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Analgesic Effect of Light-Emitting Diode (LED) Therapy at Wavelengths of 635 and 945 nm on *Bothrops moojeni* Venom-Induced Hyperalgesia

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ABSTRACT

Envenoming induced by *Bothrops* snakes is characterized by drastic local tissue damage involving hemorrhage, myonecrosis and proeminent inflammatory and hyperalgesic response. The most effective treatment is antivenom therapy, which is ineffective in neutralizing the local response. Herein, it was evaluated the effectiveness of light-emitting diode (LED) at wavelengths of 635 and 945 nm in reducing inflammatory hyperalgesia induced by *Bothrops moojeni* venom (BmV) in mice, produced by an subplantar injection of BmV (1 μg). Mechanical hyperalgesia and allodynia were assessed by von Frey filaments at 1, 3, 6 and 24 h after venom injection. The site of BmV injection (1.2 cm^2) was irradiated by LEDs at 30 min and 3 h after venom inoculation. Both 635 nm (110 mW, fluence of 3.76 J/cm^2 and 41 s of irradiation time) and 945 nm (120 mW, fluence of 3.8 J/cm^2 and 38 s of irradiation time) LED inhibited mechanical allodynia and hyperalgesia of mice alone or in combination with antivenom treatment, even when the symptoms were already present. The effect of phototherapy in reducing local pain induced by BmV should be considered as a novel therapeutic tool for the treatment of local symptoms induced after bothropic snake bites.

INTRODUCTION

Snake envenomation presents a threat to public health in tropical countries. In South America, snakes of the genus *Bothrops* are responsible for the majority of snakebites (1,2). Patients bitten by *Bothrops* snakes manifest systemic signs of envenomation, such as hemostatic disturbances, and signs at the site of bite, for example hemorrhage, myonecrosis, dermonecrosis and inflammatory reactions—such as edema, leukocyte infiltration and hyperalgesia (3,4).

Bothrops moojeni is a venomous snake responsible for most of the snakebites in the central region of Brazil (5). Despite the medical importance, there are only a few studies related to the local inflammatory reaction caused by this venom. In this sense, the literature shows that in the accidents caused by these snakes serious local complications occur such as a prominent edema formation, intense pain, swelling and pallor which may develop into more severe results such as muscle mass loss, neuropathy and amputation (6).

So far, the most effective treatment for *Bothrops* snakebites accidents is the antivenom therapy. While antivenins (AV) are efficient in reversing systemic alterations in snakebite envenomations, its administration does not prevent local effects and resultant disabilities. Consequently, there is a need to find therapeutic approaches that are able to complement AV in the neutralization of local tissue damage to minimize or prevent the progression to a severe clinical status observed after *Bothrops* snakebites (7,8). Thus, it is important to develop therapies that may be associated with AV treatment that can be effective in reducing the local effects by *Bothrops* snakes envenoming. In this scenario, phototherapy emerges as an important tool in the treatment of local reactions caused by bothropic venoms. In this sense it has been recently demonstrated that laser treatment decreases myonecrosis, edema and leukocyte influx of the gastrocnemius muscle of mice injected with *B. jararacussu* venom (8–10).

Positive effects of light-emitting diode (LED) phototherapy have been demonstrated on certain skin disease (11–14), in wound healing (15), in the treatment of retinal injury (16), in the reduction in wounds in diabetic rats (17), in chronic venous ulcers (18), in mucositis (19) and as an anti-inflammatory tool (20). LED reduction in pain has also been demonstrated (21). Experimentally it has been demonstrated that LED inhibits hyperalgesia and edema formation in an experimental model of zymosan-induced arthritis in mice (22), reduces signs of inflammation in collagenase-induced tendinitis in rats (23) and prevents muscle damage and local inflammation after exercise in an experimental model of exercise-induced muscle damage in rats (24).

Although its mechanism of action is still unknown, results suggest that during phototherapy treatment, both visible and near-infrared photons are absorbed by chromophores within the cells, resulting in increased production of cellular energy, which leads to normalization of cell function, pain relief and healing (25,26). Considering the mechanisms by which phototherapy inhibits inflammatory response, it is demonstrated, in human gingival fibroblasts, that 635 nm irradiation, can directly dissociate the

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reactive oxygen species (ROS) leading to the inhibition of phospholipase A(2) and cyclooxygenase (COX-2) expression, thus inhibiting prostaglandin E(2) release (27), and this effect involves the participation of nuclear factor-kappa B (NF-KB) (28). Similar results were observed in an experimental model of muscle damage where the inhibition of inflammatory response by 808 nm irradiation occurs through a mechanism involving NF-kB and COX-2 (29). The involvement of NF-kB in the anti-inflammatory effect of low-level laser therapy have been reported in other experimental models (30–32), thus reinforcing its participation in the anti-inflammatory effect of phototherapy.

We have previously reported that laser and LED treatment significantly reduces the edema formation and hemorrhage induced by *B. moojeni* snake venom (33). We also demonstrated that low-level laser therapy was efficient in reducing *B. jararacussu*-induced hyperalgesia (34). However, the analgesic effect of LED has not yet been demonstrated after venom injection. Although there are few data in the literature on the use of LED therapy in the treatment of snakebites, this may be an effective treatment to neutralize the local effects caused by *Bothrops* snake envenomation. In this study, we evaluated the effects of LED treatment on the outcome of *B. moojeni*-induced hyperalgesia and allodynia in mice.

MATERIALS AND METHODS

Animals. The experiments were carried out with male Swiss mice 45 days of age weighing 22–25 g. Animals were kept in plastic cages with water and food *ad libitum* and maintained under a controlled temperature on a 12-h light/dark cycle. Mice were divided into experimental groups of five animals each. For the experiments, the animals were handled considering the principles and guide use of laboratory animals involving pain and nociception (35), and approved by the Ethics Committee for the Use of Animals (CEUA2010/01) of the Hospital Sírio-Libanês.

Venom and antivenom. Bothrops moojeni venom (BmV) was supplied by the Serpentarium of the Center of Studies of Nature at UNIVAP. The BmV was lyophilized, kept refrigerated at 4°C and diluted in sterile saline solution (0.9%) immediately before use. BmV was injected into the subplantar surface of the right hind paw at the concentration of 1.0 μg/50 μL. The equine antivenom (AV) used in the experiments was a polyvalent *Bothrops* AV (lot# MS122340007) raised against a pool of venom from *B. alternatus*, *B. jararaca*, *B. jararacussu*, *B. cotiara*, *B. moojeni* and *B. neuwiedi*, and was obtained from the Butantan Institute (São Paulo, SP, Brazil). AV was injected through the intravenous route (0.2 μL of AV diluted in saline; final volume of 50 μL—considering that 1 mL of AV neutralizes 5 mg of Bothropic venom (36) 30 min after BmV injection.

Table 1. LED parameters.

	Wavelengths (nm)	
	635	945
Mean power output (m W)	110	120
Power density (m W/cm2)	0.092	0.100
Beam area (cm^2)	1.2	1.2
Fluence (J/cm^2)	3.76	3.8
Energy (J)	4.51	4.56
Energy density (J/cm^2)	3.8	3.8
Anatomical location	Subplantar/ hind paw	Subplantar/ hind paw
Number of treatments	2	2
Interval between treatments	2 h 30 min	2 h 30 min
Irradiated area (cm^2)	1.2	1.2
Irradiation time per point (sec)	41	38

A LED device (model Super Red LED-RL5-R3545; Super Bright LEDs, St. Louis, MO) was used for irradiation.

Mechanical hyperalgesia and tactile allodynia. Hyperalgesia and allodynia of the hind paw were assessed as described by Takasaki *et al.* (37). Mice were placed individually in plastic cages and 15 min later injected into the subplantar surface of the right hind paw with 1.0 μg of crude BmV diluted in 50 μL of sterile saline, or with the same volume of sterile saline. von Frey filaments with bending forces of 0.407 g (3.61 filament—allodynia stimulus), 0.692 g and 1.202 g (3.84 and 4.08 filaments—hyperalgesia stimulus) were pressed perpendicularly against the plantar skin and held for 5 s, at 1, 3, 6 and 24 h after venom injection. The stimulation of the same intensity was applied three times to each hind paw at intervals of 5 s. The responses to these stimuli were ranked

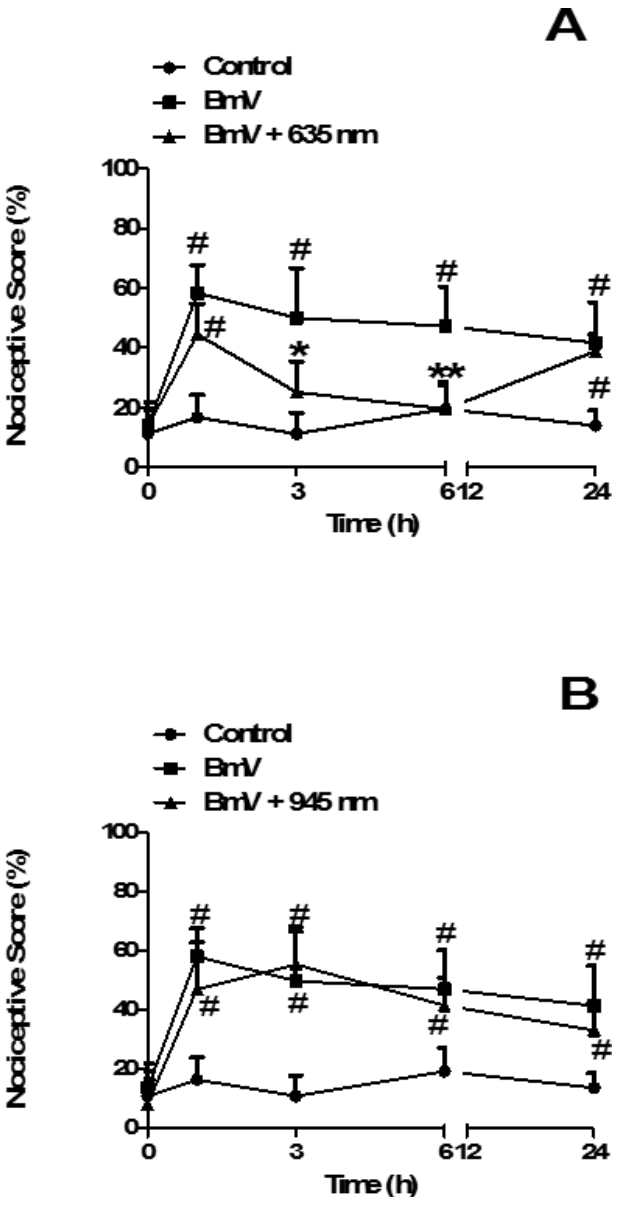

Figure 1. Effect of LED treatment on mechanical allodynia of mice. Animals were injected intraplantar with BmV (1 μg) and after 30 min and 3 h were treated with 635 nm LED (▲; **A**) or 945 nm LED (▲; **B**). Allodynia was measured by the mechanical response to tactile stimulation assessed by a von Frey filament of 0.407 g (3.61 filament). Pain threshold was determined before (baseline values, time 0) and 1, 3, 6 and 24 h after BmV injection. Animals injected only with BmV (■) or injected with saline (●; control) were submitted to the same protocol. Each point represents the mean ± SEM of five animals per group (#$P < 0.01$ *vs* control group; *$P < 0.05$ and **$P < 0.01$ *vs* BmV group).

as follows: 0, no response; 1, move away from von Frey filament and 2, immediate flinching or licking of the hind foot.

The nociceptive score was calculated as follows:

$$\text{Nociceptive score (\%)} = \frac{\sum(\text{average score of each animal})}{2 \times \text{number tested animals}} \times 100.$$

Light source, dose and treatment. A LED device (model Super Red LED-RL5-R3545; Super Bright LEDs, St. Louis, MO) was used for irradiation. The LED parameters, low enough to avoid any thermal effect, were chosen on the basis of previous studies (9,10). The LED radiation was applied to the same area as the injection of BmV or saline solution. A control group was treated using the same experimental procedure but with the LED turned off. Animals were irradiated 30 min and 3 h after subplantar injection. The experimental parameters for the LED are presented in Table 1 following Jenkins *et al.* (38).

Statistical analysis. Results are presented as mean ± standard error of mean. Statistical evaluation of data was carried out by one-way analysis of variance (ANOVA) and sequential differences among means were analyzed by Tukey test (Instat 3.01, GraphPad Software Inc, USA). Differences in results were considered statistically significant when $P < 0.05$.

RESULTS

Animals injected with *Bothrops moojeni venom* (BmV) showed significant allodynia when compared with baseline measures taken before the test, as indicated by increased basal threshold in response to stimulation by von Frey filaments. Allodynia was observed from the 1st h after BmV injection up to 24 h (Fig. 1A, B). Treatment with 635 nm LED applied 30 min and 3 h after BmV injection reversed the observed allodynia at the 3rd and 6th hours of treatment (Fig. 1A). On the other hand, treatment of animal with the 945 nm LED did not change BmV-induced allodynia (Fig. 1B).

Regarding mechanical hyperalgesia, injection of BmV caused significant increase in the nociceptive score from the 1st h after BmV injection up to 24 h after injection for both 3.84 and 4.08 von Frey filaments (Fig. 2). Treatment with 635 nm LED reversed the observed hyperalgesia for both filaments. For the 3.84 filament the inhibition of nociceptive behavior was significant for all

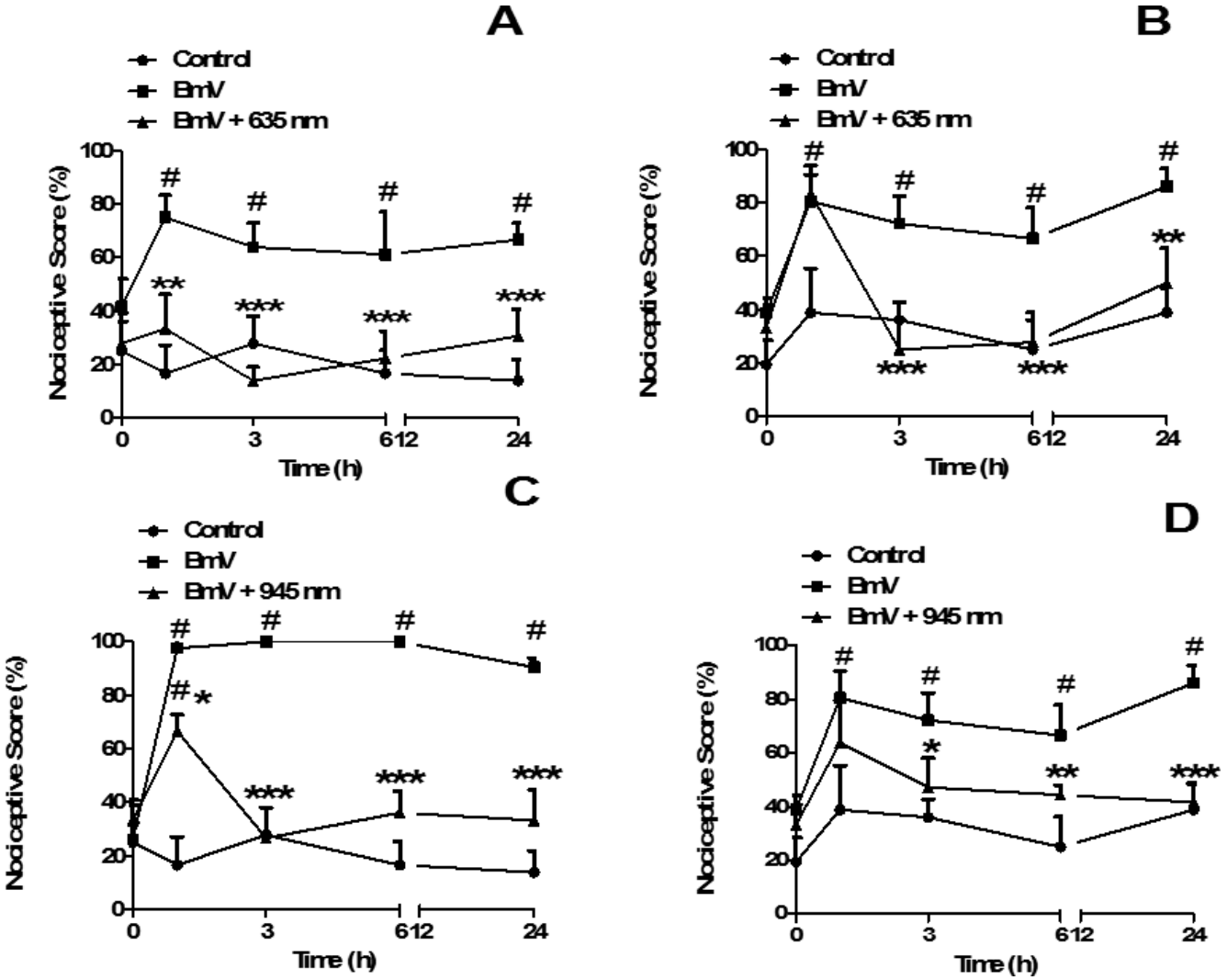

Figure 2. Effect of LED treatment on mechanical hyperalgesia of mice. Animals were injected intraplantar with BmV (1 μg) and after 30 min and 3 h were treated with 635 nm LED (▲; A/B) or 945 nm LED (▲; C/D). Hyperalgesia was measured by the mechanical response to nociceptive stimulation assessed by von Frey filaments of 0.692 g (3.84 filament; A and C) and 1.202 g (4.08 filament; B and D). Pain threshold was determined before (baseline values, time 0) and 1, 3, 6 and 24 h after BmV injection. Animals injected only with BmV (■) or injected with saline (●; control) were submitted to the same protocol. Each point represents the mean ± SEM of five animals per group (#$P < 0.01$ *vs* control group; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* BmV group).

evaluated times (Fig. 2A). For the 4.08 filament, the inhibition of nociceptive behavior was significant at the 3rd hour up to 24 h of evaluation (Fig. 2B). Similar results were observed for the 945 nm LED. However, for the 3.84 filament, besides the reversion of hyperalgesia at 3, 6 and 24 h, a partial inhibition of the nociceptive behavior at the 1st hour of evaluation was also observed (Fig. 2C). On the other hand, treatment with 945 nm LED induced only a partial reversion of nociception for the 4.08 filament (Fig. 2D).

The most effective treatment for venomous snakebites accidents is antivenom therapy, which although effective in neutralizing the systemic effects of envenomation, it does not neutralize the severe local effects induced by these accidents (8). As treatment with both 635 and 945 nm LED was effective in inhibiting painful response of animals after BmV injection, we evaluated its effect concomitant with antivenom (AV) treatment. AV treatment did not interfere with mechanical allodynia of mice when compared with control group or with animals injected with BmV (Fig. 3A, B). Concomitant treatment of AV with 635 nm LED was effective in inhibiting mechanical allodynia of mice similarly to that observed when animals were treated only with the LEDs (Fig. 3A). Also, for the 945 nm LED, once again, no changes on nociceptive score were observed (Fig. 3B).

Surprisingly, regarding mechanical hyperalgesia, when animals received AV concomitant with 635 nm LED a decrease in analgesic effect was observed for the 1st and 3rd h of treatment were only a partial reversion of hyperalgesia was detected when the 3.84 von Frey filament was evaluated (Fig. 4A). Moreover, for the 945 nm LED the loss of effect was detected for all evaluated times, in which case it was detected only as a partial reversion of mechanical hyperalgesia of mice at all evaluated times (Fig. 4C). Finally when 4.08 von Frey filament was evaluated, a loss of analgesic effect was observed for 635 nm LED for the 6th and 24th h of evaluation (Fig. 4B). Interestingly concomitant treatment of animals with AV and 945 nm LED completely inhibited mechanical hyperalgesia when the mice were evaluated with 4.08 von Frey at all evaluated times (Fig. 4D).

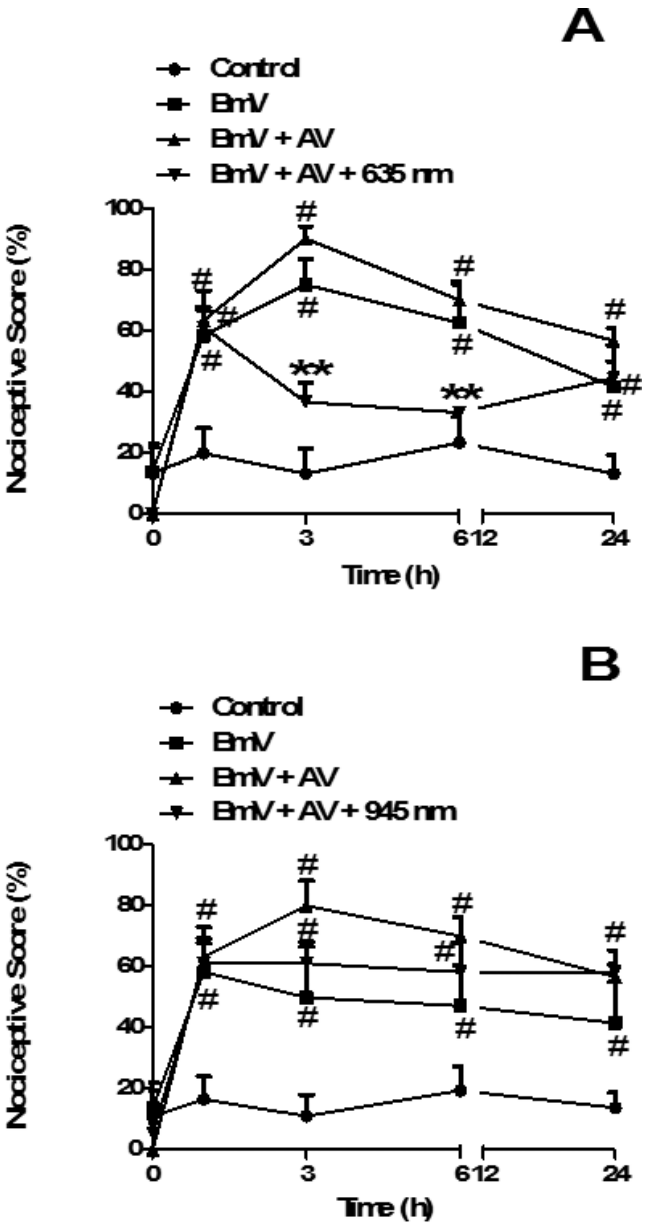

Figure 3. Effect of concomitant administration of antivenom and LED treatment on mechanical allodynia of mice. Animals were injected intraplantar with BmV (1 μg) and after 15 min received intravenous treatment with AV (0.2 μL). After 30 min and 3 h of BmV injection animals were treated with 635 nm LED (▼; A) or 945 nm LED (▼; B). Allodynia was measured by the mechanical response to tactile stimulation assessed by a von Frey filament of 0.407 g (3.61 filament). Pain threshold was determined before (baseline values, time 0) and 1, 3, 6 and 24 h after BmV injection. Groups of animals injected only with BmV (■), or injected with saline (●; control), or injected with BmV+AV (▲) were submitted to the same protocol. Each point represents the mean ± SEM of five animals per group (#$P < 0.01$ *vs* control group; **$P < 0.01$ *vs* BmV group).

DISCUSSION

In recent years, a great amount of attention has been directed toward the benefits of phototherapy as a treatment modality for reducing pain, inflammation and edema, promoting healing of wounds, deeper tissues and nerves, and preventing cell death and tissue damage (39). Although the biochemical mechanisms underlying the positive effects of phototherapy are incompletely understood, the literature demonstrates that photobiostimulation can be an effective alternative to drugs. Light-emitting diode (LED) photomodulation is a novel category of nonthermal light-based treatment designed to modulate cellular activity (40).

We showed here that LED treatment is effective in inhibiting both allodynia and hyperalgesia induced by *Bothrops moojeni* (BmV) venom, in mice. LED treatment was applied 30 min and 3 h after venom injection, to assess if LED could exert a beneficial effect even after the symptoms of envenomation become evident, as the intervention, in health centers, usually occurs only after several hours of the occurrence of the bite.

The ability that venoms from *Bothrops* snakes have to induce acute inflammatory process, have been reported by several authors (3,41). Herein, we demonstrate that the BmV was able to induce both mechanical allodynia and hyperalgesia in mice from the 1st h after injection remaining for at least 24 h. These data corroborate with data from literature demonstrating that the venoms of *B. asper* and *B. jararaca* snakes induce time-dependent mechanical hyperalgesia in rats (41–43).

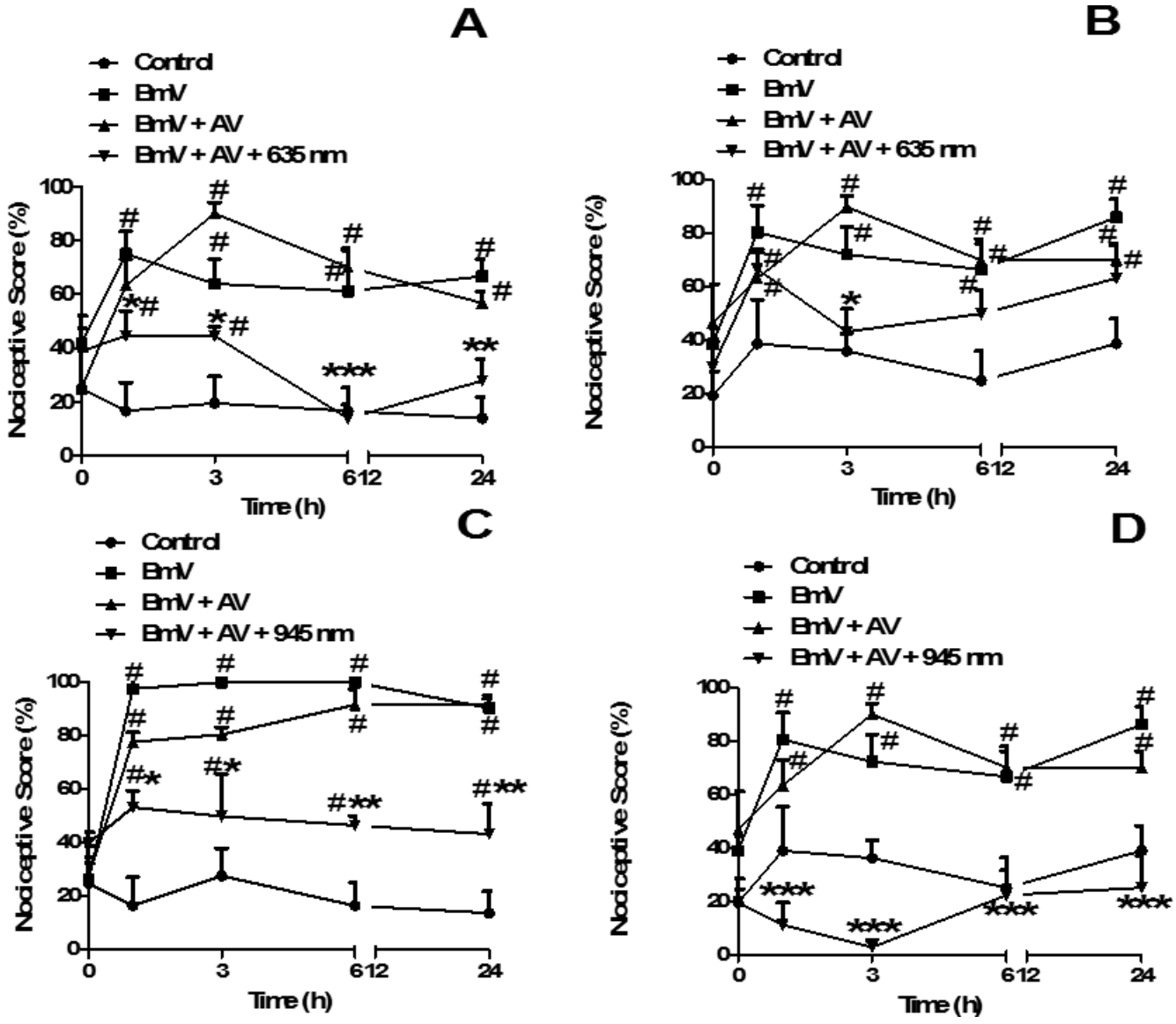

Figure 4. Effect of concomitant administration of antivenom and LED treatment on mechanical hyperalgesia of mice. Animals were injected intraplantar with BmV (1 μg) and after 15 min received intravenous treatment with AV (0.2 μL). After 30 min and 3 h of BmV injection animals were treated with 635 nm LED (▼; A/B) or 945 nm LED (▼; C/D). Hyperalgesia was measured by the mechanical response to nociceptive stimulation assessed by von Frey filaments of 0.692 g (3.84 filament; A and C) and 1.202 g (4.08 filament; B and D). Pain threshold was determined before (baseline values, time 0) and 1, 3, 6 and 24 h after BmV injection. Groups of animals injected only with BmV (■), or injected with saline (●; control), or injected with BmV + AV (▲) were submitted to the same protocol. Each point represents the mean ± SEM of five animals per group (#$P < 0.01$ *vs* control group; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* BmV group).

In this study we used two different LED wavelengths: one in the red region (635 nm) and the other in the near-infrared region (945 nm), both with a density of energy 3.8 J/cm^2. The choice of these parameters was based on the literature that demonstrates that LED irradiation is more effective around the red and infrared range (44). Results presented herein demonstrate that 635 nm LED, but not 945 nm LED, was able to inhibit mechanical allodynia in all evaluated times. Regarding hyperalgesia, both 635 and 945 nm LED were able to inhibit nociceptive behavior, however, 635 nm was more effective. These data are in accordance with data from literature demonstrating that laser treatment (at wavelength of 685 nm, 2.2 J/cm^2) inhibits hyperalgesia induced by *B. jararacussu* venom (34). One explanation of the fact that 635 nm LED was more effective than 945 nm LED could be the depth of light penetration, as the 635 nm light is absorbed more quickly than 945 nm light (45). The literature shows that the analgesic effect caused by low-level laser does not involve the participation of peripheral opioid receptors, but involves later events of prostaglandin E2 (PGE2) release during the acute inflammation (46). Also, it was demonstrated that *B. asper* and *B. jararaca* venom increase the release of PGE_2 via COX-2 expression (47). Therefore, the inhibition of PGE2 by LED could be, in our model, an important mechanism by which the phototherapy reduces the nociceptive behavior induced by *B. moojeni* venom, a hypothesis that needs to be addressed in future studies.

The most effective treatment for *Bothrops* snakebites is antivenom (AV) therapy which is very efficient in reversing venom-induced systemic effects characterized by disturbances in homeostasis, deficiency in clotting, abnormal platelet aggregation and fibrinogen depletion (48). However, AV neutralization of venom-induced local effects is difficult owing to the rapid development of tissue damage after envenomation. As a consequence, local tissue damage leading to necrosis is particularly relevant, as it is frequently followed by poor tissue regeneration, with the occurrence of permanent sequelae associated with tissue loss and dysfunction (49–51). For this reason, we also evaluated the effect of LED associated with AV therapy. Our results demonstrate that association with antivenom did not modify the effects of 635 nm LED on allodynia. However, considering hyperalgesia, a loss of effect was observed for both 635 nm and 945 nm LED when associated with antivenom therapy. This is an intriguing result as it was shown that laser therapy treatment associated with AV therapy is more effective in reducing edema in the gastrocnemius muscle after the injection of *B. jararacussu* venom when compared with either laser therapy or AV alone (9). This loss of effect could be explained by the fact that antivenoms are constituted by antibodies from immunized animals; hence, the use of heterologous proteins for human treatment involves the possibility of adverse reactions due to activation of the immune system (51). Moreover, snake venom is also a foreign antigen and can generate an immune response by itself. Therefore, one hypothesis to explain the loss of antinociceptive effect could be the presence of adverse effects that could affect the nociceptive response of animals. Another hypothesis could be that LED treatment has dissociated mechanisms for nociceptive and inflammatory response induced by the venom. This hypothesis also needs to be addressed in future studies.

Taken together results presented herein demonstrate that *B. moojeni* venom effect is able to induce mechanical allodynia and hyperalgesia in mice. Moreover, LED therapy is effective in reducing both allodynia and hyperalgesia induced by BmV even though the radiation was administered after the symptoms were already present, suggesting that phototherapy reduces local effects induced by BmV and therefore may be clinically relevant. Another interesting finding is the fact that although AV treatment reduces the effect of LED, it is still able to reduce the painful behavior of animals reinforcing the therapeutic role of LED in the treatment of patients injured by *Bothrops* snakes. Thereby, treatment with LED should be considered as a therapeutic approach for the treatment of local effects of *Bothrops* poisoning.

CONCLUSION

Both 635 and 945 nm LED are effective in inhibiting nociceptive behavior induced by *Bothrops moojeni* venom in mice.

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