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ORIGINAL ARTICLE

# Photobiomodulation exerts anti-inflammatory effects on the vascular and cellular phases of experimental inflammatory models

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## Abstract

Photobiomodulation therapy (PBMT) is a non-thermal therapeutic procedure widely used in clinical practice. It is considered an effective modality of treatment for the control of various inflammatory conditions with fewer adverse effects as compared to conventional therapy. However, despite the clinical effects, the mechanisms of action and dosimetric parameters of PBMT are not fully understood. This study was performed to describe the effects of two different doses of PBMT on experimental models of inflammation. Male Swiss mice were administered with 0.9% of saline or phlogistic agents (carrageenan, dextran, serotonin, histamine, or bradykinin) by intra-plantar injection and were treated with PBMT at a dose of 1 or 5 J/cm$^2$; right after, the variation of the paw volume was made, and histopathological analysis and myeloperoxidase assay of the carrageenan-induced edematous paw tissues were performed. The action of PBMT on carrageenan-induced vascular permeability was further evaluated. Our results showed that PBMT (1 J/cm$^2$) led to an improvement in paw edema induced by the phlogistic agents and further reduced the histological scores. Inhibition of neutrophil migration was observed following the administration of 1 and 5 J/cm$^2$ of PBMT. However, only 1 J/cm$^2$ of PBMT showed beneficial effects on carrageenan-induced edema. Laser at a dose of 1 J/cm$^2$ showed cellular and vascular effects since it was able to reverse all the inflammatory parameters, and laser at a dose of 5 J/cm$^2$ probably has only cellular effects in the presence of acute inflammation.

**Keywords** Photobiomodulation · Anti-inflammatory · Inflammation · Vascular actions · Laser · Edema

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## Introduction

Inflammation can be defined as an adaptive biological response that is triggered by harmful stimuli such as infection and cellular damage, in order to restore homeostasis [1]. Inflammation involves events coordinated by a large range of mediators, including chemokines, cytokines, and vasoactive amines, which promote structural and biochemical changes in the site of infection or injury such as vasodilatation, increase of vascular permeability, migration of immune cells, and production of oxygen and nitrogen reactive species [1]. However, although these events may ultimately result in the cell regeneration and tissue healing, uncontrolled or unsolved inflammation can lead to excessive tissue damage, dysregulation of tissue healing, and chronic inflammation [2, 3].

Currently, the therapeutic options for the treatment of inflammatory disorders focus on the use of non-steroidal anti-inflammatory drugs (NSAIDs), which have significant adverse effects [4, 5]. Besides, continuous use of these drugs may lead to gastrointestinal adverse effects, such as bleeding and ulcerations, as well as renal dysfunction [4, 6]. Therefore, the scientific community has always been interested in the discovery of new effective therapies having fewer adverse effects on the treatment of inflammatory conditions. In this context, photobiomodulation therapy (PBMT) has been suggested as a promising option [7, 8].

The photobiomodulation therapy (PBMT) involves exposing the tissues to non-thermal electromagnetic radiation with visible and/or invisible light emitted through laser and/or LED devices at a low density [9, 10]). There are positive reports on the use of PBMT in painful and inflammatory conditions [11–13]. The efficacy of PBMT was found to be similar to that of diclofenac sodium [14, 15]; in addition to that, studies show that photobiomodulation stands out as a promising agent against inflammatory conditions and pain [16, 17].

However, despite the wide use and recommendations, the biological mechanisms by which PBMT exerts its role in inflammation are not completely understood [18]. Though its influences on the regulation of transcription factors, the modulation of the levels of cytokines, and other inflammation mediators have been described [7, 8], the research on the impact of PBMT upon specific events of acute phases of inflammation, such as vascular permeability and neutrophil infiltration, is still sparse [18, 19] and hence further research is required.

Furthermore, PBMT is composed of a range of treatment parameters that include light wavelength (nm), power of the light (W), time of irradiation (s), and the total energy delivered over the target tissue (J) [7]. Of these, varying the total energy delivered seems to provide the greatest impact upon the effects of PBMT. Indeed, high exposure dosages to the target tissue(s) will suppress cell activity, while low doses show no effect [20]. Though the delivery of specific doses has been suggested as effective to inhibit some inflammatory parameters in [18, 19], the impact of different doses upon vascular and cellular events of acute inflammation triggered by distinct phlogistic agents is still unknown.

Thus, the present study aimed to evaluate the anti-inflammatory effects of two different doses of PBMT in in vivo experimental models of acute inflammation. Considering the supposed influences of PBMT to decrease inflammation, it was hypothesized that both treatment doses would positively impact upon the vascular and cellular events of acute inflammation.

## Materials and methods

### Animals

A total of 196 Male Swiss mice (25−30 g, 2 months old) were housed in cages at 22 ± 2 °C with a 12 h/12 h light/dark cycle (10 animals per cage). Food and water were provided ad libitum.

### Photobiomodulation therapy

The treatments were provided using a low-intensity Laser AsGa (Gallium Arsenide, KLD Biosystems Equipment Electronics Ltd., Brazil; Model LLT 0107) operating at a wavelength of 904 nm. For irradiating the paws of the animals, the laser parameters used were frequency, 1000 Hz; average power, 50 mW; pulse duration, 100 ns; and irradiated area, 0.6 $cm^2$. The irradiation times for groups L1 and L5 were 12 and 60 s, corresponding to the doses of 1 and 5 $J/cm^2$, respectively. The energy delivered to each point of application was 0.24 J for 12 s and 1.2 J for 60 s, calculated as described in a previous study [21] (Table 1). The irradiation was applied directly onto the skin following the contact technique. Before the initiation of the study, the power of the laser device was calibrated using a Newport Multifunctional Optical Meter (Model 1835-C).

### Paw edema induced by carrageenan

The animals were randomly divided into four groups ($n$ = 7). The saline group received sterile saline (0.9%) into the right hind paw. In the other three groups, edema was induced by carrageenan (50 μL; 500 μg/paw) into the right hind paw. Before 30 min of inducing edema, one group was treated with laser 1 $J/cm^2$, while the other was treated with laser 5 $J/cm^2$ in the paw. One group did not receive any treatment (control

**Table 1** Parameters of the equipment used in the experimental set

| Center wavelength | 904 nm ± 5% |
|---|---|
| Operating mode | Pulsed |
| Frequency | 1000 Hz |
| Pulse duration | 100 ns |
| Peak radiant power | 50 W |
| Average radiant power | 50 mW |
| Bean divergence | 0.31 rad |
| Bean spot size | 0.6 $cm^2$ |
| Exposure duration | 12 and 60 s |
| Radiant exposure | 1 and 5 $J/cm^2$ |
| Application technique | In contact to the skin |

group). The PBMT treatment procedure was repeated each 30 min during 4 h of paw measurement after the administration of the phlogistic agent. The paw volume was measured immediately before (Vo) and at 1, 2, 3, and 4 h after carrageenan treatment (Vt) using a plethysmometer (Panlab, Barcelona, Spain) as previously described [6]. The effect of the PBMT treatment was calculated as the percentage of inhibition of edema relative to the paw volume of the saline-treated controls according to the following formula: % inhibition of edema = [(Vt − Vo) Control] − [(Vt − Vo) Treated]/[(Vt − Vo) Control] × 100. The mean variation in the volume as a function of the elapsed time of 4 h after the administration of carrageenan was presented by a point and line graph.

### Histopathological analysis of the paw tissues

Segments from the sub-plantar tissue of the study groups were removed for histological evaluation shortly after the end of the carrageenan edema. The samples were fixed in 10% formalin solution for 24 h and then transferred to a solution containing 70% alcohol. The material was then embedded in paraffin and sectioned in semi-serial way. The 4-μm-thick sections were deparaffinized with xylol, stained with hematoxylin/eosin, and evaluated by a blinded experienced pathologist. The scores obtained from this test were used to verify the total scores obtained from the lesions (sum of the microscopic scores of the lesions) in the paw tissues and were presented as the stage of severity of neutrophil infiltration, edema, and tissue bleeding (variations 1−4). In summary: Score 1, connective and epithelial tissues are healthy and no inflammatory cells are visible in the area. Score 2, connective and epithelial tissues with light edema and inflammatory cells are visible in the area. Score 3, connective and epithelial tissues with moderate edema and inflammatory cells and occurrence of blood vessel congestion are visible in the area. Score 4, connective and epithelial tissues with severe edema and intense presence of inflammatory cells, occurrence of blood vessel congestion, and vascular vasodilatation are visible in the area [22].

### Paw edema induced by inflammatory mediators

The animals ($n = 7$) received either 50 μL of dextran (500 μg/paw); serotonin (5-HT, 1%, w/v); histamine (1%, w/v); bradykinin (6 nmol/paw); or sterile saline (0.9%) into the right hind paw. Before and after the administration of the inflammatory mediators, the animal groups were treated with laser 1 J/cm$^2$. The control group received no treatment. The PBMT treatment procedure was repeated every 30 min during the 4 h of paw measurement after the administration of the phlogistic agent. The paw volume was measured immediately before and at selected intervals of 30 min for 2 h after the administration of the phlogistic agent.

### Neutrophil infiltration in the paw

The neutrophil infiltration was estimated through the measurement of the myeloperoxidase (MPO) activity. It was determined based on a previously described method [23]. The results are described as MPO per milliliter of peritoneal exudate (UMPO/mL). One unit of MPO activity was defined by the conversion of 1 μmol of hydrogen peroxide to water in 1 min at 22 °C.

### Assessment of vascular permeability

The animals received saline or carrageenan intraperitoneally (Cg, i.p., 500 μg/250 μL). After 30 min, PBMT at a dose of 1 J/cm$^2$ was administered in one group, and at a dose of 5 J/cm$^2$ in the other group. The control group received no treatment. The PBMT was further administered hourly for four times. The vascular permeability was analyzed in the mice by measuring Evans blue extravasation following the administration of 50 μL of Evans blue (50 mg/kg in PBS) through the ocular plexus. The animals were euthanized 4 h after carrageenan injection, and a peritoneal wash was performed with 1.5 mL of PBS plus EDTA. The amount of Evans blue extravasation was measured by spectrophotometry at 620 nm. The vehicle (sterile 0.9% (w/v) saline) was used as the control [24].

### Statistical analyses

Data were described as mean ± SEM. Analysis of variance (ANOVA), followed by Student–Newman–Keuls test, was used to compare the means. The histopathological parameters were analyzed using the Kruskal–Wallis non-parametric test, followed by Dunn's multiple comparison test. Statistical significance was defined as $p < 0.05$.

## Results

### PBMT at a dose of 1 J/cm$^2$ reduced carrageenan-induced paw edema

According to Fig. 1 and Table 2, the administration of carrageenan on the right paw of mice led to an increase in paw volume (0.100 ± 0.013 mL and 0.082 ± 0.009 mL) at the 3rd and 4th hours, respectively, as compared to that in the saline group (0 mL at the same time points). The administration of PBMT at a dose of 1 J/cm$^2$ resulted in a significantly ($p < 0.05$) reduced paw volume (0.052 ± 0.008 mL and 0.053 ± 0.008 mL) at the 3rd and 4th hours, respectively, as compared to that in the saline group. As seen in Fig. 1 and Table 2,

**Table 2** The anti-inflammatory activity of laser on acute paw edema induced by carrageenan

| Treatment | Dose | | Paw edema in mL (time after administration of inflammatory stimulus) | | | |
|---|---|---|---|---|---|---|
| | | | 1 h | 2 h | 3 h | 4 h |
| Control(Cg) | 500 μg/paw | - | 0.038 ± 0.006$^{\#}$ | 0.090 ± 0.017$^{\#}$ | 0.100 ± 0.013$^{\#}$ | 0.082 ± 0.009$^{\#}$ |
| Saline | - | - | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| Laser | 1 J/cm$^2$ | - | 0.043 ± 0.006 (0.000%) | 0.060 ± 0.012$^{*}$ (33.33%) | 0.052 ± 0.008$^{*}$ (48.00%) | 0.053 ± 0.008$^{*}$ (35.36%) |
| | 5 J/cm$^2$ | - | 0.035 ± 0.010 (07.90%) | 0.055 ± 0.009$^{*}$ (38.88%) | 0.084 ± 0.006 (16.00%) | 0.090 ± 0.003 (0.000%) |

Values are given as the means ± SEM of ten animals, as analyzed by one-way ANOVA followed by Newman–Keuls test

$^{*}p < 0.05$ vs carrageenan group; $^{\#}p < 0.05$ vs saline group

treatment with PBMT at a dose of 5 J/cm$^2$ had no effect on carrageenan-induced paw edema.

## PBMT at doses of 1 J/cm$^2$ and 5 J/cm$^2$ reduced histopathological damages in the inflamed paws

As seen in Fig. 2, the administration of carrageenan caused a significant ($p < 0.05$) increase in the histopathological lesion score (3.667 ± 0.210) as compared to that in the saline group (1.167 ± 0.167). Figure 3 shows the corresponding changes in the paw tissues with significant ($p < 0.05$) neutrophil infiltration. The PBMT treatment (1 and 5 J/cm$^2$) reduced all the histopathological scores (1 J/cm$^2$: 2.333 ± 0.333 and 5 J/cm$^2$: 1.500 ± 0.223) as compared to that in the carrageenan group. Figure 3 shows the reconstitution of the tissue structure, as well as the reduction in inflammatory cell infiltration.

## PBMT at a dose of 1 J/cm$^2$ reduced mediator-induced paw edema

The administration of dextran at a dose of 500 μg/kg (0.076 ± 0.011 mL, Fig. 4a), histamine (0.083 ± 0.009 mL, Fig. 4b), serotonin (0.090 ± 0.007 mL, Fig. 4c), and bradykinin (0.052 ± 0.003 mL, Fig. 4d) caused significant ($p < 0.05$) edema in the paw of the mice with a maximum peak at 30 min as compared to that in the saline group. The administration of PBMT at a dose of 1 J/cm$^2$ resulted in a significantly ($p < 0.05$) reduced paw volume at 30 min as shown in Fig. 4 (dextran: 0.006 ± 0.002 mL, Fig. 4a; histamine: 0.0450 ± 0.002 mL, Fig. 4b; serotonin: 0.060 ± 0.007 mL, Fig. 4c; and bradykinin: 0.024 ± 0.002 mL, Fig. 4d).

## PBMT at doses of 1 J/cm$^2$ and 5 J/cm$^2$ reduced myeloperoxidase (MPO) activity

Figure 5 shows a significant ($p < 0.05$) increase in MPO activity in the carrageenan group (58.19 ± 5.665 UMPO/mg) as compared to that in the saline group (2.741 ± 2.314 UMPO/mg). The PBMT (1 and 5 J/cm$^2$) treatment following carrageenan-induced paw edema significantly ($p < 0.05$) reduced the MPO activity (1 J/cm$^2$: 41.81 ± 4.994 UMPO/mg; 5 J/cm$^2$: 24.91 ± 4.768 UMPO/mg).

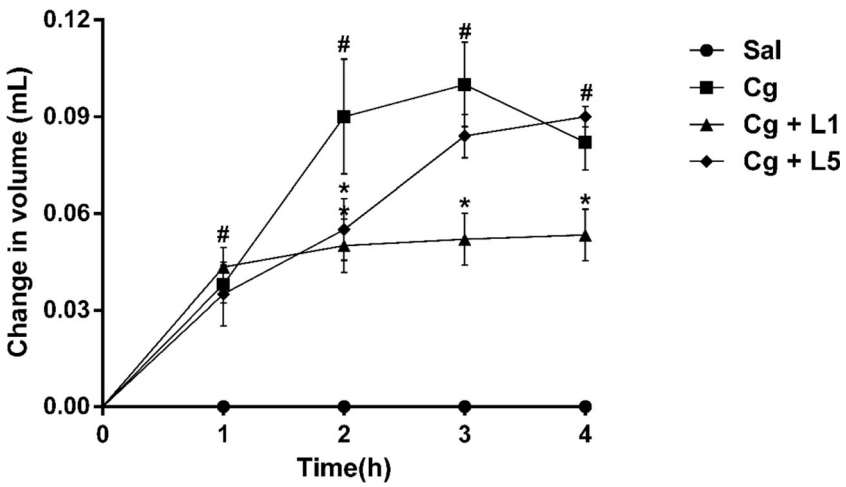


**Fig. 1** Laser effect at 1 and 5 J/cm$^2$ on paw edema induced by carrageenan in mice. The administration of carrageenan (Cg) 50 μL (500 μg/paw) promoted an increase in paw volume. However, treatment with photobiomodulation (1 and 5 J/cm$^2$) significantly reverted this effect. Results are expressed as the mean ± SEM of seven animals per group. $^{\#}p < 0.05$ versus saline group; $^{*}p < 0.05$ versus carrageenan group. ANOVA and Newman–Keuls test were used for evaluation

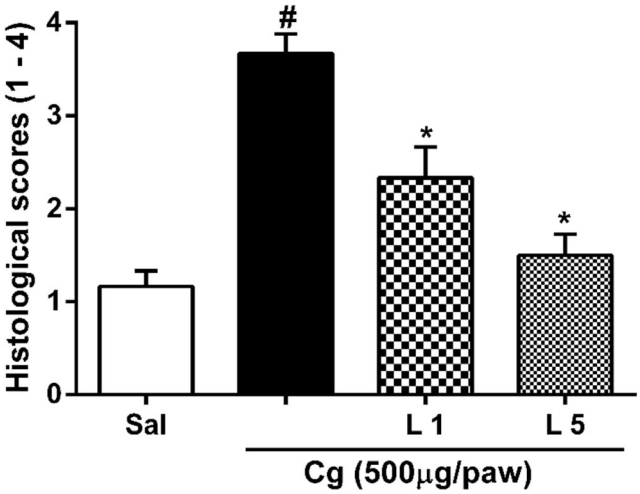


**Fig. 2** Laser effect at doses 1 J/cm$^2$ and 5 J/cm$^2$ on the histological microscopic paw lesion scores. Lesion scores were measured after carrageenan-induced (Cg) paw edema (500 μg/paw). The laser groups 1 J/cm$^2$ (L1) and 5 J/cm$^2$ (L5). *$p < 0.05$ compared to group Cg. #$p < 0.05$ compared to saline group

### PBMT at a dose of 1 J/cm$^2$ reduced carrageenan-induced vascular permeability

According to Fig. 6, administration of carrageenan into the mice peritoneum promoted a significant ($p < 0.05$) increase in the vascular permeability (215.6 ± 11.00 μg/mL of exudate) as compared to that in the saline group (112.4 ± 6.396 μg/mL of exudate). The administration of PBMT at a dose of 1 J/cm$^2$ in the specific peritoneal areas promoted a significant ($p < 0.05$) reduction in the vascular permeability (150.2 ± 22.00 μg/mL of exudate). The PBMT (5 J/cm$^2$) played no role in carrageenan-induced vascular permeability.

## Discussion

The PBMT has shown promising effects in the treatment of inflammatory processes as well as in the reduction of triggering parameters of acute inflammation [16, 17]. For these reasons, in this study we decided to test the PBMT with low-intensity laser on inflammatory parameters that are considered key in the acute inflammatory process.

The current study showed that PBMT at a dose of 1 J/cm$^2$ promoted amelioration of paw edema induced by carrageenan and other inflammatory mediators. Laser at a dose of 1 J/cm$^2$ also reduced carrageenan-induced neutrophil infiltration in the paw. The PBMT using a dose of 5 J/cm$^2$ did not show positive effects on the alleviation of edema or vascular permeability, although it showed positive histopathological results accompanied by a significant reduction in neutrophil migration resulting in an improvement of the inflammatory process in the paw. Moreover, both doses of PBMT reduced the capillary permeability induced by carrageenan and promoted an improvement in the histopathological damage leading to the reorganization of the inflamed paw tissue structures. However, laser AsGa 904 nm at a dose of 5 J/cm$^2$ did not show positive effects on the alleviation of edema or vascular permeability, although it showed positive histopathological results accompanied by a significant reduction in neutrophil migration resulting in an improvement of the inflammatory process in the paw.

The development of edema induced by carrageenan is described as a biphasic event [25]. The initial phase is marked by activation and degranulation of mast cells that are involved in the release of histamine, serotonin, bradykinin, and other mediators acting to alter the vascular permeability [26]. Albertini et al., using a dose of 2.5 J/cm$^2$, have already evidenced the effectiveness of PBMT on reducing paw edema induced by carrageenan in rats [14]. Likewise, in another investigation, Albertini et al. also demonstrated that PBMT at the 7.5 J/cm$^2$ dosage reduced cytokine mRNA expression levels 3 h after inflammation induced by this phlogistic agent [27]. The current study, in addition to corroborating the finding of the aforementioned studies, also evidenced that PBMT with a dose of 1 J/cm$^2$ was capable of reducing paw edema induced by dextran, histamine, serotonin, and bradykinin, reinforcing the assumption that PBMT with a dose of 1 J/cm$^2$ is protective in the vascular phase of paw edema.

In the second phase of edema, carrageenan triggers a cascade of inflammatory mediators, including prostaglandins and reactive oxygen species, resulting in the migration of leukocytes toward the injured site [28]. The current study demonstrated that although PBMT at a dose of 5 J/cm$^2$ did not show positive effects on the alleviation of edema or vascular permeability, it reduced the neutrophil migration to the tissue, assessed by MPO enzyme activity. Similar findings were described by Correa e al., using PBMT to treat induced peritonitis in rats, observing reductions in the number of neutrophil cells in the peritoneal cavity with doses of 3 (−42%) and 7.5 J/cm$^2$ (−70%) in comparison to the control group [29]. Besides, Pallotta et al. observed that PBMT with 50, 150, and 300 J/cm$^2$, in addition to reducing MPO enzyme activity in an experimental model of knee inflammation, also reduced the total number of leukocytes infiltrated to the knee region [13]. Merging these results, PBMT seems to impact upon acute inflammation in different ways, depending on the dosage used, acting on both vascular and cellular events at low dosages (< 3 J/cm$^2$) and, predominantly, on the cellular events as the dose used increases (> 3 J/cm$^2$). Indeed, a previous study has demonstrated that both doses of 1 J/cm$^2$ and 5 J/cm$^2$ impact upon the cellular phase of acute peritonitis induced by carrageenan [30]. However, this hypothesized dose-dependent characteristic of the effects of PBMT on specific events of acute inflammation needs to be better investigated by future research.

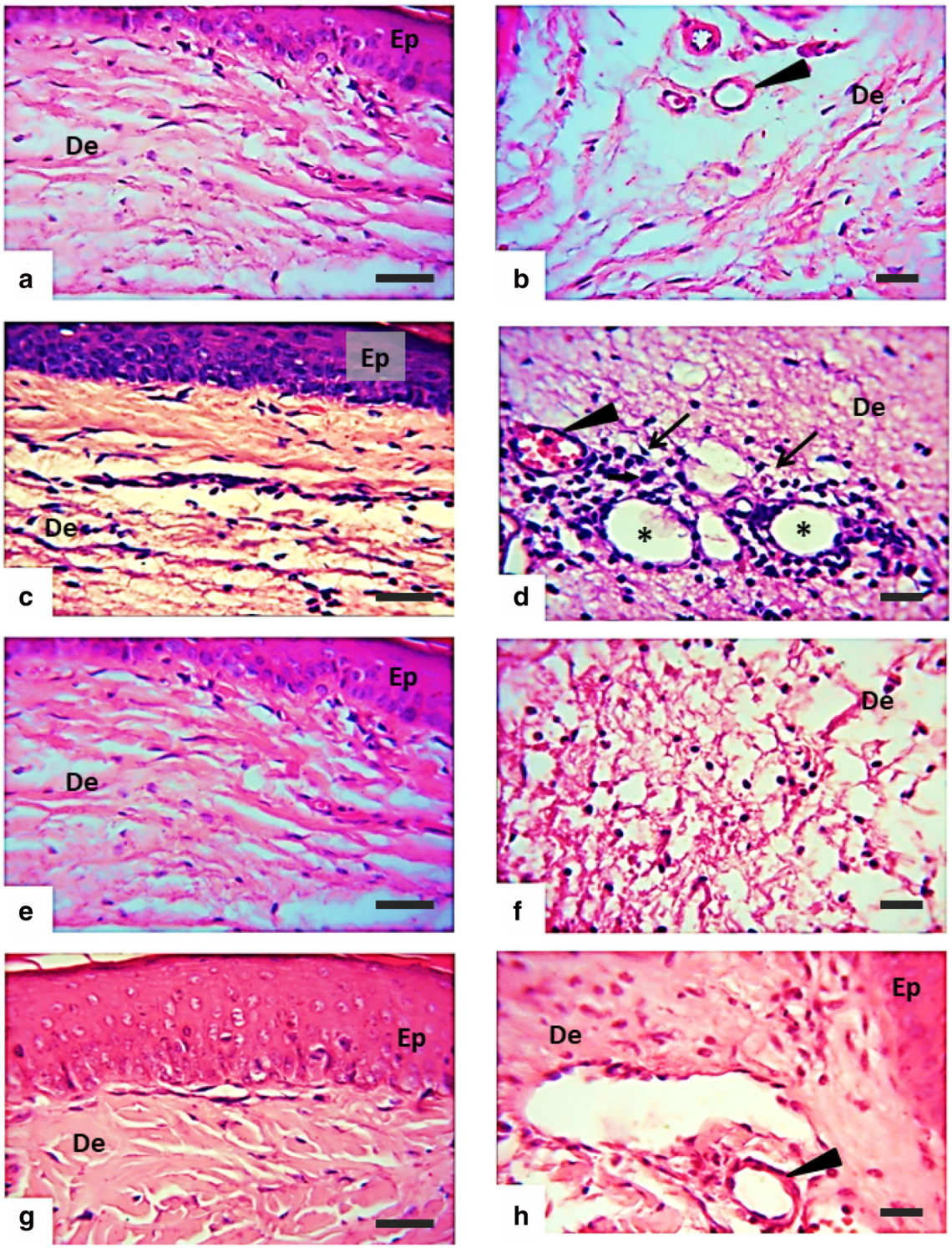


**Fig. 3** Microphotograph representing the effect of laser at doses of 1 and 5 J/cm$^2$ on the microscopic damage of tissue damage caused by carrageenan-induced paw edema. Saline group (**a**, **b**); carrageenan (Cg) group (**c**, **d**); laser 1 J/cm$^2$ (**e**, **f**); laser 5 J/cm$^2$ (**g**, **h**). Epidermis, *Ep*; dermis (*De*); arrows indicate inflammatory cells; asterisk "*" indicates vasodilatation vascular; arrowhead indicates blood vessel. All images were stained with hematoxylin and eosin; images **a**, **c**, **e**, and **g** are at × 400 original magnification; images **b**, **d**, **f**, and **h** are at × 500 original magnification; bar = 50μm; arrowhead indicates blood vessel

Nevertheless, in spite of the different results comparing the impact of 1 J/cm$^2$ and 5 J/cm$^2$ doses on vascular and cellular events of acute inflammation, the current study demonstrated that both the doses of PBMT presented satisfactory results in improving the microscopic histopathological scores in carrageenan-induced paw edema. Similar effects were

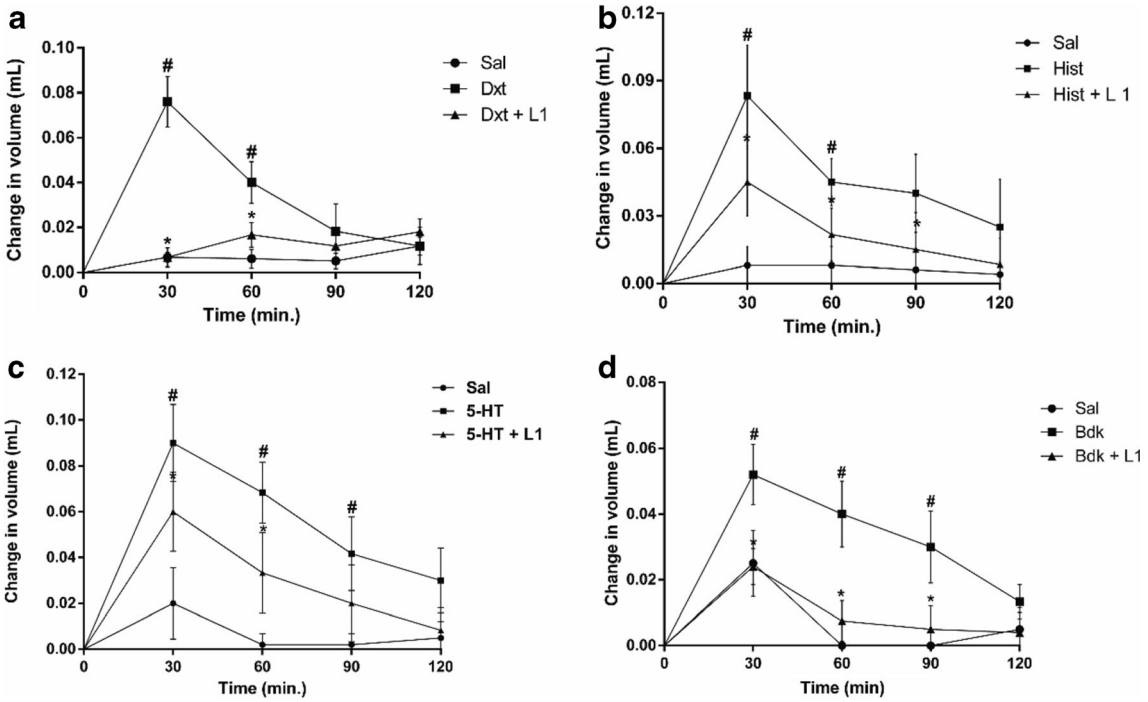


**Fig. 4** Effect of laser 1 J/cm$^2$ on paw edema induced by various inflammatory mediators. Paw edema was induced by dextran (Dxt: 500 μg per paw) (**a**); histamine (Hist: 500 μg per paw) (**b**); serotonin (Ser: 500 μg per paw) (**c**); bradykinin (Bk: 6 nmol, w/v, per paw) (**d**). Injections into the plantar surface of the right paw. The change in paw volume was measured at the indicated time intervals. Animals were treated with laser 1 J/cm$^2$ (L1). The values given are means ± SEM ($n$ = 5). *Statistical difference ($p < 0.05$) compared to inflammatory stimuli treatment (one-way ANOVA followed by Newman–Keuls post test). Each column represents the mean ± SEM of seven animals per group. $^{\#}p < 0.05$ versus control group (saline); $^{*}p < 0.05$ versus carrageenan group. One-way analysis of variance (ANOVA) followed by Kruskal–Wallis (non-parametric data)

described by Tomazoni et al., who observed that PBMT at 107.1 J/cm$^2$ was effective in improving morphological changes induced by trauma in muscle tissue of rats [31]. Likewise, these authors also demonstrated in the aforementioned study and in another investigation that PBMT was more effective in improving inflammation than was diclofenac sodium, a classical pharmacological agent used in these conditions [32]. Taken altogether, these findings reinforce the ability of PBMT on positively impacting upon inflammation. In fact, such benefits have already been

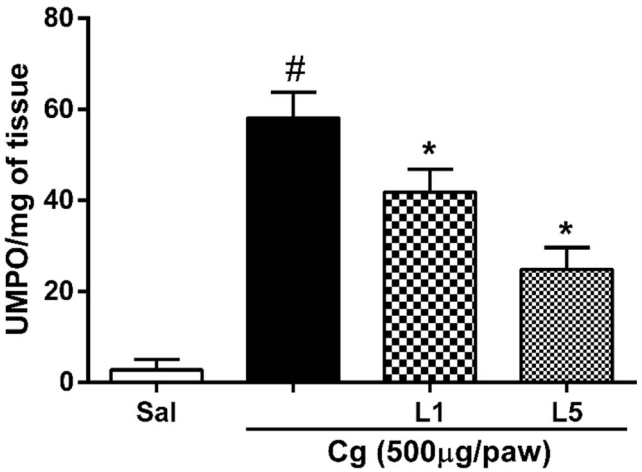


**Fig. 5** Effect of laser at doses 1 J/cm$^2$ and 5 J/cm$^2$ reduces myeloperoxidase (MPO) activity in paw edema induced in mice. Carrageenan (Cg) was administered 50 μL (500 μg/paw). In the saline group, 50 μL saline solution at 0.9% only was administered. After 30 min, the laser treatment was started with doses of 1 J/cm$^2$ (L1) and 5 J/cm$^2$ (L5). In treatment groups (L1 and L5), there was a reduction in neutrophil concentration. Each column represents the mean ± SEM of seven animals per group. $^{\#}p < 0.05$ versus control group (saline); $^{*}p < 0.05$ versus carrageenan group. One-way analysis of variance (ANOVA) followed by Newman–Keuls test

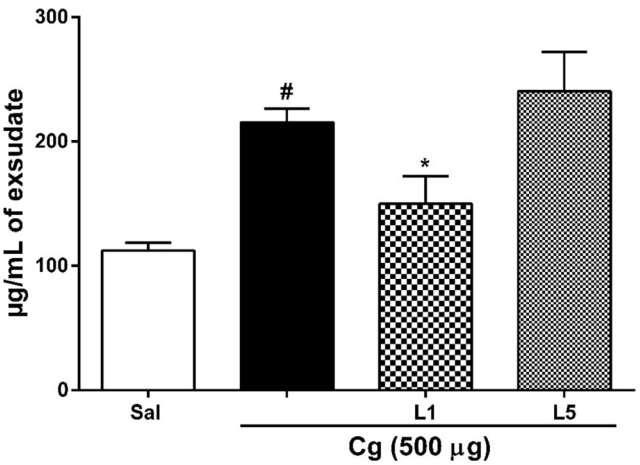


**Fig. 6** Effect of laser at doses 1 J/cm$^2$ and 5 J/cm$^2$ on vascular permeability. The laser groups 1 J/cm$^2$ (L1) and 5 J/cm$^2$ (L5). $^{*}p < 0.05$ compared to group Cg. $^{\#}p < 0.05$ compared to saline group

demonstrated in clinical trials investigating the impact of PBMT on wound healing [33] and postoperative situations [34].

Last, the current study demonstrates that although PBMT was effective in improving inflammation induced by carrageenan and other distinct phlogistic agents, its impact on specific events of acute inflammation seems to have a dose-dependent characteristic. Our results expand previous investigations and collectively provide a solid foundation for the continued research that will lead to the standards for PBMT usage in inflammation scenarios.

## Conclusion

Based on the results presented, we can suggest that laser at a dose of 1 J/cm$^2$ exerts anti-inflammatory effects in both the cellular and vascular phases of carrageenan-induced paw edema, whereas laser at a dose of 5 J/cm$^2$, although effective in reducing leukocyte migration, has no effects on the vascular permeability. Thus, we can suggest that laser at a dose of 1 J/cm$^2$ is an effective anti-inflammatory resource as it works by alleviating both phases of inflammation.

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## Declarations

**Ethics approval** All experiments were conducted in accordance with the currently established principles for the care and the use of COBEA, Brazil. The Animal Studies Committee of the Federal University of Piauí approved the experimental protocol (038/15).

**Conflict of interest** The authors declare no competing interests.

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