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Influence of the HPA axis on the inflammatory response in cutaneous wounds with the use of 670-nm laser photobiomodulation

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ABSTRACT

This study evaluated the influence of hypothalamic–pituitary–adrenal (HPA) axis in cutaneous wounds subjected to laser biomodulation. A total of 48 rats were divided into two groups: Group I (GI) with 24 adrenalectomized animals and Group II (GII) with 24 non-adrenalectomized animals. Each group was divided into two subgroups: the irradiated subgroup which laser was applied to four points at the edges of the wound (670 nm laser, 9 mW) and control subgroup. Rats in each subgroup were sacrificed at 24 or 72 h. Adrenal glands were only removed from GI rats. Three days after adrenalectomy, a cutaneous wound was made. An immunohistochemical analysis was performed using anti-CD45 and anti-CD8 antibodies. Flow cytometry was used to count T lymphocytes and their subpopulations in blood. Decreases in the number of CD45-positive inflammatory cells and in the total numbers of CD8- and CD45-positive cells were observed in histological sections of adrenalectomized animals subjected to laser biomodulation at 24 h. Similar results were observed for distribution of total lymphocytes in blood ($p < 0.05$). The action of 670 nm laser does not depend exclusively on HPA axis. It is believed that corticosteroid-promoting enzymes liberated in non-adrenal tissues may influence immune response under the influence of this type of phototherapy.

1. Introduction

Therapy with low-power lasers is a therapeutic modality that was first introduced by Mester and collaborators. They noted an improvement in wound healing from the application of a ruby laser [1]. Since that study, various lasers with different wavelengths, powers, and energy densities have been used for therapeutic ends.

Studies using lasers with low energy densities have shown that this treatment modality has a photobiological effect and accelerates wound healing [2–6]. There is evidence of local biological events, such as vascular and cellular alterations, and of modifications in extracellular-matrix synthesis [7,8]. There are also systemic effects; data in the literature suggest that laser treatment stimulates adrenal glands to produce endogenous cortisol, which attenuates the inflammatory response [9]. In fact, it is known that activation of the hypothalamic–pituitary–adrenal axis (HPA) is triggered by injuries of various kinds, which increase the levels of circulating glucocorticoids and possibly modulate local and systemic events during the process of wound repair [10].

Although previous studies have described local alterations induced by laser treatment in vivo and in vitro experimental models, there is little in the literature that specifically investigates the systemic effects of lasers in absence of adrenal glands. Thus, the present study aimed to investigate local and systemic effects in rats of a low-power laser in the presence and absence of adrenal glands.

2. Materials and methods

All the animal handling procedures used in this study were performed in accordance with norms and directives of Ethics Committee on the Use of Animals of the Bahia School of Medicine and Public Health, as approved in ruling number 016/2009.

2.1. Animals

Forty-eight male Wistar rats, weighing between 150 and 200 g, were kept in individual cages with free access to water and a balanced diet. The animals were randomly divided into two experimental groups, each with 24 rats. A second random allocation

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was performed in each group, resulting in two subgroups with 12 animals each.

2.2. Surgical procedures

A solution of 5% ketamine (2.5 ml) (Vetanarcol – Konig – Lot 007 07, Brazil) and 2% xylazine (0.5 ml) (Sedomin – Konig – Lot 002 08, Brazil) diluted in saline solution (1.0 ml) was prepared. The solution was administered intraperitoneally at 0.2 ml per 100 g of body weight, and the rats' dorsum were shaven under anesthesia. Two parallel incisions on the lower back of all animals, each measuring 3 cm, were then made under aseptic conditions for access to the kidneys. The subcutaneous and muscular tissues were separated, and the adrenal glands were located [11]. Depending on the experimental group, adrenal glands were removed or preserved. The surgical procedure was carried out by an experienced surgeon.

Three days after the first surgical intervention, a circular wound was made under intraperitoneal anesthesia in dorsum cutaneous tissue between the front paws of the animal and close to the cervical region. The experimental protocol has been described elsewhere [12,13]. A 6 mm diameter punch (Stiefel Tabe, São Paulo, Brazil) was placed perpendicular to the previously shaved tissue and was used to generate standard-size wounds.

2.3. Experimental groups

Group I (GI) – Non-adrenalectomized animals. The animals from this group were subjected to surgical intervention mimicking adrenalectomy, but the adrenal glands were not removed. After 3 days, the procedure to create a circular dorsal cutaneous wound was performed. This experimental group was made up of two subgroups of twelve animals each, with six animals from each subgroup sacrificed 24 and 72 h after cutaneous surgery.

Subgroup 1. Control. The wounds and adjacent skin of control animals were subjected to stationary contact with laser probe with the beam turned off to simulate similar stress conditions of the animals from the laser subgroup without laser's therapeutic effects.

Subgroup 2. Laser. The device used was an AsGaAl semiconductor diode with continuous emission (9 mW, 670 nm, 0.031 W/cm^2) at the spot size (0.28 cm^2) of the beam (Laser VR-KC-610 – Dentoflex, Brazil). For the animals sacrificed after 24 h, laser was applied immediately after cutaneous surgery using a total single dose of 4 J/cm^2 divided into four 124-s point applications of 1 J/cm^2 at diametrical vertices of the circular wound. Animals sacrificed after 72 h received a total dose of 8 J/cm^2, with part of the dose immediately after cutaneous surgery (4 J/cm^2 for 124 s) and part 48 h after surgery (4 J/cm^2 for 124 s), giving a total exposure time of 248 s.

Group II (GII) – Adrenalectomized animals. The animals in this group were subjected to surgical intervention for removal of adrenal glands in accordance with an established protocol. A standardized circular wound on the dorsum of the rat was made 3 days after adrenalectomy, following the same procedure as for Group I. This experimental group was made up of two subgroups of animals (laser and control) sacrificed 24 and 72 h after the formation of cutaneous wound, following the same procedures described for subgroups of Group I (non-adrenalectomized animals).

2.4. Blood collection

Blood was drawn from animals on the day of sacrifice, before euthanasia by an overdose of anesthetic substances by a experienced phlebotomist. Under deep anesthesia, the ventral region was shaved and an aseptic ventral incision was made along the midline from the peritoneum to thoracic region. With the use of blunt scissors, a cut was made in the sternum to open the chest. A blood puncture was performed in the area of the right ventricle with a disposable 3 ml syringe (BD® brand, Curitiba, Brazil) containing a drop of 10% EDTA. A total of 2 ml of blood was removed from each animal.

2.5. Histological processing and immunohistochemical techniques

Fragments of skin, including the four edges of the lesion and subcutaneous tissue, were collected, fixed in 10% buffered formaldehyde, and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin.

Slides that had been previously treated with a solution of organosilane (A 3648 – Sigma) were used for immunohistochemical studies. Histological samples were deparaffinization, dehydrated, and washed with tap water and distilled water. For the slides stained with anti-CD45 and anti-CD8 antibodies, antigen retrieval was performed using a citrate buffer solution (pH 6.0) in a water bath at approximately 97.6 °C for 30 min. Endogenous peroxidase activity was blocked with a ready-to-use peroxidase blocking reagent (DAKO, Denmark – S2001) at room temperature. Nonspecific binding was prevented by blocking with a solution of 10% skim milk in Phosphate Buffer Solution for 20 min. Tissues were then incubated with anti-CD45 monoclonal primary antibodies (1:10, PHARMINGEN) for staining granulocytes, macrophages, lymphocytes, and monocytes and with anti-CD8 antibodies (1:10, BIOSOURCE INTERNATIONAL) for staining T lymphocytes. Antibodies were incubated overnight at 4 °C in a moist chamber. The polymer was applied with HRP from EnVision Kit (DAKO, Denmark, K4061) for 30 min at room temperature. The reaction was developed with diaminobenzidine (Liquid DAB Substrate Chromogen System, DAKO, Denmark, K3466). The slides were then counter-stained rapidly with Harris hematoxylin and rinsed with tap water. Finally, the sections were dehydrated, dipthongized, and mounted in Canada balsam.

Granulation tissue and sections of lymph node with known positivity for lymphocytes, macrophages, monocytes, and granulocytes were used as positive controls in all of immunohistochemical reactions. We also used the primary antibody with Bovine Serum Albumin (BSA) as a negative control.

2.6. Flow cytometry

A total of 50 μl of the previously collected total blood was used for flow cytometry. Anti-lymphocyte antibodies recognizing CD4, CD8, and CD3 were added to homogenized blood and incubated for 20 min at 4 °C. A lysing solution and PBS were also added to the mixture before mechanical agitation. Mixture was then incubated for another 10 min. The material was subjected to centrifuging (1000 rpm for 5 min at 4 °C), and the supernatant was discarded. Immediately afterward, we added 1 ml of a solution of PBS, BSA, and azide 0.01%, followed by further centrifugation. After adding 200 μl of PBS and azide solution to the mixture, an automated reading was performed on the same day as the preparation using a flow cytometer. The quantity of lymphocytes in blood was calculated using this automated reading.

2.7. Data analysis

For the analysis of histological sections, we used a Motic B5 Professional Series microscope with an attached camera that was connected to the Motic Image Advance 3.0 computer program. The slides were examined by randomly selecting four 0.1-mm^2 areas. An image of each area was then captured at 40× and saved in the JPEG format.

Morphometric studies were created using tissue sections subjected to immunohistochemistry. The area of the cells stained with anti-CD45 and anti-CD8 antibodies was estimated.

After collecting histomorphometric data, we prepared Excel spreadsheets for flow cytometry, and the results were then analyzed using the non-parametric Mann–Whitney test to determine differences between groups. The level of statistical significance was set at $p \leqslant 0.05$.

3. Results

3.1. Local alterations

In histological sections subjected to CD45 immunohistochemistry, we observed cytoplasmic staining with a granular aspect during the evolution of the acute inflammatory process in all of the groups. In the animals with intact adrenal glands, we observed a significant decrease in CD45-positive cells at both times in the tissues subjected to light therapy. In animals without adrenal glands, we noted a significantly smaller number of CD45-positive cells in laser group 24 h after surgery than in control group ($p = 0.004$). We further observed a significant increase in the number of CD45-positive cells in animals subjected to laser biomodulation between the death periods that are time points 1 and 2 ($p = 0.041$) (Table 1). The absence of adrenal glands was associated with an increase in the CD45-positive cells compared to the group with intact adrenal glands that was statistically significant at 24 h ($p = 0.026$) (Table 1, Figs. 1 and 2).

In the animals with intact adrenal glands, we observed an increase in CD8-positive T cells in laser-treated group that was statistically significant at 24 h ($p = 0.04$) (Figs. 3 and 4). The differences between the subgroups with and without laser treatment were not statistically significant in animals without adrenal glands. A significant decrease in the number of CD8-positive T cells was observed in animals subjected to laser biomodulation between the death periods ($p = 0.002$) (Table 2). There was a significant increase in CD8-positive T cells at 24 h between the animals with and without intact adrenal glands in both subgroups with ($p = 0.041$) and without ($p = 0.002$) laser biomodulation (Figs. 5 and 6, Table 2).

We also evaluated the number of all anti-CD45 and anti-CD8 stained cells (Table 3). In the animals with intact adrenal glands, differences were statistically significant only in relation to the control groups at the different time points 1 and 2 ($p = 0.004$). For animals without adrenal glands, we observed lower numbers of CD45- and CD8-positive inflammatory cells at 24 h, with a statistically significant difference between control and laser groups ($p = 0.02$). There was a significant increase in inflammatory cells at 24 h between the animals with and without intact adrenal glands in control subgroup ($p = 0.002$).

3.2. Systemic alterations

The number of CD4-positive T lymphocytes in blood collected at 24 h from the animals with intact adrenal glands was increased in laser subgroup compared to the non-laser subgroup, although a statistically significant difference was not observed. This trend was also observed in adrenalectomized animals, with a statistically significant increase observed in the number of cells in blood of laser-irradiated animals ($p = 0.03$). In addition, we noted a significant difference between adrenalectomized control animals and those with the intact adrenal glands ($p = 0.009$). At 72 h, the

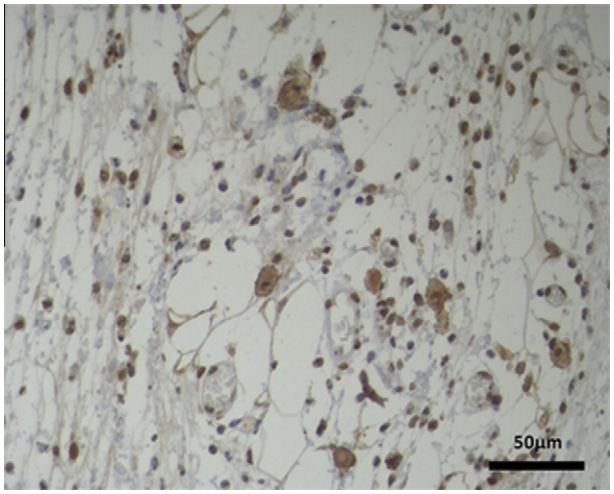

Fig. 1. CD45-positive cells. Laser group at 24 h with adrenal glands preserved. EnVision. Scale = 50 µm.

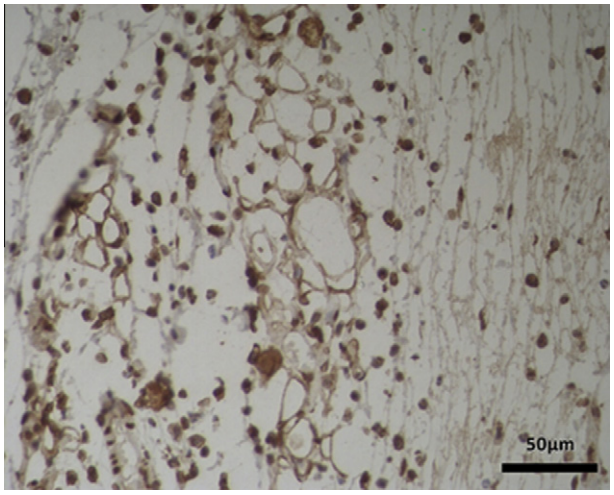

Fig. 2. CD45-positive cells. Laser group at 24 h with adrenal glands removed. EnVision. Scale = 50 µm.

Table 1
The distribution of CD45-positive cells (by immunohistochemistry) at 24 and 72 h in the control group and the laser-treated group for the animals with and without adrenal glands.

CD45-positive cells median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
		Groups	p^A		Groups	p^A
24 h	Control[a]	1.3 (1.1–1.9)[**](*ae*)	0.3	Control[e]	2.1 (1.9–2.5)	0.004[*](*ef*)
	Laser[b]	1.6 (1.4–1.7)		Laser[f]	1.7 (1.4–1.9)[***](*fh*)	
72 h	Control[c]	1.7 (1.6–2.0)	0.9	Control[g]	2.0 (1.6–2.2)	0.8
	Laser[d]	1.9 (1.5–2.0)		Laser[h]	2.0 (1.8–2.1)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
[*] (*ef*): Statistically significant between the laser and control groups with adrenal glands removed at 24 h ($p = 0.004$).
[**] (*ae*): Statistically significant between the control group with and without adrenal glands at 24 h ($p = 0.026$).
[***] (*fh*): Statistically significant between the laser groups with adrenal glands removed at 24 and 72 h ($p = 0.041$).

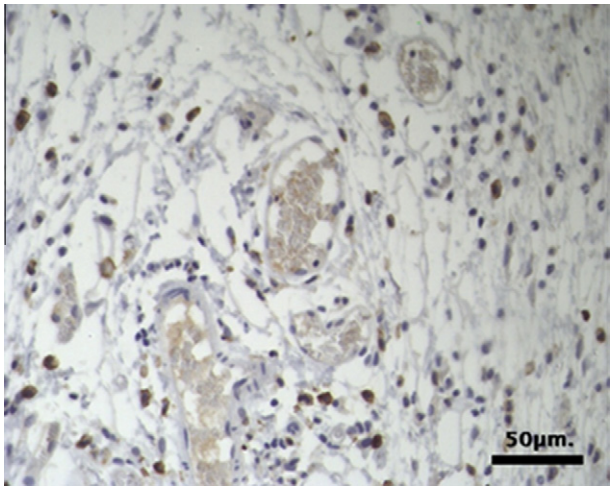

Fig. 3. CD8-positive T lymphocytes. Control group at 24 h with adrenal glands retained. EnVision. Scale = 50 µm.

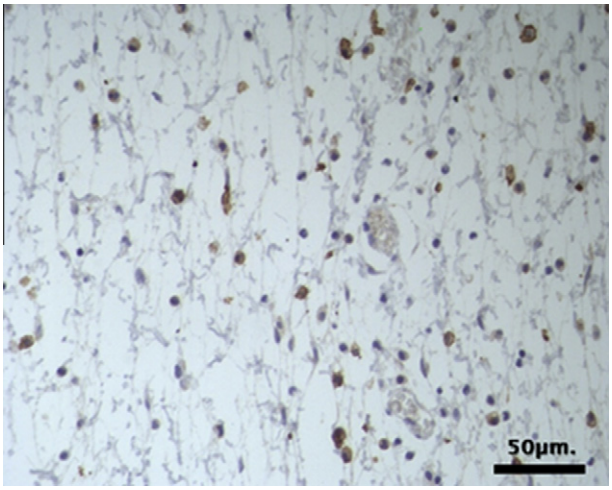

Fig. 5. CD8-positive T lymphocytes. Laser group at 24 h with adrenal glands retained. EnVision. Scale = 50 µm.

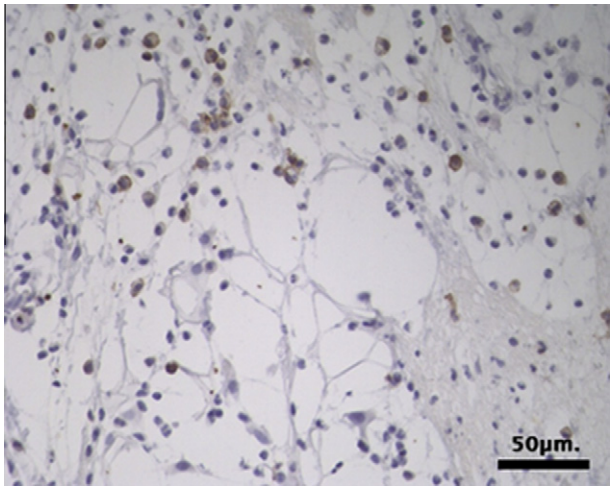

Fig. 4. CD8-positive T lymphocytes. Laser group at 24 h with adrenal glands retained. EnVision. Scale = 50 µm.

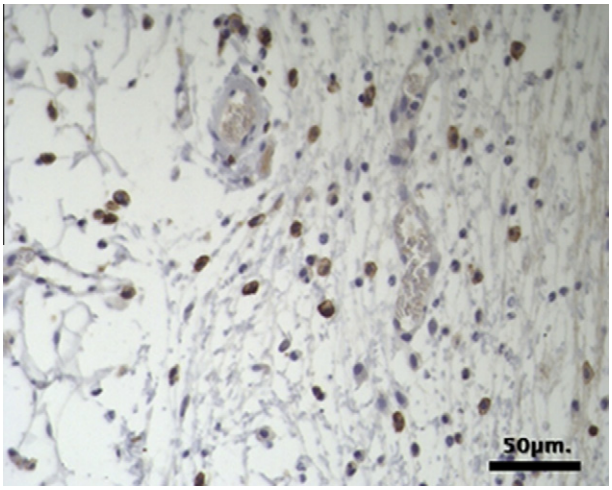

Fig. 6. CD8-positive T lymphocytes. Laser group at 24 h with adrenal glands removed. EnVision. Scale = 50 µm.

number of CD4-positive T lymphocytes decreased in laser-irradiated animals compared to controls, although this difference was not statistically significant. In adrenalectomized animals, the laser group showed a distinct but non-significant increase in CD4-positive T cells (Table 4).

In animals with intact adrenal glands, there was a non-significant increase in the number of CD8-positive T lymphocytes in laser subgroup when compared to control group. At 72 h, however, this increase was significant ($p = 0.02$). In the irradiated animals, the number of CD8-positive T lymphocytes at 72 h was significantly

Table 2
The distribution of CD8-positive T cells (by immunohistochemistry) at 24 and 72 h in the control group and the laser-treated group for the animals with and without adrenal glands.

CD8-positive T cells median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
	Group		p^A	Group		p^A
24 h	Control[a]	1.1 (1.0–1.5)	0.04*(*ab*)	Control[e]	2.3 (2.0–2.5)**(*ae*)	0.8
	Laser[b]	1.7 (1.6–1.9)		Laser[f]	2.2 (2.1–2.4)***(*bf*)	
72 h	Control[c]	1.8 (1.7–2.0)	0.06	Control[g]	1.3 (1.0–2.0)	0.8
	Laser[d]	1.2 (0.9–1.7)		Laser[h]	1.4 (1.3–1.5)****(*fh*)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
* (*ab*): Statistically significant between the laser and control groups with adrenal glands retained at 24 h ($p = 0.04$).
** (*ae*): Statistically significant between the control groups with and without adrenal glands at 24 h ($p = 0.002$).
*** (*bf*): Statistically significant between the laser groups with and without adrenal glands at 24 h ($p = 0.041$).
**** (*fh*): Statistically significant between the laser groups with adrenal glands removed at 24 and 72 h ($p = 0.002$).

Table 3
The distribution of CD8- and CD45-positive T cells (by immunohistochemistry) at 24 and 72 h in the control group and the laser-treated group for the animals with and without adrenal glands.

CD8- and CD45-positive T cells median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
		Group	p^A		Group	p^A
24 h	Control[a]	2.8 (2.3–3.1)[***](*ac*)	0.06	Control[e]	4.5 (4.1–4.7)[**](*ae*)	0.02[*](*ef*)
	Laser[b]	3.4 (3.0–3.8)		Laser[f]	4.0 (3.6–4.1)	
72 h	Control[c]	3.7 (3.4–3.9)	0.13	Control[g]	3.2 (2.8–4.3)	0.58
	Laser[d]	3.2 (2.4–3.8)		Laser[h]	3.4 (3.1–3.6)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
[*] (*ef*): Statistically significant between the laser and control groups with adrenal glands removed at 24 h ($p = 0.02$).
[**] (*ae*): Statistically significant between the control groups with and without adrenal glands at 24 h ($p = 0.002$).
[***] (*ac*): Statistically significant between the control groups with adrenal glands retained at 24 and 72 h ($p = 0.004$).

Table 4
Distribution of CD4-positive T cells in the blood of animals with and without adrenal glands at 24 and 72 h for the control and laser-treated groups.

CD4-positive T cells median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
		Group	p^A		Group	p^A
24 h	Control[a]	44.1 (42.3–46.9)	0.4	Control[e]	37.7 (32.8–42.3)[**](*ae*)	0.03[*](*ef*)
	Laser[b]	46.1 (39.8–50.1)		Laser[f]	43.2 (40.6–44.1)	
72 h	Control[c]	47.5 (40.5–51.6)	0.6	Control[g]	38.5 (36.1–47.0)	0.9
	Laser[d]	43.4 (40.9–48.7)		Laser[h]	39.4 (36.4–43.1)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
[*] (*ef*): Statistically significant between the laser and control groups with adrenal glands removed at 24 h ($p = 0.03$).
[**] (*ae*): Statistically significant between the control groups with and without adrenal glands at 24 h ($p = 0.009$).

Table 5
Distribution of CD8-positive T cells in the blood of animals with and without adrenal glands at 24 and 72 h for the control and laser-treated groups.

CD8-positive T cells median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
		Group	p^A		Group	p^A
24 h	Control[a]	15.5 (13.5–20.2)	0.3	Control[e]	15.4 (14.1–16.4)	0.06
	Laser[b]	17.0 (16.4–18.8)		Laser[f]	12.0 (9.6–13.8)	
72 h	Control[c]	15.0 (13.5–16.7)	0.5	Control[g]	14.7 (12.4–16.5)	0.3
	Laser[d]	14.3 (12.0–15.9)[*](*bd*)		Laser[h]	15.1 (13.8–16.6)[**](*fh*)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
[*] (*bd*): Statistically significant between the laser 24-h group and the laser 72-h group with adrenal glands retained ($p = 0.01$).
[**] (*fh*): Statistically significant between the laser 24-h group and the laser 72-h group with adrenal glands removed ($p = 0.02$).

Table 6
Distribution of CD4/CD8 double positive T cells in the blood of animals with and without adrenal glands at 24 and 72 h for the control and laser-treated groups.

CD4/CD8 double positive T cells median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
		Group	p^A		Group	p^A
24 h	Control[a]	0.9 (0.2–1.1)	0.1	Control[e]	2.4 (1.1–14.3)[**](*ae*)	0.2
	Laser[b]	1.1 (0.9–1.5)		Laser[f]	1.2 (1.0–1.6)	
72 h	Control[c]	1.1 (0.8–1.8)	0.6	Control[g]	2.3 (2.0–3.5)[***](*cg*)	0.05[*](*gh*)
	Laser[d]	1.0 (0.9–1.1)		Laser[h]	0.8 (0.6–2.1)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
[*] (*gh*): Statistically significant between the laser and control groups with adrenal glands removed at 72 h ($p = 0.05$).
[**] (*ae*): Statistically significant between the control groups with and without adrenal glands at 24 h ($p = 0.02$).
[***] (*cg*): Statistically significant between the control groups with and without adrenal glands at 72 h ($p = 0.01$).

greater in animals without adrenal glands than in those with intact adrenal glands, although there was a decrease the doubly positive T cells in laser subgroup 24 h after surgery ($p = 0.01$) (Table 5).

There were no statistically significant differences in doubly positive T cells (CD4/CD8) among the different experimental groups with intact adrenal glands at either time. In adrenalectomized

Table 7
Distribution of total lymphocytes in the blood of animals with and without adrenal glands at 24 and 72 h for the control and laser-treated groups.

Total lymphocytes median ($mm^2 \times 10^{-2}$) (Q1–Q3)						
Time post-treatment	Adrenal glands retained			Adrenal glands removed		
		Group	p^A		Group	p^A
24 h	Control[a]	61.8 (55.6–65.8)	0.9	Control[e]	53.5 (44.9–59.1)	0.6
	Laser[b]	64.3 (56.5–67.3)		Laser[f]	53.6 (52.7–56.1)*(*bf*)	
72 h	Control[c]	61.1 (54.7–66.7)	0.2	Control[g]	50.8 (48.2–62.7)	0.2
	Laser[d]	56.5 (53.8–63.6)		Laser[h]	53.7 (52.8–57.0)	

[A] Exact Mann–Whitney test, $p \leqslant 0.05$.
* (*bf*): Statistically significant between the laser groups with and without adrenal glands at 24 h ($p = 0.009$).

animals, however, there was a significant decrease in the doubly positive T cells in the irradiated animals at 72 h. Difference between adrenalectomized controls and non-adrenalectomized controls was also statistically significant, both at 24 and 72 h after surgery ($p = 0.02$) (Table 6).

The total number of lymphocytes diminished at both time points in adrenalectomized group compared to non-adrenalectomized group. Although an increase in total lymphocytes in the laser subgroup of adrenalectomized animals was observed, this difference was not statistically significant in relation to control animals. However, at 24 h, there was a difference between irradiated animals that were adrenalectomized and those with intact adrenal glands ($p = 0.009$) (Table 7).

4. Discussion

Steroid hormones are released after tissue injury. These hormones influence the immune response concomitantly with the cells participating in inflammatory process [10,14]. A considerable number of studies have demonstrated that immune and neuroendocrine systems are interconnected through mechanisms that lead to reciprocal regulation. The course of inflammation is modulated by plasma concentration of glucocorticoids. These hormones suppress the immune system at higher doses and produce a notable reduction in the production of lymphocytes, especially T lymphocytes [15,16]. Laser irradiation is also an effective modulator of inflammatory response that is capable of activating immune cells, depending on the dose and on the phlogistic phase in which it is applied [15]. Thus, the goal of this study was to verify the essential cell populations of acute inflammation in the presence or absence of adrenal glands.

According to Abertini et al. [10], the anti-inflammatory effects of low-power lasers are related to the release of corticosteroid hormones from intact adrenal glands; therefore, the absence of these glands should inhibit the migration of inflammatory cells to the wound. This conclusion was tested by our examination of the action of a 670-nm laser in animals with and without adrenal glands. In the histological sections of adrenalectomized animals subjected to laser biomodulation, we observed decreases in the numbers of CD45-positive and combined CD8- and CD45-positive inflammatory cells that were statistically significant at 24 h compared to the control group. Similar results were obtained for the distribution of total lymphocytes in blood, with a significant difference between the irradiated adrenalectomized and non-adrenalectomized animals at 24 h.

Some studies have reported the possible synthesis of endogenous cortisol by extra-adrenal tissues, such as brain [17], intestinal cells [18], and heart [19]. Freel et al. [20] evaluated the synthesis of endogenous cortisol in 10 patients subjected to bilateral adrenalectomy. After 3 days, urine samples were collected to measure cortisol levels. Their data demonstrated that cortisol, which is usually produced by the adrenal cortex, could be detected in patients without adrenal glands. Thus, the authors suggested that cortisol synthesis in extra-adrenal tissues is possible.

The thymus is the principal lymphoid organ in which T cells develop. Although the thymus regresses with age and the organ is virtually absent after puberty, some T-cell development continues to occur during adult life [21]. There are many controversies concerning the production of cortisol by thymocytes to maintain thymic homeostasis when the levels of glucocorticoids produced by adrenal gland are low, such as in adrenalectomized animals. Some authors have reported that there is a considerable increase in the number of thymocytes after adrenalectomy [22–24]. In addition, the expression of a specific receptor for adrenocorticotropic hormone in adrenal gland, the melanocortin receptor type 2, has already been described in the literature. This receptor triggers the signals necessary for the production of endogenous cortisol [25]. In addition, Pruett and Padgett [26] evaluated the effect of adrenalectomy on the population of thymic cells and concluded that glucocorticoids stemming from the thymus are insufficient to maintain normal thymic homeostasis in adult rats, results that were corroborated by Qiao et al. [27]. Therefore, the results observed in the present study strongly suggest that other pathways for cortisol production can be activated to partially compensate for the absence of this hormone in adrenalectomized animals.

This study also compared the inflammatory response to a 670-nm laser in adrenalectomized animals to that in animals with intact adrenal glands. Histological sections showed an increase in the number of CD8- and CD45-positive T cells for both the control and laser groups, especially at 24 h. This result suggests that the increased release of extra-adrenal corticosterone through other mechanisms is not sufficient to minimize the local inflammatory response. Our results showed that adrenalectomized animals had lower levels of total lymphocytes in blood when compared to the animals with intact adrenal glands, but the differences were not statistically significant.

The dosages used in low-power laser studies vary from 0.2 to 30 J/cm^2. Recent studies using a dosage of 4 J/cm^2 have shown impressive changes in the inflammatory response and accelerated tissue repair [8,12,28–31]. In this study, the animals sacrificed 24 h after surgical wound received only one dose of 4 J/cm^2, whereas those sacrificed 72 h after surgery were treated with a total dose of 8 J/cm^2. The animals irradiated with 8 J/cm^2 did not show significant differences in the number of CD45- or CD8-positive T cells in either adrenalectomized or non-adrenalectomized groups.

In addition to local effects, some authors have suggested that systemic effects produced by laser act as coadjutants in the healing process [10,32,33]. Rodrigo et al. [32] evaluated the systemic effects of a GaAlAs laser (830 nm, 20 J/cm^2) in a rat model that involved three cutaneous wounds on the lumbar area of each animal, of which only the first was irradiated. Improved healing at non-irradiated wounds suggested a possible systemic modulation by the laser. A systemic evaluation of the effects of a HeNe laser (632.8 nm,

30 J/cm^2) on ulcers and gangrene in diabetic patients showed a significant improvement in local microcirculation in the irradiated wounds, as determined by infrared thermography [34]. However, these studies did not evaluate the circulating levels of the characteristic components of systemic inflammatory response, such as cytokines, chemokines, growth factors, and polymorphic and mononuclear cells. In the present study, we did not observe the systemic effects from the laser that were suggested by the authors cited above. In the irradiated animals with intact adrenal glands, a distinct increase in inflammatory cells in the blood was noted at 24 h. Afterward, there was a decrease in CD4CD8-positive T lymphocytes. At both time points, the differences were not statistically significant. The absence of adrenal glands was not a determining factor in the distribution of the cells studied, with the exception of the CD4-positive T lymphocytes in blood of irradiated animals at 24 h. It is known that T cells, mainly CD4-positive subpopulation, are the greatest producers of cytokines [35,36]. During the processes of infection and wound healing, these cells are transferred to the tissue through the bloodstream, become activated, and regulate the immune response. They also influence proliferation of fibroblasts [37].

It may be concluded from our results that the response to the 670 nm laser at the dosage we used does not depend exclusively on the activation of hypothalamic–pituitary–adrenal axis or, by consequence, on the release of endogenous corticosterone secreted by adrenal glands. It is believed that corticosteroid enzymes released in extra-adrenal tissues may influence the immune response under the action of this type of phototherapy.

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