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Effect of Low-level Laser Therapy on Osteoarthropathy in Rabbit

HYUNG-JIN CHO[1], SUNG-CHUL LIM[2,3], SU-GWAN KIM[4], YOUNG-SOO KIM[1], SEONG-SOO KANG[1], SEOK-HWA CHOI[5], YONG-SEONG CHO[6] and CHUN-SIK BAE[1,7]

[1]College of Veterinary Medicine and [7]Biotechnology Research Institute, Chonnam National University, Gwangju; [2]Department of Pathology, [3]Research Center for Resistant Cells, Chosun University, Medical School, Gwangju; [4]Department of Oral and Maxillofacial Surgery, Oral Biology Research Institute, College of Dentistry, Chosun University, Gwangju; [5]College of Veterinary Medicine, Chungbuk National University, Cheongju; [6]College of Veterinary Medicine, Konkuk University, Seoul, Korea

Abstract. *The aim of this study was to determine whether low-level laser therapy (LLLT) aided the recovery of damaged articular cartilage in joints with artificially induced osteoarthropathy (OA). OA was induced by injecting hydrogen peroxide (H_2O_2) into the articular spaces of both knees in rabbits, twice a week for 4 weeks. The induction of OA and the effect of LLLT were evaluated by biochemical, radiological and histopathological analysis. Superoxide dismutase (SOD) activity increased about 40% in the OA group, as compared to the controls. Although SOD activity in the OA group was not significantly different from the 2-week groups, it was significantly different from the 4-week control and treatment groups. There was also a significant difference between the 4-week control and treatment groups. Simple radiographs and three-dimensional computed tomographs (3D CT) did not show detectable arthropathy in the OA group, nor any paricular changes in the 2-week groups. In contrast, distinct erosions were seen in the distal articular cartilage of the femur, with irregularity of the articular surface, in the 4-week control group, while the erosions were reduced and arthropathy improved slightly in the 4-week treatment group. Grossly, erosions formed on the articular surface in the OA group. In comparison, severe erosions damaged the articular cartilage in the 4-week control group, but not in the 2-week control and treatment groups. Regeneration of articular cartilage was seen in gross observations in the 4-week treatment group. Histopathologically, there was slight irregularity of the articular surface and necrosis in the OA group, and serious cartilage damage, despite slight chondrocyte regeneration, in the 4-week control group. Conversely, the 4-week treatment group showed chondrocyte replacement, with sometimes close to normal articular cartilage on the articular surface. These results suggest that LLLT was effective in the treatment of chemically-induced OA.*

Correspondence to: Chun-Sik Bae, D.V.M., Ph.D., Department of Veterinary Surgery, College of Veterinary Medicine, Biotechnology Research Institute, Chonnam National University, #300, Yongbong-dong, Puk-gu, Gwangju, 500-757, Republic of Korea. Tel: (82-62)530-2876, Fax: (82-62)530-2809, e-mail: csbae210@chonnam.ac.kr

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Although it is a common non-inflammatory, degenerative disease occurring in synovial joints, the pathology of osteoarthropathy (OA) is still uncertain (1). This disease induces cartilage damage and deformity, mainly due to weakened articular cartilage, and brings about local degenerative changes characterized by secondary inflammation with abnormal bone formation on the joint surface and surrounding area (2,3). The degenerative changes are thought to be due to cartilage metabolic dysfunction in joints that support the body weight (4,5).

Growing interest in the pathology of this disease has led to a number of studies. Various methods have been developed to artificially induce OA in animals, including dogs, rabbits, mice, horses and sheep (6-9). These include the injection of papain (7,10), fluorescein (11), collagenase (12,13), H_2O_2 (9,14) or complete Freund's adjuvant (6,15) into the articular capsule, and dissection of the anterior cruciate ligament (16-18) or semilunar valve (19,20). Experimentally-induced destruction of the articular cartilage can be classified into 3 types: 1) a reduction in the number of stroma or chondrocytes without macroscopic detachment of cartilage tissue or physical destruction, 2) physical destruction of limited cartilage tissue, and 3) physical destruction of cartilage tissue affecting subchondral bone. These types of damage are similar to those which occur during the early, middle and late stages of degenerative arthritis (9,21). OA develops from degenerative changes in the articular cartilage and there is no cure for this disease.

The treatment of OA focuses on pain relief and functional recovery by inhibiting the factors causing cartilage deformity as much as possible, since this results in

a loss of cartilage and articular deformity, unlike rheumatoid arthritis. Various approaches are used to treat OA (22) including synovectomy, weight control and the use of non-steroidal anti-inflammatory drugs (NSAIDs), steroids and immunosuppressants (6,17,23,24). However, most of these methods have complications or side-effects, necessitating the development of drugs or treatment methods that are not harmful (25). Many clinicians prefer low-level laser therapy (LLLT), a form of electrotherapy.

Introduced 10 years ago, LLLT constitutes a non-invasive alternative for treating joint disease (26-28). LLLT is also used to treat soft tissue injuries, rheumatoid arthritis, musculoskeletal pain and dental problems (29,30). Although there is much controversy over its efficacy, positive clinical results have been reported (25,29,31,32).

There are many methods for inducing and treating artificial OA. After we confirmed the induction of OA in rabbits by injecting 4% H_2O_2 into the articular spaces in both knees, we examined whether transcutaneous irradiation with LLLT aided the recovery of the damaged articular cartilage in the knee joints.

Materials and Methods

Animals. Twenty-eight, healthy, 10-month-old New Zealand white rabbits (Damool Science, Korea), weighing 2.5 ~ 3.0 kg, were reared in individual cages at 20±2˚C and a relative humidity of 60±5%, and given solid rabbit feed (Purina) and water *ad libitum*. Three rabbits were placed in the normal control group, while the remaining 25 were used for OA induction and LLLT. These were divided into 5 groups of 5 rabbits each: OA induced only (OA group); the rabbits were treated with low-level laser for 2 or 4 weeks starting 1 day after the confirmation of induced OA (2- and 4-week treatment groups); and the rabbits were not treated with laser (2- and 4-week control groups). The Animal Care Committee at Chonnam National University, Korea, approved the protocols in this report.

Induction of osteoarthropathy. OA was induced by injecting 0.5 ml of 4% hydrogen peroxide (H_2O_2) into the articular spaces of both knee joints of restrained, unanesthetized rabbits twice weekly for 4 weeks, using a 23-gauge needle.

Laser irradiation. For the control group, the knee joint was not irradiated. For the animals in the treatment group, LLLT was done transcutaneously for 5 minutes every day, using a SCAN-BIO LASER (TS-1003A, TMC, Korea) for 2 or 4 weeks, starting the day after the confirmation of OA. This machine emits a complex laser that is a combination of an IR (GaAs, gallium arsenide) semiconductor laser and a He-Ne laser. The machine was set at 100% duty cycle and 2,500 Hz frequency.

Superoxide dismutase (SOD) activity analysis. After sacrificing the animals, the separated end of the tibia was obtained for the antioxidative enzyme SOD analysis. The sample was ground in liquid nitrogen and homogenized by adding 1 ml of phosphate-buffered saline (pH 7.4) to a 0.5g bone sample using a homogenizer (PT-3100, Swiss). The homogenized sample was centrifuged for 1 min at 20,000g at 4˚C and the supernatant was carefully collected. Using 0.05 ml of prepared sample mixed with 0.85 ml of mixed substrate placed in a cuvette, the absorbance was measured at 505 nm 30 sec and 3 min after adding 0.125 ml of xanthine oxidase. The amount of protein was quantified using the modified Bradford Method (30), and the total protein volume was obtained from each sample. SOD activity was measured using the standard sample provided in the kit to obtain a standard curve, and the % inhibition calculated from each sample was compared with the standard curve to obtain the calculated value (SOD units). Then, the total protein was divided by the SOD units to calculate SOD unit /g total protein to measure SOD activity.

Radiography. In 28 rabbits, both knees were radiographed in the cranio-caudal and medio-lateral projection at intervals varying from 1 week to 8 weeks. The results were evaluated using the following parameters: increased synovial mass, altered thickness of the joint space, subchondral bone opacity change, subchondral bone cyst formation, altered perichondral bone opacity, perichondral bone proliferation, intra-articular calcified bodies and mineralization of joint soft tissues (33).

Three-dimensional computed tomography (3D CT). After the rabbits had been anaesthetized, 3D CT images of both knee joints were obtained using a CT scanner (CT-HiSpeed Advantage, USA) by performing an axial scan at 1 mm thickness. 3D CT images were obtained using custom software (Advantage Windows 2.0, SUN, USA).

Gross appearance. In specimens where the distal end of the femur and the proximal end of the tibia were separated, the gross appearance of the joint surfaces and the periarticular soft tissues was observed. The soft tissues from the distal ends of the femora and the proximal ends of the tibiae were carefully removed and the gross appearance of the joint surfaces was evaluated and recorded photographically.

Histopathological analysis. Histological sections were made of both knees of 28 animals. The separated end of the femur was fixed in 10% formalin and was decalcified in nitric acid (DeCal Rapid solution, Pational Diagnosis, Atlanta, USA) for 12 h. Then, the samples were embedded in paraffin in the usual way. Serial 4-μm-thick sections were stained with hematoxylin and eosin.

Histopathological alterations of the joints were evaluated by an observer, blinded to the experimental design, in consultation with two pathologists after standardization of the measurements. A minimum of 10 sections per joint were assessed for the presence of the following: erosion and necrosis of articular cartilage, fissuring and flaking of articular surfaces; subchondral bone destruction; subsynovial inflammation; and synovial hyperplasia.

Statistical analysis. The SOD activity is expressed as the mean±SEM. The groups were compared using the Student's *t*-test and one-way ANOVA to determine statistical significance. A *p*-value less than 0.05 was deemed significant.

Results

Superoxide dismutase (SOD) activity. The SOD activity was 54.39±3.46 U/g in the normal group, which was significantly lower that the 76.54±4.19 U/g in the OA group ($p<0.05$). This activity increased in the LLLT groups, as compared

with the control groups. The increase became more significant with increasing treatment period; it was 66.17±3.48 *vs.* 59.78±1.97 U/g in the treatment *vs.* controls at 2 weeks and 60.50±3.93 *vs.* 46.07±1.13 U/g at 4 weeks, respectively (Figure 1). With increasing time, the SOD activity decreased in both the control and treatment groups. However, the treatment groups had higher SOD activity than the control groups, and this difference was significant at 4 weeks ($p<0.05$).

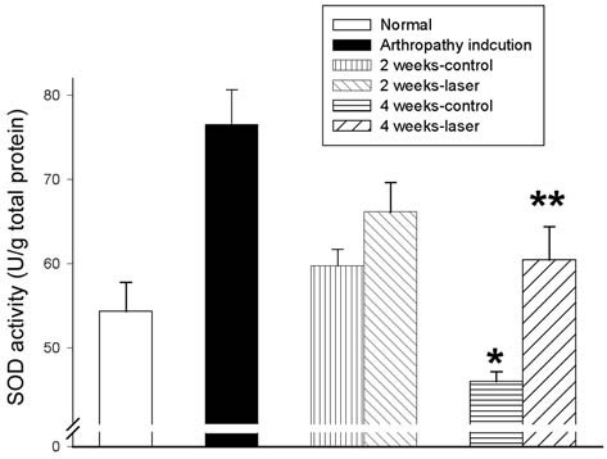

Figure 1. *Comparison of superoxide dismutase(SOD) in the stifle joint of rabbit after treatment with hydrogen peroxide(H_2O_2) and/or low level laser therapy. *p<0.05 against H_2O_2-induced arthropathy. **p<0.05 against 4-week control after arthropathy induction. Normal: 54.4±3.5, H_2O_2 induced arthropathy: 76.5±4.2, 2-week control: 59.8±2.0, 2-week laser: 66.2±3.5, 4-week control: 46.1±1.1, 4-week laser: 60.5±3.9*

Radiography. A smooth normal contour and bony trabecular shadow were seen on the articular surface of normal rabbits. In the induced OA group, the contour of the lateral femoral condyle was irregular, but no definite findings of OA were observed. However, the articular surface had an irregular contour in the distal portion of the femur in the 4-week control group. Several translucent areas of decreased bone density were seen in the craniocaudal view in the lower portion of the cartilage, indicating that OA had been induced. By contrast, the bone density was close to normal, as were the joint surface and contour, in the 4-week treatment group.

Three-dimensional computed tomography (3D CT). The femur and articular surface of the fibula in the knee joint were smooth even on 3D CT. When OA was induced, the articular surface was slightly irregular and not as smooth as a normal knee joint. Furthermore, there was slight damage to the femoral epicondyle, while the trochlear lip was irregular and damaged. In the 4-week control group, there was marked irregularity of the articular surface, in the area where the femur and tibia met the trochlear lip had an irregular articular surface and outline, and the femur epicondyle was severely damaged (Figure 2A). By contrast, in the 4-week treatment group, the trochlear lip was slightly damaged, the articular surface was close to normal and the overall damage was significantly improved, while no damage was observed in the femoral epicondyle (Figure 2B). In contrast, no particular changes were seen in the 2-week control and treatment groups.

Gross appearance. Inflammation and swelling were observed in the knee joints of rabbits treated with H_2O_2, as compared with normal. These findings worsened with time in the control groups, but decreased in the treatment groups. Grossly, the articular surface was even, smooth and clear, and viscous synovial fluid was seen in normal rabbits not injected with H_2O_2. In the induced OA group, the articular surface was irregular, the lateral femoral condyle slightly eroded and the synovial fluid was less translucent, although there were no significant gross changes. In the control groups, the synovial fluid was not clear, but brownish-yellow, and the amount and opacity increased with time. The degree of deformity differed with time at the femoral and tibial articular surfaces. The degree of deformity in the 2- and 4-week control groups worsened and severe erosion, redness and irregularity were seen at the articular surface. Ulceration was seen in the 4-week control group (Figure 2C). However, no osteophyte formation was observed. In contrast, the degree of deformity of the articular surface was inhibited in the LLLT treatment groups and significant cartilage proliferation was observed in the 4-week treatment group (Figure 2D).

Histopathological analysis. The normal articular surface was even and smooth. The normal articular cartilage was very cellular and the chondrocytes were aligned vertically. In the induced OA group, the articular surface was irregular with fibrillation, fissuring and flakings and the superficial zone was mostly lost. The articular cartilage showed eosinophilic changes with the disappearance of cell nuclei in most layers due to necrosis. In the 2-week control group, several lumps of chondrocytes were seen in an eosinophilic subchondral area, whereas a band of chondrocyte proliferation reached towards the articular surface in the 2-week treatment group. Conversely, chondrocytes were more proliferated, even in the surface layers sometimes, in the 4-week control group (Figure 2E), whereas the articular surface was replaced with chondrocytes but showed no organized alignment, as in normal articular cartilage, in the 4-week treatment group (Figure 2F) (Table I).

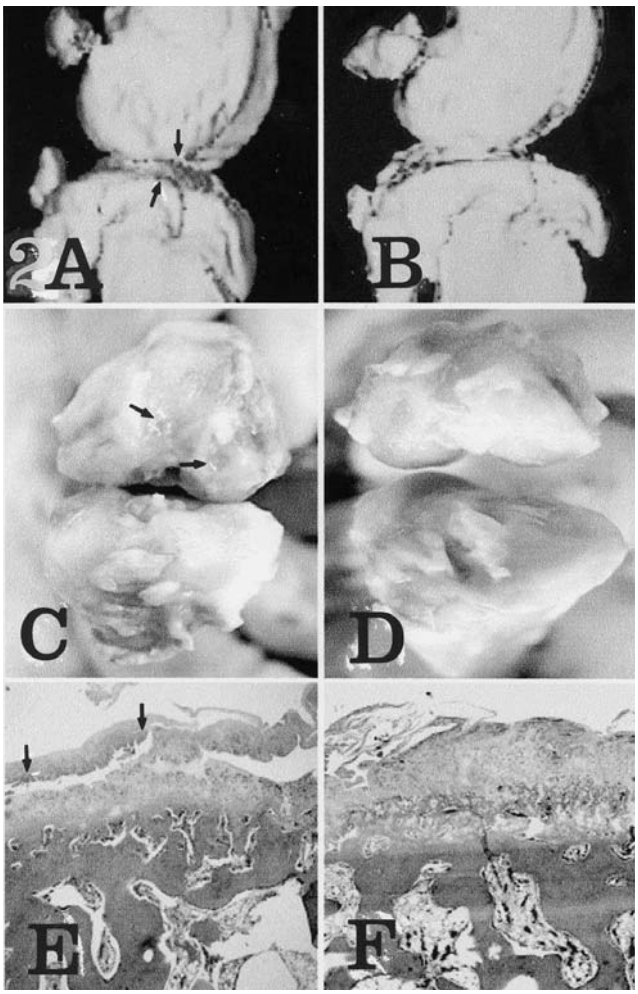

Figure 2. *3D CT finding: A. In the 4-week control group, there was marked irregularity (arrows) of the articular surface, in the area where the femur and tibia met the trochlear lip had an irregular articular surface and outline, and the femur epicondyle was severely damaged. B. The trochlear lip was slightly damaged, the articular surface was close to normal and the overall damage was significantly improved, and no damage was observed in the femoral epicondyle in the 4-week treatment group. Gross finding: C. Ulceration (arrows) was seen in the 4-week control group. D. The degree of deformity of the articular surface was improved in the LLLT treatment groups and significant cartilage repair was observed in the 4-week treatment group. Histological finding: E. Proliferation of chondrocytes was noted in the lower 2/3 of the articular cartilage. Some eosinophilic degeneration (arrows) of the articular surface was still noted in the 4-week control group (hematoxylin-eosin, original magnification X100). F. The articular surface was completely replaced with chondrocytes but showed no organized alignment, as in normal articular cartilage, in the 4-week treatment group (hematoxylin-eosin, original magnification X100).*

Discussion

OA is characterized by a decrease in proteoglycan, the major component of cartilage, and by the decomposition of collagen fibrils, which is due to a dysfunction in cartilage metabolism. The mechanism leading to the early stages of articular cartilage damage is not clear. In this study, we induced OA in rabbits by injecting 0.5 ml of 4% H_2O_2 into both knee joints. The concentration and amount of H_2O_2 used to induce OA differs according to the study and other authors (9,14) injected 0.5 ml of 5% H_2O_2 into both knee joints twice a week for 5 weeks to induce OA, while we followed the same schedule with 4% H_2O_2 for 4 weeks. Although it was difficult to confirm OA using simple radiographs and 3D CT images, we confirmed that OA had been induced by measuring SOD activity and by reviewing the gross changes and histopathology.

H_2O_2 induces oxidative changes in the articular capsule and produces activated oxygen in the form of OH and $^1O_2$, due to continuous reaction by superoxide. These oxygen forms cause proteoglycan depolymerization, collagen degradation and proteolytic enzyme discharge of hyaluronic acid due to chondrocyte damage and lipid peroxide formation. This series of processes induces degenerative changes in the joint; a repetitive mechanical load on the joint enhances the degenerative process, ultimately causing OA (4,34).

The SOD activity was significantly increased in the induced OA group (76.54±4.11 U/g) as compared to the normal group (54.39±3.46 U/g), due to the effect of free radicals formed with the introduction of 4% H_2O_2. This was similar to the result reported by Bae *et al.* (9). Furthermore, the SOD activity was significantly higher in the LLLT groups, as compared to the controls. It decreased to 66.17±3.48 and 60.50±3.93 U/g in the 2- and 4-week treatment groups, respectively. It also decreased in the control groups to 59.78±1.97 and 46.07±1.13 U/g at 2 and 4 weeks, respectively. We believe that these decreases probably occurred because LLLT inhibited the toxic effect of free radicals formed *in vivo* with the introduction of H_2O_2, by decreasing the activity and amount of free radicals. In the control groups, the formation and activity of SOD were significantly decreased by a mechanism that compensates for the action of free radicals. This activity was significantly decreased, especially in the 4-week control group, where it was about 20%, as compared to the control group.

The radiographs and 3D CT images showed minimal evidence of OA in the induced OA group, even when H_2O_2 was introduced into the knee joint twice a week for 4 weeks. We could not find many differences between the radiographs obtained from normal rabbits and those obtained from rabbits with induced OA. Moreover, with LLLT there was little difference between the 2-week treatment and control groups. However, articular cartilage erosion, articular surface irregularity, perichondral bone opacity and subchondral opacity were seen on simple radiographs and 3D CT images in the 4-week control group, and these changes were improved significantly in the 4-week treatment group.

Table I. *Histopathological findings of each group.*

Micro-	Normal	H_2O_2 induced	Laser treatment		No treatment	
finding	control	OA	2 wks	4 wks	2 wks	4 wks
articular surface	smooth	fissuring, flaking, fibrillation	fissuring, fibrillation	relatively smooth, focal fissuring	fissuring, fibrillation	relatively smooth, focal flaking
articular cartilage	well organized	degenerated thinned	degenerated	more improved organization	degenerated	improved organization
degeneration of cartilage	absent	present in full thickness	present in upper 4/5	present in upper 1/3	present in full-thickness	present in upper 3/4
tidemark	relatively straight, monolayered	loss or irregular/ multilayered	irregular/ multilayered	irregular/ multilayered	irregular/ multilayered	irregular/ multilayered
subarticular bone	well preserved	damaged	regenerating	more regenerated	regenerating	more regenerated
cellularity	hypercellular	hypocellular	hypocellular	more improved cellularity	hypocellular	improved cellularity

OA: osteoarthropathy

Since Spaeth *et al.* (35) reported that magnetic resonance imaging (MRI) is more sensitive for diagnosing early-stage joint diseases using periosteum responses than CT or simple radiography. MRI was considered sufficient for the diagnosis of OA. Clearer signs of OA might have seen on simple radiographs or 3D CT images if we had also applied a mechanical factor, such as a running load (14) for a set period each day, along with the H_2O_2 injection, if we had increased the H_2O_2 concentration, or if we had used chemicals such as complete Freund's adjuvant to induce inflammatory changes.

Grossly, the articular surface of the normal rabbit knee was even and smooth. In this study, we did not observe osteophyte formation in any of the rabbits in the induced OA group or in the 2- and 4-week control groups. However, we observed more prevalent articular cartilage erosion, fibrillation, irregularity of the articular surface and swelling and inflammation of the joint in the control groups with time, although the changes in the 4-week control group were not significant. The area of damage was also increased from the lateral femoral condyle and the entire articular surface showed severe changes. Furthermore, articular cartilage erosion in all rabbits in the induced OA and control groups was seen at the lateral joint surface initially, the area and degree increasing with time, as reported by Mitrovic *et al.* (3). The degree of damage differed slightly in each joint, probably because of differences in the stimulation caused by the injection needle and by technical error in injecting H_2O_2 into the articular spaces. As compared with the control groups, a decreased area of damage, reduction of erosion formation and decreased irregularity of the articular surface were observed in the treatment groups. Slight fibrillation was also seen on the articular surface in a few cases. The regeneration of articular cartilage was observed visually in the 4-week treatment group, showing that LLLT improves joint deformity, while treating the lesion.

Histologically, articular cartilage can be divided into the superficial or tangential, transitional, radiate or deep, and mineralized zones in normal rabbits. The radiate and mineralized zones were separated by a single tidemark, as reported by Bae *et al.* (9). Normal articular cartilage was very cellular. The chondrocytes were aligned vertically, with an organized alignment. In the induced OA group, the articular surface was irregular and exposed, so that the superficial zone was lost. The entire layer of articular cartilage underwent necrosis, with eosinophilic changes and absent nuclei. The tidemark was unclear and it was multilayered. In the 2-week control group, we observed several lumps of chondrocytes under the eosinophilic articular cartilage, whereas we sometimes observed a band of chondrocyte proliferation reaching the articular surface in the 2-week treatment group. Evidence of chondrocyte

proliferation was seen in surface areas covered with deformed cartilage tissue fragments in the 4-week control group. The induced OA group showed articular cartilage softening, fibrillation and subchondral bone damage in severe cases. These results are similar to those of Kikuchi *et al.* (12) and Bae *et al.* (9), who observed a multilayer tidemark, instead of the single line tidemark seen in normal rabbits, irregularity, radiate zone loss, subchondral bone damage and hypocellularity. In this study, the 4-week treatment group lacked the organic arrangement of chondrocytes seen in normal articular cartilage, but the articular surface was sometimes replaced by regenerating chondrocytes, as reported in the studies mentioned above.

Brosseau *et al.* (28) reported that LLLT is effective for rheumatoid arthritis, but not for osteoarthritis. Amano *et al.* (36) reported that LLLT is effective in rheumatoid arthritis due to a direct photochemical effect, which increases the density of the synovial stroma, promotes connective tissue proliferation, and prevents primary and secondary immune responses. Although our study differed somewhat from the studies mentioned above, as we induced non-inflammatory OA, we believe that our results are similar to those of previous studies, since edema and heat sensation were significantly decreased and no inflammatory cells were observed histologically in the groups treated with LLLT, as compared to the control groups.

The effectiveness of combining IR and He-Ne lasers is controversial. Beckerman *et al.* (29) reported that a combined laser was more effective for musculoskeletal diseases than a placebo group, whereas Ulugol *et al.* (15) reported the opposite. Although we did not directly compare our results with the use of LLLT in weight control (24), NSAIDs or antioxidants (6,8,15,17), or surgical treatment (5,23), we believe that, based on previous reports, the treatment method can cause significant differences in the results. McLaughlin (37) compared various non-surgical methods of treating rheumatoid arthritis and OA, including the use of anti-inflammatory drugs, analgesics, antioxidants, diet and weight control. Drugs cause side-effects, immunosuppressive effects and have limited use (25). Therefore, we used LLLT to treat OA. Basford (30) deemed LLLT safe, as long as the eyes are not exposed to the laser directly.

Based on our results and the literature review, LLLT is effective in treating OA. This claim is based on the SOD activity, simple radiographs, 3D CT images, gross observations and histopathology, after we confirmed the induction of OA with the injection of 4% H_2O_2 into both knees of 25 normal rabbits. No significant treatment effect was seen after 2 weeks, but we found a significant improvement after 4 weeks of treatment. Therefore, we believe that OA should be treated with LLLT for at least 3 weeks.

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