Lasers Med Sci
DOI 10.1007/s10103-013-1467-2

ORIGINAL ARTICLE

Comparative analysis of two low-level laser doses on the expression of inflammatory mediators and on neutrophils and macrophages in acute joint inflammation

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Received: 25 February 2013 / Accepted: 13 October 2013

Abstract Synovial membrane inflammation plays an important role in osteoarthritis (OA) pathophysiology. The synovial tissue of patients with initial OA is characterized by mononuclear cell infiltration and the production of pro-inflammatory cytokines and other mediators of joint injury. The study aims to evaluate the effect of low-level laser therapy (LLLT) at doses of 2 and 4 J on joint inflammation in rats induced by papain through histopathological analysis, differential counts of inflammatory cells; gene expression of IL-1β, IL-6, and IL-10; and TNF-α protein expression. Male Wistar rats (20) were randomly divided (5 animals each) into a negative control group, an inflammation injury positive control group, a 2-J LLLT group subjected to injury and treated with 2 J of LLLT, and a 4-J LLLT group subjected to injury and treated with 4 J of LLLT. The animals were subjected to joint inflammation (4 % papain solution) and treated with LLLT. On the day of euthanasia, articular lavage was collected and centrifuged. The supernatant was analyzed for TNF-α protein expression by ELISA and IL-1β, IL-6, and IL-10 mRNA by RT-PCR. The joint tissue was also examined histologically. ANOVA with Tukey's post hoc test was used for comparisons. All data were expressed as means ± S.D. ($p < 0.05$). Both laser modalities were efficient in reducing cellular inflammation and decreasing the expression of IL-1β and IL-6. However, the 2-J treatment led to more reduction in TNF-α than the 4-J treatment. A single application of LLLT with 2 J was more efficient in modulating inflammatory mediators and inflammatory cells.

Keywords LLLT · Acute inflammation · Musculoskeletal disorders

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Introduction

Under normal physiological conditions, the synovial lining consists of a thin layer of cells that exhibit phenotypic features of macrophages and fibroblasts. These cells and the underlying vascularized connective tissue stroma form a complex structure that is an important source of synovial fluid (SF) components, which is essential for normal cartilage and joint function [1].

Published online: 31 October 2013

Synovial inflammation or synovitis is a frequently observed phenomenon in joints that are affected by osteoarthritis (OA). It contributes to the pathogenesis of OA through the formation of various catabolic and pro-inflammatory mediators by altering the balance of cartilage matrix degradation and repair. Catabolic mediators that are produced by inflamed synovium include pro-inflammatory cytokines, nitric oxide, prostaglandin E(2), and several neuropeptides which further contribute to the pathogenesis of OA by increasing cartilage degradation [2].

Cytokines such as IL-1 and TNF-α, which are produced by activated synoviocytes, mononuclear cells, or by the articular cartilage itself, significantly upregulate matrix metalloproteinase (MMP) gene expression. Cytokines also blunt chondrocyte compensatory synthesis pathways that are required to restore the integrity of the degraded extracellular matrix (ECM). Moreover, in the OA synovium, a relative deficit in the production of natural antagonists of the IL-1 receptor (IL-1Ra) has been demonstrated, and it could possibly be related to the excess production of nitric oxide in OA tissues [3].

The pattern of synovial reaction varies with the duration of the disease and is associated with metabolic and structural changes in other joint tissues. The ensuing synovial reaction can lead to the synthesis and release of a wide variety of cytokines and chemokines. Some of these inflammatory mediators can be detected in joint tissues and SF in OA and have catabolic effects on chondrocytes [1].

Certain cytokines have anti-inflammatory properties. Three such cytokines (IL-4, IL-10, and IL-13) have been identified as being able to modulate various inflammatory processes. Their anti-inflammatory potential, however, appears to depend greatly on the target cell. IL-4 has been tested in vitro in OA tissue and shown to suppress the synthesis of both TNF-α and IL-1β in the same manner as low-dose dexamethasone. Naturally occurring anti-inflammatory cytokines such as IL-10 inhibit the synthesis of IL-1 and TNF-α and could be potential targets for therapy in OA [3].

According to Bondeson et al. [4], histological studies have demonstrated that OA synovial macrophages exhibit an activated phenotype and that they produce both pro-inflammatory cytokines and vascular endothelial growth factor. Synovial macrophage differentiation differs between inflammatory and noninflammatory OA.

Studies have shown that low-level laser therapy (LLLT) is capable of acting on the inflammatory process in various clinical situations [5, 6, 7, 8,]. In addition, it can modulate pro-inflammatory mediators, thereby decreasing the expression of cytokines such as IL-1β [9], IL-6 [10], and TNF-α [11], and can increase the expression of anti-inflammatory cytokines such as IL-10 [12].

This study aimed to evaluate the effect of LLLT at doses of 2 and 4 J on joint papain-induced inflammation in rats. In addition, we analyzed histopathological aspects; gene expression of IL-1β, IL-6, and IL-10; and the protein expression of TNF-α.

Materials and methods

Animals

The sample population consisted of 20 90-day-old male Wistar rats (*Norvegicus albinus*) that weighed 250–300 g. The animals were obtained from the animal housing facility of the Universidade Nove de Julho (Brazil) and kept under controlled conditions of light and temperature with free access to water and chow. All of the experimental procedures were approved by the Institutional Research Ethics Committee (AN 0016/2011). They were in accordance with the guidelines of the Brazilian College for Animal Experimentation as well as the standards of the International Council for Laboratory Animal Science.

Experimental groups

Twenty animals were randomly distributed into four groups of five animals each. The first group (control) did not receive any kind of intervention. The second group (injury) received induction but did not receive any treatment. The third group was treated with LLLT at 2 J. The rats in the fourth group were treated with LLLT at 4 J. All of the groups were evaluated at 24 h post-injury (five animals per group at each experimental time point).

Papain-induced inflammation

The animals were anesthetized with an intramuscular injection of 7 % ketamine (Cetamin, Syntec, Cotia, SP, Brazil) and 0.3 % xylene (Xilazin, Syntec, Cotia, SP, Brazil) solution at a ratio of 2:1 (0.2 mL per 100 g). The induction of OA was then performed following a previously published method [13]. Specifically, 200-μL injections were administered on the right knee of the hind leg of each animal with a 4 % papain solution that was dissolved in 10 mL of saline solution to which 10 mL of a cysteine solution (0.03 M) was added. This solution was used as the activator to produce cartilage injury. The animals were then immediately administered LLLT.

LLLT

An AsGaAl-type diode laser with a wavelength (λ) of 808 nm from Photon Laser III DMC (Sao Carlos, SP, Brazil) was used. The optical power was calibrated using a Newport multifunction optical meter model 1835C. The stability of the laser during laser irradiation was measured collecting light

with a partial reflect (4 %). The optical power output of the laser unit was measured before, halfway through, and after the experiment. All measurements to state parameters were performed at the laser aperture, and the manufacturer gave the laser beam information. The doses and parameters are summarized in Table 1.

Irradiation

Laser irradiation was carried out transcutaneously at two points: medial and lateral. It was performed immediately after the papain–cysteine injection on the right knee in groups at doses of 2 and 4 J. The control and injury groups received no treatment and served as negative and positive control groups, respectively, for comparative histomorphometric analysis. The animals were immobilized by means of a grip and were irradiated at an angle of 90° at the surface of the tissue.

Sample collection

After receiving the treatment, on the day of euthanasia, a procedure for obtaining washed articular synovial fluid was performed. The articular cavity was washed with 1 mL of physiologic serum into the intracapsular knee space. The material was immediately centrifuged at 1,500 rpm for 5 min, and the supernatant was stored at −80 °C for the analysis of inflammatory mediators.

Total and differentiated cell counts

Articular synovium was washed by injecting 200 μL of phosphate buffered saline (PBS) into the joint cavity, and as much material (washed) as possible was recovered (usually around 20–30 μL). The recovered lavage was centrifuged at 1, 500 rpm for 5 min, and the cell pellet was suspended in 200 μL of PBS. Total cells were then counted in a hemocytometer (Neubauer chamber, 10 μL; diluting solution was Turk's solution at a ratio of 1:5). The remaining articular material was washed thoroughly and used for differential cell counts (neutrophils, eosinophils, macrophages, and lymphocytes). For this, the washed joint was processed in a cytocentrifuge (Bio Research, 450 rpm for 6 min), and the slides that contained the cell pellet were stained with Diff-Quick. As shown in a previous study, 300 cells were counted on each slide [14].

Evaluation of inflammatory mediator (TNF-α)

The amount of TNF-α in the washed articular synovium was quantified using an enzyme-linked immunosorbent assay as per manufacturer's instructions (R&D Systems, USA). For this purpose, 96-well plates were coated with 100 mL of monoclonal antibody for each cytokine: anti-TNF-α diluted in sodium carbonate buffer (0.1 M, pH 9.6). The plates were incubated at 4 °C for 18 h. For blocking, the plates were washed four times with PBST (PBS containing 0.05 % Tween 20) and then filled with 300 μL/well of blocking solution (3 % gelatin in PBST, Sigma) at 37 °C for 3 h before being subjected to a new cycle of washes. Next, 100 mL of properly diluted samples or standards of recombinant cytokines were added to the plate and incubated for 18 h at 4 °C. After washing, 100 μL of the respective biotinylated antibodies that were specific for the detection of each cytokine was added and left for 1 h at room temperature (RT). After washing the plates, 100 μL of streptavidin–peroxidase was added and left for 1 h at RT (22 °C) followed by further washes. The reaction was visualized by adding 100 μL/well solution of 3,3′,5,5′-tetramethylbenzidine and stopped by adding 50 μL/well of sulfuric acid (2 N). The readings were performed in a Spectrum Max Plus 384 spectrophotometer (Sunnyvale, CA, USA) at a wavelength of 450 nm with correction at 570 nm. The sample concentrations were calculated from standard curves that were obtained for recombinant cytokines. The limit of detection for TNF-α was 1.95 pg/mL [10].

Quantitative real-time reverse transcription PCR (IL-1β, IL-6, and IL-10)

One microgram of total RNA was used for cDNA synthesis and real-time PCR gene expression analysis. Initially, contaminating DNA was removed using DNase I (Invitrogen) at a concentration of 1 unit/μg RNA in the presence of 20 mM Tris–HCl at pH 8.4 that contained 2 mM $MgCl_2$ for 15 min at 37 °C. This was followed by incubation at 95 °C for 5 min to bring about enzyme inactivation. Then, reverse transcription PCR was carried out in a 200-μL reaction mixture in the presence of 50 mM Tris–HCl at pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus reverse transcriptase

Table 1 Low-level laser therapy parameters

Group	Wavelength (nm)	Type of diode laser	Mean power output (mW)	Spot size (cm^2)	Power density (W/cm^2)	Energy (J)	Energy density (J/cm^2)	Time per point (s)
2-J LLLT	808	AsGaAl	50	0.028	1.78	2	71.4	40
4-J LLLT	808	AsGaAl	50	0.028	1.78	4	142.8	80

(Invitrogen). The reaction conditions were as follows: 20 °C for 10 min, 42 °C for 45 min, and 95 °C for 5 min. The reaction product was amplified by real-time PCR on a 7000 Sequence Detection System (CA, USA) by using the SYBRGreen Core Reaction System (ABI Prism, Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The experiments were performed in triplicate for each data point. IL-1β, IL-6, and IL-10 mRNA abundances were quantified as relative values compared with an internal reference (β-actin) whose abundance did not change between the varying experimental conditions. The primers that were used for real-time PCR were as follows: IL-1 (GenBank™ accession number M98820), forward primer 5′-CACCTCTCAAGCAGAGCACAG-3′ and reverse primer 5′-GGGTTCCATGGTGAAGTCAAC-3′; IL-6 (GenBank accession number E02522), forward primer 5′-TCCTACCCCAACTTCCAATGCTC-3′ and reverse primer 5′-TTGGATGGTCTTGGTCCTTAGCC-3′; and IL-10 (GenBank accession number NM012854), forward primer 5′-AAGCAAGGCAGTGGAGCAG-3′ and reverse primer 5′-TCAAACTCATTCATGGCCTTGT-3′ [11].

Euthanasia

At the end of each treatment, the animals in each group were identified, weighed, and subsequently euthanized by inhalation of carbon dioxide (CO_2). This method confers rapid loss of consciousness in response to hypoxia that is attributed to the depression of vital centers and is performed in a CO_2 chamber. The tibiofemoral articulation of the right hind leg of each animal was separated for analysis of the cartilaginous tissue of the knee. The material was immediately fixed using a 10 % buffered formaldehyde solution and submitted to histological procedures.

Histological procedures and histopathological analysis

The material was decalcified with EDTA and submitted to a classic histological method for embedding paraffin. This comprised dehydration in increasing concentrations of alcohol, clearing with xylol in order to allow the penetration of paraffin, impregnation in paraffin baths, insertion into molds, producing cross-sectional cuts to a thickness of 5 μm, and mounting the sections on synthetic balsam. Then, the sections were stained with hematoxylin and eosin in order to perform histopathological analysis.

Statistical analysis

The data were tabulated using Microsoft Excel 2007 software and initially assessed for normality using the Shapiro–Wilk test. Since a normal distribution was observed, ANOVA with Tukey's post hoc test was used for comparisons between experimental groups. All of the data are expressed as mean and standard deviation values. GraphPad Prism 5 software program was used. Significant differences from the null hypothesis were considered to exist when $p<0.05$.

Results

Differential inflammatory cell counts

The data that are presented demonstrate the results for total counts of washed joint fluid viable cells in a Neubauer chamber and allowed us to observe the following: control group (6.8±0.5), injury group (72.8±5.3), 2-J LLLT group (11.4±3.2), and 4-J LLLT group (32.3±0.8). In the differential analysis for neutrophils, we observed that the groups that were treated with LLLT of 2 J (6.49±1.2) and 4 J (18.88±5.3) showed reductions in the absolute number of neutrophils compared with the injury group (48.7±7.3). However, only the 2-J LLLT group showed no statistical difference ($p>0.05$) when compared with the control group (4.1±0.9), which demonstrates that this dose reduced the number of neutrophils at the level of the control group (Fig. 1a).

Figure 1b showed the effect of LLLT on the differential count for the macrophages. It was observed that the LLLT groups are significantly different than the injury group ($p<0.001$); however, no difference was observed compared to the control group ($p>0.05$). Additionally, no difference was observed between the LLLT groups ($p>0.05$).

Effect of LLLT on IL-1β-α mRNA expression in articular synovial lavage

As seen in Fig. 2a, we observed that there was a significant difference between the control group and the group that was challenged with joint injury ($p<0.05$) in terms of the expression of IL-1β. Treatment with 2 J of LLLT reduced the expression of IL-1β to values that were similar to that of the control group and significantly lower than that of the injury group ($p<0.05$). However, treatment with 4 J was not able to decrease IL-1β levels.

Effect of LLLT on IL-6 mRNA in articular synovial lavage

Figure 2b presents the effect of LLLT on IL-6 mRNA expression. In this experiment, IL-6 expression was significantly increased ($p<0.05$) after papain injection when compared to the animals from the control groups. Only the LLLT treatment with a dose of 4 J was markedly efficient at reducing papain-induced IL-6 mRNA expression compared to the injury group ($p<0.05$).

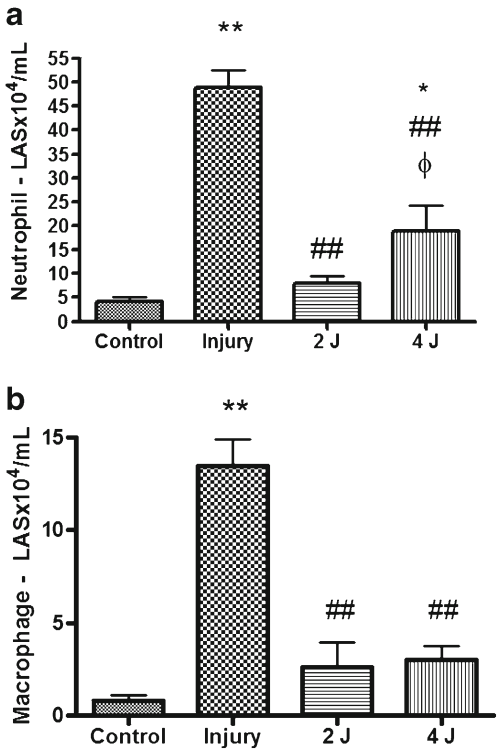

Fig. 1 Analysis of the articular lavage at 24 h after induced papain injury. **a** The absolute neutrophil count was calculated from a differential count that was determined in cytospin preparations that were stained with Diff-Quik stain. $*p<0.05$ and $**p<0.001$ for Tukey's test compared with the control group. $^{\#\#}p<0.001$ for Tukey's test compared with the injury group. $p<0.05$ for Tukey's test compared with the 2-J LLLT group. **b** The absolute macrophage count was calculated from a differential count that was determined for cytospin preparations that were stained with Diff-Quik stain. $**p<0.001$ for Tukey's test compared with the control group. $^{\#\#}p<0.001$ for Tukey's test compared with the injury group. The results are expressed as mean ± SEM

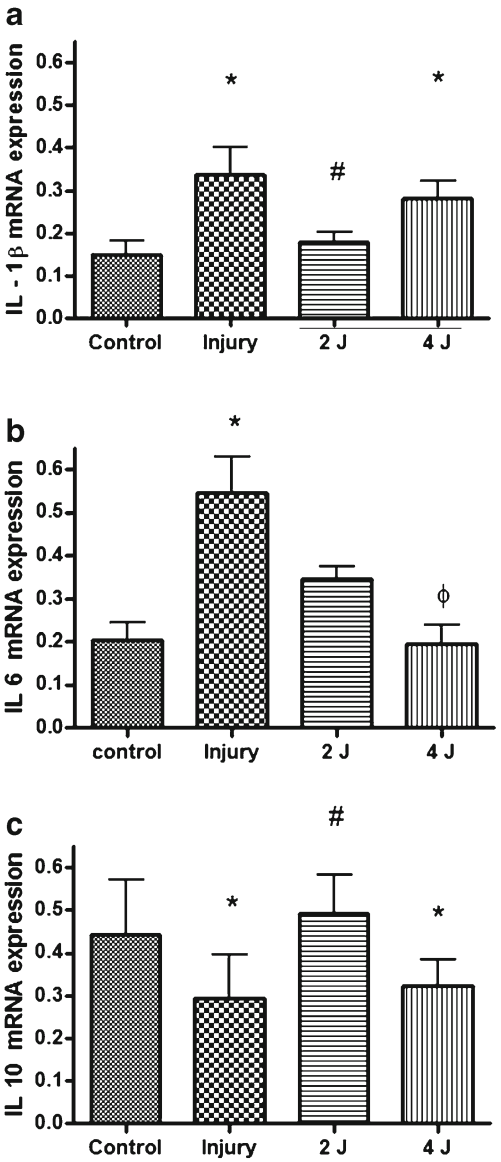

Fig. 2 **a** IL-1β expression measured by real-time PCR of articular wash fluid. $*p<0.05$ for Tukey's test compared with the control group. $^{\#}p<0.05$ for Tukey's test compared with the injury group. **b** IL-6 RNA expression measured by real-time PCR of articular lavage fluid. $*p<0.05$ for Tukey's test compared with the control group. $p<0.05$ for Tukey's test compared with the 2-J LLLT group. **c** IL-10 expression measured by real-time PCR of articular wash fluid. $*p<0.05$ for Tukey's test compared with the control group. $^{\#}p<0.05$ for Tukey's test compared with the injury group. $p<0.05$ for Tukey's test compared with the 2-J LLLT group. The results are expressed as mean ± SEM

Effect of LLLT on IL-10 mRNA in articular synovial lavage

Figure 2c shows the effect of LLLT on IL-10 mRNA expression. In this experiment, IL-10 expression was significantly lower ($p<0.05$) after papain injection when compared to the animals from the control group. Only treatment with 2-J LLLT was efficiently and markedly increased for IL-10 mRNA expression ($p<0.05$). Data of 2-J LLLT was not statistically different compared to that of the control group ($p>0.05$).

Effect of LLLT on the protein expression of TNF-α in articular synovial lavage

Figure 3 shows the results of the analysis of TNF-α expression in the articular synovial lavage. We observed that papain-induced inflammation leads to a statistically significant increase in the expression of TNF-α compared to the control group ($p<0.05$). Only treatment with 2-J dose significantly decreased TNF-α expression compared to the injury group ($p<0.05$). No difference was observed between 2-J LLLT and control groups ($p>0.05$).

Histological analysis

For histological examination, the control group had general features that were consistent with synovial joint normality. The joint spaces did not have inflammatory exudate, and the synovial membranes had thickened intima and subintima that

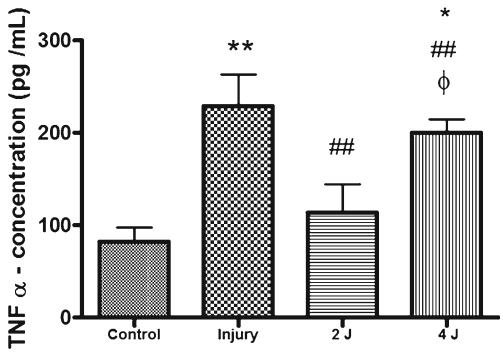

Fig. 3 Comparison of the mean and standard deviation of the TNF-α concentration that was obtained by enzyme-linked immunosorbent assay in articular lavage fluid at 24 h after the induction of papain injury. $*p < 0.05$ and $**p < 0.001$ for Tukey's test compared with the control group. $^{\#\#}p < 0.001$ for Tukey's test compared with the injury group. $p < 0.05$ for Tukey's test compared with the 2-J LLLT group. The results are expressed as mean ± SEM

showed typical characteristics. The surfaces of the coated articular hyaline cartilage were homogeneous, and there was active endochondral ossification of the epiphysis. The control group also had epiphyseal bone marrow with a normal pattern that was filled with red bone marrow. The articular meniscus consisted of thick collagen fibers and chondrocytes, and ossification was present (Fig. 4a, b).

In the injury group (induction of inflammatory process without treatment), the synovial joint showed the occurrence of an acute inflammatory process. The joint spaces had fibrinous and hyaline material that adhered to the surface of the synovial membrane, and some areas had an acute inflammatory infiltrate. The synovial membrane had an intima of normal thickness, but the subintimal layer showed signs of acute inflammatory infiltrate and had dilated blood vessels. Similar to that of the control group, the surfaces that were coated with articular hyaline cartilage were homogeneous, and the epiphyseal bone marrow had a normal fill of red bone marrow (Fig. 4c, d).

As seen in Fig. 4e, f, the group that was treated with 2-J LLLT had synovial joints. We observed the presence of an acute inflammatory process of low intensity in the connective tissue underlying the synovial membrane. The articular surfaces showed normal characteristics as well as marrow spaces (Fig. 4f).

The group that was treated with 4-J LLLT had synovial joints. The only major difference with the control group was the presence of discrete inflammatory cells (a few neutrophils) in the underlying connective tissue of the anterior cruciate ligament (i.e., the joint capsule) as seen in Fig. 4g, h.

Discussion

Firstly, it is important to clarify that in this study we choose to use dosages inside the "therapeutic window" for inflammatory conditions observed in previous studies [5–11], and the choice of 808-nm wavelength is due to the better penetration of infrared light. Several studies that involved the use of LLLT as a therapeutic approach demonstrated the ability of laser light to modulate inflammatory biomarkers in various situations [5–11]. Currently, there is a growing consensus that various pro-inflammatory cytokines that are produced by stimulated synovial macrophages are partly responsible for triggering the morphological changes that are found in joint cartilage caused by MMPs [14]. The literature has also shown that suppression or decrease of these cytokines (IL-1β and TNF-α) may increase production of anti-inflammatory cytokines such as IL-10 [15, 16] and might be an alternative for the treatment of OA [17, 18]. In this sense, we aimed to investigate the effect of LLLT as a modulatory tool of inflammatory process.

Our results showed a reduction in both IL-1β and IL-6 expression for the two LLLT doses. However, 2-J LLLT led to a statistically significant reduction in the expression of IL-1β and IL-6 to values that were observed for the control group. This was consistent with several studies that showed the modulation of these cytokines by LLLT [8, 19, 20].

Pallotta et al. [20] conducted a study to examine the response to LLLT at different times and doses in a model of joint inflammation that is induced by kaolin plus carrageenan. They analyzed several inflammatory mediators, including IL-1β and IL-6, and concluded that LLLT acts by modulating the inflammatory process and possibly stimulates the production of anti-inflammatory mediators.

We also observed in our study that the two groups that were treated with LLLT had lower neutrophil counts compared with the injury group and that the counts were similar to those that were obtained for the control group. This indicates that LLLT was able to reduce the migration of neutrophils during the initial inflammatory phase. This was also observed via the histological analysis, although those results indicated a higher efficiency for the 2-J LLLT treatment in attenuating the general inflammatory process.

Our results point to an increase in the modulation of cytokines and inflammatory cells (neutrophils and macrophages) when they are irradiated by LLLT at a dose of 2 J. However, we cannot ignore the results of the 4-J LLLT group, in particular with regard to the expression of TNF-α.

Pezelj-Ribaric et al. [8] studied the conditions of subjects with burning mouth syndrome before and after treatment with LLLT (685-nm continuous wave, 30-mW output power, 3.0 J/cm^2) by analyzing the expression of TNF-α and IL-6 in their saliva. They found a statistically significant reduction in their salivary levels of both cytokines after LLLT treatment.

Guo et al. [21] performed a study whereby they evaluated and compared the effects of millimeter waves, pulsed electromagnetic fields, ultrasounds, LLLT, and short-wave diathermy on the serum levels of TNF-α, chondrocyte

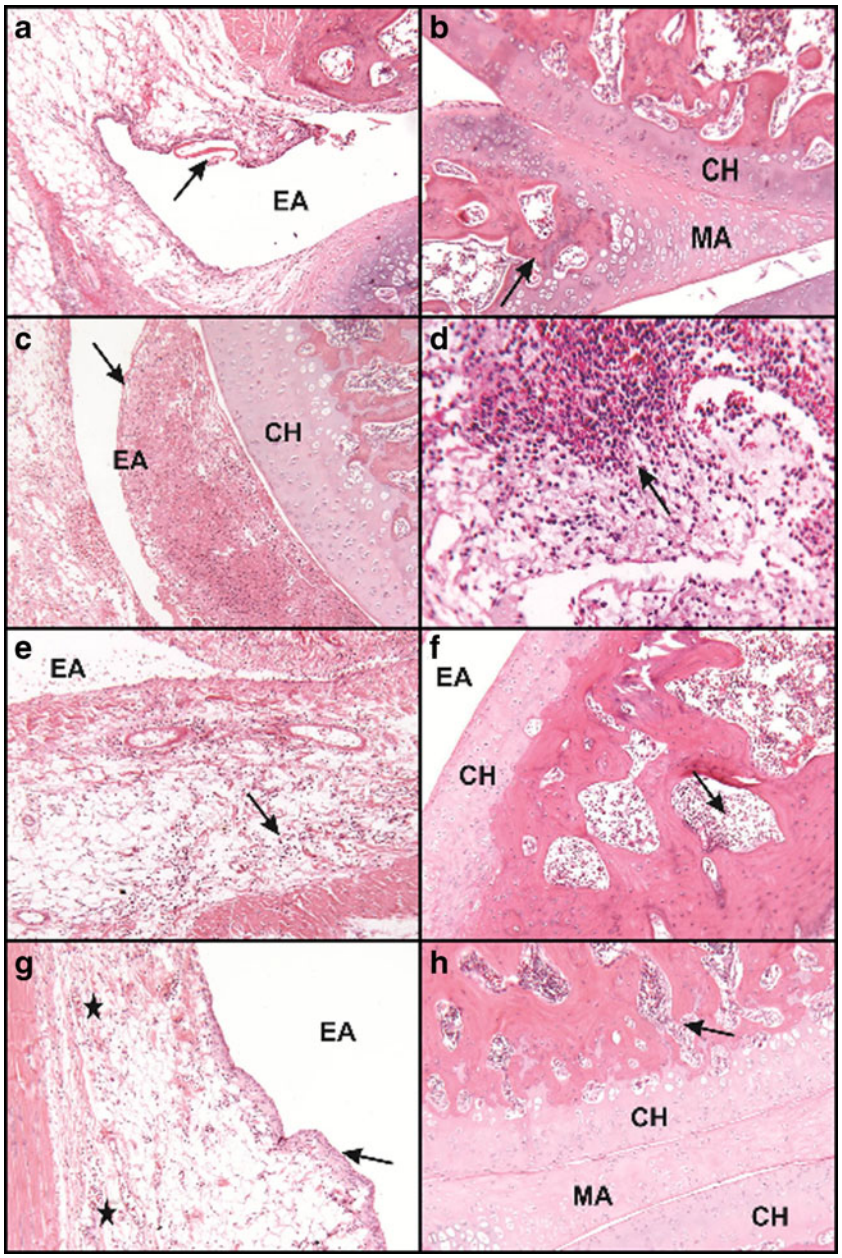

Fig. 4 Montage of the photomicrographs of the histological preparations at 24 h after. **a** Control group without induction of inflammation process with papain at 4 %. Notice the slight presence of hyaline material in the joint space due to plasma extravasation (*arrow*). **b** Control group without induction of inflammation process with papain at 4 %; observe joint surfaces under conditions of normal meniscus and articular signs of ossification. **c** Group with induction of inflammation process with papain at 4 % without treatment; note the intense acute inflammatory exudate filling the part of the joint space (*arrow*). **d** Group with induction of inflammation process with papain at 4 % without treatment; observe the presence of neutrophil accumulation in the connective tissue underlying the synovial membrane which can be observed (*arrow*). **e**, **f** Group with induction of inflammation process with papain at 4 % and treated with 2 J of energy had synovial joints. We can observe the presence of an acute inflammatory process of low intensity in the connective tissue underlying the synovial membrane. The articular surfaces show normal characteristics, as well as the marrow spaces. **g** Group with induction of inflammation process with papain at 4 % and treated with 4 J of energy; note the intimal layer of the synovial membrane presenting usual thickness (*arrow*), whereas few inflammatory cells are isolated as observed in the underlying layer (*star*). **h** Group with induction of inflammation process with papain at 4 % and treated with energy of 2 J. Note that the meniscus and the articular joint surfaces show signs of tissue integrity. Scale bar, 20 μm. Hematoxylin and eosin, ×40

apoptosis, caspase-3, and caspase-8 in an experimental model of OA in rabbit knees. They suggested that their findings may shed light on the efficacy of various physical treatments in terms of managing OA. The same authors suggested that LLLT is not an ideal treatment modality for OA. This contrasts with our findings, which demonstrate that TNF-α protein

expression was reduced in both groups treated with LLLT, although only the 2-J LLLT group reached similar levels compared to that observed for the control group.

Our results provide evidence of LLLT-dependent reductions in IL-1β, IL-6, and TNF-α and the ability of LLLT to inhibit the proliferation of inflammatory cells. This makes LLLT a suitable treatment for synovitis that is associated with the early stages of OA. Our results also indicate that a better understanding of the role of LLLT in modulating these mediators could be a basis for future therapeutic interventions. We conclude that a single application of LLLT with a dose of 2 J is more efficient in modulating inflammatory mediators (IL-1β, IL-6, TNF-α, and IL-10) and inflammatory cells (macrophages and neutrophils) and that its effects can be observed by histological signs of attenuation of inflammatory processes.

Acknowledgments The authors would like to thank FAPESP for the master degree scholarship that was granted to Ana Carolina Araruna Alves (FAPESP process number 2011/04834-8) and to CNPq for the Institutional Program of Scientific Initiation Scholarships (PIBIC) granted to Solange Almeida dos Santos (CNPQ process number 139733/2011-6). Professor Ernesto Cesar Pinto Leal-Junior would like to thank FAPESP for the financial support (process number 2010/52404-0).

Conflict of interest Professor Ernesto Cesar Pinto Leal-Junior receives research support from Multi Radiance Medical (Solon, OH, USA), a laser device manufacturer. Multi Radiance Medical had no role in the planning of this study, and the laser device used was not theirs. They had no influence on study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remaining authors do not have any conflict of interest.

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