Effects of phototherapy on cartilage structure and inflammatory markers in an experimental model of osteoarthritis

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Abstract. The aim of this study was to evaluate the effects of laser phototherapy on the degenerative modifications on the articular cartilage after the anterior cruciate ligament transaction (ACLT) in the knee of rats. Eighty male rats (Wistar) were distributed into four groups: intact control group (IG), injured control group (CG), injured laser treated group at 10 J/cm² (L10), and injured laser treated group at 50 J/cm² (L50). Animals were distributed into two subgroups, sacrificed in 5 and 8 weeks postsurgery. The ACLT was used to induce knee osteoarthritis in rats. After 2 weeks postsurgery, laser phototherapy initiated and it was performed for 15 and 30 sessions. The histological findings revealed that laser irradiation, especially at 10 J/cm², modulated the progression of the degenerative process, showing a better cartilage structure and lower number of chondrocytes compared to the other groups. Laser phototherapy was not able to decrease the degenerative process measured by Mankin score and prevent the increase of cartilage thickness related to the degenerative process. Moreover, it did not have any effect in the biomodulation of the expression of markers IL1β, tumor necrosis factor-α, and metalloprotein-13. Furthermore, laser irradiated animals, at 50 J/cm² showed a lower amount of collagen type 1.

Keywords: articular cartilage; chondrocytes; laser irradiation; osteoarthritis.

1 Introduction

Osteoarthritis (OA) is a progressive degenerative disease characterized by loss of articular cartilage, subchondral bone remodeling, joint space narrowing, and osteophyte formation. According to the World Health Organization (WHO) Scientific Group on Rheumatic Diseases, 10% of the world’s population older than 60 years has significant clinical problems that can be attributed to OA. Obesity, trauma, age, genetic factors, and mechanical forces constitute risk factors and can initiate the process of cartilage degeneration. Furthermore, clinical symptoms involve joint pain, stiffness, local inflammation, and loss of mobility, which lead to a significant reduction of the quality of life.

The OA treatment options include painkillers and anti-inflammatory drugs, physical exercises and, in the most serious cases, surgical interventions. In this context, there is a critical need to develop innovative technologies capable of treating the degenerative process related to OA and enhancing cartilage repair.

Low level laser therapy (LLLT) has been indicated for several therapeutic purposes, including cartilage repair. It is shown that the LLLT has anti-inflammatory effects, increases tissue metabolism and neoangiogenesis, and stimulates collagen production by fibroblasts and tissue regeneration.

In cartilage tissue, in vitro and in vivo studies demonstrated positive effects of laser therapy. Kushihi et al. showed an increased chondrocyte differentiation and higher chondrogenic messenger RNA expression in prechondrogenic cells after laser irradiation. Furthermore, the LLLT is able of reducing swelling, modulating inflammation, decreasing cartilage destruction, stimulating angiogenesis, and reducing fibrosis formation in OA animal models. Lin et al. demonstrated that 810-nm LLLT can improve cartilage structure, prevent articular cartilage degradation, and significantly decrease the expression of caspase-3 in the knees of the rats submitted to an anterior cruciate ligament transaction (ACLT).

Despite the positive effects of the LLLT on tissue regeneration, there are limited evidences demonstrating the effects of this therapeutic approach on cartilage repair. Moreover, the mechanism by which the LLLT acts on cartilage is not fully understood and for many, the use of LLLT as a treatment modality is still controversial. Also, different authors use a wide range of doses which make difficult the comparison of published results and the choice for an ideal protocol.

In this context, it was hypothesized that laser therapy could biomodulate the inflammation and prevent the degenerative process on an OA, providing a treatment with additional advantages.
for clinical use. Consequently, the present study was carried out in order to analyze the effects of laser therapy at two different fluencies, on cartilage damage in an OA experimental model in knees of rats. Histology analysis was used to evaluate the dose responses of laser application in cartilage repair, after 5- and 8-weeks postsurgery. Also, immunohistochemistry evaluation of inflammatory markers involved in the process of cartilage degeneration [tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β), proteolitic enzymes related to the matrix degradation metalloprotein 13 (MMP 13) and Collagen type 1 (Col-1)].

2 Methods

2.1 Experimental Groups

This study was approved and conducted in accordance with the Animal Care Committee guidelines of the Federal University of São Carlos (CEP 040/2010). Animals were maintained at 19°C to 23°C on a 12:12 h light-dark cycle in the Animal Experimentation Laboratory of the Federal University of São Carlos. Rats were housed in plastic cages and had free access to water and standard food.

Eighty male Wistar rats (weighing 300 ± 20 g, 12 to 13 weeks) were randomly distributed into four groups (n = 20): intact control group (IG), injured control group (CG), injured laser treated group at 10 J/cm² (L10), and injured laser treated group at 50 J/cm² (L50). The animals were distributed into two subgroups, with different periods of sacrifice (5- and 8-weeks postsurgery).

2.2 Anterior Cruciate Ligament Transection (ACLT)

The animals were submitted to general anesthesia induced by intraperitoneal injection of xilazin (Syntec®, R. Soluções Lar, Cotia, São Paulo, Brasil, 20 mg/kg, IP) and ketamin (Agener®, Av. do Café, São Paulo, Brasil at 40 mg/kg, IP) and subjected to the ACLT of the left hind paw. The left knee was shaved, then sterilized and draped in sterile fashion. A medial arthrotomy was performed. Then, the patella was displaced and the anterior cruciate ligament was isolated and transected. The ACLT was confirmed with Lachman testing by the surgeon and an observer. After being irrigated with sterile saline solution, the wounds were closed in layers and antiseptically treated. Rats were given appropriate postoperative care and allowed free activities in individual cages.

2.3 Treatments

Laser treatment started 2 weeks after the surgery and it was performed for 15 and 30 sessions for each subgroup, using the following protocol: five consecutive days of treatment with an interval of 2 days, for 3 and 6 weeks, respectively. The LLLT was applied at two points (on the medial and lateral sides of the joint), using the punctual contact technique. A low-energy GaAl-As laser (Theralase, DMC® São Carlos, São Paulo, Brazil) was used at 830 nm, continuous wave diode, with a 0.026-cm² beam diameter, a power output of 30 mW, fluence at 10 J/cm² (irradiation time of 10 s, energy point 0.3 J), and fluence at 50 J/cm² (irradiation time of 47 s, energy point 1.4 J). On the respective days, animals were euthanized individually by carbon dioxide asphyxia. The knee joints were removed for analysis.

2.4 Histological Analysis

After harvesting, the specimens were fixed in 4% formaldehyde for 2 days, followed by decalcified in 4% ethylenediaminetetraacetic acid. 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 done. Therefore, thin sections (6 μm) were prepared in the sagittal plane, starting from the medial margin of the joint using a microtome (Leica RM—2145, Germany). Laminas were stained with hematoxylin and eosin (HE, Merck, Germany), Safranin-O (Merck, Germany), and Picrosirius-Red (Merck, Germany). Moreover, three sections were obtained for the immunohistochemical analysis.

2.5 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 cartilage structure, amount of cells and cellular organization. The specimens were examined using a light microscopy (100x) (Leica Microsystems AG, Wetzlar, Germany).

2.6 Semi-Quantitative Analysis

The severity of the OA lesion was graded on a scale using the Mankin scoring system (Table I). Samples were stained with Safranin-O and histological evaluation system was performed along all of the extension. At least three sections of each specimen were examined using light microscopy (100x) (Leica Microsystems AG, Wetzlar, Germany). The mean Mankin score of the two observers was calculated.

2.7 Morphometric Analysis

The morphometric study was carried out using one randomized slide stained with HE. 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 μm², at the anterior, central, and posterior region of each slide were chosen. Within each area, cells were marked and the chondrocytes average was calculated. Thickness was also measured in three regions, one central and two laterals (300-mm left and right from the first region), from subcondral bone to articular surface.

2.8 Picrosirius Red Analysis

Histological sections stained by the Picrosirius-polarization method were viewed under polarized light (Carl Zeiss, Oberkochen, Germany) to assess the collagen organization in the cartilage tissue. Similar to the morphometric analysis, three areas of 80,000 μm² (anterior, central, and posterior region) of each slide were chosen to quantify the amount of collagen using the software ImageJ (Version 1.45, National Institutes of Health, Bethesda, Maryland). In each field, an indirect evaluation of the total collagen fibers organization based on the birefringence of the collagen fiber bundles after staining with Picrosirius was performed. Two experienced observers (PO and AR) performed the scoring in a blinded manner.
Table 1  Mankin score for the histological grading of cartilage degeneration.

<table>
<thead>
<tr>
<th>Grade</th>
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<tbody>
<tr>
<td>I. Structure</td>
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<tr>
<td>a. Normal</td>
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<tr>
<td>b. Surface irregularities</td>
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<tr>
<td>c. Pannus and surface irregularities</td>
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<tr>
<td>d. Clefts to transitional zone</td>
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<tr>
<td>e. Clefts to radial zone</td>
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<tr>
<td>f. Clefts to calcified zone</td>
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<tr>
<td>g. Complete disorganisation</td>
</tr>
<tr>
<td>II. Cells</td>
</tr>
<tr>
<td>a. Normal</td>
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<tr>
<td>b. Diffuse hypercellularity</td>
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<tr>
<td>c. Cloning</td>
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<tr>
<td>d. Hypocellularity</td>
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<tr>
<td>III. Safranin-O staining</td>
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<tr>
<td>a. Normal</td>
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<tr>
<td>b. Slight reduction</td>
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<tr>
<td>c. Moderate reduction</td>
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<tr>
<td>d. Severe reduction</td>
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<td>e. No dye noted</td>
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<td>IV. Tidemark integrity</td>
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<tr>
<td>a. Intact</td>
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<tr>
<td>b. Crossed by blood vessels</td>
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2.9 Immunohistochemistry

The TNF-α, IL-1β, MMP 13, and Col-1 immunoexpression were determined using the streptavidin-biotin-peroxidase method. Sections at 3 μm were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol to distilled water. For antigen retrieval, slides were placed in 0.01 M citrate-buffer pH 6.0 and heated in a microwave for three cycles of 5 min each at 850 W. Endogenous peroxidases were quenched by incubation in 3% H2O2 for 20 min at room temperature. Sections were incubated overnight at 4°C with primary antibody: TNF-α (polyclonal rabbit anti-rat, ab6671, Abcam, Cambridge, MA, UK), interleukin-1 (IL-1β) (polyclonal rabbit anti-rat, sc-7884, Sta Cruz biotechnology, California, USA), metalloprotein 13 (MMP 13) (polyclonal rabbit anti-rat, ab75606, Abcam, Cambridge, MA, UK) and Collagen type 1 (Col-1) (anti-Col 1A monoclonal primary antibody, Sta Cruz Biotechnology, USA). Subsequently, sections were incubated. Which tissue was used as positive control for each antibody tested in immunohistochemistry technique with biotinylated secondary antibody (LSA B, Dakocytomation) for 30 min, washed in phosphate-buffered saline, and incubated with streptavidin peroxidase conjugate (LSA B, Dakocytomation) for 30 min. Finally, the reaction was developed using 3,3′-Dianobenzidine tetrahydrochloride (Sigma, St. Louis, Missouri) for 5 min. Slides were briefly counterstained in hematoxylin and rehydrated, and cover slips added. Negative and positive controls were to run simultaneously. Positive controls were represented by mammary tissue. Negative controls were made by eliminating the primary antibody as established in previous studies conducted by our group. The TNF-α, IL-1β, MMP 13, and Col-1 immunoexpressions were evaluated both qualitatively (presence of the immunomarkers) and quantitatively in predetermined fields using a light microscope ((Leica Microsystems AG, Wetzlar, Germany).

2.10 Immunohistochemical Semiquantitative Analysis

Sections stained using immunohistochemistry were analyzed for the percentages of immunopositive cells in control and experimental animals. A total of 1000 cells were evaluated in 3 to 5 fields at 400× magnification. These values were used as labeling indices.

2.11 Statistical Analysis

The normality of all variables’ distribution was verified using the Shapiro–Wilk W test. For the variable that exhibited normal distribution, comparisons among the groups were made using one-way analysis of variance (ANOVA) with post hoc Tukey’s test. For the variable that exhibited nonnormal distribution, Kruskal Wallis test was used. STATISTICA version 7.0 was used to carry out the statistics analysis. Values of p < 0.05 were considered statistically significant.

3 Results

3.1 General Findings

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

3.2 Histological Analysis

3.2.1 Descriptive analysis

Histopathological analysis revealed that after 5 weeks, cartilage tissue of IG presented a normal structure, without signs of fibrillation. Moreover, in the superficial region, the chondrocytes were displayed in a parallel arrangement and in the intermediate region they were organized in columns [Fig. 1C]. The CG presented signs of fibrillation, irregularities in the articular surface and chondrocytes displayed in a disorganized disposition [Fig. 1C]. In the laser treated groups, at both fluencies, the articular surface showed initial signs of fibrillation and chondrocytes organized in a normal orientation, resembling the intact animals [Figs. 1E and 1F].

After 8 weeks, the histological findings revealed that IG presented normal cartilage structure [Fig. 1B]. In the control animals, the degenerative process progressed, presenting intense fibrillation, surface irregularities and hypocellularity, with
chondrocytes displayed in a disorganized way [Fig. 1(d)]. The L10 presented a better tissue organization compared to CG, with a moderate amount of cells, with slight fibrillation, and irregularities [Fig. 1(f)]. Cartilage structure of the L50 was more disorganized compared to L10, with a moderate presence of chondrocytes and fibrillation in articular surface [Fig. 1(h)].

### 3.2.2 Semi-quantitative analysis

The semi-quantitative analysis demonstrated that, after 5 weeks, IG presented significantly lower scores in comparison to CG and L50. After 8 weeks, Mankin scores observed in intact animals were significantly lower compared to CG, L10 and L50 (Fig. 2). No other difference was observed.

### 3.3 Morphometric Analysis

#### 3.3.1 Cellularity

Five weeks postsurgery, similar results in the cellularity analysis were found for all groups. In the second period evaluated, the number of chondrocytes in the CG was higher compared to IG, L10 and L50 (Fig. 3).

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**Fig. 1** Representative photomicrographs the experimental groups. Organization of chondrocytes (thin arrow →), fibrillation and irregularities (thick arrow ↑), cartilage joint (hash), subchondral bone (asterisk). (a) IG 5 weeks; (b) IG 8 weeks; (c) CG 5 weeks; (d) CG 8 weeks; (e) L10 5 weeks; (f) L10 8 weeks; (g) L50 5 weeks; (h) L50 8 weeks. Scale bar, 100 μm.

**Fig. 2** Mankin score. IG: intact control group; CG: injured control group; L10: injured laser treated group at 10 J/cm²; L50: injured laser treated group at 50 J/cm². * p ≤ 0.05.

**Fig. 3** Results of quantitative analysis. (a) Cellularity and (b) Thickness. IG: intact control group; CG: injured control group; L10: injured laser treated group at 10 J/cm²; L50: injured laser treated group at 50 J/cm². * p ≤ 0.05.
L10 and L50 ($p = 0.0052, 0.00017, 0.00015$, respectively) [Fig. 3(a)].

3.3.2 Thickness

After 5 weeks, the IG presented lower cartilage thickness compared to CG ($p = 0.029$) and L50 ($p = 0.00050$) [Fig. 3(a)]. After 8 weeks, IG had significantly lower cartilage thickness compared to the other groups ($p = 0.0031, 0.0021, 0.00019$ to CG, L10, and L50, respectively) [Fig. 3(b)].

3.3.3 Collagen fibers

No difference was observed in collagen fiber evaluation comparing the results found in the experimental groups at the two periods analyzed (Fig. 4).

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**Fig. 4** Results of the collagen fiber evaluation. IG: intact control group; CG: injured control group; L10: injured laser treated group at 10 J/cm²; L50: injured laser treated group at 50 J/cm².

**Fig. 5** Representatives sections of IL-1β immunohistochemistry. (a) IG 5 weeks; (b) IG 8 weeks; (c) CG 5 weeks; (d) CG 8 weeks; (e) L10 5 weeks; (f) L10 8 weeks; (g) L50 5 weeks; (h) L50 8 weeks. Scale bar, 100 μm.
3.4 Immunohistochemistry

Figures 5–8 showed the qualitative immunoexpression for IL-1β, TNF-α, MMP-13, and Col-1, respectively. It is possible to observe that all the immunoreactivities were observed in the nucleus of chondrocytes.

Figure 9 reveals the results quantitative of IL-1β, TNF-α, MMP-13, and Col-1 immunoexpression. Results indicated that a lower IL-1β expression was observed in the IG compared to the other groups at both experimental periods analyzed (5- and 8-weeks postsurgery) [Fig. 9(a)]. Similar findings of IL-1β expression were demonstrated in the other experimental injured groups (treated and control groups).

Quantitative analysis of TNF-α showed that no difference was observed between the experimental groups after 5 weeks. Eight weeks postsurgery, the expression of TNF-α was higher in the CG, L10, and L50 compared to IG. No other difference was detected [Fig. 9(c)].

Similar results were observed in the MMP-13 immunoexpression evaluation. At the first experimental period, no difference was observed. After 8 weeks, the expression of MMP-13 was higher in the treated and nontreated OA groups compared to IG [Fig. 9(c)].

The analysis of Col-1 showed that 5-weeks postsurgery, a significant increase of this protein was found in the L10 compared to the other groups. In the second experimental period, intact animals showed a lower expression of col-1 compared to the other groups. Moreover, CG and L10 showed a significantly higher expression compared to L50 [Fig. 9(d)].

4 Discussion

In this study, the effects of 830-nm laser on the degeneration of the articular cartilage of knees of rats submitted to an OA experimental model were investigated. The main histological findings revealed that lasertherapy, at 10 J/cm², modulated the progression of the degenerative process, showing a better cartilage structure and lower number of condrocytes compared to the other OA animals. However, lasertherapy was not able to decrease the degenerative process measured by Mankin score.

Fig. 6 Representatives sections of tumor necrosis factor (TNF)-α immunohistochemistry. (a) IG 5 weeks; (b) IG 8 weeks; (c) CG 5 weeks; (d) CG 8 weeks; (e) L10 5 weeks; (f) L10 8 weeks; (g) L50 5 weeks; (h) L50 8 weeks. Scale bar, 100 μm.
and the increase of cartilage thickness related to the arthritic process. Moreover, similar findings in the expression of IL1β, TNF-α, and MMP-13 were found in all experimental groups, and col-1 expression was decreased in irradiated animals, at 50 J/cm².

Many studies have recently demonstrated the positive effects of the LLLT on tissue metabolism. This therapy has been shown to accelerate cartilage cell proliferation in vitro studies and to stimulate cartilage repair in an OA experimental model. Also, it was demonstrated that the LLLT associated with exercises is effective in decreasing pain and improve articular function in patients with OA of the knees.

Authors affirm that in the presence of a degenerative process, chondrocytes have their metabolic rate increased in an attempt to recover the damaged tissue. These modifications lead to an abnormal increase in the number of cells, which culminate in apoptosis. In this study, the positive effects of a laser, especially at 10 J/cm² on the qualitative histological findings and in the reduction of the number of cartilage cells, support the idea that laser therapy had a positive influence in cell metabolism, preventing the hypercellularity. These findings corroborate those of da Rosa et al who observed that 808-nm laser stimulated angiogenesis and reduced the formation of fibrosis in an experimental model of the OA in rats.

Structural modifications and increase of articular thickness are important indicators of cartilage degeneration. However, in this study, injured laser treated animals, at both fluencies, showed similar results in the Mankin evaluation and thickness measurement compared to CG. These results are in accordance with Bayat et al who found an increase in the cartilage thickness after 632-nm laser irradiation, at 13 J/cm² in an OA experimental model. The authors considered this fact as a beneficial response in relation to tissue structure and attributed this to the laser having stimulated cellular metabolism.

Picrossirius analysis showed that no difference in the amount of collagen was observed in the present study. However, immunochemistry showed that, 8-weeks postsurgery, a higher expression of Col-1 was observed in control animals and laser treated animals, at 10 J/cm², suggesting a positive effect of the

Fig. 7 Representatives sections of metalloprotein (MMP)-13 immunohistochemistry. (a) IG 5 weeks; (b) IG 8 weeks; (c) CG 5 weeks; (d) CG 8 weeks; (e) L10 5 weeks; (f) L10 8 weeks; (g) L50 5 weeks; (h) L50 8 weeks. Scale bar, 100 μm.
LLLT at 50 J/cm². It is well known that the degenerative process culminates in a substitution of collagen type II for collagen type I.29,38 In this context, our immunohistochemistry findings after 50 J/cm² irradiation appeared to be a predictive factor for response to treatment. Furthermore, a series of anti-inflammatory markers are activated during the progression of OA, including IL-1, TNF, and MMP-13. The action of these inflammatory mediators lead to an increase in the catabolic pathways, inhibit matrix synthesis, and promote cellular apoptosis.39 The analysis of the immunexpression of IL-1β, TNF-α, and MMP-13 showed that laser irradiated animals presented similar findings compared to untreated animals, suggesting that the LLLT did not have any effect in the modulation of the inflammatory process related to OA. The anti-inflammatory effects of the LLLT on the degenerative process have been demonstrated by many authors, who observed decreased prostaglandin E2 and TNF expression after LLLT in OA experimental models.7,12 The disparate nature of our results may be due to a range of factors, including cellular physiology, fluences, and/or the LLLT wavelength employed. The disparity of the findings cited here supports the notion of probable cell/tissue and dose/wavelength specificities and the hypothesis that the parameters used in the present study were not capable of modulating the inflammatory process.

Due to the lack of established parameters to treat the degenerative process in the cartilage tissue and based on the existence of a curve dose-response, the present study investigated the biological performance of two different fluences. Our results showed that the lower fluency (10 J/cm²) was more likely to produce morphological modifications in the tissue than the higher fluence (10 J/cm²). It is important to stress that this parameter is extremely variable in laser therapy studies relating to cartilage regeneration, and a wide range of doses are used for different authors.

Although the effects of the LLLT have been demonstrated by many authors, the regulatory mechanisms of laser on tissues are poorly understood. It may involve photochemical signaling, with laser light enhancing cell proliferation through changes in cellular physiology.

Fig. 8 Representatives sections of collagen (Col)-1 immunohistochemistry. (a) IG 5 weeks; (b) IG 8 weeks; (c) CG 5 weeks; (d) CG 8 weeks; (e) L10 5 weeks; (f) L10 8 weeks; (g) L50 5 weeks; (h) L50 8 weeks. Scale bar, 100 μm.
in mitochondrial physiology, subsequently affecting RNA synthesis. Such effects then alter the expression of various cell regulatory proteins. It may be due also to the redox potential of the target cells, which is associated with stimulation of cell function if it shifts toward oxidation and with inhibition if towards reduction. However, the reasons for the stimulatory and inhibitory effects of laser on cartilage repair remain unclear and warrant further investigation.

5 Conclusion

In conclusion, the results found in present study indicate that 830-nm laser phototherapy modulates the proliferation of chondrocytes and prevented the increase of Coll-1 in the experimental model of OA in rats, but had no effect on the modulation of the inflammatory process. Further investigations are required to investigate possible response mechanisms that may explain the contrasting outcomes obtained when examining laser irradiation, especially related to the modulation of the inflammatory process. Such future studies will undoubtedly contribute to a better understanding of the safety and efficacy of the LLLT to be used in clinical studies.

Acknowledgments

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