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ORIGINAL ARTICLE

Low-level laser therapy prevents degenerative morphological changes in an experimental model of anterior cruciate ligament transection in rats

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Abstract The aim of this study was to analyze the effects of low-level laser therapy (LLLT) on the prevention of cartilage damage after the anterior cruciate ligament transection (ACLT) in knees of rats. Thirty male rats (Wistar) were distributed into three groups (n=10 each): injured control group (CG); injured laser-treated group at 10 J/cm^2 (L10), and injured laser-treated group at 50 J/cm^2 (L50). Laser treatment started immediately after the surgery and it was performed for 15 sessions. An 808 nm laser, at 10 and 50 J/cm^2, was used. To evaluate the effects of LLLT, the qualitative and semi-quantitative histological, morphometric, and immunohistochemistry analysis were performed. Initial signs of tissue degradation were observed in CG. Interestingly, laser-treated animals presented a better tissue organization, especially at the fluence of 10 J/cm^2. Furthermore, laser phototherapy was able of modulating some of the aspects related to the degenerative process, such as the prevention of proteoglycans loss and the increase in cartilage area. However, LLLT was not able of modulating chondrocytes proliferation and the immunoexpression of markers related to inflammatory process (IL-1 and MMP-13). This study showed that 808 nm laser, at both fluences, prevented features related to the articular degenerative process in the knees of rats after ACLT.

Keywords LLLT · Articular cartilage · Proteoglycan · Metalloproteinase 13 · Interleukin-1

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Introduction

Osteoarthritis (OA) is a common chronic disease among older people which leads to a loss of articular cartilage [1]. It is characterized by gradual degeneration of the joint, progressive destruction of articular cartilage, and new bone formation at the joint surface and surrounding areas [2, 3]. It is estimated that 10 % of the population in the world older than 60 years demonstrated signals of OA [4, 5]. In general, patients with OA present joint stiffness, loss of mobility, and pain [4, 6].

Treatments of OA involve the administration of nonsteroidal anti-inflammatory drugs and physical therapy, such as muscle strengthening and stretching, in the early or intermediate stages and surgical intervention for joint replacement in late stages. However, these therapies are introduced only after the appearance of the first symptoms [7, 8]. In this context, due to the very limited cartilage regenerative capacity and consequently the limited efficacy of the standard treatments, it would be of great interest the investigation of strategic innovative approaches to prevent the development of the clinical condition of OA [8].

One promising treatment is the use of low level laser therapy (LLLT) mainly due to its anti-inflammatory and regenerative effects on biological tissues [1, 9, 10]. The action of LLLT is based on the absorption of the light by tissues, which modulates cell biochemical reactions and stimulates mitochondrial respiration [11, 12]. These effects can increase the synthesis of DNA, RNA, and cell-cycle regulatory proteins, therefore promoting cell proliferation [11].

In cartilage tissue, in vivo studies demonstrated the positive effects of laser phototherapy [13]. Kushibiki et al. [9] showed increased chondrocyte differentiation and higher chondrogenic mRNA expression in prechondrogenic cells after laser irradiation. Furthermore, it was demonstrated that LLLT is able of reducing swelling [13, 14], inhibiting

inflammation, and reducing fibrosis formation [15] in OA animal models. Also, Lin et al. [1] affirm that 810 nm LLLT can improve cartilage structure, prevent articular cartilage degradation in the knees of the rabbits submitted to anterior cruciate ligament transection (ACLT), and significantly decrease the expression of caspase-3, which is a protein related to apoptosis of chondrocyte and plays an important role in the development of OA.

Despite the positive effects of LLLT on tissue regeneration, the cellular mechanisms of this therapeutic approach on the prevention of cartilage degeneration have not yet been clarified. Also, the wide range of fluences used by different authors make the use of LLLT as a treatment modality still controversial. In this context, based on the stimulatory effects of LLLT, it was hypothesized that light energy could biomodulate cartilage metabolism and prevent degenerative process after the ACLT, providing a treatment with additional advantages for clinical use. Consequently, the present study was carried out in order to evaluate the effects of LLLT, at two different fluences, on the prevention of cartilage damage after the ACLT in the knees of rats. Histology and immunohistochemistry analysis were used to evaluate the dose response of laser application in cartilage tissue.

Materials and methods

Experimental groups

This study was approved and conducted in accordance with the Animal Care Committee guidelines of the Federal University of São Paulo (1933/2010). Animals were maintained at 19–23 °C on a 12:12 h light–dark cycle in the Animal Experimentation Laboratory of the Federal University of São Paulo. Rats were housed in plastic cages and had free access to water and standard food.

Thirty male Wistar rats (weighing 300±20 g, 12–13 weeks) were randomly distributed into three groups (n=10 each): injured control group (CG); injured laser-treated group at 10 J/cm^2 (L10); and injured laser-treated group at 50 J/cm^2 (L50).

Anterior cruciate ligament transection

The animals were submitted to general anesthesia induced by intra-peritoneal injection of xilazin (Syntec®, 20 mg/kg, IP) and ketamine (Agener®, at 40 mg/kg, IP) and subjected to ACLT of the left hind paw. The left knee was shaved and sterilized. The joint cavity was approached by a lateral parapatellar incision, the patella was dislocated and the anterior cruciate ligament was transected. The anterior drawer test was performed by the surgeon and an observer to verify the success of the surgery procedure [16]. The incision was closed in layers and antiseptically treated. Further, the animals were observed for signs of pain, infection, and proper activity.

Laser application

Laser treatment started immediately after the surgery and in five consecutive days of irradiation with an interval of 2 days, for 3 weeks (in a total of 15 sessions). LLLT was applied at two points (on the medial and lateral sides of the joint), using the punctual contact technique. A low-power Ga-Al-As laser (Theralaser, DMC® São Carlos, São Paulo, Brazil) was used at 808 nm, continuous wave diode, with a 0.028 cm^2 spot area, a power output of 30 mW, fluence at 10 J/cm^2 (irradiation time of 10s, energy per point 0.3 J) and fluence at 50 J/cm^2 (irradiation time of 47 s, energy per point 1.4 J). On the respective day, animals were euthanized individually by carbon dioxide asphyxia and the knee joints were removed for analysis [17].

Histological analysis

A standard histological protocol was used. Briefly, the specimens were fixated in 4 % formaldehyde for 2 days, followed by decalcification in 4 % EDTA. The specimens were divided into two pieces, using a blade, at the mean point between both condyles, perpendicular to the articular surface. Samples were embedded in paraffin blocks and histological sections were obtained (6 μm), in the sagittal plane, starting from the medial margin of the joint using a microtome (Leica RM-2145, Germany). Samples were stained with hematoxylin and eosin (HE—Merck, Darmstadt, Germany) and Safranin-O (Merck, Darmstadt, Germany). Moreover, three sections were obtained for the immunohistochemical analysis.

Histological descriptive analysis

Histopathological alterations in the articular cartilage were evaluated by two blinded observers. For descriptive analysis, the samples were stained with HE to evaluate cellular organization, cartilage structure, and amount of cells. The specimens were examined using a light microscopy (×100; Leica Microsystems AG, Wetzlar, Germany).

Semi-quantitative analysis

A Modified Mankin Score [18] was used as a histopathologic grading system to assess cartilage damage (Table 1). The HE- and Safranin-O-stained samples were used for cellularity and for proteoglycans analyses, respectively. At least three sections of each animal were examined using light microscopy (×100; Leica Microsystems AG, Wetzlar, Germany). Two experienced observers (LA and CB) performed the scoring in a blinded manner.

Table 1 Modified Mankin Score

Cellularity	
Normal	0
Hypercellularity	1
Severe hypercellularity	2
Hypocellularity	3
Safranin-O staining	
Normal	0
Slight reduction	1
Up to half of total area	
Slight reduction	2
On total area or total surface	
Severe reduction	3
Up to half of total area	
Severe reduction	4
On total area or total surface	

Morphometric analysis

The morphometric study was carried out using one slide stained with HE per animal. The cartilage thickness and number of chondrocytes in each area were quantitatively scored using the computer-based image analysis Axiovision 3.1 Image Analysis (Carl Zeiss, Oberkochen, Germany). To count the number of chondrocytes, three areas of 80.000 μm^2, at the anterior, central, and posterior region of each slide were chosen. Within each area, cells were marked and the chondrocytes average was calculated. Total cartilage area was also measured from subchondral bone to articular surface [18]. Two experienced observers (LA and CB) performed the scoring in a blinded manner [18].

Immunohistochemistry analysis

Paraffin was removed with xylene from serial sections and were rehydrated in graded ethanol, then pretreated in a microwave oven with 0.01 M citric acid buffer (pH 6) for three cycles of 5 min each at 850 W for antigen retrieval. The material was pre-incubated with 0.3 % hydrogen peroxide in phosphate-buffered saline (PBS) solution for 5 min for inactivation of endogenous peroxidase and then blocked with 5 % normal goat serum in PBS solution for 10 min. The specimens were then incubated with primary antibodies interleukin-1 (IL-1β; polyclonal rabbit anti-rat, sc-7884, Sta Cruz biotechnology, California, USA) and metalloprotein 13 (MMP-13; polyclonal rabbit anti-rat, ab75606, abcam, Cambrige, MA, UK). The tissue sections were deparaffinized and rehydrated, and incubate with prepared 30 % hydrogen peroxide diluted in phosphate-buffered saline for 30 min. This was followed by application of biotin-labeled secondary antibody (ABC kit, PK-6200, Vector laboratories, Burlingame, CA, USA) at 1:5 dilution for 30 min. Colorimetric detection with a diaminobenzidine substrate (DAB, SK-4100, Vector laboratories, Burlingame, CA, USA) and hematoxylin. For a negative control, the primary antibody was omitted and PBS alone applied. Digital images of the ×100 magnification were captured by optical microscope. Brown marked cells was considered positive for IL1-β and MMP-13 expression. The results were evaluated both qualitatively (presence of the immunomarkers) and semi-quantitatively according to the percentage of positive cells in the randomly selected fields in each sample using a light microscopy (Leica Microsystems AG, Wetzlar, Germany), according to a previously described scoring scale from 1 to 4 (1=absent, 2=weak, 3=moderate, and 4=intense) [19, 20] for immunohistochemical analysis. Two experienced observers (LA and CB) performed the scoring in a blinded manner.

Statistical analysis

The normality of all variable's distribution was verified using the Kolmogorov–Smirnov test. For the variables that exhibited normal distribution (morphometric evaluation of the cellularity and cartilage area), comparisons among the groups were made using one-way analysis of variance with post hoc Tukey's test. For the variables that exhibited non-normal distribution (semi-quantitative analysis of cellularity, proteoglycans, IL1-β, and MMP-13), Kruskal–Wallis test was used. PRISMA 5.0 was used to carry out the statistics analysis. Values of $p<0.05$ were considered statistically significant.

Results

General findings

Neither postoperative complications nor behavioral changes were observed. The rats returned rapidly to their normal diet and showed no loss of weight during the experimentation. None of the animals died during the experiment and no infection in the surgical site was observed.

Histological descriptive analysis

Qualitative histological findings were showed in Fig. 1. Histopathologic analysis revealed that in the CG, 3 weeks post-surgery, presented a disorganized and irregular tissue, with initial signs of fibrillation along the entire articular surface and lower amount of cells in comparison to treated groups (Fig. 1a). L10 showed a more organized tissue organization in comparison to CG and L50 groups, with chondrocytes arranged in parallel in the superficial region and in columns in the intermediate region. Furthermore, L10 group presented slight irregularities and absence of fibrillation along the

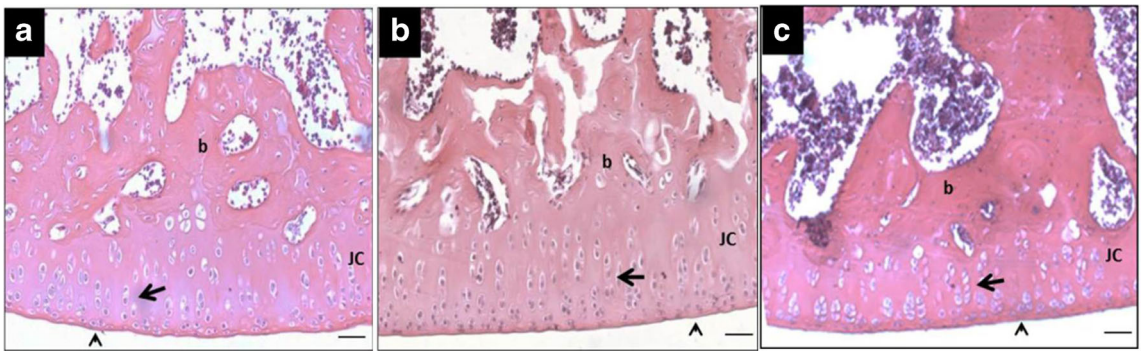

Fig. 1 Representative photomicrographs of the experimental groups (HE). Organization of chondrocytes (*arrow*), fibrillation and irregularities (*arrowhead*), joint cartilage (*JC*), subchondral bone (*b*). **a** CG: injured control group; **b** L10: injured laser-treated group at 10 J/cm^2; **c** L50: injured laser-treated group at 50 J/cm^2. *Bar* 200 μm (magnification, ×100)

articular surface, with intense presence of cells (Fig. 1b). Interestingly, L50 demonstrated a slight tissue disorganization in comparison to L10 group, however a better organization in comparison with CG. L50 showed slight irregularities and initial signs of fibrillation only at the anterior and posterior extremities of the joint cartilage surface, with a higher concentration of cells (Fig. 1c).

Semi-quantitative analysis

Figure 2 shows the results of the histological semi-quantitative analysis. The cellularity analysis demonstrated that no significant difference was observed between the experimental groups (Fig. 2). Furthermore, the results of the statistical analysis revealed a significantly higher value of the proteoglycans content in the L10 and L50 compared to the CG ($p<0.001$). Similar findings for both treated groups were observed in the proteoglycans analysis (Fig. 3b).

Morphometric analysis

Figure 4a shows the morphometric evaluation of the cellularity. The statistical analysis showed that similar findings were found in the parameter in all experimental groups. Furthermore, CG group showed a significantly higher cartilage area compared to both treated groups ($p<0.001$; Fig. 4b).

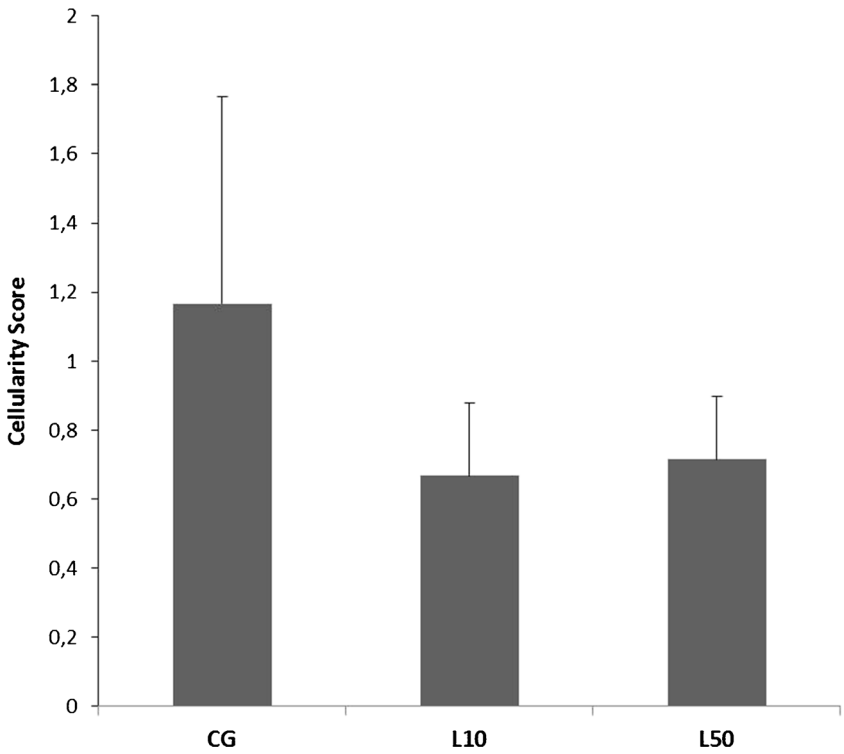

Fig. 2 Results of the semi-quantitative analysis of cellularity. *CG* injured control group, *L10* injured laser-treated group at 10 J/cm^2, *L50* injured laser-treated group at 50 J/cm^2

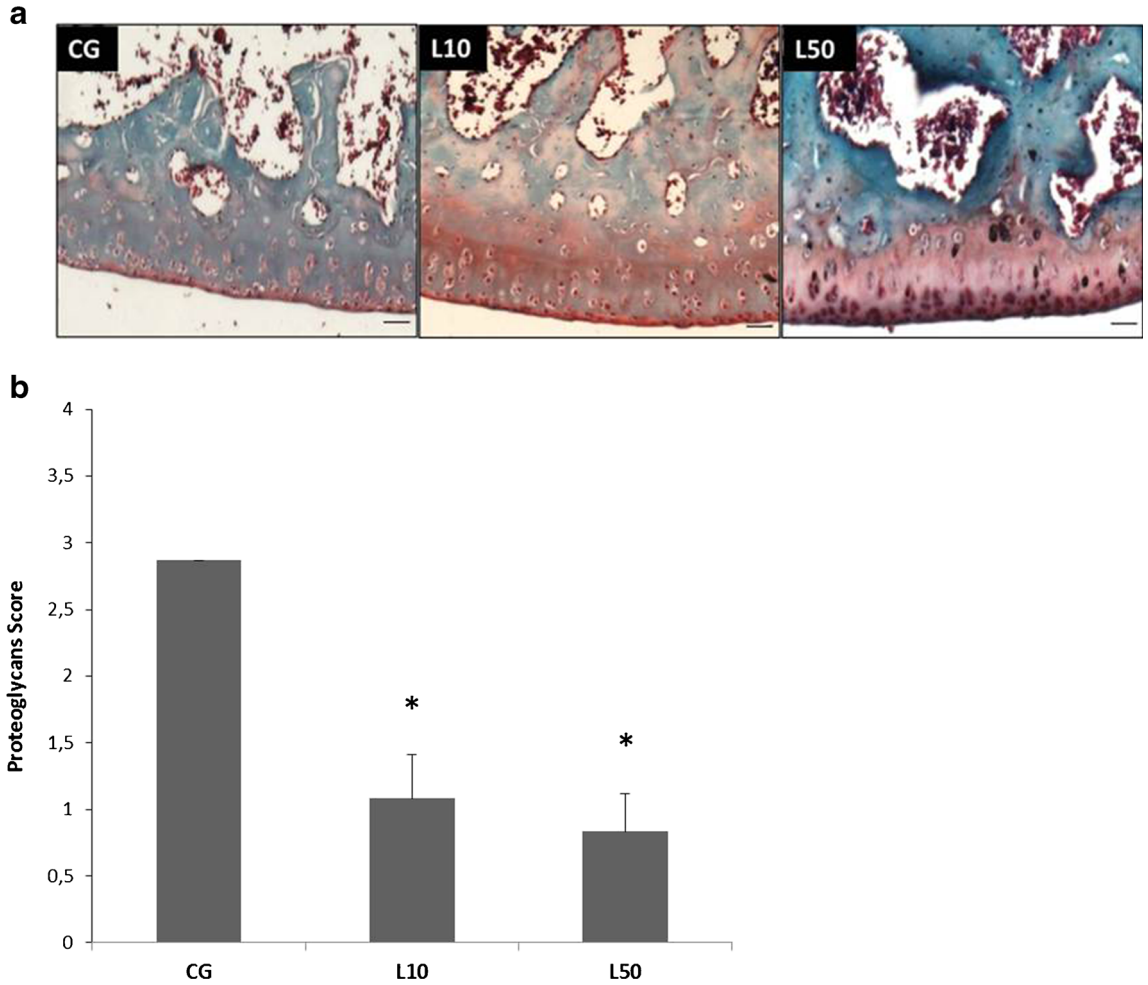

Fig. 3 **a** Representative photomicrographs of the experimental groups (Safranin-O). L10 and L50 sections highlight the significantly higher value of the proteoglycan content compared to the CG. **b** Results of the semi-quantitative analyses of proteoglycans. *CG* injured control group, *L10* injured laser-treated group at 10 J/cm^2, *L50* injured laser-treated group at 50 J/cm^2. *$p \leq 0.001$ vs. CG. *Bar* 200 μm (magnification, ×100)

Immunohistochemistry analysis

Qualitative

Figure 5 shows the qualitative analyses of IL1-β immunohistochemistry. Immunolabeling of this marker was observed mainly in the nucleus of the cells, at the anterior and posterior extremities of the joint cartilage for all the groups.

Also, the qualitative analysis of MMP-13 immunohistochemistry showed the presence of this marker mainly in the nucleus of the cells distributed along the joint cartilage for all groups (Fig. 6).

Semi-quantitative

Semi-quantitative analysis of IL1-β and MMP-13 revealed that no significant difference was found between the experimental groups (Fig. 7a, b).

Discussion

The present study investigated the effects of 808 nm laser, used at two different fluences (10 and 50 J/cm^2) in the prevention of the degenerative process in the articular cartilage after the ACLT in the knee of rats. The histological results demonstrated that initial signs of tissue degradation were observed in CG. Interestingly, laser-treated animals presented a better cartilage tissue organization. Furthermore, laser phototherapy was able of modulating some of the aspects related to the degenerative process, such as the prevention of proteoglycans loss and the increase in cartilage area. However, LLLT was not able of modulating chondrocytes proliferation and the immunoexpression of markers related to inflammatory process (IL-1 and MMP-13).

The pathogenesis of OA highlighted the importance of the development of therapeutic strategies to treat and to prevent the clinical symptoms of the degenerative process [21]. In this context, some authors demonstrated that laser therapy is

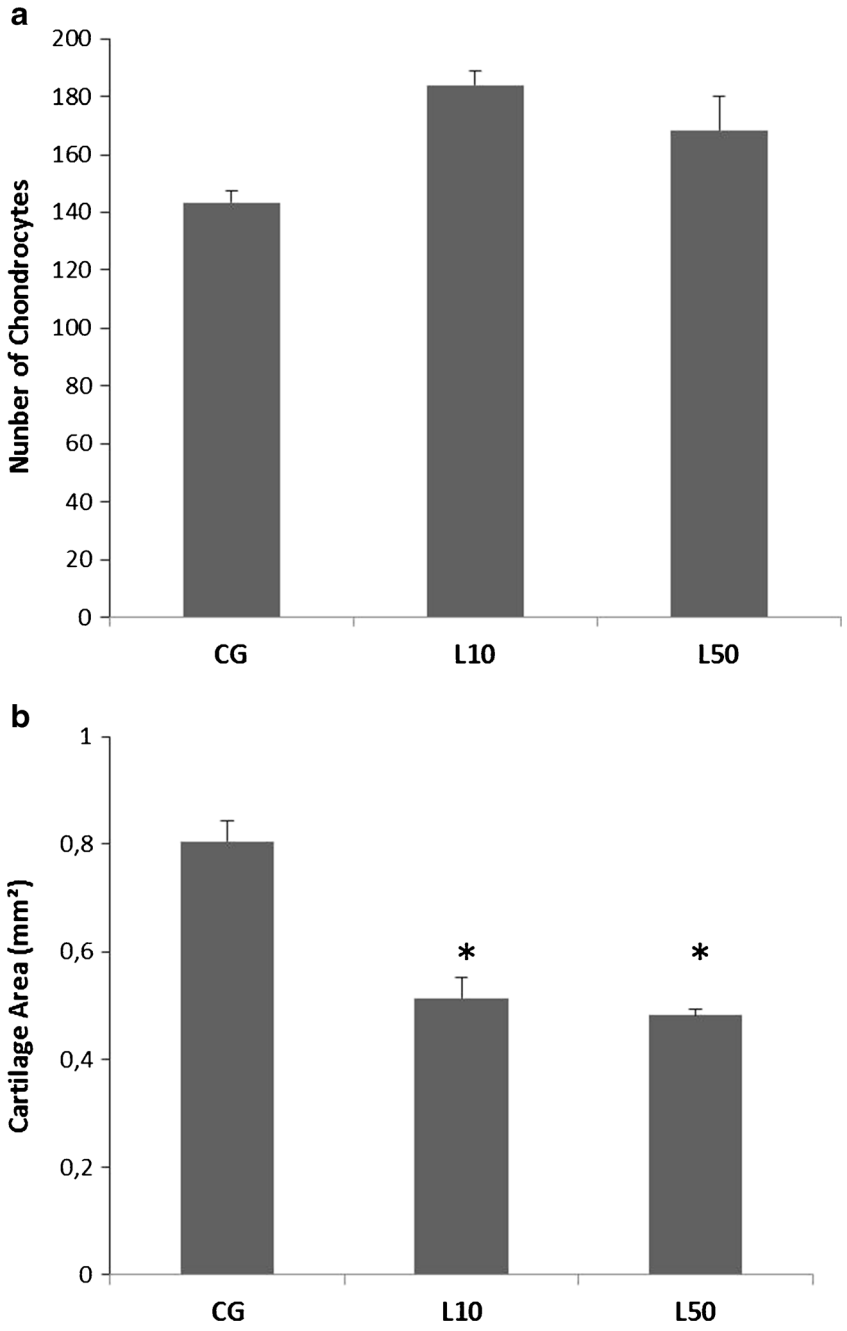

Fig. 4 **a** Results of the morphometric analysis of cellularity. **b** Results of the morphometric analysis of cartilage area. *$p \leq 0.001$ vs. CG. *CG* injured control group, *L10* injured laser-treated group at 10 J/cm^2, *L50* injured laser-treated group at 50 J/cm^2

efficient to stimulate cartilage metabolism and modulate the inflammatory process related to OA [22, 23].

The degeneration of the cartilage tissue is followed by a series of physiological modifications in an attempt of protecting tissue [24, 25]. An increased tissue metabolism and accelerated cell proliferation are common histological findings in experimental models of OA [26]. In the present study, the 808 nm laser irradiation, at both fluences, was efficient to promote a better tissue organization in the injured rats. It can be hypothesized that the energy intake of LLLT may lead to a return to tissue homeostasis, avoiding some of the typical aspects of the degeneration. These findings corroborate those of da Rosa et al. [15] who observed that 808 nm laser stimulated angiogenesis and reduced the formation of fibrosis in an experimental model of OA in rats.

In addition, the accelerated cell metabolism in the presence of OA leads to an abnormal increase in the number of cells, cellular disorganization, and cell death by apoptosis [27, 28]. In the present study it was found that the semi-quantitative and quantitative analysis of the chondrocyte number demonstrated similar findings in control and treated groups. It may be hypothesized that the experimental period (3 weeks post-surgery) was not sufficient to initiate the alterations in cell proliferation related to the cartilage degenerative process.

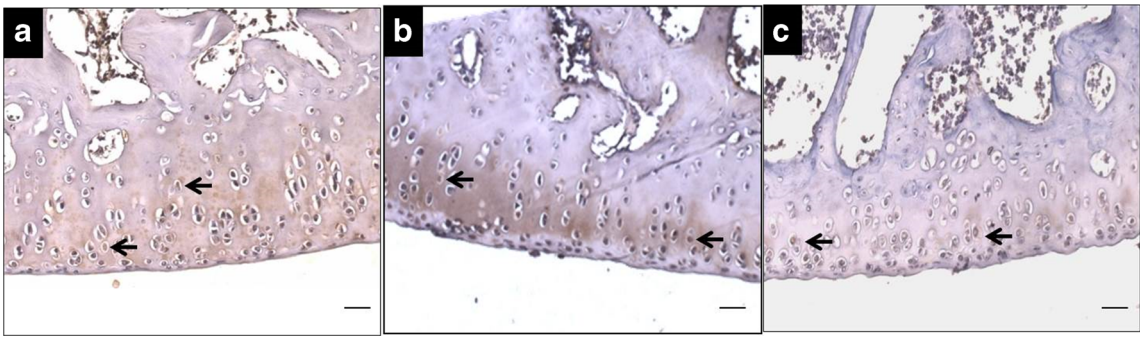

Fig. 5 Representative sections of IL1-β immunohistochemistry. Immunolabeled chondrocytes (*arrow*). **a** *CG* injured control group; **b** *L10* injured laser-treated group at 10 J/cm^2; **c** *L50* injured laser-treated group at 50 J/cm^2. *Bar* 100 μm (magnification, ×200)

Furthermore, proteoglycan loss in the cartilage tissue is another abnormality often observed during the course of degenerative process [29] and it appears before macroscopic signs of deterioration [30]. Also, there is a progressive decrease in the rate of glycosaminoglycan synthesis by chondrocytes with the evolution of the degenerative process. Interestingly, in the present study, the 808 nm laser therapy, at both fluences, prevented proteoglycan loss, indicating that this therapy may have modulated articular cartilage metabolism. These results corroborate those of Lin et al. [1] who found an increased production of proteoglycans amount after He-Ne 632 nm laser irradiation in a model of OA in rabbits. Moreover, Gottlieb et al. [31] also observed cartilage regeneration after laser stimulation due to increased proteoglycan synthesis in arthritic cartilage of rabbits.

At an early stage of OA process, there is an apparent increase in cartilage volume due to swelling which supports a pathophysiological role of inflammation [25]. In the present study, LLLT was able of modulating the increase of cartilage area related to the initial phase of cartilage degradation, at both fluences analyzed. This phenomenon may be related to LLLT capacity of modulating cell metabolism in an attempt to delay the evolution of the degenerative process in the articular cartilage.

Many authors demonstrated the degenerative process related to the OA is accompanied by joint inflammation [32]. The balance of cartilage matrix remodeling is altered by the overproduction of cytokines and catabolic factors, which includes IL1-β and may lead to chondrocytes apoptosis [26, 33]. Also, the degradation of extracellular matrix is regulated by the matrix metalloproteinase's (MMPs) family, mainly MMP-1, 3, and 13 [34]. Data of the literature show that 810 nm LLLT produced anti-inflammatory effects in OA experimental model of rabbits [35]. In this study, laser therapy, at both fluences, did not have any effect on the expression of IL1-β and MMP-13 markers. Possibly, a longer period of irradiation might be necessary to achieve a positive response in the modulation of the inflammatory markers expression.

Also, it has been suggested that the effect of laser irradiation on tissues is wavelength and dose-dependent [36]. The present study demonstrate that both fluences used, combined with 808 nm laser, at the period analyzed produced similar effects on cartilage tissue organization after ACLT. It is important to stress that the fluence is extremely variable in laser therapy studies relating to tissue regeneration and a wide range of doses is used for different authors [17]. For example, investigations comparing the effects of 810 nm laser irradiation at different dosage regimes (3.75 to 25 J/cm^2) on red blood cell membranes have shown that a dose of 15 J/cm^2 increased ATPase activity [37] whereas other researchers have found that the mitotic rate in carcinoma cells of the gingival mucosa decrease after 805 nm diode laser irradiation at

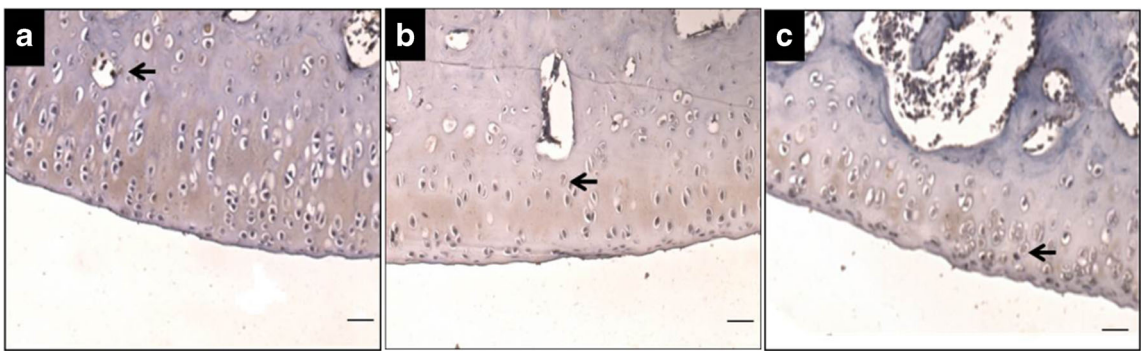

Fig. 6 Representative sections of MMP-13 immunohistochemistry. Immunolabeled chondrocytes (*arrow*). **a** *CG* injured control group; **b** *L10* injured laser-treated group at 10 J/cm^2; **c** *L50* injured laser-treated group at 50 J/cm^2. *Bar* 100 μm (magnification, ×200)

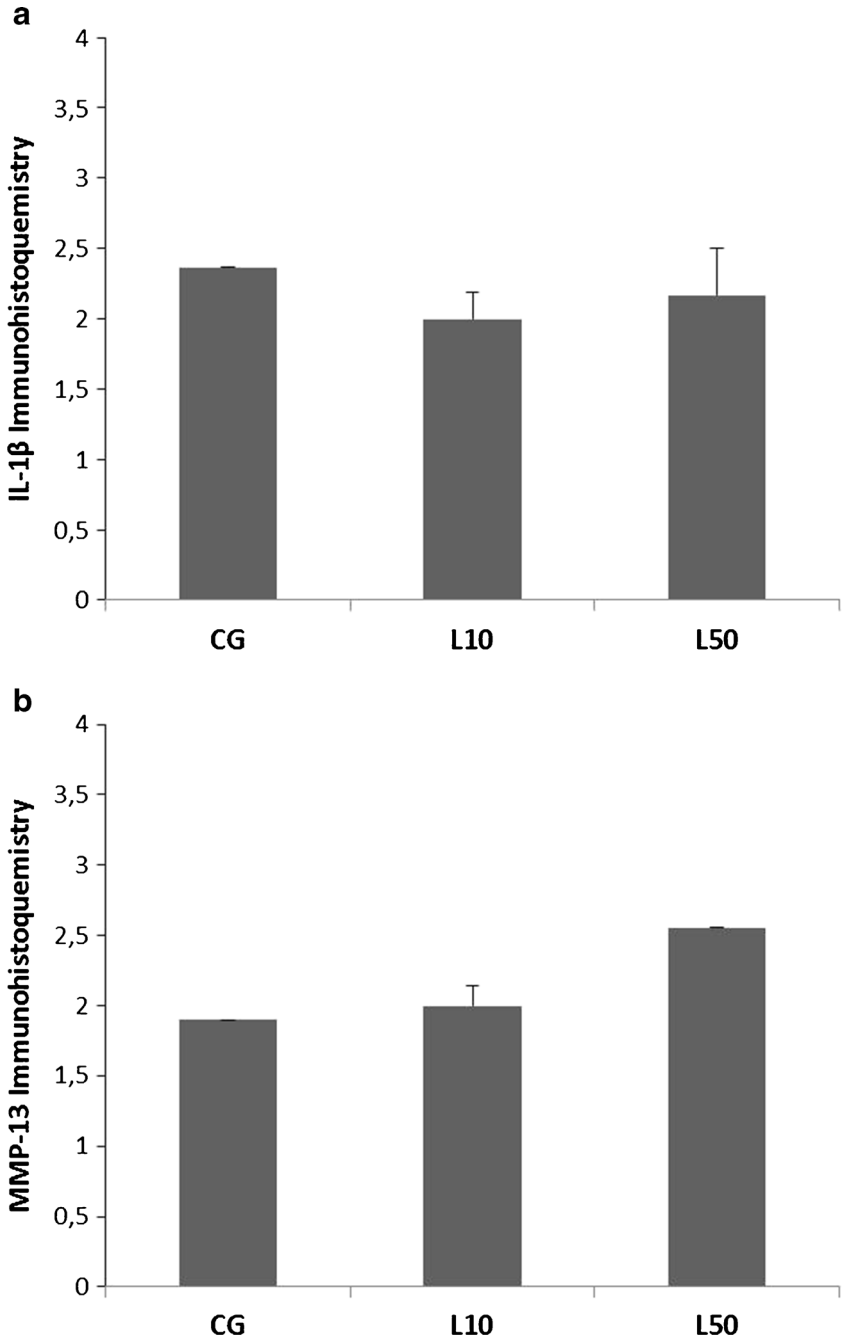

Fig. 7 **a** Results of the IL-1β expression. **b** Results of the MMP-13 expression. *CG* injured control group, *L10* injured laser-treated group at 10 J/cm^2, *L50* injured laser-treated group at 50 J/cm^2

intensities of 4 and 20 J/cm^2 [38]. The same scenario occurs in the field of cartilage repair where authors used fluences from 3 to 142 J/cm^2 [1, 15]. At this time, it is difficult to define an ideal protocol of laser treatment to prevent cartilage degradation yet. However, it can be suggested that the amount of energy offered to the tissue by the two fluences used in the present study were able to interact properly with cartilage tissue, restoring homeostasis, and preventing degradation. Further studies are needed to evaluate the exact mechanisms of action of different laser dosages on cartilage to determine the best treatment.

Conclusion

In conclusion, this study showed that 808 nm laser, at 10 and 50 J/cm^2, prevented the progression of morphological modifications related to the articular damage in the knees of rats after ACLT. Consequently, these data highlight the potential of the use of this therapy to improve the biological performance for cartilage regeneration applications. Further long-term studies should be carried out to provide additional information concerning the late stages of interaction between LLLT and cartilage degenerative process.

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Competing interests No competing financial interest exists.

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