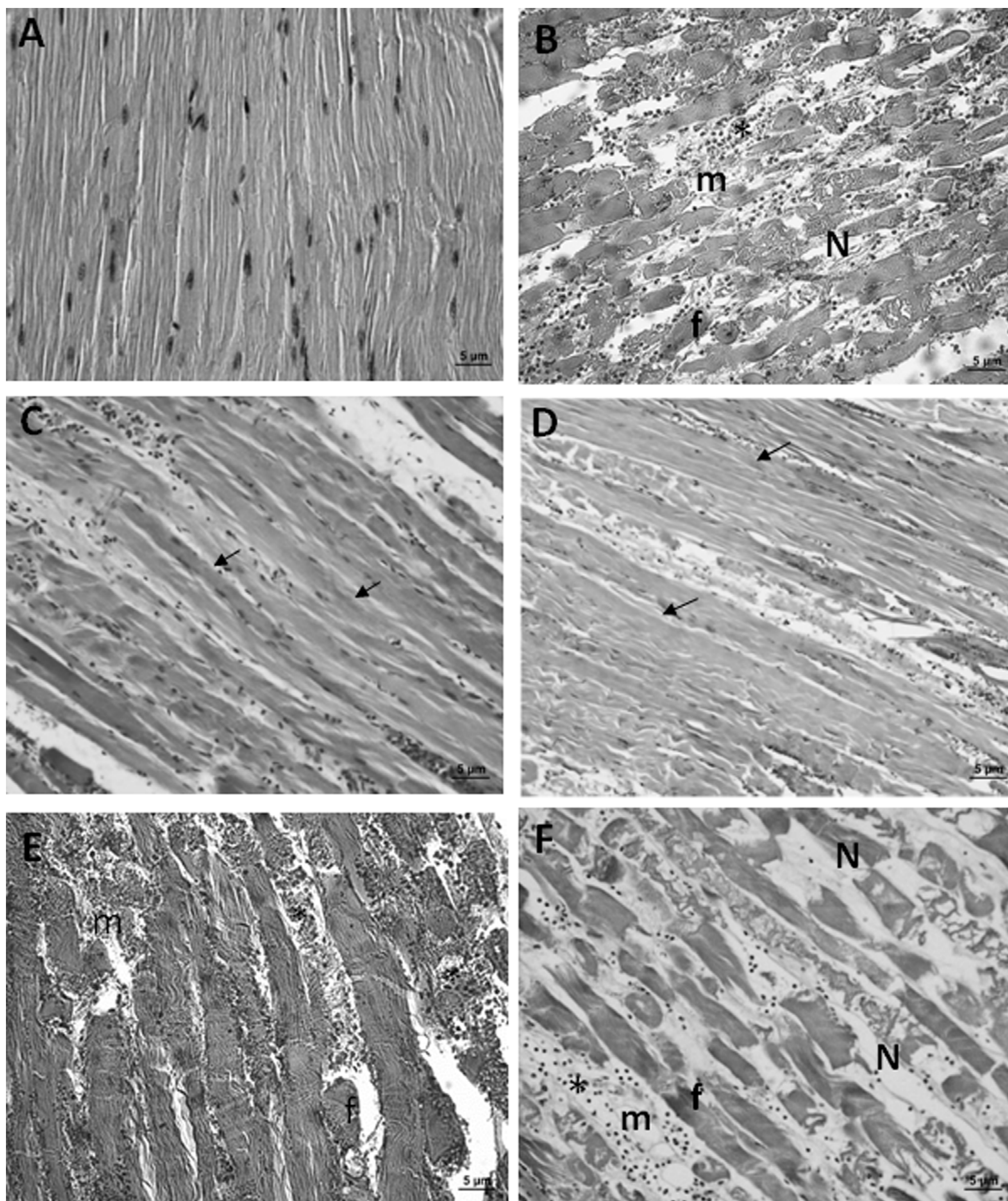

Fig. 6. Effect of red LED (635 nm) or infrared LED (945 nm) in the myotoxic effect in gastrocnemius muscle injected with Bav or iBav. (A): Control gastrocnemius muscle injected with saline solution presents normal fibers; Muscle injected with (B): Bav, (C): iBav red LED and (D): iBav Infrared LED. Note typical alterations due to myonecrosis, such as loss of myofibrils (m); hypercontracted fibers (f) and presence of inflammatory cells (*).Muscle injected with Bav and irradiated with (E): red LED or (F): infrared LED present most of the fibers with normal morphology (arrows) and fewer inflammatory cells as compared to venom-injected muscle. Haematoxylin/eosin (HE). Scale bar 5 µm.

wavelength consistently produced positive outcomes in different models of venom-induced local injury.

Prominent local edema is a common clinical finding in envenomations by Bav, occurring in 95% of the cases (Otero-Patiño, 2009). In this study, the edema formation induced by Bav, measured on plantar and gastrocnemius muscle, was significantly reduced by both LED treatments used, when compared to venom-injected mice that did not receive irradiation. This result is similar to those of Nadur-Andrade et al. (2012) that showed an effective reduction in edema formation by LED treatment after injection of *B. moojeni* venom. Olivo et al. (2007) reported that Bav releases PGE_2 via expression of COX-2, contributing to the edema formation in mice. Therefore, we suggest that the LED therapy acts by reducing the levels of PGE_2 induced by Bav, with the consequent effect on edema formation; this hypothesis is based on studies showing a reduction of PGE_2 after PBMT irradiation in animals injected with myotoxins isolated from *B. jararacussu* venom (Santos et al., 2018).

Another important event of the inflammatory response evoked by Bav is the leukocytes influx into the inflammatory focus (Zamuner et al., 2005). In our study, LED irradiation was significantly effective in reducing the total number of leukocytes as well as of neutrophils in the peritoneal cavity. Zamuner et al. (2005) suggested that the leukocyte influx caused by Bav is related to increased levels of pro-inflammatory cytokines, particularly IL-6. In this regard, Nadur-Andrade et al. (2016) showed a modulating effect of pro- and anti-inflammatory cytokines IL-6, TNF and IL-10, as well as kinin B1 and B2 receptors, at mRNA transcriptional level, in paw tissue, after injection of *B. moojeni* venom. From this point of view, it is likely that, in our experimental model, the LED irradiation acts by decreasing the synthesis of pro-inflammatory mediators, important for the recruitment of inflammatory cells, and thus, reducing the number of these cells at the site of venom injection.

Pain is another characteristic manifestation in Bav envenomations in humans and experimental animals (Arroyo et al., 1999; Chacur et al., 2001). We have shown here that LED treatment is effective in reducing nociception induced by Bav. The mechanism by which PBMT reduces nociceptive effects after venom-induced hyperalgesia is unknown; however, Nadur-Andrade et al. (2016) demonstrated that PBMT is effective in decreasing nociceptor activation at the spinal cord level after *B. moojeni* venom injection.

Local myonecrosis is a prominent effect of envenomings by Bav and

the consequence is the loss of muscle mass with impaired regeneration (Hernández et al., 2011, Gutiérrez et al., 2009a). The ability of LED to reduce myonecrosis caused by Bav was evaluated by biochemical and histological techniques. LED treatment was able to reduce the increments in CK activity in plasma and increased the CK content in muscle. Histological examination of muscle tissue injected with Bav showed an intense myonecrosis, with interstitial edema, vascular congestion, and a massive infiltrate of inflammatory cells, characteristic of *Bothrops* venoms (Gutiérrez et al., 2009a). The treatment with the two LEDs reduced skeletal muscle damage caused by the venom, corroborating the biochemical analyses. Other studies showed reduced myonecrosis after LLL irradiation in experimental models of envenomation (Aranha de Sousa et al., 2013; Barbosa et al., 2009; Doin-Silva et al., 2009). This protection of acute muscle damage may be related to an increasing resistance of muscle fibers to the action of myotoxic PLA_2s present in Bav, perhaps conferring the plasma membrane of muscle cells with increased stability, a hypothesis that needs to be evaluated.

In conclusion, our data demonstrate that PBM with LED light in red and infrared wavelengths, when applied after Bav injection, reduces the extent of myonecrosis, local edema, hyperalgesia, and inflammatory infiltrate in mice. The effect is likely to depend on the protective action of irradiation in tissue components, and not in a direct inactivation of venom toxins, since irradiated venom showed similar toxicity as non-irradiated venom. Although the mechanisms involved in the effects of LED therapy are not fully understood, the results hereby presented, suggest that PBM may become a complementary tool for the treatment of snakebite envenomations, especially in the reduction of the extent of local tissue damage.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

Aranha de Sousa, E., Bittencourt, J.A., Seabra de Oliveira, N.K., Correia Henriques, S.V., dos Santos Picanço, L.C., Lobato, C.P., Ribeiro, J.R., Pereira, W.L., Carvalho, J.C., da Silva, J.O., 2013. Effects of a low-level semiconductor gallium arsenide laser on local pathological alterations induced by *Bothrops moojeni* snake venom. Photochem. Photobiol. Sci. 12 (10), 1895–1902.

Arroyo, O., Rojas, G., Gutiérrez, J.M., 1999. Envenenamiento por mordedura de serpiente en Costa Rica en 1996: epidemiología y consideraciones clínicas. Acta Med. Costarric. 41, 23–29.

Barbosa, A.M., Villaverde, A.B., Guimarães-Souza, L., Ribeiro, W., Cogo, J.C., Zamuner, S.R., 2008. Effect of low-level laser therapy in the inflammatory response induced by *Bothrops jararacussu* snake venom. Toxicon 51, 1236–1244.

Barbosa, A.M., Villaverde, A.B., Sousa, L.G., Munin, E., Fernandez, C.M., Cogo, J.C., Zamuner, S.R., 2009. Effect of low-level laser therapy in the myonecrosis induced by *Bothrops jararacussu* snake venom. Photomed. Laser Surg. 27 (4), 591–597.

Camey, K.U., Velarde, D.T., Sanche, E.F., 2002. Pharmacological characterization and neutralization of the venoms used in the production of Bothropic antivenom in Brazil. Toxicon 40 (5), 501–509.

Chacur, M., Picolo, G., Gutierrez, J.M., Teixeira, C.F., Cury, Y., 2001. Pharmacological modulation of hyperalgesia induced by Bothrops asper (terciopelo) snake venom. Toxicon 39 (8), 1173–1181.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of allodynia in the rat paw. J. Neurosci. Meth. 53, 55–63.

Chugh, K., Aikat, B., Sharma, B., Dash, S., Mathew, M., Das, K., 1975. Acute renal failure following snakebite. Am. J. Trop. Med. Hyg. 24 (4), 692.

Chung, H., Dai, T., Sharma, S.K., Huang, Y.Y., Carroll, J.D., Hamblin, M.R., 2012. The nuts and bolts of low-level laser (light) therapy. Ann. Biomed. Eng. 40 (2), 516–533.

de Oliveira Melo, M., Pompeo, K.D., Baroni, B.M., Vaz, M.A., 2016. Effects of neuromuscular electrical stimulation and low-level laser therapy on neuromuscular parameters and health status in elderly women with knee osteoarthritis: a randomized trial. J. Rehabil. Med. 48 (3), 293–299.

Doin-Silva, R., Baranaukas, V., Rodrigues-Simioni, L., da Cruz-Hofling, M.A., 2009. The ability of low level laser therapy to prevent muscle tissue damage induced by snake venom. Photochem. Photobiol. 85 (1), 63–69.

Dourado, D.M., Favero, S., Baranauskas, V., Cruz-Hofling, M.A., 2003. Effects of the GaAs laser irradiatio non myonecrosis caused by *Bothrops moojeni* snake venom. Lasers Surg. Med. 33, 352–357.

Dourado, D.M., Matias, R., Barbosa-Ferreira, M., da Silva, B.A.K., de Araujo Isaias Muller, J., Vieira, W.F., da Cruz-Höfling, M.A., 2017. Effects of photobiomodulation therapy on Bothrops moojeni snake-envenomed gastrocnemius of mice using enzymatic biomarkers. Lasers Med. Sci. 32 (6), 1357–1366.

Dourado, D.M., Fávero, S., Matias, R., Carvalho, P.de T., da Cruz-Höfling, M.A., 2011. Low-level laser therapy promotes vascular endothelial growth factor receptor-1 expression in endothelial and nonendothelial cells of mice gastrocnemius exposed to snake venom. Photochem. Photobiol. 87 (2), 418–426.

Gutiérrez, J.M., 2010. Snakebite envenomation in Central America. In: Mackessy, S.P. (Ed.), Handbook of Venoms and Toxins of Reptiles. CRC Press, Florida, pp. 491–507.

Gutiérrez, J.M., Escalante, T., Rucavado, A., 2009a. Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom. Toxicon 54, 976–987.

Gutiérrez, J.M., León, G., Rojas, G., Lomonte, B., Rucavado, A., Chaves, F., 1998. Neutralization of local tissue damage induced by *Bothrops asper* (terciopelo) snake venom. Toxicon 36, 1529–1538.

Gutiérrez, J., Rojas, G., Lomonte, B., Gené, J., Cerdas, L., 1986. Comparative study of the edema-forming activity of Costa Rican snake venoms and its neutralization by a polyvalent antivenom. Comp. Biochem. Physiol. C Comp. Pharmacol. 85 (1), 171–175.

Gutiérrez, J.M., Rucavado, A., Chaves, F., Díaz, C., Escalante, T., 2009b. Experimental pathology of local tissue damage induced by Bothrops asper snake venom. Toxicon 54 (7), 958–975 1.

Hernández, R., Cabalceta, C., Saravia-Otten, P., Chaves, A., Gutiérrez, J.M., 2011. Rucavado A. Poor regenerative outcome after skeletal muscle necrosis induced by Bothrops asper venom: alterations in microvasculature and nerves. PLoS One 6 (5), e19834.

Herrera, C., Macêdo, J.K., Feoli, A., Escalante, T., Rucavado, A., Gutiérrez, J.M., Fox, J.W., 2016. Muscle tissue damage induced by the venom of Bothrops asper: identification of early and late pathological events through proteomic analysis. PLoS Negl. Trop. Dis. 10 (4), e0004599.

Jorge, M.T., Ribeiro, L.A., O'Connell, J.L., 1999. Prognostic factors for amputation in the case of envenoming by snakes of the Bothrops genus (Viperidae). Ann. Trop. Med. Parasitol. 93 (4), 401–408.

Lizano, S., Domont, G., Perales, J., 2003. Natural phospholipase A(2) myotoxin inhibitor proteins from snakes, mammals and plants. Toxicon 42 (8), 963–977.

Magri, L.V., Carvalho, V.A., Rodrigues, F.C., Bataglion, C., Leite-Panissi, C.R., 2017. Effectiveness of low-level laser therapy on pain intensity, pressure pain threshold, and SF-MPQ indexes of women with myofascial pain. Lasers Med. Sci. 32 (2), 419–428.

Nadur-Andrade, N., Barbosa, A.M., Carlos, F.P., Lima, C.J., Cogo, J.C., Zamuner, S.R., 2012. Effects of photobiostimulation on edema and hemorrhage induced by *Bothrops moojeni* venom. Lasers Med. Sci. 27, 65–70.

Nadur-Andrade, N., Dale, C.S., Oliveira, V.R.S., Toniolo, E.F., Feliciano, R.S., Silva Jr., J.A., Zamuner, S.R., 2016. Analgesic Effect of photobiomodulation on *Bothrops moojeni* venom-induced hyperalgesia: a mechanism dependent on neuronal inhibition, cytokines and kinin receptors modulation. PLoS Negl. Trop. Dis. 17 (10), e0004998.

Nadur-Andrade, N., Dale, C.S., Santos, A.S., Soares, A.M., Lima, C.J., Zamuner, S.R., 2014a. Photobiostimulation reduces edema formation induced in mice by Lys-49 phospholipases A2 isolated from *Bothrops moojeni* venom. Photochem. Photobiol. Sci. 13, 1561–1567.

Nadur-Andrade, N., Zamuner, S.R., Toniolo, E.F., de Lima, C.J., Cogo, J.C., Dale, C.S., 2014b. Analgesic effect of light-emitting diode (LED) therapy at wavelengths of 635 and 945 nm on *Bothrops moojeni* venom-induced hyperalgesia. Photochem. Photobiol. 90, 207–213.

Olivo, R., Teixeira, C., Wallace, J., Gutierrez, J., Zamuner, S.R., 2007. Role of cyclooxygenases in oedema-forming activity of bothropic venoms. Toxicon 49 (5), 670–677.

Otero-Patiño, R., 2009. Epidemiological, clinical and therapeutic aspects of Bothrops asper bites. Toxicon 54, 998–1011.

Picolo, G., Chacur, M., Gutierrez, J.M., Teixeira, C.F., Cury, Y., 2002. Evaluation of antivenoms in the neutralization of hyperalgesia and edema induced by Bothrops jararaca and *Bothrops asper* snake venoms. Braz. J. Med. Biol. Res. 35 (10), 1221–1228.

Rostelato-Ferreira, S., Leite, G.B., Cintra, A.C., da Cruz-Höfling, M.A., Rodrigues-Simioni, L., Oshima-Franco, Y., 2010. Heparin at low concentration act as antivenom against Bothrops jararacussu venom and bothropstoxin-I neurotoxic and myotoxic. J. Venom Res. 15 (1), 54–60.

Ruaro, J.,A., Fréz, A.R., Ruaro, M.B., Nicolau, R.A., 2014 Nov. Low-level laser therapy to treat fibromyalgia. Lasers Med. Sci. 29 (6), 1815–1819.

Rucavado, A., Escalante, T., Franceschi, A., Chaves, F., León, G., Cury, Y., Ovadia, M., Gutiérrez, J.M., 2000. Inhibition of local hemorrhage and dermonecrosis induced by *Bothrops asper* snake venom: effectiveness of early in situ administration of the peptidomimetic metalloproteinase inhibitor batimastat and the chelating agent $CaNa_2EDTA$. Am. J. Trop. Med. Hyg. 63, 313–319.

Rucavado, A., Soto, M., Escalante, T., Loria, G.D., Arni, R., Gutierrez, J.M., 2005. Thrombocytopenia and platelet hypoaggregation induced by Bothrops asper snake

venom. Toxins involved and their contribution to metalloproteinase-induced pulmonary hemorrhage. Thromb Haemostasis 94 (1), 123–131.
Saborío, P., González, M., Cambronero, M., 1998. Snake bite accidents in children in Costa Rica: epidemiology and determination of risk factors in the development of abscess and necrosis. Toxicon 36 (2), 359.
Saldarriaga-Córdoba, M., Parkinson, C.L., Daza, J.M., Wüster, W., Sasa, M., 2017. Phylogeography of the central American lance head Bothrops asper (Serpentes: Viperidae). PLoS One 12 (11), e0187969 27.
Santos, A.S.D., Guimarães-Sousa, L., Costa, M.S., Zamuner, L.F., Sousa, N.C., Hyslop, S., Soares, A.M., Chavantes, M.C., Cogo, J.C., Zamuner, S.R., 2018. Photobiomodulation of local alterations induced by BthTX-I, a phospholipase A2 myotoxin from Bothrops jararacussu snake venom: in vivo and in vitro evaluation. Int. J. Biol. Macromol. 107 (Pt B), 2020–2025.
Souza, L.G., Dale, C.S., Nadur-Andrade, N., Barbosa, A.M., Cogo, J.C., Zamuner, S.R., 2011. Low-level laser therapy reduces edema, leukocyte influx and hyperalgesia induced by *Bothrops jararacussu* snake venom. Clin. Exp. Med. Lett. 52 (3–4), 97–102.
Tribuiani, N., Tavares, M.O., Santana, M.N., Fontana Oliveira, I.C., Amaral Filho, J.D., Silva, M.G., Dos Santos, M.G., Cogo, J.C., Floriano, R.S., Cogo-Müller, K., Oshima-Franco, Y., 2017. Neutralizing ability of Terminalia fagofolia extract (Combretaceae) against in vitro neuromuscular effects of *Bothrops jararacussu* venom. Nat. Prod. Res. 31 (23), 2783–2787.
Yeh, N.G., Wu, Chia-Hao, Cheng, T.C., 2010. Light-emitting diodes—their potential in biomedical applications. Renew. Sustain. Energy Rev. 14, 2161–2166.
Zamunér, S.R., da Cruz-Höfling, M.A., Corrado, A.P., Hyslop, S., Rodrigues-Simioni, L., 2004. Comparison of the neurotoxic and myotoxic effects of Brazilian Bothrops venoms and their neutralization by commercial antivenom. Toxicon 44 (3), 259–271 1.
Zamuner, S.R., Zuliani, J.P., Fernandes, C.M., Gutiérrez, J.M., Teixeira, C.F.P., 2005. Inflammation induced by Bothrops asper venom: release of proinflammatory cytokines and eicosanoids, and role of adhesion molecules in leukocyte infiltration. Toxicon 46 (7), 806–813 1.
Zamuner, S.R., Gutiérrez, J.M., Muscará, M.N., Teixeira, S.A., Teixeira, C.F., 2001. Bothrops asper and Bothrops jararaca snake venoms trigger microbicidal functions of peritoneal leukocytes in vivo. Toxicon 39 (10), 1505–1513.