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ORIGINAL ARTICLE

Low-level laser therapy improves the inflammatory profile of rats with heart failure

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Abstract Following heart failure (HF), immune activation leads to an imbalance between pro-inflammatory and anti-inflammatory cytokines. Low-level laser therapy (LLLT) has been used as an anti-inflammatory treatment in several disease conditions. However, the effect of LLLT on the skeletal muscle of rats with HF remains unclear. The present report aimed to evaluate the influence of LLLT on the inflammatory profile of rats with HF. The left coronary artery was ligated to induce HF and a sham operation was performed in the control groups. Male Wistar rats (n=49) were assigned to one of six groups: placebo sham rats (P-Sham; n=8), LLLT at a dose of 3 J/cm^2 sham rats (3 J/cm^2-Sham; n=8), LLLT at a dose of 21 J/cm^2 sham rats (21 J/cm^2-Sham; n=8), placebo HF rats (P-HF; n=9), LLLT at a dose of 3 J/cm^2 HF rats (3 J/cm^2-HF; n=8), and LLLT at a dose of 21 J/cm^2 HF rats (21 J/cm^2-HF; n=8). Four weeks after myocardial infarction or sham surgery, rats were subjected to LLLT (InGaAlP 660 nm, spot size 0.035 cm^2, output power 20 mW, power density 0.571 W/cm^2, energy density 3 or 21 J/cm^2, exposure time 5.25 s and 36.75 s) on the right gastrocnemius for 10 consecutive days. LLLT reduced plasma IL-6 levels (61.3 %; P<0.01), TNF-α/IL-10 (61.0 %; P<0.01) and IL-6/IL-10 ratios (77.3 %; P<0.001) and increased IL-10 levels (103 %; P<0.05) in the 21 J/cm^2-HF group. Moreover, LLLT reduced the TNF-α (20.1 % and 21.3 %; both P<0.05) and IL-6 levels (54.3 % and 37.8 %; P<0.01 and P<0.05, respectively) and the IL-6/IL-10 ratio (59.7 % and 42.2 %; P<0.001 and P<0.05, respectively) and increased IL-10 levels (81.0 % and 85.1 %; both P<0.05) and the IL-10/TNF-α ratio (171.5 % and 119.8 %; P<0.001 and P<0.05, respectively) in the gastrocnemius in the 3 J/cm^2-HF and 21 J/cm^2-HF groups. LLLT showed systemic and skeletal muscle anti-inflammatory effects in rats with HF.

Keywords Cytokines · Inflammation · LLLT · Myocardial infarction

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Introduction

Heart failure (HF) is currently recognized as a multisystemic disorder that, besides the cardiovascular system, affects systems such as the musculoskeletal and immune systems [1, 2]. Following HF, activation of the immune system results in an imbalance between pro-inflammatory and anti-inflammatory cytokines, leading to a persistent pro-inflammatory state [3, 4].

Plasmatic levels of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are elevated in patients [2, 5, 6] and experimental models of

HF [7] whereas levels of anti-inflammatory cytokines such as interleukin-10 (IL-10) are reduced in both patients [8] and rats with HF [9]. There are few studies showing the imbalance between pro-inflammatory and anti-inflammatory cytokines in skeletal muscles [10–12].

Several studies have suggested that cytokines could be associated with morphological and metabolic alterations in peripheral skeletal muscles, early fatigue, muscle weakness, exercise intolerance [13–15] and cachexia [16]. Recently, the skeletal muscle has been described as an important source of cytokines, contributing to the local and systemic pro-inflammatory state in HF [10, 12]. Thus, the production of cytokines by skeletal muscle may represent an important biological mechanism responsible for worsening the condition of HF patients.

Low-level laser therapy (LLLT) has been widely used in clinical practice for purposes such as pain relief, reducing inflammation and healing, with a dose-dependent response [17, 18], and data on the use of LLLT for treatment of the inflammatory process is increasing, particularly regarding the modulation of inflammatory cytokines. For example, experimental studies have shown a decrease in the expression and synthesis of inflammatory cytokines after LLLT in vitro [19] and in vivo [20–22].

However, no studies have evaluated the influence of LLLT on systemic and skeletal muscle inflammation in the animal model of HF following myocardial infarction. We hypothesized that LLLT can modulate local and systemic cytokine production and concentration in rats with HF. Therefore, the aim of the present report was to evaluate the effect of LLLT on the skeletal muscle and plasmatic pro- and anti-inflammatory cytokines in rats with HF.

Materials and methods

Animals

This animal experimental controlled study was performed on 49 male Wistar rats weighing between 200 and 230 g (~90 days of age), obtained from the Animal Breeding Unit of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). The rats were housed three per cage, and received food and water ad libitum in an animal room under a 12:12-h light–dark cycle, at 22°C. The study protocol followed the ethical rules established by the *Guide for Care and Use of Experimental Animals* published by the National Institutes of Health (NIH publication no. 85–23, revised in 1996). All procedures outlined in this study were approved by the UFCSPA Ethics and Research Committee (protocol 620/08).

Surgery to induce MI

Rats were anaesthetized with xylazine (12 mg/kg, ip) and ketamine (90 mg/kg, ip), intubated, and artificially ventilated (SamWay VR 15) with a breathing rate of 60 breaths/min and an oxygen inspired fraction of 100 %. MI was induced as previously described [23, 24]. The heart was briefly exposed through left thoracotomy between the fourth and fifth ribs. In the animals in which MI was induced, a mononylon suture 6–0 was passed around the main left descending coronary artery, at a point between 1 and 2 mm distal to the edge of the left atrium, and the left coronary was ligated. Sham-operated animals underwent the same procedure without tying the suture and served as control rats. The thorax was closed, the skin was sutured with mononylon suture 3–0, and the pneumothorax was drained using a continuous aspiration system. The rats received a single dose of penicillin (20,000 U, ip) and naproxen (40 mg/kg, im). After surgery, the rats were placed in a heated environment for recovery [25].

Experimental design

After MI, rats were allowed to recover for at least 4 weeks [23]. Then, they were allocated to one of six experimental groups: placebo sham rats (P-Sham, $n=8$), LLLT at a dose of 3 J/cm^2 sham rats (3 J/cm^2-Sham, $n=8$), LLLT at a dose of 21 J/cm^2 sham rats (21 J/cm^2-Sham, $n=8$), placebo HF rats (P-HF, $n=9$), LLLT at a dose of 3 J/cm^2 HF rats (3 J/cm^2-HF, $n=8$), and LLLT at a dose of 21 J/cm^2 HF rats (21 J/cm^2-HF, $n=8$).

Laser irradiation protocol

Four weeks after the MI or sham surgery, the rats were started on the LLLT protocol or placebo. An InGaAlP type, continuous wave diode laser (model Endophoton-LLT-0107; KLD Biosistemas Equipamentos Eletrônicos Ltda., São Paulo, Brazil) with an output power of 20 mW and a wavelength 660 nm (visible red) was used. The spot size was 0.035 cm^2 and the power density was fixed at 0.571 W/cm^2. Two different doses were used for each of the four groups that received the laser irradiation, the 3 J/cm^2-Sham group (two laser points, 3 J/cm^2 each), the 3 J/cm^2-HF group (two laser points, 3 J/cm^2 each), the 21 J/cm^2-Sham group (two laser points, 21 J/cm^2 each) and the 21 J/cm^2-HF group (two laser points, 21 J/cm^2 each). In the irradiated groups, the total energy delivered per treatment was 0.21 J (3 J/cm^2-Sham and 3 J/cm^2-HF groups) and 1.47 J (21 J/cm^2-Sham and 21 J/cm^2-HF groups), over 10.5 and 73.5 s, respectively (see Table 1). Before starting the experiments, the laser equipment was calibrated using a power meter (ILX Lightwave OMN-6810B Optical Multimeter; ILX Lightwave

Table 1 Protocol table of laser radiation

Model	Laser InGaAlP (660 nm)	
Output power (mW)	20 (continuous)	
Spot size (cm^2)	0.035	
Power density (W/cm^2)	0.571	
Groups	3 J/cm^2-Sham and 3 J/cm^2-HF	21 J/cm^2-Sham and 21 J/cm^2-HF
Energy density per point (J/cm^2)	3	21
Time per point (s)	5.25	36.75
Number of points	2	2
Dose per treatment (J/cm^2)	6	42
Total energy per point (J)	0.105	0.735
Total energy per treatment (J)	0.21	1.47
Number of treatments (once a day)	10	10
Total final energy (J)	2.1	14.7
Application mode	Spot held stationary in skin contact at 90° angle with slight pressure	

Corporation, Bozeman, MT, USA). The animals received the irradiation medially and laterally to the belly of the gastrocnemius muscle, approximately 3 cm from the beginning of the paw, one point in each belly. All experimental groups were irradiated with LLLT (probe held stationary in contact with the skin at a 90° angle, maintaining slight pressure; the skin had was been shaved and cleaned) every day for 10 consecutive days. Hair removal minimized reflection and refraction, and consequently, increased the laser's effectiveness. Placebo LLLT-exposed animals underwent the same handling procedures although without the laser treatment and were used as controls. Table 1 shows the laser parameters used in our experiment.

Surgical preparation for haemodynamic evaluation

After the LLLT protocol, animals were anaesthetized with xylazine (12 mg/kg, ip) and ketamine (90 mg/kg, ip), and a small incision was made in the anterior cervical region in order to insert a polyethylene catheter (PE-50) into the right carotid artery. Arterial pressure was first recorded for 5 min by connecting the arterial cannula to a pressure transducer (strain-gauge; Narco Byosystem Miniature Pulse Transducer RP-155, Houston, TX, USA), coupled to a pressure amplifier (Stemtech). Then the catheter was positioned inside the left ventricle, and the pulse wave was monitored by graphical registration of ventricular pressure for 5 min. Analogical pressure signals were digitalized by a data acquisition system (CODAS-Data Acquisition System) with a sampling rate of 2,000 Hz. These recordings were used to determine the mean arterial pressure (MAP), heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular maximum change in pressure over time (dP/dt_{max}), left ventricular minimum change in pressure over time (dP/dt_{min}) and left ventricular end-diastolic pressure (LVEDP). The last parameter was determined manually by detecting the point of inflection to the end of diastole via analysis of the ventricular pressure wave.

Blood samples

After haemodynamic evaluation, blood samples were drawn from the catheter positioned in the right carotid artery, and collected into a 2-ml tube containing ethylenediaminetetraacetic acid (EDTA, 1:9 vol/vol). The blood samples were centrifuged at 1,000×*g* for 10 min at 4°C. The plasma was collected, aliquoted into a 2-ml tube and stored at −80°C.

Skeletal muscle collection

After blood collection, the animals were euthanized with an overdose of anaesthetic (thiopental 80 mg/kg, ip) and the right gastrocnemius muscle was collected, frozen in liquid nitrogen, and stored at −80°C.

Heart hypertrophy, lung and hepatic congestion

The heart, lungs and liver were removed and weighed. The right ventricle (RV) and left ventricle (LV) were dissected, separated and weighed. The heart-to-body weight (H/BW), LV-to-body weight (LV/BW) and RV-to-body weight (RV/BW) were determined and used as an indication of heart hypertrophy. The lungs and liver of each animal were dehydrated (80°C) for 48 h and then reweighed to determine the water content. Lung and liver wet-to-dry weight ratios were used to determine the percentage of water in those tissues, as an indication of congestion.

Infarct size

Left ventricles were filled with an insufflating latex balloon and placed in buffered formaldehyde for 24 h for subsequent analysis of the size of the infarction area. The total left

ventricle area and myocardial infarction scar were manually drawn on the scanned images and measured automatically using a computer program (IMAGE Pro-plus 6.1; Media Cybernetics, Silver Spring, MD, USA). The infarction area, expressed as a percentage, was calculated by dividing the sum of the infarcted area from all sections by the sum of the area of the left ventricle (including those without infarction) and multiplying by 100, as previously described [23].

Determination of plasmatic TNF-α, IL-6 and IL-10 protein levels

Plasmatic TNF-α, IL-6 and IL-10 protein levels were determined by multiplex bead array using Milliplex™ MAP rat cytokine kits (RCYTO-80 K) (Millipore, Billerica, MA, USA). Milliplex™ MAP is based on Luminex® xMAP™ technology. Frozen samples were thawed immediately prior to analysis, and then maintained on ice throughout the assay setup. Briefly, according to procedures recommended by the manufacturers and previously described [26], all plasma samples were diluted 1:5 in sample diluent and then incubated in duplicate overnight with capture beads specific for TNF-α, IL-6 and IL-10. The beads were subsequently washed and incubated for 2 h with a biotin-conjugated detection antibody and then for 30 min with streptavidin–phycoerythrin. Bead fluorescence was then analysed on a Luminex 100 IS Multiplex Bio-Assay Analyser. The concentrations of the cytokines were determined from standard curves of recombinant rat cytokines in which four-parameter logistic curve fitting analysis was used. All cytokines are reported in picograms per millilitre.

Skeletal muscle sample preparation and determination of tissue TNF-α, IL-6 and IL-10 protein levels

For cytokine assays, gastrocnemius samples were homogenized in phosphate-buffered saline (PBS, ph7.4), containing 0.4 mol/l NaCl, 0.05 % Tween-20, 0.5 % bovine serum albumin, 10 mmol/l EDTA and 20 Kl/ml aprotinin. The homogenates were centrifuged at 12,000×*g* for 60 min at 4 °C. The supernatant was removed and TNF-α, IL-6 and IL-10 levels were determined, as described above for plasma.

Statistical analysis

The mean and standard deviation (SD) were calculated for each variable and group. The Kolmogorov–Smirnov test was performed to evaluate normality for all variables. Comparisons were made between groups using two-way ANOVA followed by the Tukey post hoc test. Pearson's correlation test was used to examine the relationship between the values obtained for systemic and local TNF-α, IL-6 and IL-10 protein levels. A P value <0.05 was considered statistically significant. SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) for Windows was used as a computational tool in the data analysis. GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) for Windows was used for complementary analysis and to construct charts.

Results

Mortality, body weight, heart hypertrophy, infarct size, pulmonary and hepatic congestion

Table 2 summarizes the data on mortality, body weight, heart hypertrophy, infarct size, pulmonary and hepatic congestion. The data suggest that rats with HF had significant lung and liver congestion. Rats with HF had a higher heart-to-body weight ratio compared to sham rats. Total mortality in MI-induced HF rats was 32 %. No behaviour associated with stress or adverse effects was identified in rats that participated in the LLLT protocol.

Table 2 Body, heart and ventricular weight ratios, tissue and infarct characteristics of sham-operated rats and rats with left ventricular dysfunction

Groups	Initial body weight (g)	Final body weight (g)	Infarcted area (%)	H/BW (mg/g)	LV/BW (mg/g)	RV/BW (mg/g)	Pulmonary congestion (%)	Hepatic congestion (%)
P-Sham	220.6±7.2	292±21.9	–	2.8±0.2	2.2±0.2	0.6±0.1	69.6±3.8	70.8±0.8
3 J/cm^2-Sham	224.4±4.9	299.5±11.4	–	2.9±0.3	2.2±0.3	0.7±0.2	70.4±3.1	72.1±0.9
21 J/cm^2-Sham	227.1±6.8	310.1±18.9	–	3.0±0.3	2.3±0.3	0.7±0.3	70.9±2.9	72.4±1.2
P-HF	221.9±9.0	295.6±32.3	45.9±8.3	3.6±0.7‡	2.6±0.5	1.0±0.6	73.9±2.8‡	72.4±1.4*
3 J/cm^2-HF	226.6±8.2	311.3±23.8	41.7±5.7	3.4±0.6*$^\#$	2.4±0.2	1.0±0.7	74.0±2.4†	71.9±0.8
21 J/cm^2-HF	218.4±12.8	286.9±32.3	43.6±5.2	4.1±0.4‡	2.7±0.3‡	1.4±0.4‡	76.0±1.7+	73.7±2.4†

Values are means ± SD. Groups and abbreviations are described in Materials and methods section

*P<0.05 compared to P-sham

† P<0.05 compared to P-sham and 3 J/cm^2 -Sham

‡ P<0.05 compared to all sham groups

$^\#$ P<0.05 compared to 21 J/cm^2 -HF

Haemodynamic variables

Table 3 presents the values for the haemodynamic variables. Rats with HF demonstrated left ventricular dysfunction. There were no differences between placebo or LLLT HF rats in haemodynamic variables.

Plasmatic TNF-α, IL-6 and IL-10 levels

There was no significant difference in the plasmatic TNF-α protein levels between groups (Fig. 1a). Plasmatic IL-6 was higher in the P-HF group compared with the P-Sham. Plasmatic IL-6 was lower in the 21 J/cm^2-HF group than the P-HF group, suggesting that LLLT at a dose of 21 J/cm^2 decreases systemic levels of IL-6 in rats with HF. Moreover, plasmatic IL-6 was lower in the 21 J/cm^2-HF group compared to the 3 J/cm^2-HF group (Fig. 1b).

Plasmatic IL-10 was lower in the P-HF group than the P-Sham group, and higher in the 21 J/cm^2-HF group than the P-HF gruop. There was no difference in plasmatic IL-10 levels between the HF groups that received different doses of LLLT. However, the 3 J/cm^2-Sham group had higher plasmatic IL-10 levels compared to the P-Sham, 3 J/cm^2-HF and 21 J/cm^2-Sham groups (Fig. 1c).

Plasmatic TNF-α/IL-10 and IL-6/IL-10 ratio

The plasmatic TNF-α/IL-10 ratio was higher in the P-HF group than in the P-Sham group. In the 21 J/cm^2-HF group, there was a decrease in the plasmatic TNF-α/IL-10 ratio compared to the P-HF group. The decrease in the TNF-α/IL-10 ratio was associated with an increase in the plasmatic levels of IL-10, suggesting an important systemic anti-inflammatory effect of LLLT at a dose of 21 J/cm^2 (Fig. 2a).

The plasmatic IL-6/IL-10 ratio was higher in the P-HF than the P-Sham group. In the HF group, LLLT at 21 J/cm^2 reduced the IL-6/IL-10 ratio compared to both the placebo HF group and 3 J/cm^2-HF group (Fig. 2b). This reduction was associated with a decrease in plasmatic IL-6 and increase in plasmatic IL-10.

Skeletal muscle TNF-α, IL-6 and IL-10 levels

The levels of TNF-α and IL-6 skeletal muscle were higher in the P-HF group than in the P-Sham group (Fig. 3a and b, respectively). The level of IL-10 skeletal muscle was lower in the P-HF group compared to the P-Sham group (Fig. 3c). At both doses (3 and 21 J/cm^2), LLLT decreased the levels of TNF-α and IL-6 in skeletal muscle in the HF compared to P-HF group (Fig. 3a and b, respectively) and increased the IL-10 levels in the HF compared to the P-HF group (Fig. 3c).

Skeletal muscle IL-10/TNF-α and IL-6/IL-10 ratio

The skeletal muscle IL-10/TNF-α ratio was lower in the P-HF group than in the P-Sham group. At both doses (3 and 21 J/cm^2), LLLT increased the skeletal muscle IL10/TNF-α ratio in the HF compared to the P-HF group. These results were associated with an increase in tissue levels of IL-10 and a decrease in the tissue levels of TNF-α (Fig. 4a).

The skeletal muscle IL-6/IL-10 ratio was higher in the P-HF than the P-Sham group. At both doses (3 and 21 J/cm^2), LLLT decreased the IL-6/IL-10 ratio in the HF groups compared to the placebo HF group. The decrease in the IL-6/IL-10 ratio in the LLLT HF groups was associated with a decrease in tissue IL-6 and an increase in tissue IL-10 (Fig. 4b).

Correlation between systemic and local TNF-alpha, IL-6 and IL-10

There was no correlation between the systemic and local protein levels of TNF-α (r=−0.34; P>0.05), IL-6 (r=−0.13; P>0.05) or IL-10 (r=−0.05; P>0.05) in the HF placebo group.

Table 3 Hemodynamic variables of sham-operated rats and rats with MI

Groups	LVEDP (mmHg)	LVSP (mmHg)	dP/dt_{max} (mmHg/s)	dP/dt_{min} (mmHg/s)
P-Sham	1.4±2.2	128.2±14.3	7,534±2426	−4,239±1,185
3 J/cm^2-Sham	2.3±2.7	117.4±13.7	5,590±399.4	−3,178±946.8
21 J/cm^2-Sham	2.7±2.9	108.9±15.5	5,422±932.8	−3,547±309.6
P-HF	26.4±7.6‡	109.9±14.6*	4,614±1406*	−2,937±1,140*
3 J/cm^2-HF	25.9±2.4‡	111.7±14.7*	4,916±1091*	−3,279±5,934
21 J/cm^2-HF	24.6±7.0‡	92.9±11.0*†	3,690±931.1*	−2,520±466.3*

Values are means ± SD. Groups and abbreviations are described in Materials and methods section

*P<0.05 compared to P-sham

† P<0.05 compared to 3 J/cm^2 –Sham

‡ P<0.05 compared to all sham groups

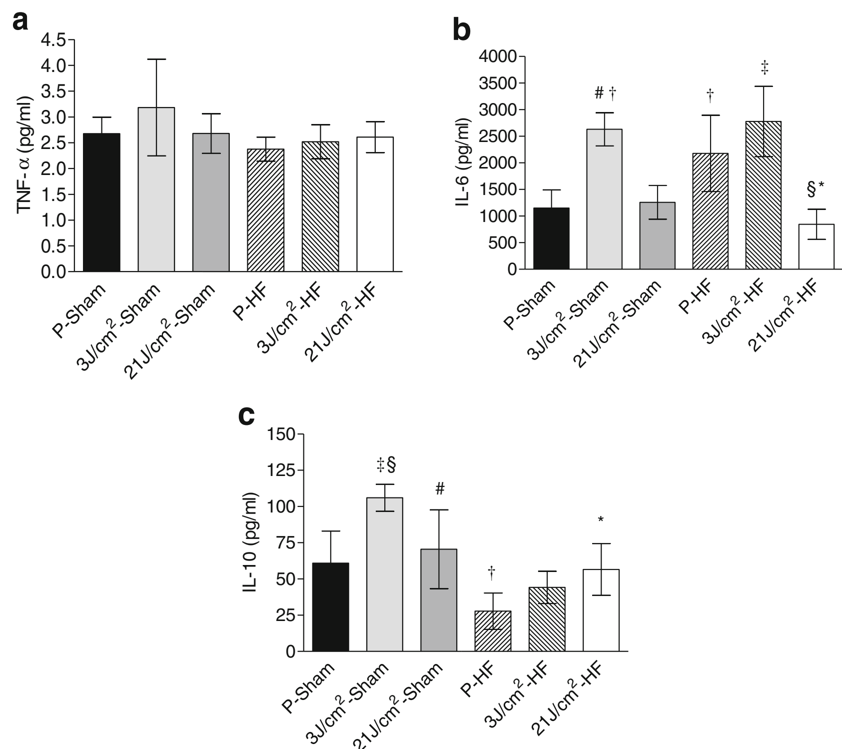

Fig. 1 Effects of LLLT on plasmatic cytokine protein levels. Groups are described in the Materials and methods section. **a** TNF-α. **b** IL-6. #$P<0.05$ compared to 21 J/cm^2-Sham. †$P<0.01$ compared to P-Sham. §$P<0.01$ compared to P-HF. ‡$P<0.001$ compared to P-Sham, 21 J/cm^2-HF and 21 J/cm^2-Sham. *$P<0.001$ compared to 3 J/cm^2-Sham c: IL-10. *$P<0.05$ compared to P-HF. #$P<0.05$ compared to 3 J/cm^2-Sham and 3 J/cm^2-HF. †$P<0.01$ compared to P-Sham and 21 J/cm^2-Sham. ‡$P<0.001$ compared to P-Sham and 3 J/cm^2-HF. §$P<0.001$ compared to P-HF and 21 J/cm^2-HF. Values are means ± SD

Discussion

To the best of our knowledge, this is the first study to demonstrate that LLLT can modulate the pro-inflammatory response of rats with HF after MI. Evidence of this effect was provided by local and systemic changes in inflammatory markers, such as the reductions in the TNF-α and IL-6 levels and the IL-6/IL-10 ratio, as well as by the increase in the IL-10 level and IL-10/TNF-α ratio in the gastrocnemius muscle. Moreover, there was a reduction in the IL-6 level and TNF-α/IL-10 and IL-6/IL-10 ratios as well as an increase in plasmatic IL-10 after LLLT.

Immune activation is responsible in part for the development and progression of HF [27], besides being an important indicator of poor prognosis in these patients [5, 6]. In this context, new therapeutic modalities for the management of HF, particularly focusing on immunomodulatory treatment modalities and anticytokine therapy [28, 29], have been investigated. However, discouraging and sometimes harmful results have been reported [30]. On the other hand, LLLT has been used in inflammatory diseases as a new anti-inflammatory therapy without any side effect [31].

The results of our study indicate an important systemic immunomodulatory effect of LLLT. There was a systemic 61.3 % reduction in IL-6 levels in the 21 J/cm^2-HF group

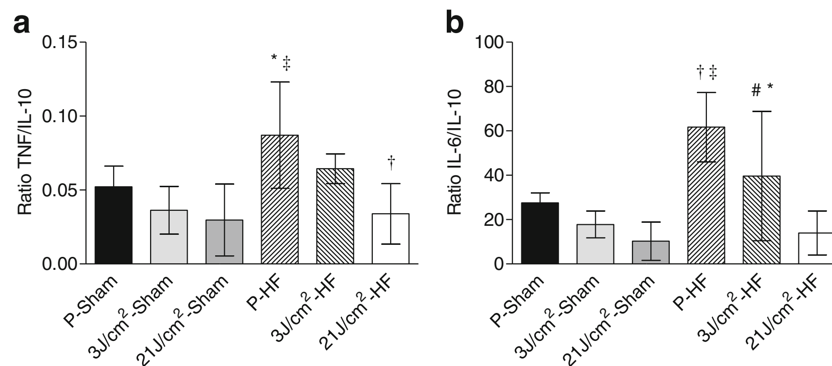

Fig. 2 Effects of LLLT on plasmatic TNF-α/IL-10 and IL-6/IL-10 ratio. Groups are described in the Materials and methods section. **a** TNF-α/IL-10 ratio. *$P<0.05$ compared to P-Sham. †$P<0.01$ compared to P-HF. ‡$P<0.001$ compared to 3 J/cm^2-Sham and 21 J/cm^2-Sham. **b** IL-6/IL-10 ratio. †$P<0.01$ compared to P-Sham and 3 J/cm^2-Sham. ‡$P<0.001$ compared to 21 J/cm^2-HF and 21 J/cm^2-Sham. #$P<0.05$ compared to 21 J/cm^2-HF. *$P<0.01$ compared to 21 J/cm^2-Sham. Values are means ± SD

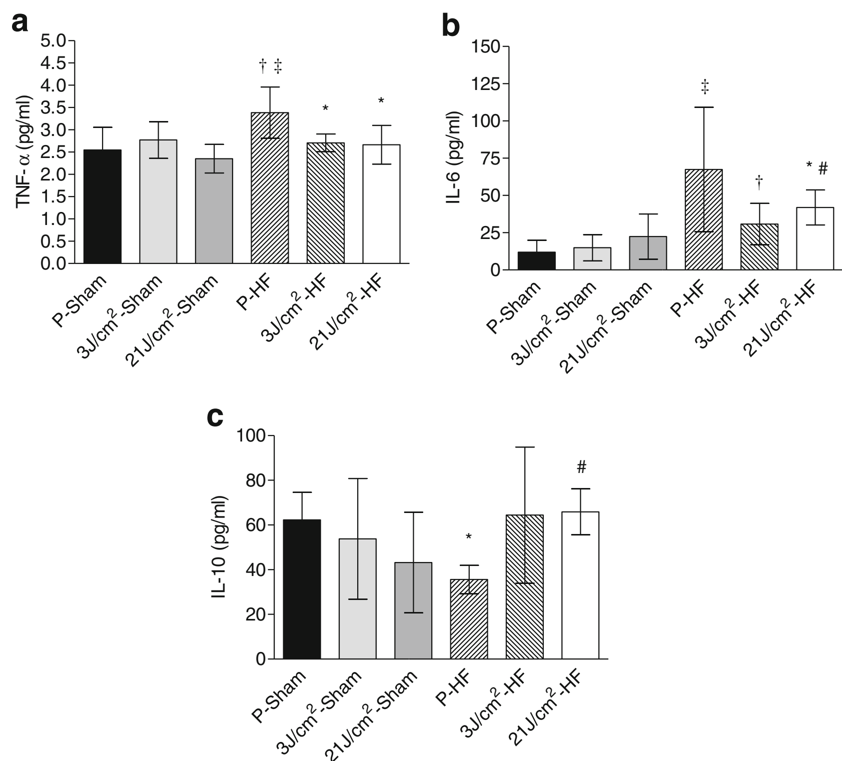

Fig. 3 Effects of LLLT on gastrocnemius cytokine protein levels. Groups are described in the Materials and methods section. **a** TNF-α. †$P<0.01$ compared to 3 J/cm^2-Sham. ‡$P<0.001$ compared to P-Sham and 21 J/cm^2-Sham. *$P<0.05$ compared to P-HF. **b** IL-6. *$P<0.05$ compared to P-HF and 3 J/cm^2-Sham. †$P<0.01$ compared to P-HF. ‡$P<0.001$ compared to P-Sham, 3 J/cm^2-Sham and 21 J/cm^2-Sham. #$P<0.01$ compared to P-Sham. **c** IL-10. *$P<0.05$ compared to P-Sham, 3 J/cm^2-HF and 21 J/cm^2-HF. #$P<0.05$ compared to 21 J/cm^2-Sham. Values are means ± SD

compared to the P-HF group. Similarly, in a study involving an animal model of brain damage [32], a reduction in systemic levels of IL-6 in animals subjected to a LLLT protocol was observed. Another study, using visible and infrared polarized light (VIP), observed a significant decrease in IL-6 levels in individuals with elevated levels of inflammatory cytokines [33]. In this context, LLLT applied to lower limb muscles was recently found to systemically decrease oxidative stress biomarkers in humans [34]. In the present study, we found a 103 % increase in systemic IL-10 after LLLT (21 J/cm^2) in the HF group compared to the P-HF group. Similar results were reported in another study that observed a significant increase in IL-10 levels after irradiation with VIP light [33]. In contrast, another study found no changes in systemic IL-10 levels after LLLT [32]. Interestingly, there appeared to be a pro-inflammatory effect (increased levels of IL-6) at lower laser doses (3 J/cm^2). However, this effect was counterbalanced by increased levels of IL-10 at the same dose, keeping the IL-6/IL-10 ratio normalized.

In addition to a systemic immunomodulatory effect, the results of our study suggest that LLLT has an important local immunomodulatory effect. We demonstrated here that the levels of TNF-α in the gastrocnemius muscle were 21.3 % lower in the 21 J/cm^2-HF group and 20.1 % lower in the 3 J/

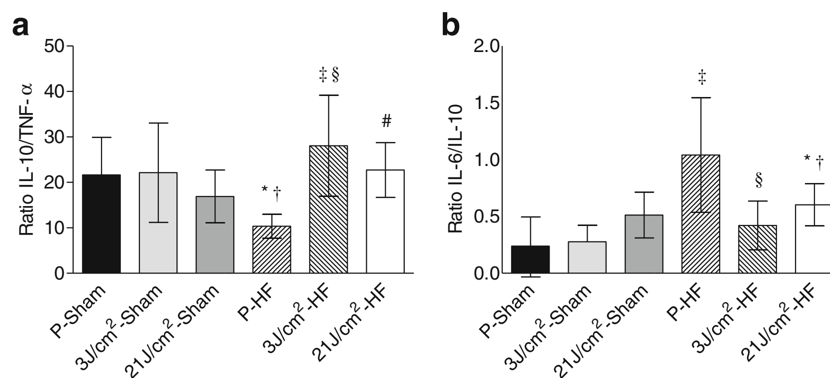

Fig. 4 Effects of LLLT on gastrocnemius IL-10/TNF-α and IL-6/IL-10 ratio. Groups are described in the Materials and methods section. **a** IL-10/TNF-α ratio. *$P<0.05$ compared to P-Sham. #$P<0.05$ compared to P-HF. †$P<0.01$ compared to 3 J/cm^2-Sham. §$P<0.01$ compared to 21 J/cm^2-Sham. ‡$P<0.001$ compared to P-HF. **b** IL-6/IL-10 ratio. *$P<0.05$ compared to P-HF. †$P<0.01$ compared to P-Sham and 3 J/cm^2-Sham. ‡$P<0.001$ compared to P-Sham, 3 J/cm^2-Sham and 21 J/cm^2-Sham. §$P<0.001$ compared to P-HF. Values are means ± SD

cm^2-HF group, compared to the P-HF group. Similarly, at both doses (21 and 3 J/cm^2), LLLT decreased the local levels of IL-6 by 37.8 % and 54.3 %, respectively, compared to the P-HF group. Moreover, the 21 J/cm^2-HF and 3 J/cm^2-HF groups showed higher values of IL-10 (85.1 % and 81.0 %, respectively) compared to the P-HF group.

LLLT may be administered using different wavelengths of the visible and near-infrared spectra [17]. A wavelength similar to that used in this study (660 nm) is known for its anti-inflammatory effect (modulation of the synthesis and expression of pro- and anti-inflammatory cytokines) both in clinical studies [35] and animal model studies [20, 36–38], in particular in skeletal muscle [22].

The lack of knowledge regarding the dosimetry required to achieve the maximal efficiency of LLLT precludes the wide employment of this therapy in several pathologies [39]. A well-designed study using the rat pleurisy model showed that an LLLT protocol (InGaAlP 660 nm) employing the same parameters used in our study (3 and 21 J/cm^2) produced a dose-dependent anti-inflammatory effect, as demonstrated by the modulation of pro- and anti-inflammatory cytokines [20]. Other evidence suggests that the LLLT effect is dose-dependent [17, 18]. Thus, we chose to use two distinct doses to examine the influence of this parameter on the anti-inflammatory effect at different levels (muscle and plasma).

Systemically, the present study showed an increase of 47.1 % in IL-6 levels and a reduction of 119.4 % in IL-10 levels in the placebo HF group. Similarly, other experimental studies showed high levels of IL-6 [7] and reduced levels of IL-10 [7, 9], after myocardial infarction. Clinical studies have also shown an increase in IL-6 levels [2, 5, 6] and a decrease in IL-10 levels [8] in patients with HF. Interestingly, in our study there was no increase in systemic levels of TNF-α in the placebo HF groups. However, other studies involving different durations of HF and different animal species observed increases in TNF-α after myocardial infarction [7, 40]. Clinical studies have also demonstrated higher levels of TNF-α in patients with HF [2, 5, 6]. Such differences may in part be explained by the use of different durations of HF and different animal species, as well as by the severity of cardiac dysfunction.

The present study showed an increase of 24.8 % in TNF-α and 82.36 % in IL-6 in the gastrocnemius muscle. In contrast, there was a reduction of 74.85 % in IL-10 in the placebo HF group. Our results are similar to other studies that found an increase in TNF-α in quadriceps [11] and soleus, together with a reduction of IL-10 [12, 41]. Clinical studies have also shown an increase in pro-inflammatory cytokines in peripheral muscles of patients with HF [10, 15]. Regarding skeletal muscle IL-6, to our knowledge, this is the first study to have demonstrated an increased IL-6 level in rats with chronic HF.

The instability in the balance between pro-inflammatory and anti-inflammatory mediators in HF has been shown in animal [7, 42] and clinical studies [8, 43] through the TNF-α/IL-10 or IL-10/TNF-α ratios. In our model, a systemic increase in TNF-α/IL-10 and IL-6/IL-10 ratios was observed in the P-HF group compared to the P-Sham group. The LLLT treatment (21 J/cm^2) reduced the TNF-α/IL-10 and IL-6/IL-10 ratios compared to the P-HF group, due to both the increased IL-10 levels and reduced IL-6 levels. In skeletal muscle, we observed a reduction in the IL-10/TNF-α ratio and an increase in the IL-6/IL-10 ratio in the P-HF group compared to the P-Sham group. At both doses (3 and 21 J/cm^2), LLLT increased the IL-10/TNF-α ratio and reduced the IL-6/IL-10 ratio compared to the P-HF group. Regarding the IL-6/IL-10 ratio, we have demonstrated for the first time an increase in this ratio in both plasma and skeletal muscle in rats with HF. The balance between pro- and anti-inflammatory cytokines may be the major determinant in the prognosis after myocardial infarction [12].

Our study found no correlation between systemic and local TNF-α, IL-6 and IL-10 levels. This is consistent with the results of studies in animals and humans showing that the increase in the local inflammatory profile occurs independently of changes in plasma levels [10, 41, 44]. Thus, it has been proposed that the increase in the local expression of inflammatory cytokines precedes the increase in circulating levels of these cytokines [12]. It is clear that skeletal muscle is not only affected by HF, but contributes significantly to triggering systemic inflammation and participates actively in the onset of the chronic consequences of this syndrome [12]. The observation that the expression of TNF-α in skeletal muscle occurs in the absence of infiltration of macrophages and monocytes [10] indicates that the skeletal muscle fibre itself can produce TNF-α [45] and thus the skeletal muscle may be an important site of production of inflammatory mediators in HF. As a consequence, TNF-α could act in an autocrine and paracrine manner and in sufficient quantities to mediate a series of morphological and functional changes in skeletal muscle [12, 46].

The present study does have limitations that warrant discussion. Firstly, comparison of our results is restricted by the lack of similar studies on the influence of LLLT on skeletal muscle in the HF model. Studies on the ability of LLLT to modulate the production of pro- and anti-inflammatory cytokines in skeletal muscle are also lacking [22]. Our results are similar to those of studies that demonstrated the ability of LLLT to modulate TNF-α and IL-6 levels in vitro [19] and in vivo [20]. A smaller number of studies in other experimental models noted the influence of LLLT on IL-10 [32]. Secondly, in the present report, even though LLLT improved the local and systemic inflammatory profile, there was no improvement in haemodynamic or morphological alterations or in pulmonary and hepatic

congestion. This may be attributed to the fact that improvement in haemodynamic variables is related to improved cytokine levels in a time-dependent manner. It is possible that the duration of improvement in cytokine profile was not long enough to promote haemodynamic improvements.

Conclusions

In conclusion, this study provides a noteworthy contribution to the understanding of the benefits obtained from the use of LLLT in the pro-inflammatory state of HF. Considering the importance of inflammatory activation and its systemic and local impact on skeletal muscles in HF, a safe, simple and inexpensive strategy such as LLLT could be used as a new non-pharmacological form of treatment for the pro-inflammatory state in HF syndrome. Thus, our results show that LLLT can modulate the balance between pro- and anti-inflammatory cytokines, both systemically and peripherally.

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Conflicts of interest None.

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