Histological evaluation of low-intensity pulsed ultrasound on osteochondritis dissecans of the humeral capitellum

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Abstract

Background: The clinical use of low-intensity pulsed ultrasound (LIPUS) was recently evaluated in cases of osteochondritis dissecans of the humeral capitellum (elbow OCD). However, the mechanism underlying the effect of LIPUS in elbow OCD is not well understood. The aim of this study was to histopathologically evaluate the effect of LIPUS irradiation on elbow OCD.

Methods: Fifteen patients with elbow OCD were enrolled in this study. All patients were juvenile baseball players (average age, 13.1 years). LIPUS was performed under the same conditions as the fracture treatment for an average length of 15.1 days in the preoperative period in seven patients (LIPUS group). Cylindrical tissue specimens obtained at the time of surgery were stained with hematoxylin and eosin and alcian blue, and were also immunostained to detect type 1 collagen (Col-1), osteopontin (OPN), and Runx2. The cartilage and subchondral bone findings in histological evaluations did not differ significantly between the LIPUS and control groups. The distribution of OPN expression levels in the two groups was as follows: Grade 0—LIPUS group, zero patients, and control group, five patients; Grade 1—LIPUS group and control group, two patients each; Grade 2—LIPUS group, five patients and control group, one patient; Grade 3—LIPUS group, one patient and control group, zero patients. OPN expression was significantly higher in the LIPUS group than in the control group (p = 0.04).

Conclusion: LIPUS stimulation increased the expression levels of OPN in elbow OCD.

Keywords: immunohistochemistry; low-intensity pulsed ultrasound; osteochondritis dissecans of the humeral capitellum; osteopontin

Introduction

Osteochondritis dissecans of the humeral capitellum (elbow OCD) frequently occurs in adolescent athletes who have played baseball since childhood. Although no single cause has been identified, the main factor contributing to elbow OCD is presumed to be microtrauma, caused by repetitive compression and shear force on the radiocapitellar joint as a result of throwing. OCD is a localized disorder of the subchondral bone resulting in separation and fragmentation of the articular surface and underlying bone. Patients with early-stage elbow OCD can generally be managed with nonsurgical treatment (e.g., prohibition of heavy use of the joint and...
throwing), leading to good results.\textsuperscript{4,5} However, the time from initial examination to healing is 4–60 months,\textsuperscript{5} during which time the patients are forced to limit their sports activities.

Low-intensity pulsed ultrasound (LIPUS) has been used to treat fresh fractures, delayed unions, and nonunions, and it can stimulate healing in a relatively short period.\textsuperscript{6,7} Fracture healing is a physiological process that involves the participation of several cell types, all of which multiply, differentiate, migrate, and synthesize the extracellular matrix. In bone regeneration, it is important to assess the changes in the presence of some key proteins and the expression of bone differentiation markers. In a recent in vitro study, the expression of the bone differentiation markers, type 1 collagen (Col-1), osteopontin (OPN), osteocalcin, and osteonectin, was increased by LIPUS stimulation in various cell types.\textsuperscript{8–10} Additionally, mRNA expression of the transcription factors associated with osteoblast differentiation, Runx2, osterix, and bone morphogenetic protein-2, were reported to be elevated because of LIPUS stimulation.\textsuperscript{8,11,12}

Clinical studies have also documented the use of LIPUS for treatment of elbow OCD.\textsuperscript{13,14} The studies describing elbow OCD concluded that LIPUS led to good clinical outcomes for patients with early-stage lesions who had not responded to nonsurgical treatment. These reports comprised a case series that evaluated the healing process by radiographic and ultrasound findings. Therefore, it remains unclear whether the healing was the result of the natural course of elbow OCD healing or if there was an effect of LIPUS on the rate of healing. The aim of this study was to evaluate the effect of LIPUS on elbow OCD, both histopathologically and immunohistochemically. Our hypothesis was that the bone differentiation markers and transcription factors associated with osteoblast differentiation increased as a result of LIPUS stimulation in patients with elbow OCD.

Materials and methods

Between April 2010 and June 2012, a series of 17 consecutive patients with elbow OCD underwent reconstruction of the articular surface with osteochondral autograft transplantation from their lateral femoral condyle. Of these patients, 15 were included in this study; one patient with recurrent elbow OCD and one patient from whom it was not possible to harvest the tissue using a harvester in the lesion were excluded. Fifteen patients were juvenile, male baseball players with dominant-arm involvement. Magnetic resonance imaging (MRI) and preoperative radiographs (anteroposterior and lateral views and tangential views of the elbow in 45° flexion) were obtained for all patients. Surgical treatment was indicated by the diagnostic imaging as well as by the symptoms, which included catching, locking, and persistent pain in the elbow. The OCD lesions were classified by MRI, according to the Nelson staging system.\textsuperscript{15} Evidence of fluid accumulation at the interface between the fragment and its bed, best detected in the T2-weighted images, indicated an unstable lesion. Unstable OCD lesions (Nelson Grades 3 and 4), and stable OCD lesions (Nelson Grade 2) that did not heal with adequate nonsurgical treatment were candidates for surgical intervention. Patients were randomly assigned, prior to the surgery, into either the LIPUS or control groups (block randomisation). This study was approved by the Hirosaki University Graduate School of Medicine Institutional Review Board (Hirosaki, Japan), and each participant provided written informed consent.

LIPUS treatment

A sonic-accelerated fracture healing system (Teijin Pharma Ltd, Tokyo, Japan) was used in this study. The system was set to deliver a 30mW/cm² intensity at a 1.5-MHz frequency, repeated at 1 kHz, with a pulse width of 200 ms. This wave-form was the same as the conditions recommended for the treatment of fractures. LIPUS was applied through the anterior side of the elbow, confirmed OCD lesion by ultrasound, for 20 minutes daily until the day of the surgery. The mean length of LIPUS irradiation was 15.1 ± 5.6 days (range, 11–27 days).

Surgery and tissue sampling

Tissue sampling was performed during surgery, under general anesthesia. The posterior joint capsule was incised, and the lesion was directly identified and categorized according to the International Cartilage Repair Society’s (ICRS) OCD classification. The Osteochondral Autograft Transfer System (Arthrex, Naples, FL, USA) was used to harvest and transfer the osteochondral autografts. A cylindrical sample of osteochondral tissue, 5–10 mm in diameter and 10–15 mm in length, was obtained, vertically, from the articular surface of the central portion of the lesion using the harvester (Fig. 1). The drilling was done in the base of the socket, prior to inserting the graft for bone marrow stimulation. The osteochondral graft was harvested arthroscopically from the intercondylar notch of the lateral femoral condyle or directly from the lateral side of the patellofemoral joint, and then the graft was transferred.\textsuperscript{16}

Histological assessment of osteochondral specimens

The cylindrical tissue samples were fixed with 10% buffered formalin, decalcified, and cut longitudinally along their midline. These specimens were embedded in paraffin and cut into consecutive 5-μm-thick sections, and were stained either with hematoxylin and eosin or with alcian blue. Additional sections were processed for immunohistochemical evaluation.

The variance of the articular cartilage and subchondral bone in the specimens was evaluated histologically, as previously described, using the following parameters: (1) discontinuity of the cartilage surface; (2) cloning of chondrocytes; (3) fibrosis of the cartilage matrix, demonstrated by a reduction in alcian blue staining; or (4) necrosis of subchondral bone, demonstrated by empty lacunae in the bone trabeculae. The specimens were graded from 0 to 2 (0 = no or minimal change; 1 = mild or focal; 2 = marked or diffuse).\textsuperscript{3} In addition, the osteoid formation and cartilaginous metaplasia in the
reparative tissue was assessed with the same grading system by two pathologists who were blinded to the treatment received prior to sampling.

**Immunohistological evaluation**

To evaluate the ossification of the reparative tissue, the specimens were immunostained for Col-1, OPN, and Runx2, using streptavidin–biotin methods (Nichirei Histofine; Nichirei Co., Tokyo, Japan). Rabbit polyclonal antibodies to Col-1 (1:100; Rockland Inc., Gilbertsville, PA, USA), Runx2 (1:100; Abcam, Cambridge, UK), and mouse monoclonal antibodies to OPN (1:100; IBL, Gunma, Japan) were used. Briefly, deparaffinized sections were subjected to heated antigen retrieval using a PASCAL pressure jar (Dako, Glostrup, Denmark). The slides were incubated with each primary antibody overnight at 4°C. Then, the slides were incubated with the secondary antibodies (biotinylated antimouse or antirabbit immunoglobulin) and peroxidase-conjugated streptavidin for 30 minutes each. Finally, the reaction products were visualized using a diaminobenzidine staining kit (Dako) after the nuclei had been lightly counterstained with hematoxylin. The intensity of the immunoreactions to the Col-1, OPN, and Runx2 proteins within the interstitial tissue was graded semiquantitatively, as follows: negative (score 0); weakly positive (lightly stained but clearly differentiated from negative background: score 1); moderately positive (between weak and strong: score 2); and strongly positive (dark brown with high contrast: score 3). Scoring agreement between the two pathologists evaluating the samples was >85%.

**Statistical analysis**

Each histological and immunohistological parameter in the specimens was compared using Fisher's exact test. The significance level was set at \( p < 0.05 \). The SPSS software (version 16.0; SPSS, Chicago, IL, USA) was used to conduct the statistical analyses.

**Results**

The results were obtained for 15 patients, of which seven patients with elbow OCD were enrolled in the LIPUS group (mean age, 13.1 ± 1.6 years) and eight in the control group (mean age, 13.0 ± 1.2 years). There were no statistically significant differences between the two groups with respect to age, length of time from onset of symptoms to surgery, MRI findings, or ICRS OCD classification (Table 1).

**Histological evaluation with hematoxylin and eosin and alcian blue staining**

All specimens were evaluated histologically; however, in the control group, one patient with bone fragments in the joint (ICRS OCD Grade 4) was only evaluated for subchondral bone. For the remaining 14 patients (LIPUS group, 7 cases; control group, 7 cases), the recovered cylindrical tissue samples, consisting of cartilage and subchondral bone, were assessed. In both groups, histological inspection of the cartilage (discontinuity and fibrosis of cartilage, cloning of chondrocytes) revealed similar results. Fibrosis of the cartilage was found in most of the specimens (Grade 1). Discontinuity of the cartilage and cloning of chondrocytes (Grade 1 or 2) was also observed in most of the specimens. There were no statistically significant differences in the cartilage, by histological inspection, between the two groups (discontinuity of the

**Table 1**

Demographic data and characteristics of study sample patients.

|                      | LIPUS (n=7) | Control (n=8) | \( p \) |
|----------------------|-------------|--------------|--------|
| Mean age, y (range)  | 13.1 (11–16) | 13.0 (12–15) | 0.95   |
| Mean length of time from onset of symptoms to surgery, mo (range) | 19.9 (2–48) | 12.6 (2–36) | 0.33   |
| MRI classification   |             |              | 0.71   |
| Grade 1              | 0           | 0            |        |
| Grade 2              | 0           | 1            |        |
| Grade 3              | 6           | 7            |        |
| Grade 4              | 1           | 0            |        |
| ICRS OCD             |             |              | 0.67   |
| Grade 1              | 1           | 1            |        |
| Grade 2              | 5           | 4            |        |
| Grade 3              | 1           | 2            |        |
| Grade 4              | 0           | 1            |        |

ICRS OCD = International Cartilage Repair Society Osteochondritis Dissecans classification; LIPUS = low-intensity pulsed ultrasound; MRI = magnetic resonance imaging classification according to the Nelson staging system.

\(^{a}\) Mann–Whitney \( U \) test.

\(^{b}\) Fisher's exact test.
cartilage surface, cloning of chondrocytes, and fibrosis of the cartilage matrix: $p > 0.99$, $p > 0.99$, and $p = 0.46$, respectively. Necrosis of the subchondral bone was also found in many specimens from both groups, and there were no statistically significant differences between the two groups ($p = 0.20$). Osteoid formation, osteoblast, and osteoclast in the reparative tissue were also identified around the separation site in most specimens from both groups (Fig. 2A and B). Although five of the seven LIPUS specimens and two of the eight control specimens were Grade 2, with regard to osteoid

Table 2
Histological and immunohistochemical findings of osteochondritis dissecans cylindrical specimens.

| Age (y) | LIPUS (d) | MRI | ICRS OCD | Discontinuity of the cartilage surface | Cloning of chondrocytes | Fibrosis of the cartilage matrix | Necrosis of subchondral bone | Osteoid formation | Cartilaginous metaplasia | Col-1 | OPN | Runx2 |
|---------|-----------|-----|----------|--------------------------------------|------------------------|-------------------------------|----------------------------|-----------------|--------------------------|-------|-----|------|
| 1       | 13        | 17  | 2        | 1                                    | 2                      | 2                             | 1                          | 1               | 1                        | 2     | 2   | 3    |
| 2       | 14        | 13  | 3        | 2                                    | 1                      | 1                             | 1                          | 1               | 1                        | 2     | 2   | 2    |
| 3       | 16        | 11  | 3        | 2                                    | 2                      | 1                             | 1                          | 1               | 1                        | 2     | 2   | 3    |
| 4       | 11        | 14  | 3        | 2                                    | 2                      | 1                             | 1                          | 2               | 1                        | 2     | 1   | 1    |
| 5       | 12        | 27  | 3        | 2                                    | 0                      | 2                             | 1                          | 1               | 1                        | 2     | 2   | 2    |
| 6       | 12        | 12  | 3        | 2                                    | 2                      | 1                             | 1                          | 1               | 0                        | 0     | 2   | 0    |
| 7       | 14        | 12  | 4        | 3                                    | 1                      | 2                             | 2                          | 2               | 2                        | 2     | 3   | 1    |
| 8       | 15        | —   | 2        | 1                                    | 2                      | 2                             | 1                          | 1               | 1                        | 2     | 0   | 0    |
| 9       | 12        | —   | 3        | 2                                    | 0                      | 1                             | 1                          | 0               | 1                        | 2     | 2   | 1    |
| 10      | 12        | —   | 3        | 2                                    | 1                      | 1                             | 0                          | 0               | 2                        | 2     | 1   | 0    |
| 11      | 14        | —   | 3        | 2                                    | 2                      | 1                             | 1                          | 2               | 1                        | 2     | 0   | 1    |
| 12      | 13        | —   | 3        | 2                                    | 2                      | 1                             | 1                          | 1               | 1                        | 2     | 0   | 2    |
| 13      | 12        | —   | 3        | 3                                    | 1                      | 1                             | 1                          | 0               | 1                        | 2     | 0   | 0    |
| 14      | 14        | —   | 3        | 3                                    | 2                      | 0                             | 0                          | 1               | 1                        | 2     | 2   | 0    |
| 15      | 12        | —   | 3        | 4                                    | —                      | —                             | —                          | —               | —                        | 1     | 1   | 2    |

Col-1 = type 1 collagen; ICRS OCD = International Cartilage Repair Society osteochondritis dissecans classification; LIPUS = low-intensity pulsed ultrasound; MRI = magnetic resonance imaging, according to the Nelson staging system (15); OPN = osteopontin.

a Grade of histological findings: 0 = no or minimal change; 1 = mild or focal; 2 = marked or diffuse.

b Grade of immunohistochemical findings: 0 = negative; 1 = weakly positive; 2 = moderately positive; 3 = strongly positive.
Fig. 3. Immunohistochemical findings of cylindrical specimens. (A, B) Type 1 collagen. (A) LIPUS group (Case 2); (B) control group (Case 14). (C–F) Osteopontin. (C, D) LIPUS group (positive; Case 5); (E, F) control group (negative; Case 11). (G–J) Runx2. (G, H) LIPUS group (positive; Case 3); (I, J) control group (negative; Case 13). Separation site (*), reparative tissue (dagger) and high-power field (square). LIPUS = low-intensity pulsed ultrasound.
formation, a statistical difference between the two groups was not detected \( (p = 0.063) \). Similarly, there was no significant difference between the two groups with respect to the extent of cartilaginous metaplasia in the reparative tissue \( (p = 0.61; \text{Fig. 2C and D; Table 2}) \).

**Col-1, OPN, and Runx2 immunohistology**

In the 15 cylindrical osteochondral specimens, Col-1 immunostaining was found to be widespread in the collagen fibers of the reparative tissue, below the separation level (Fig. 3A and B). There were no statistical differences in the extent of Col-1 in the interstitial tissue between the two groups \( (p = 0.28) \). OPN was found in the fibroblasts and metastatic chondrocytes in the reparative tissue, which is located in the subchondral bone near the separation level (Fig. 3C-F). Runx2 was expressed in the capillary endothelial cells, in some of the osteocytes and fibroblasts, and in the regenerative chondrocytes located in the subchondral bone (Fig. 3G-J). OPN expression tended to be significantly higher in LIPUS patients than in control patients \( (p = 0.04) \). Although Runx2 expression was seen in six of the seven LIPUS specimens and three of the eight control specimens, there was no significant difference between the two groups with regard to Runx2 expression \( (p = 0.21) \) (Table 2).

**Discussion**

In this study, the effect of LIPUS on elbow OCD healing was assessed histologically and immunohistochemically. There were no statistically significant differences observed between the groups with respect to the histological evaluations of the cartilage, subchondral bone, reparative tissue around the separation level, or with respect to the amount of osteoblast transcription factors that were detected immunohistochemically. However, the osteoblast transcription factor, OPN, was expressed to a significantly greater extent in the repair tissue \( \text{OPN, was expressed to a significantly greater extent in the} \) than in control patients \( (p = 0.04) \). Although Runx2 expression tended to be expressed to a higher frequency \( (6 \text{ out of 7 specimens}) \) in the interstitial tissue of the subchondral bone as a result of LIPUS stimulation, as compared to control tissues. Although many kinds of cytokines and transcription factors participate in bone regeneration and remodelling, the increase in Runx2 appeared to be a key factor in osteoblast differentiation and may support the healing response in elbow OCD.

The present study has several limitations. First, the number of patients in both groups was small and inhibited the identification of differences between the groups, potentially leading to false conclusions. In particular, Runx2 also tends to be expressed to a higher frequency in the interstitial tissue of the subchondral bone as a result of LIPUS stimulation than in the control group, but there were no statistically significant differences. Second, the LIPUS irradiation period was short and may not have been enough to demonstrate LIPUS effects, histopathologically. Third, expressions of Col-1, OPN, and Runx2 were not quantified by in situ hybridisation or real-time polymerase chain reaction. Fourth, OPN has several roles and is involved in mineralisation, bone remodelling, and immune function. Therefore, upregulation of OPN by LIPUS irradiation is not necessarily an indication of accelerated bone remodelling in patients with elbow OCD. Other markers of osteoblast differentiation, such as bone sialoprotein and osteocalcin, and transcription factors such as osterix, should also be assessed to provide evidence that LIPUS irradiation is associated with bone remodelling in elbow OCD. Fifth, the immunohistochemical evaluation was not compared prior to and after irradiation in the same LIPUS-treated patient, owing to the nature of the harvesting procedure. Finally, it remains unclear up to what extent LIPUS may shorten the healing process. Although LIPUS may have resulted in an increased expression of OPN in tissue specimens from elbow OCD patients, it is unknown whether OPN increased the bone formation rate. Although this study had these limitations and our results did not reveal the effectiveness of LIPUS for elbow OCD, if LIPUS has some positive effect on osteogenesis in elbow OCD patients, it may be added to conventional therapy to accelerate the healing process, as there were no adverse effects on the epiphyseal cartilage. In conclusion, there were no statistical differences between the LIPUS and control groups with regard to the histological observations. In the immunohistological evaluation, OPN expression was significantly increased by LIPUS stimulation.
Conflicts of interest

There are no conflicts of interest to declare.

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