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ORIGINAL ARTICLE

Effects of low-level laser therapy on joint pain, synovitis, anabolic, and catabolic factors in a progressive osteoarthritis rabbit model

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Abstract The aim of this study was to investigate the effect of low-level laser therapy (LLLT) on short-term and long-term joint pain, synovitis, anabolic, and catabolic factors in the cartilage of a rabbit model with progressive osteoarthritis (OA) induced by anterior cruciate ligament transection (ACLT). A total of 160 New Zealand white rabbits were randomly assigned into two groups (ACLT group and LLLT group). All rabbits received ACLT surgery, and 2-, 4-, 6-, and 8-week treatment after the surgery, with 20 rabbits being tested biweekly over every study period. The LLLT group received LLLT with a helium–neon (He–Ne) laser (830 nm) of 1.5 J/cm^2 three times per week, and the ACLT group received placebo LLLT with the equipment switched off. Long-term and short-term pain was tested via weight-bearing asymmetry; synovitis was assessed histologically; and knee joint cartilage was evaluated by gross morphology, histology, and gene expression analysis of anabolic and catabolic factors. The histological assessment of pain and synovitis showed that at least 6-week intermittent irradiation of LLLT could relief knee pain and control synovium inflammation. Gross morphologic inspection and histological evaluation showed that 6 weeks of LLLT could decrease cartilage damage of medical femoral condyle and 8 weeks of LLLT could decrease cartilage damage of medical and lateral femoral condyles and medical tibial plateau. Gene expression analysis revealed two results: At least 6 weeks of LLLT could decrease production of catabolic factors, for example, interleukin 1β (IL-1β), inducible nitric oxide synthase (iNOS), and MMP-3, and slow down the loss of anabolic factors, mainly TIMP-1. Eight weeks of LLLT treatment could slow down the loss of collagen II, aggrecan, and anabolic factors, mainly transforming growth factor beta (TGF-β). The study suggests that LLLT plays a protective role against cartilage degradation and synovitis in rabbits with progressive OA by virtue of the regulation of catabolic and anabolic factors in the cartilage.

Keywords Osteoarthritis · Catabolic factors · Anabolic factors · Synovitis · Low-level laser therapy

Introduction

Osteoarthritis (OA) is the most common form of degenerative joint disease, associated with pain, stiffness, and chronic physical and functional disability in older adults [1]. Complex interactions and cross-talk involving numerous anabolic and catabolic factors play a key role in the progression of cartilage degradation and pathogenesis of OA [2–5]. Transforming growth factor beta (TGF-β), insulin-like growth factors-1 (IGF-1), and bone morphogenetic proteins (BMPs), especially BMP-2 and BMP-7, are regarded as essential anabolic factors in the formation and maintenance of the cartilage [6, 7]. Cytokines interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), and consequent matrix metalloproteinases (MMPs) are seen as catabolic factors for the erosion and proteolysis of extracellular matrix components of the cartilage including collagen type II and aggrecan [6, 7]. Consequently, how to coordinate, upregulate, or downregulate these anabolic and catabolic factors has become an important target for research on optimal treatments of OA [8, 9].

Low-level laser therapy (LLLT) has been listed as a nonpharmacological and noninvasive treatment option for OA [10]. A current systematic review demonstrated that intensive treatment (2–4 weeks) with LLLT seems to offer clinically relevant short-term pain relief for OA of the knee [11]. Animal experiments suggested that LLLT may promote regeneration of the articular cartilage [12, 13], enhance

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stiffness of repairing tissue [14], and inhibit apoptosis of chondrocytes [15, 16] and inflammation of synovial membrane [17, 18]. However, other research produced conflicting results [19, 20].

Moreover, the therapeutic effect and mechanism of LLLT regarding the regulation of catabolic and anabolic factors in progressive OA is not well understood at the moment. Most importantly, an optimal duration of treatment with LLLT in progressive OA regarding effects on pain and cartilage repair needs to be yet determined. Using a rabbit model of progressive OA, this study aimed to investigate effect and mechanism of LLLT administered over different periods of time on joint pain and synovitis as well as anabolic and catabolic factors in the cartilage.

Methods

Animals and treatment

One hundred sixty adult New Zealand white rabbits (3.5±0.8 kg; 6 months old) were purchased from the animal center of Sichuan University and used for this study. After having excluded joint pathology by radiographs of both femorotibial joints, all rabbits were randomly assigned to two groups: experimental group (LLLT group *n*=80) and control group (anterior cruciate ligament transection (ACLT) group *n*=80). Within the two groups, animals were further randomized into 2-, 4-, 6- and 8-week subgroups in order to determine the duration of therapy in the LLLT group and the time points when the rabbits would be sacrificed for obtaining tissue samples.

All animals were housed in individual cages for a 12-h light–dark cycle at 25 °C in the standard animal laboratory of the Science Center of West China Hospital of Sichuan University. This study fully complied with the national legislation and the *Guide for the Care and Use of Laboratory Animals* issued by the Ministry of Health of the People's Republic of China and was approved by the local research ethical committees.

All rabbits were given intraperitoneal injection of 5 % chloral hydrate (3 ml/kg). Left knee OA was induced with ACLT [21–23]. After the surgery, 3 days, the left knees of the rabbits from the LLLT group were medially and laterally exposed to a low-level laser (applied by CL). The parameters of the laser used (HJZ-2, Guoxiong Photoelectric Technology Co., Ltd., Chengdu, China) were as follows: laser source, 50 mW; helium–neon laser tube; Wavelength, 830 nm; frequency, continuous; irradiation points, three points per side; surface area of laser beam, 0.028 cm^2; duration of LL He–Ne LT per session, 300 s per side; energy density, 4.8 J/cm^2; irradiation energy per point 0.13 J; and timing: three times a week. Animals from the ACLT group received placebo LLLT

Table 1 Histological assessment of articular cartilage changes

Parameter	
Safranin O-fast green staining	
0	Uniform staining throughout articular cartilage
1	Loss of staining in superficial zone of hyaline cartilage<50 % the length of the condyle or plateau
2	Loss of staining in superficial zone of hyaline cartilage ≥50 % the length of the condyle or plateau
3	Loss of staining in the upper 2/3 of hyaline cartilage<50 % the length of the condyle or plateau
4	Loss of staining in the upper 2/3 of hyaline cartilage ≥50 % the length of the condyle or plateau
5	Loss of staining in all hyaline cartilage<50 % the length of the condyle or plateau
6	Loss of staining in all hyaline cartilage ≥50 % the length of the condyle or plateau
Structure	
0	Normal
1	Surface irregularities
2	Fissures in <50 % surface
3	Fissures in ≥50 % surface
4	Erosion 1/3 hyaline cartilage<50 % surface
5	Erosion 1/3 hyaline cartilage ≥50 % surface
6	Erosion 2/3 hyaline cartilage<50 % surface
7	Erosion 2/3 hyaline cartilage ≥50 % surface
8	Full depth erosion hyaline cartilage<50 % surface
9	Full depth erosion hyaline cartilage ≥50 % surface
10	Full depth erosion hyaline and calcified cartilage to the subchondral bone <50 % surface
11	Full depth erosion hyaline and calcified cartilage to the subchondral bone ≥50 % surface
Chondrocyte density	
0	No decrease in cells
1	Focal decrease in cells
2	Multifocal decrease in cells
3	Multifocal confluent decrease in cells
4	Diffuse decrease in cells
Cluster formation	
0	Normal
1	<4 clusters
2	≥4 but <8 clusters
3	≥8 clusters
Total score 0–24	

Definitions for the recommended grading system. For the purposes of this assessment, articular cartilage is divided into four zones: zone 1 superficial zone upper 1/3 hyaline cartilage; zone 2 middle zone, middle 1/3 hyaline cartilage; zone 3 deep zone, deep 1/3 hyaline cartilage; zone 4 the calcified cartilage. Artefact definition: fissure but no hypocellularity or loss of safranin O adjacent to the cleft; Focal: observed at one site on section; Multifocal: observed at more than one site; Multifocal confluent: observed at multiple sites that are in contact

Table 2 Polymerase chain-reaction primer information

Gene	Sequence	GenBank
ECM gene		
Aggrecan	GCTACGGAGACAAGGATGAGTTC CGTAAAAGACCTCACCCTCCAT	L38480
Collagen type II	CCTGTGCGACGACATAATCTGT GGTCCTTTAGGTCCTACGATATCCT	AF027122
Anabolic factors' gene		
TGF-β 1	AAGGGCTACCACGCCAACTT CCGGGTTGTGCTGGTTGTAC	AF000133
IGF-1	AGCTGGTGGATGCTCTTCAGTT GAAGCAGCACTCATCCACGAT	U75390
TIMP-1	AGCAGAGCCTGCACCTGTGT CCACAAACTTGGCCCTGATG	J04712
BMP-2	CGCCTCAAATCCAGCTGTAAG GGGCCACAATCCAGTC GTT	AF041421
BMP-7	GCCGAGTTCCGGATCTACAA CTCCTGCAGCACCTGGTACAC	AF413111
Catabolic factors' gene		
IL-1β	GCCGATGGTCCCAATTACAT ACAAGACCTGCCGGAAGCT	M26295
iNOS	CTGTGACGTCCAGCGCTACA GCACGGCGATGTTGATCTCTCGCCT	AF469048
MMP-1	TCAGTTCGTCCTCACTCCAG TTGGTCCACCTGTCATCTTC	AH005676
MMP-3	ACACCGGATCTGCCAAGAGA CTGGAGAACGTGAGTGGAGTCA	NM001082280
MMP-13	TTCGCTTAGAGGTGACAGG ACTCTTGCCGGTGTAGGTGT	NM001082037
GAPDH	GGAGAAAGCTGCTAA ACGACCTGGTCCTCGGTGTA	L23961

with the equipment switched off. All interventions and evaluations were performed between 9 and 10 am.

Assessment

All assessments were conducted by two researchers (QW and QDJ) who were blinded with regard to allocation to experimental groups. QW performed the short- and long-term pain assessment, the macroscopic examination of the cartilage, and microscopic histological examination of the cartilage and synovium. QDJ performed the gene expression analysis.

In order to evaluate the effect of LLLT on OA short-term pain, a pain behavior test was used before surgery and after intervention at days 1, 3, 5, 7, 9, and 11 and at 2, 4, 6, and 8 weeks. To determine the effect of LLLT on cartilage damage in OA, macroscopic and microscopic histological examinations of the cartilage and synovium were performed according to the OARSI (Osteoarthritis Research Society International) histopathology initiatives/recommendations for histological assessments of osteoarthritis in the rabbit [24]. Gene expression analysis was used to quantify anabolic and catabolic factors in the cartilage.

Pain behavior test

To evaluate pain behavior, weight-bearing asymmetry between contralateral and ipsilateral knees was measured using an incapacitance tester (Linton Instrumentation, Diss, UK) which can independently measure weight bearing for each leg (weight distribution between the left (LW) and right legs (RW)). Measurements were repeated three times, and the percent weight distribution on the left hind paw (LWD) was calculated by applying the following formula:

$$\mathrm{LWD} = \mathrm{LW}/(\mathrm{LW} + \mathrm{RW}) \times 100$$

The value of the percent weight distribution on the left leg was defined as the mean of the three calculations [25–27].

Macroscopic examination of the cartilage

After the animals were sacrificed, all of the left lateral and medial femoral condyles (LFC and MFC) as well as the lateral and medial tibial plateau (LTP and MTP) could be macroscopically scored with scoring systems as described

previously by Pritzker et al. [28]. Digital images of the specimens with a scale marker were obtained with a camera (Canon EOS1100D). These photographs were used for section-cut direction so as to capture the most severe lesions in each compartment.

Microscopic histological examination of the cartilage and synovium

The specimens of each femoral condyle and tibial plateau (n=10 in each group) were fixed in 10 % neutral-buffered formalin, decalcified in 10 % ethylenediaminetetraacetic acid (EDTA) (in 0.1 M phosphate buffer 7–8), embedded in paraffin, and cut into 4-μm-thick sections on a microtome in a parasagittal plane of the femur (LFC and MFC) and in coronal plane of the tibia plateau (LTP and MTP) for histological evaluation. Two sections were stained with hematoxylin and eorin (H & E) and Safranin O and fast green. Severity of chondropathy was then evaluated using the grade and stage system of OARSI [29] (Table 1). The final score corresponds to the score found for the most severe lesions.

The specimens of the synovium (n=20 in each group) of the suprapatellar pouch that were distant from the arthrotomy site were fixed in 10 % neutral-buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections for histological evaluation. The sections were stained with H & E. The synovial alterations were assessed according to the histological scoring system based on hyperplasia of synovial lining cells, hypertrophy of synovial lining layer, infiltration of inflammatory cells, proliferation of granulation tissue, and vascularization [30]. The final score corresponds to the score of the most severe lesions with the highest possible score being 15.

Anabolic and catabolic factors gene expression analysis of cartilage

We choose TGF-β, IGF-1, TIMP-1, BMP-2, and BMP-7 as anabolic factors in the cartilage and IL-1β, iNOS, MMP-1, MMP-3, and MMP-13 as catabolic factors (n=10 in each group). ECM inherent components in the cartilage (n=10 in each group) were collagen type II and aggrecan. The primers for the rabbit specific genes were designed according to the published sequences available in GenBank using Primer Express (Applied Biosystems) as shown in Table 2. Total RNA was extracted from cartilage from the left femur and tibia using Trizol-Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR (RT-PCR) was performed using a FTC-2000 Real-Time PCR machine (Funglyn, Toronto, Canada). For the analysis of each sample, gene expression levels were calibrated using GAPDH expression levels as an internal control.

Statistical analysis

We used paired t tests to analyze change in percent weight distribution on the left hind paw as compared to baseline. Student's independent sample t tests were used for between-group comparisons for all outcomes at each timepoint. All RT-PCR data were normalized to the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) to facilitate equal loading of gels for quantitative comparisons of amplified PCR products. All data are expressed as mean±SD. Data were analyzed using SPSS version 17.0 software for Windows (SPSS Inc, Chicago, IL) and presented using GraphPad Prism version 4.0 (GraphPad Software). Alpha error level was set to 0.05. All data analysis was conducted by a researcher (LY) who was blinded with regard to experimental groups and effect assessment.

Results

Effects of LLLT on inflammation and pain behavior in ACLT-induced synovitis

Figure 1 shows short-term and long-term pain assessments at left hind paws by weight-bearing asymmetry at sequential intervals. As compared to baseline, the percent weight distribution on the left hind paw was gradually and significantly

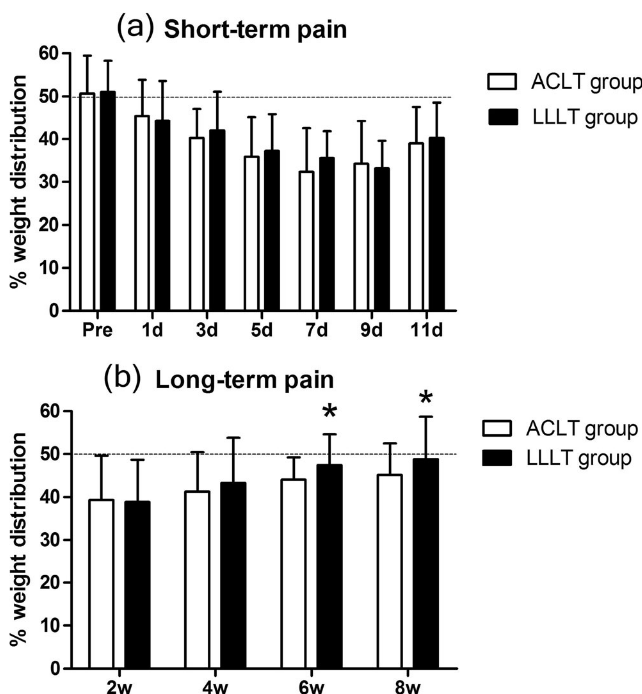


Fig. 1 Short-term (**a**) and long-term (**b**) pain assessments at left hind paws by weight-bearing asymmetry at sequential intervals

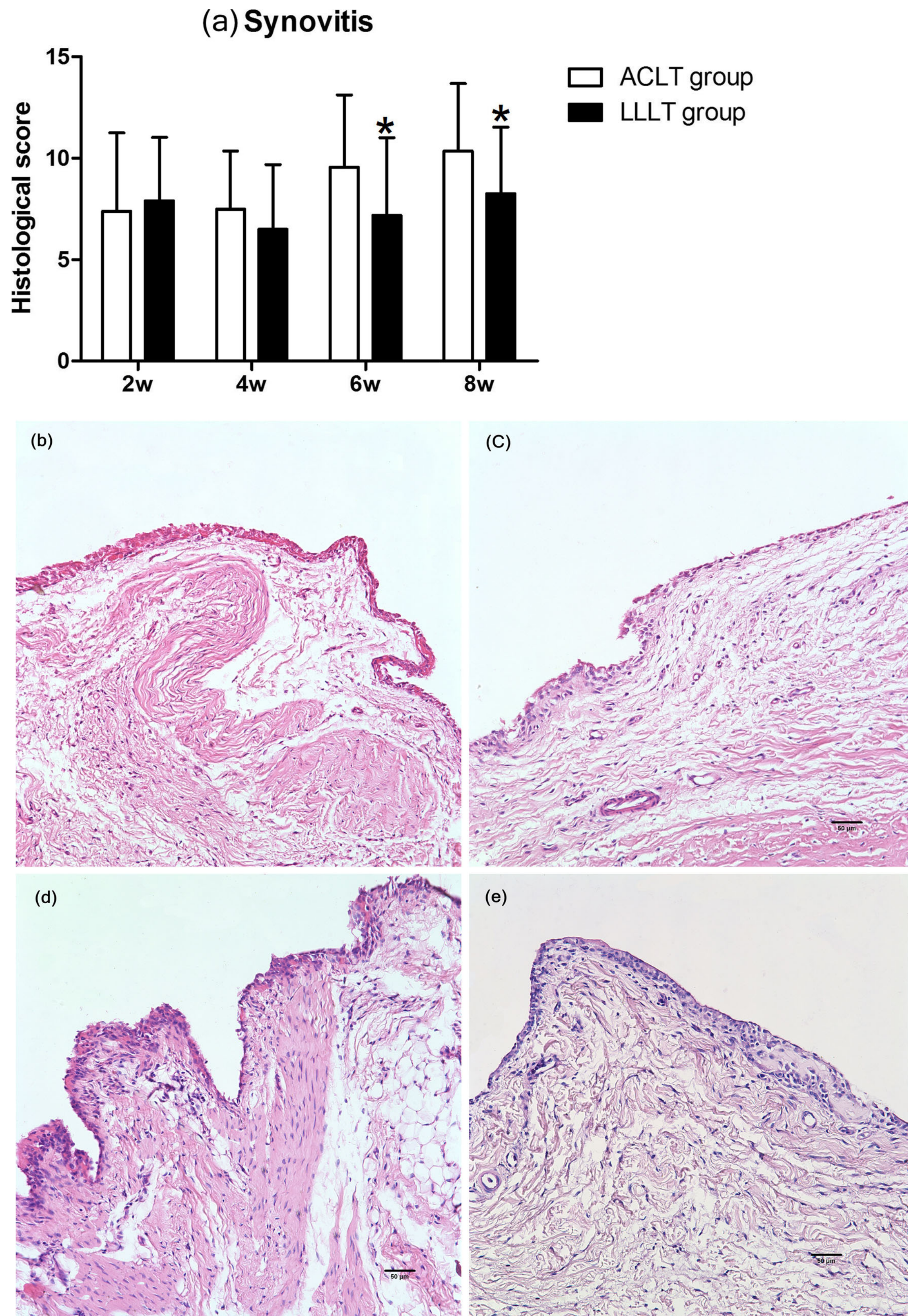


Fig. 2 **a**–**e** Representative pictures of the inflammatory reaction of synovium assessed by H & E staining at sequential intervals

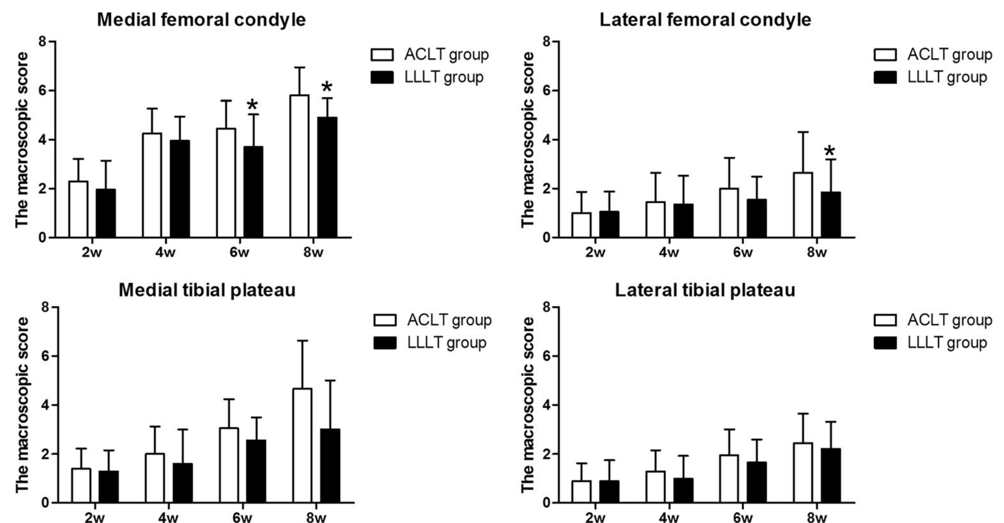


Fig. 3 Representative pictures of macroscopic histological examinations of cartilage from ACLT and LLLT groups at sequential intervals

reduced in the ACLT group 7 days after surgery and in the LLLT group 9 days after surgery. In both groups, the reduced percent weight distribution started to increase again at day 11. At 2 and 4 weeks after LLLT treatment, animals of the LLLT groups did not differ significantly from ACLT groups. After 6 and 8 weeks of LLLT treatment, weight bearing on the left hind paw was significantly increased in the LLLT groups as compared to the respective ACLT groups (48.5±6.23 vs. 45.3 ±4.28 % at 6 weeks, t=4.343, p<0.05, and 49.2±9.88 vs. 45.7±6.41 % at 8 weeks, t=3.182, p<0.05).

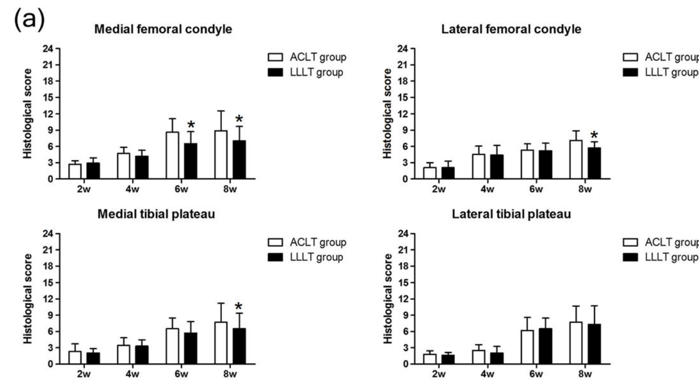
Figure 2 shows the representative pictures of the inflammatory reaction of synovium assessed by H & E staining at sequential intervals. After 2 and 4 weeks, no significant differences between ACLT and the LLLT groups were found for most of the histological parameters (Fig. 2a and b). At 6 weeks after ACLT surgery, specimens of synovium from the respective ACLT group showed mild-to-moderate inflammatory reaction including mild hyperplasia of synovial lining cells, mild-to-moderate hypertrophy of synovial lining layers, moderate infiltration of inflammatory cells, mild proliferation of granulation tissue, and mild vascularization. After 6 weeks of LLLT treatment, specimens of synovium from LLLT group showed mild synovitis according to most of the histological parameters. At 8 weeks, specimens of the synovium from the ACLT group showed moderate to severe synovitis. After 6 and 8 weeks of LLLT treatment, the degree of synovitis showed significant alleviation of lesion in the LLLT groups as compared with the ACLT groups (7.2±3.82 vs. 9.55± 3.58 at week 6, t=2.153, p<0.05; and 8.25±3.29 vs. 10.35± 3.34 at week 8, t=2.106, p<0.05).

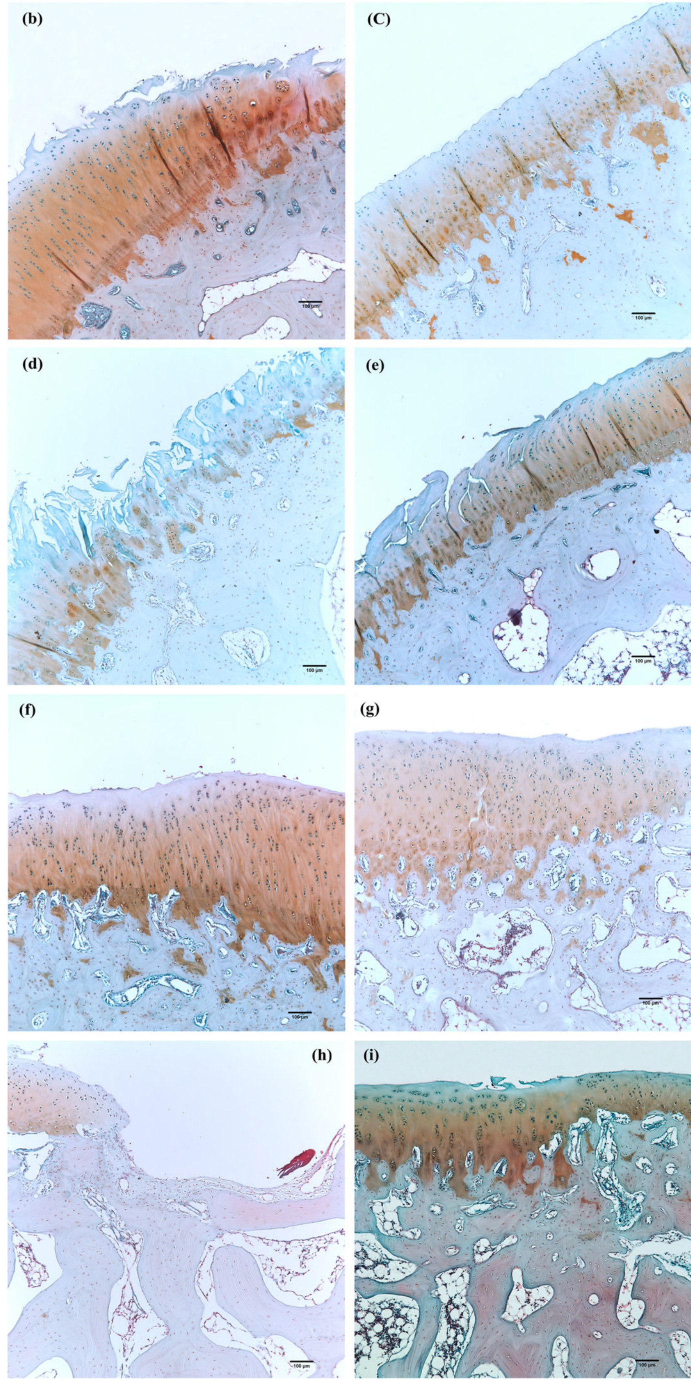
Effects of LLLT on progressive degeneration and erosion of cartilage induced by ACLT

Figure 3 shows representative pictures of macroscopic histological examinations of cartilage from ACLT and LLLT groups at sequential intervals. All specimens from the ACLT and LLLT knees showed complete transection of the anterior cruciate ligament at death. With progressing time, degrees of degeneration and erosion of cartilage increased in both ACLT and LLLT groups. The most severe area of cartilage degeneration usually occurred in medial femoral condyles. Generally, condyles and plateau in the LLLT group showed less severe cartilage damage than those in the ACLT group. However, no significant difference was found between the two groups at 2 and 4 weeks. At 6 weeks, MFC of animals from the LLLT group showed significantly less cartilage damage as compared with the ACLT group (3.7±1.34 vs. 4.5±1.15, t=2.210, p<0.05). After 8 weeks of LLLT treatment, degeneration of cartilage of MFC and LFC were significantly lower in the treatment group (MFC 4.9±0.79 vs. 5.8±1.15, t=2.592, p<0.05 and LFC 1.9±1.34 vs. 2.7±1.66, t=2.320, p<0.05). Gross analysis revealed that damage in the LLLT groups was lower than that in the ACLT groups at the 6th and the 8th weeks, but no significant differences were found between the two groups.

Figure 4 shows representative pictures from microscopic histological examinations of cartilage of ACLT groups and LLLT groups at sequential intervals. No significant differences were observed among all the histological parameters and the total histological scores between the ACLT and LLLT groups at 2 and 4 weeks. The mean values of Safranin O and fast green staining, surface structure of cartilage, chondrocyte density, and total histological scores of MFC of the LLLT group were significantly lower than the mean values of those of the ACLT group after 6 and 8 weeks of administration (6.5 ±2.22 vs. 8.6±2.50 at week 6, t=2.272, p<0.05 and 7.0±2.67

Fig. 4 a–i Representative pictures from microscopic histological examinations of cartilage of ACLT groups and LLLT groups at sequential intervals ▶


(a)
Medial femoral condyle
Lateral femoral condyle
Medial tibial plateau
Lateral tibial plateau
Histological score
ACLT group
LLLT group
2w
4w
6w
8w
*


(b)
(C)
(d)
(e)
(f)
(g)
(h)
(i)

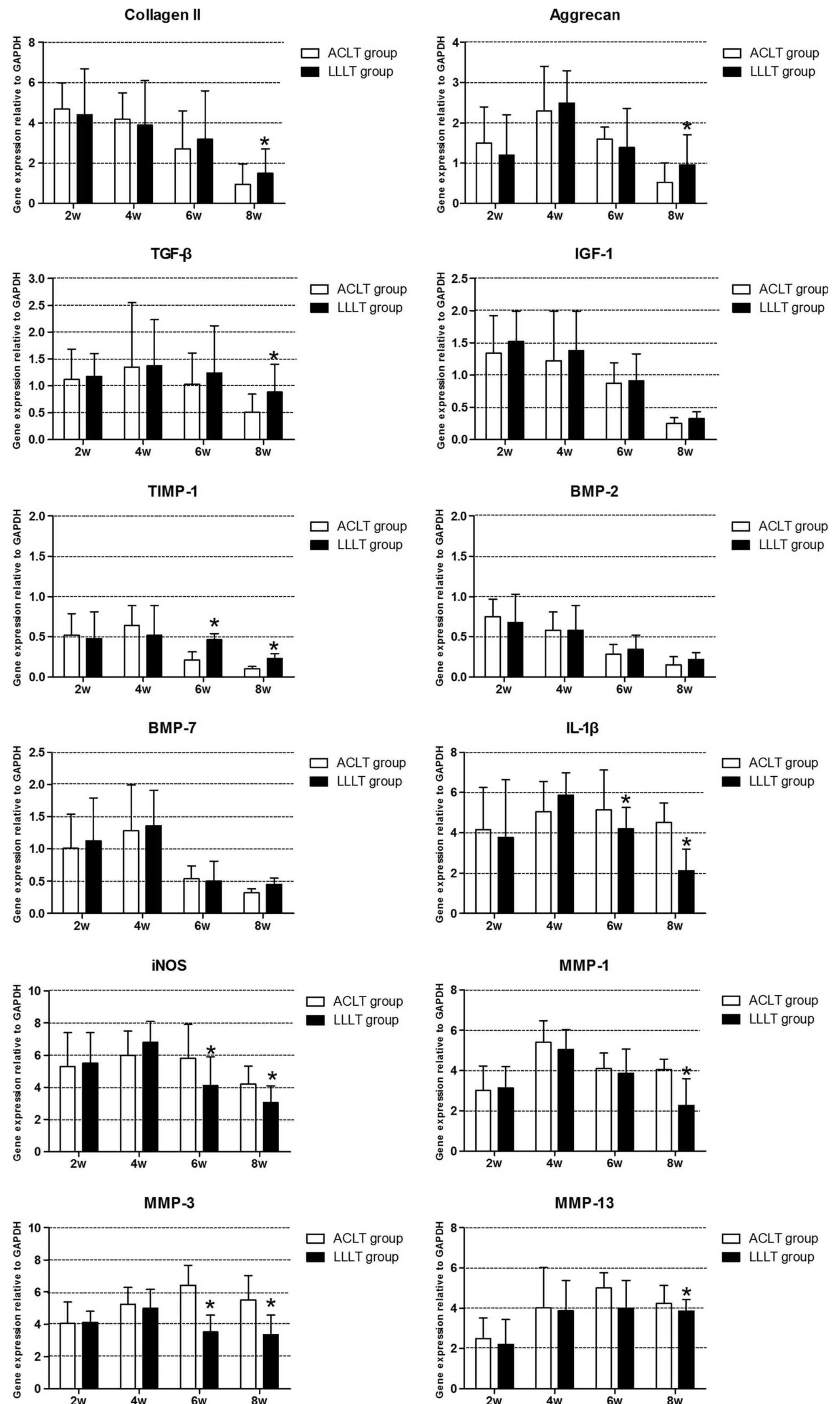


Fig. 5 Anabolic and catabolic factors' gene expression in cartilage at sequential intervals

vs. 8.9±3.67 at week 8, t=2.752, p<0.05. After 8 weeks of LLLT treatment, degeneration of cartilage of LFC and MTP showed significant decrease in the LLLT group (LFC 5.7±1.16 vs. 7.1±1.79, t=2.492, p<0.05 and 6.5±2.84 vs. 7.7±3.49, t=2.343, p<0.05).

Effects of LLLT on anabolic and catabolic factors gene expression in cartilage

Figure 5 shows anabolic and catabolic factors' gene expression in cartilage at sequential intervals. At 4 weeks after ACLT surgery, agrrecan of ECM inherent components, TGF-β and BMP-7 of anabolic factors, and all catabolic factors increased significantly as compared with the respective groups at week 2. There was a significant increase in the expression of collagen II and aggrecan genes in the cartilage from LLLT at week 8 as compared with ACLT cartilage. Regarding anabolic factors, expression of TIMP-1 gene in cartilage from LLLT was increased significantly at weeks 6 and 8 in comparison with ACLT cartilage (p<0.05). Expression of TGF-β gene in cartilage from LLLT was increased significantly at week 8 (p<0.05). In contrast, expression of IGF-1, BMP-2 and BMP-7 in LLLT cartilage showed no significant difference at all intervals as compared with ACLT cartilage. Regarding catabolic factors, expression of IL-1β, iNOS, and MMP-3 gene was decreased significantly in LLLT groups at weeks 6 and 8 as compared to ACLT groups (p<0.05). Furthermore, expression of MMP-1 and MMP-13 gene was decreased significantly at week 8 in comparison with ACLT cartilage (p<0.05).

Discussion

In this study, we examined the effects of LLLT on short-term and long-term joint pain and synovitis in an OA rabbit model. We found that at least 6-week intermittent irradiation of 830 nm and 4.8 J/cm^2 LLLT with He–Ne laser in three points per side of the knee could relief knee pain and control synovial inflammation in rabbits with progressive OA. Although, effects of LLLT on pain had been demonstrated previously by some studies [31, 32], other researches failed to produce respective evidence [33, 34]. These inconsistent findings may be related to differential laser parameters (the energy-generated, wavelength, time of illumination) as well as duration of therapies [11]. While we kept the laser parameters constant in this study, we varied treatment duration. Our findings suggest time-dependent effects of LLLT on relieving knee pain and controlling synovial inflammation. The effects of LLLT on pain may be due to the control of synovial inflammation as previously reported [35].

In our study, 6 weeks of LLLT treatment improved cartilage damage of the medial femoral condyle according to macroscopic and microscopic examinations. Eight weeks of LLLT treatment improved knee cartilage damage and erosion at all locations expect LMP and LTP from gross observation and LTP from microscope, suggesting that protecting effects of LLLT on progressive cartilage lesion may be location dependent. Moreover, we found that 8 weeks of LLLT treatment slowed down loss of collagen II, aggrecan, and anabolic factors, mainly TGF-β, while at least 6 weeks of LLLT treatment decreased the production of catabolic factors, for example, IL-1β, iNOS, and MMP-3, and slowed down the loss of anabolic factors, mainly TIMP-1.

TGF-β is a catabolic factor in OA, which is essential for the formation and maintenance of cartilage and affects cartilage homeostasis at multiple levels [36]. It possibly promotes chondrogenesis of stem cells and the synthesis of anti-catabolic factors such as TIMPs. TGF-β may also attenuate the cellular response to inflammatory cytokines containing IL-1β and inhibit terminal differentiation and hypertrophy of chondrocytes [37]. Pro-inflammatory cytokines such as IL-1β exert catabolic effects on the chondrocyte metabolism, decreasing proteoglycan collagen synthesis and increasing aggrecan release via blocking proteases [38]. IL-1β may also induce chondrocytes and synovial cells to produce other inflammatory mediators such as IL-8, IL-6, nitric oxide, and prostaglandin E2 [39]. Nitric oxide, produced by the inducible isoform of nitric oxide synthase (iNOS), is a major catabolic factor produced by chondrocytes in response to pro-inflammatory cytokines such as IL-1β and TNF [40]. MMPs as an array of proteases may break down type II collagen and proteoglycans, which leads to proteolysis of cartilage, especially MMP-1, MMP-3, and MMP-13 [41]. This study suggested that LLLT impacts on cartilage protection due to decreased loss of TGF-β and TIMP-1 and decreased production of IL-1β, iNOS, and MMP-3 in cartilage. However, our study did not confirm that LLLT decreases the loss of other catabolic factors, such as insulin-like growth factor (IGF) and bone morphogenetic proteins (BMPs).

Our study has some limitations that need to be mentioned. Firstly, as we kept laser parameters constant, our findings cannot be generalized to different types of LLLT. Secondly, we cannot exclude that longer duration of treatment may have produced additional effects. Thirdly, as we did not perform immunohistochemical examinations, we do not know the exact location where the contributing factors express within the cartilage.

Conclusion

Our findings demonstrated that anabolic and catabolic regulation may be responsible for positive time- and location-

dependent effects of LLLT on progressive OA. Our results suggest an optimal treatment duration of at least 6 weeks, preferably 8 weeks. Clinical trials are however needed to confirm our findings in human patients with progressive OA.

Conflicts of interest None

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