Histologic Evaluation of Osteochondral Loose Bodies and Repaired Tissues After Fixation

Yoriko Touten, M.D., Nobuo Adachi, M.D., Masataka Deie, M.D., Nobuhiro Tanaka, M.D., and Mitsuo Ochi, M.D.

Purpose: The purpose of this study was to evaluate histologic changes in osteochondral loose bodies in the rabbit knee joint and histologic changes in repaired tissue after fixation of osteochondral loose bodies following isolation periods of varying length. Methods: We harvested osteochondral fragments from the patellar groove in rabbit knee joints and left them in the lateral gutters of the joints for periods of varying duration to create osteochondral loose bodies. We then evaluated histologic and immunohistochemical changes within these loose bodies. Next, we fixed osteochondral loose bodies that had been isolated for various periods within the joints to the osteochondral defect in the patellar groove. Twelve weeks after fixation, repaired tissues were evaluated histologically and immunohistochemically, and results were analyzed according to the varying isolation periods of fragments. Results: Extracellular matrix and type II collagen expression of osteochondral loose bodies deteriorated with increased duration of fragment isolation periods. A significantly negative correlation was noted between length of isolation periods and histologic grading scores. After osteochondral loose bodies had been fixed, repaired tissues deteriorated significantly in accordance with duration of fragment isolation periods. However, in some cases, even when osteochondral fragments had been isolated for 12 weeks, repaired tissues showed dense extracellular matrix stained by safranin O and abundant type II collagen expression, which indicated regeneration of the cartilage layer. Conclusions: Osteochondral loose bodies and repaired tissues deteriorated after they were fixed to osteochondral defects. Although a direct correlation was noted between isolation periods of fragments and time to their deterioration, some osteochondral loose bodies showed regeneration of cartilage after fixation. Clinical Relevance: Clinically, reduction of osteochondral loose bodies should be performed as early as possible, if these can be found. However, even if the fragment seems to be old, fragment fixation is worthy of consideration. Key Words: Articular cartilage—Cartilage repair—Osteochondral loose body—Fixation—Histologic evaluation.

Fixation of an osteochondral loose body to an osteochondral defect has often been performed in the treatment of patients with osteochondral fracture or osteochondritis dissecans (OCD), if the condition of the osteochondral loose body was considered adequate for fixation. However, controversy continues regarding the efficacy of this approach, because whether the osteochondral fragment can be united or not may depend largely on the quality of the fragment. Some reports have shown that the cartilage layer in osteochondral loose bodies that had been isolated for a long time deteriorated over time, and fragments calcified. In 1977, Milgram et al. reported that degenerative calcification appeared to occur in all osteochondral loose bodies from 119 different patients that remained free in a joint for longer than 3 weeks. Pei et al. reported that expression of collagen type III was identified in all 10 loose bodies in osteoarthritis, suggesting dedifferentiation of chondrocytes within loose bodies. Attarian and Guilak reported a case of 2 osteochondral loose bodies in a patient’s knee over the course of a decade. They found that loose bodies...
possessed a large number of viable cells and had undergone growth encapsulation of the niduses through multiple layers of fibrocartilaginous tissue. It is clear that fixation of degenerated loose bodies is not adequate for repair of articular cartilage.

To date, no reports have evaluated postoperative histologic differences between repaired tissues after fixation of loose bodies that had been isolated in the joint for various periods. The purposes of this study were to evaluate the histologic changes that occurred in osteochondral loose bodies isolated for various periods and to evaluate changes seen in repaired tissue after osteochondral defects in the rabbit knee joint were fixed. The hypothesis of this study was that, although histologic features of isolated osteochondral loose bodies and repaired tissues deteriorated after they had been fixed to osteochondral defects, in accordance with the duration of isolation periods of the fragment, some osteochondral loose bodies may show regeneration of cartilage after fixation.

METHODS

Twenty-eight experimental male Japanese White rabbits (12 to 13 weeks old, weighing 2.5 to 3.0 kg) that were used in this study were kept in the research facilities for laboratory animal science at our university. The research protocol of this experiment was reviewed and approved by the ethical committee at the university.

Production of Osteochondral Loose Bodies

Surgery was performed on rabbits under combined general anesthesia through the intravenous administration of sodium pentobarbital solution (30 mg/kg body weight, Nembutal; Dainippon Pharmaceutical, Osaka, Japan) and intramuscular injection of ketamine hydrochloride (2 mg/kg body weight, Ketalar; Sankyo-Yell Pharmaceuticals, Tokyo, Japan). Both knee joints were opened through medial parapatellar incisions. The patella was dislocated laterally to expose the patellar groove. An osteochondral fragment (width, 6 mm; length, 6 mm; thickness, 2 mm) was harvested from the patellar groove with a chisel and was placed in the lateral gutter of the knee joint to make it a loose body. This osteochondral fragment, the thickness of the cartilage layer was about 0.4 mm. Loose bodies were harvested immediately after surgery and then at 3, 6, and 12 weeks after surgery (normal, 3w group, 6w group, and 12w group, respectively). If adhesion between the fragment and the joint capsule was observed, the fragment was excluded from the evaluation. After this exclusion, 6 loose bodies from 6 knees were evaluated macroscopically and histologically.

After macroscopic observation, osteochondral loose bodies were fixed with 10% buffered formalin for 1 day. Each specimen was decalcified with 0.25 M ethylenediaminetetraacetic acid in phosphate buffered saline (PBS) at pH 7.5, dehydrated in graded alcohols, and embedded in paraffin wax. Specimens were cut sagittally into 5-μm-thick sections, and the center areas of the fragments were stained with safranin O/fast green. Histologic sections of the fragments were examined with the histologic grading scale described by Mankin et al. The examiner (one of the authors) was an orthopaedic surgeon who was familiar with the grading system. He was not provided with information on the source group of the fragment. In this grading system, the cartilage of the loose body is evaluated for (1) structure (0 to 6 points), (2) cells (0 to 3 points), (3) safranin O staining (0 to 4 points), and (4) tidemark integrity (0 to 1 points) (Table 1).

Expression of type II collagen within loose bodies was analyzed immunohistochemically. Sections (5 μm) were cut, air dried, deparaffinized, rehydrated, and incubated for 3 min in PBS. To abolish endogenous peroxidase activity, sections were incubated with 0.3% H2O2 in methanol for 10 min. After 3 washes in PBS with 0.2% Tween 20 at pH 7.4, sections were enzymati-

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<th>Table 1. Histologic Grading Scale*</th>
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<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>
</tr>
<tr>
<td>d. Clefs to transitional zone</td>
</tr>
<tr>
<td>e. Clefs to radial zone</td>
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<tr>
<td>f. Clefs to calcified zone</td>
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<tr>
<td>g. Complete disorganization</td>
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<tr>
<td>II. Cells</td>
</tr>
<tr>
<td>a. Normal</td>
</tr>
<tr>
<td>b. Diffuse hypercellularity</td>
</tr>
<tr>
<td>c. Cloning</td>
</tr>
<tr>
<td>d. Hypocellularity</td>
</tr>
<tr>
<td>III. Safranin O staining</td>
</tr>
<tr>
<td>a. Normal</td>
</tr>
<tr>
<td>b. Slight reduction</td>
</tr>
<tr>
<td>c. Moderate reduction</td>
</tr>
<tr>
<td>d. Severe reduction</td>
</tr>
<tr>
<td>e. No dye noted</td>
</tr>
<tr>
<td>IV. Tidemark integrity</td>
</tr>
<tr>
<td>a. Intact</td>
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<tr>
<td>b. Crossed by blood vessels</td>
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*Modified from the scale described by Mankin et al.15

In this grading system, zero points are given for normal articular cartilage.
cally digested with testicular hyaluronidase type VIII (0.2% in PBS, pH 7.4) for 30 min at 37°C. Sections were then incubated overnight in a 1:100 dilution of mouse monoclonal anti-rabbit type II collagen antibody (Oncogene Research Products, San Diego, CA) at 4°C. Immunoreactivity was detected through serial incubation of sections with biotinylated goat anti-mouse antibody, followed by streptavidin-horseradish peroxidase (LASB kit; Dako, Hamburg, Germany). The signal was developed as a brown reaction product with the use of peroxidase substrate 3, 3’-diaminobenzidine, and H2O2. Sections were counterstained with Harris hematoxylin and were dehydrated, cleared, and mounted.

Fixation of Osteochondral Loose Bodies to the Osteochondral Defect

We produced osteochondral loose bodies through the procedure described earlier. If adhesion between the fragment and the joint capsule was observed, the fragment was excluded. After this exclusion, 18 knees in 9 rabbits were randomly divided into 3 groups. Six loose bodies from 6 knees were harvested in each group at 3 weeks, 6 weeks, and 12 weeks after surgery (3w group, 6w group, and 12w group, respectively). We then fixed the loose bodies to the osteochondral defect on the patellar groove after we performed curettage at the base of the defect with a single poly-L-lactate pin (diameter, 2 mm; length, 10 mm; Zimmer, Tokyo, Japan). After the operation had been completed, all animals were allowed to walk freely without cast fixation. Rabbits were sacrificed at 12 weeks after fixation through intravenous injection of a fatal dose of sodium pentobarbital. In the remaining 6 knees, the same osteochondral defect was created and was left untreated for 24 weeks. After 24 weeks, tissues in the defect were evaluated histologically and immunohistochemically, as described earlier (defect group). Knees were assessed for contractures and adhesions, and the surfaces of repaired tissue were inspected for color, integrity, contour, and smoothness. Degree of synovitis was evaluated according to the assessment protocol suggested by Grande et al. (Table 2).16

Immunohistochemical Staining

For immunohistochemical evaluation of the expression of type II collagen in repaired tissue, sections were deparaffinized, rehydrated in 3% H2O2 in ethanol, and incubated in testicular hyaluronidase (0.2% in PBS, pH 7.5) for 30 minutes at room temperature (RT). Sections were dipped in goat serum for 10 minutes and were then stained with antibodies to type II collagen. Secondary antibody was biotinylated goat anti-mouse (Histofine; Nichirei, Tokyo, Japan). Samples incubated in biotinylated secondary antibody were then incubated in avidin/peroxidase complex (Histofine) for 60 minutes. Slides were washed in PBS and were contrasted with a solution of 0.05% diaminobenzidine (Dojindo, Kumamoto, Japan) in PBS with 0.009% H2O2.

Statistical Analysis

All statistical analyses were conducted with Statview 5.0 (SAS Institute, Cary, NC). All data are shown as mean ± standard error of the mean (SEM). Histologic scores of loose bodies and repaired tissue after fixation of each group were statistically analyzed by means of analysis of variance (ANOVA) with Bonferroni/Dunn post hoc comparison. P values less than .05 were regarded as statistically significant.

**Table 2. Grading of Synovitis**

<table>
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<tr>
<th>Degree</th>
<th>Description</th>
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<tr>
<td>Absent</td>
<td>No synovitis</td>
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<tr>
<td>Mild</td>
<td>25%-50% of the synovium exhibits inflammation with a predominantly red color.</td>
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<tr>
<td>Moderate</td>
<td>Inflamed area covers 50%-74% of the total synovial surface.</td>
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<tr>
<td>Severe</td>
<td>75% or more of the total synovial surface exhibits synovitis, and osteophyte formation is found on the femoral condyles or the patella.</td>
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RESULTS

Evaluation of Osteochondral Loose Bodies

One osteochondral fragment each in the 6w group and the 12w group was excluded from this study because of adhesion between the fragment and the joint capsule.

Macroscopic Findings

Osteochondral loose bodies from the 3w group showed slight fibrillation and erosion of the cartilaginous surface. However, no ulceration of the cartilage was seen. The brilliance and smoothness of the surface were lost slightly. In 2 fragments from the 6w group, fibrillation and erosion of the cartilage were observed in some areas. The other 4 fragments from the 6w group showed full-thickness ulceration, and the borderline between the cartilage and the subchondral bone was clear. In the 12w group, overt calcification was seen in the cartilage layer and in subchondral cancellous bone. The borderline between them was unclear in all fragments.

Histologic Findings

Histologically, the layered structure of normal cartilage deteriorated over time. Normal articular cartilage forms several layers, which consist of a superficial layer, a middle layer, a deep layer, a tidemark, a calcified layer, and subchondral bone (Fig 1A). Although the cartilage in the 3w group consisted of all layers seen in normal cartilage, fibrous cartilage covered the cartilaginous surface, borders between layers were unclear, and cohesive cells within the deep layers were reduced compared with normal cartilage. The tidemark and the calcified layer were clear, as in normal cartilage (Fig 1B). In the 6w group, a thick layer of fibrous tissue covered the cartilaginous layer, and cell density was sparser than in the 3w group. The border of each layer was unclear, and the extracellular matrix stained by safranin O was significantly decreased (Fig 1C). In the 12w group, almost no cartilage layer was observed (Fig 1D). Average histologic scores for each group were zero for normal cartilage, 4.7 ± 0.7 in the 3w group, 7.7 ± 0.5 in the 6w group, and 12 ± 1.0 in the 12w group; statistically significant differences were noted between groups ($P < .01$) (Fig 2).

Immunohistochemical Expression of Type II Collagen

Histologically, normal hyaline cartilage matrix was stained strongly by safranin O, and strong expression of type II collagen was detected immunohistochemically (Fig 3A). In the cartilage layer in the 3w group, abundant expression of type II collagen was seen (Fig 3B). However, the extent of the stained area and the intensity of type II collagen expression decreased significantly in the 6w group (Fig 3C). At 12 weeks after surgery, no stained areas could be detected in the fragments (Fig 3D).

Evaluation of Repaired Tissue After Fixation

When osteochondral loose bodies were produced before fixation of the fragment, 1 fragment in the 6w group and 2 fragments in the 12w group were excluded from this study because of adhesion between the fragment and the joint capsule.

Macroscopic Findings

When sacrificed, all models had a thickened joint capsule with slight adhesion to subcutaneous tissue and
retinaculum. However, no contracture and no apparent synovitis were observed in any of the rabbits. The border between transplanted osteochondral loose bodies and normal articular cartilage was distinguishable by a dent or a change in color between them. In general, the surface regularity of the repaired tissue deteriorated according to the isolation period of the osteochondral loose bodies, that is, the longer the fragment was isolated in the joint, the worse were the macroscopic findings observed.

**Histologic Findings**

The extracellular matrix of repaired tissue in the 3w group was well stained by safranin O. However, a thin layer of fibrous tissue covered its surface. Cell density in the middle and deep layers was less than that of normal cartilage (Fig 4A). In 4 knees in the 3w group, the thickness of the cartilage layer was greater than two thirds the thickness of normal articular cartilage, although in the other 2 knees, the thickness of the cartilage layer was only one half to two thirds that of normal articular cartilage. In the 6w group, more fibrous tissue covered the cartilage layer, and cell density was sparse. The extracellular matrix stained by safranin O was decreased. In 4 knees in the 6w group, the cartilage layer of the repaired tissue had deteriorated severely compared with that in the 6w group, showing surface irregularity or decreased thickness of the cartilaginous layer (Fig 4C). However, it is interesting to note that in 2 knees of the 12w group, hyaline cartilage–like regeneration was observed, although the cartilage layer was thinner than normal cartilage (Fig 5). In the cartilage defect group, almost no repaired tissue could be seen.

**Figure 1.** Histologic findings of loose bodies. (A) Normal cartilage: Several layers, consisting of a superficial layer, a middle layer, a deep layer, a tidemark, a calcified layer, and subchondral bone, were observed. (B) 3w group: Fibrous cartilage covered the cartilaginous surface. The layered structure was unclear, and cohesive cells in the deep layers were less firm than in normal cartilage. (C) 6w group: Thick fibrous tissue covered the cartilage layer, and cell density was sparse. The extracellular matrix stained by safranin O was decreased. (D) 12w group: Almost no cartilage layer was observed (safranin O/fast green staining, original magnification ×50).

**Figure 2.** Histologic grading scores of loose bodies. Average histologic scores were zero for normal cartilage, 4.7 in the 3w group, 7.7 in the 6w group, and 12 in the 12w group; statistically significant differences were noted between groups.
Average scores were 5.0 ± 0.9 in the 3w group, 6.9 ± 0.6 in the 6w group, 9.9 ± 0.6 in the 12w group, and 13.5 ± 0.2 in the cartilage defect group; significant differences were noted between groups ($P < .01$) (Fig 6).

**Expression of Type II Collagen in Repaired Tissue**

Abundant type II collagen expression was observed in the cartilage layer of the 3w group (Fig 7A). The area and intensity of staining for type II collagen decreased in the 6w group and the 12w group (Fig 7B, 7C). In the 12w group, although we detected no stained area in 2 of the fragments and only a little expression in 2 fragments, abundant expression was observed in 2 fragments (Figs 5, 7C). No type II collagen expression was observed in the defect group (Fig 7D).

**DISCUSSION**

Fixation of an osteochondral loose body to a cartilage defect has been performed to treat patients with osteochondral fracture or OCD. Fixation of an osteochondral lesion can preserve the normal contour of the distal femur. Recently, bioabsorbable pins or screws have frequently been used for fixation of the osteochondral fragment. To date, favorable results have been reported after fixation of unstable OCD or osteochondral lesions. In 1998, Dervin et al. treated 9 patients with unstable OCD lesions with the use of internal fixation with polylactic acid rods. Investigators reported that 8 fragments united radiographically, whereas 1 loose body remained ununited at 26 months after fixation. Recently, Nakagawa et al. reported clinical results of 8 knees in 7 patients with OCD, including 5 loose bodies treated by internal fixation of the fragments. At a mean follow-up of 5 years, 7 fragments were united, whereas 1 loose body lesion was not united; arthroscopic removal of the fragment was required. It is reasonable to consider that whether the osteochondral loose body can be united or not may depend largely on the viability of the fragment. Therefore, it is essential that the changes that occur in osteochondral loose bodies are understood.

In 1977, Milgram et al. investigated osteochondral loose bodies from 119 different patients. They showed that the phenomena of proliferation of new layers of bone and cartilage, surface resorptive activity, and degenerative calcification appeared to occur in all osteochondral loose bodies that remained free within the sy-
Joint fluid in the joint cavity for longer than 3 weeks. Pei et al.,13 performed collagen typing of 10 loose bodies related to osteoarthritis, although the pathology of loose bodies in osteoarthritis is different from that in OCD or osteochondral fracture. Investigators reported that collagen type III expression was detected in all 10 loose bodies. Expression was located primarily in cartilage, as well as in a layer of fibrous tissue on the surface, suggesting dedifferentiation of chondrocytes within loose tissue.

![Figure 1](image1)

**Figure 1.** Histologic findings in repaired tissue. (A) In the 3w group, thin fibrous tissue covered the surface of repaired tissue, and cell density seen in the middle and deep layers was reduced compared with that in normal cartilage. (B) In the 6w group, more fibrous tissue covered the surface of repaired tissue, and cell density was less than in the 3w group. (C) In the 12w group, the cartilage layer of repaired tissue had deteriorated severely compared with that in the 6w group; surface irregularity or decreased thickness of the cartilaginous layer was noted. (D) In the cartilage defect group, virtually no repaired tissue could be seen. In each figure, the arrow shows the border between normal cartilage and repaired tissue. The right side of the arrow indicates normal cartilage, and the left side of the arrow shows repaired tissue (original magnification ×50).

![Figure 2](image2)

**Figure 2.** Two of the repaired tissues showed good histology after loose bodies that had been isolated in the joint for 12 weeks were fixed; extracellular matrix was abundant. (A) Safranin O/fast green staining (original magnification ×50). (B) Immunohistochemical staining for type II collagen (original magnification ×50). In each figure, the arrow indicates the border between normal cartilage and repaired tissue. The area between the arrows shows repaired tissue.

![Figure 3](image3)

**Figure 3.** Histologic grading scores of repaired tissue. Average scores were 5.0 ± 0.9 in the 3w group, 6.9 ± 0.6 in the 6w group, 9.9 ± 0.6 in the 12w group, and 13.5 ± 0.2 in the cartilage defect group; significant differences between groups were observed.
bodies. Attarian and Guilak\textsuperscript{14} reported 2 osteochondral loose bodies in a patient’s knee over the course of a decade. They found that loose bodies possessed a large number of viable cells and had undergone growth encapsulation of the niduses in multiple layers of fibrocartilaginous tissue. Thus, several former studies on osteochondral loose bodies showed degenerative and proliferative changes within the loose bodies. This study clearly showed that extracellular matrix stained by safranin O and characterized by expression of type II collagen within the loose bodies decreased significantly over time, and no or almost no cartilage layer was apparent in the 12w group. Loose bodies isolated for longer periods were covered with fibrocartilaginous tissue and were calcified, that is, the longer the isolating period of the loose bodies lasted, the greater was the deterioration that was seen in the hyaline cartilage. Results are consistent with those of former studies\textsuperscript{11-14} and those recalled in the clinical experiences of the authors of the present study.

Even though several histologic evaluations of loose bodies have been performed, no study has investigated the histologic changes that occur after fixation of osteochondral loose bodies following isolation periods of varying duration. Our study shows that osteochondral loose bodies isolated for shorter periods had better histologic findings, that dense extracellular matrix was stained with safranin O, and that type II collagen expression was abundant. These results were reasonable and clinically compatible. Histologic scores in the 12w group were significantly better than those in the defect group. Therefore, if surgeons find an osteochondral loose body, it is clinically important that the fragment be fixed as early as possible so the best possible condition can be attained in the cartilage.

Our study also shows that some of the repaired tissue fixed to loose bodies that had been isolated in the joint for 12 weeks showed good histology, with abundant extracellular matrix (Fig 7). This finding is very interesting and suggests the possibility that an already deteriorated loose body may demonstrate cartilage regeneration. We have no apparent explanation for these findings; however, it is possible that deteriorated cartilage within the loose bodies could regenerate through normal biomechanical conditions, such as weight bearing or range of motion of the joint.

Although fixation of osteochondral loose bodies requires several clinical studies, the procedure is worthy of consideration, even if the surgeon finds an osteochondral fragment that may have been isolated in the joint for longer periods. It is believed that preoperative or postoperative knowledge of cartilage viability within loose bodies is very important. However, because we did not
investigate the relationship between cartilage viability in loose bodies and the histologic findings of repaired tissue after fixation, further study will be necessary to clarify this matter.

We recognize several limitations in this study. Because we produced osteochondral loose bodies and fixed them to osteochondral defects with the use of young rabbits (12 to 13 weeks old), this was an experimental model of acute osteochondral fracture. The pathology of an OCD lesion is different from that seen in an acute fracture, and the regeneration potential may differ according to various ages and species. Therefore, we must be very careful in adapting the results obtained in this study to the occurrence of OCD lesions in humans of various ages. Another limitation is the fact that most evaluations, including macroscopic and histologic evaluations, were based on objective findings alone. These were not quantified, although we used a semiquantitative grading score when conducting histologic evaluations. Interobserver and intraobserver differences were not investigated.

Another shortcoming of this study was that investigators did not directly compare histologic findings in the cartilage layer of osteochondral loose bodies with those in repaired tissue after fixation to the osteochondral defects was performed. Evaluation of cartilaginous layers before and after fixation within the same specimens should provide more convincing information about cartilage regeneration of osteochondral loose bodies. We also investigated histologically and immunohistochemically osteochondral loose bodies and repaired tissue after fixation was performed. Additional biomechanical or biologic studies, including quantitative analyses, are definitely needed. Another weak point of this study is that investigators evaluated the histology of repaired tissue after fixation to the osteochondral defects with the use of young rabbits (12 to 13 weeks old), this was an experimental model of acute osteochondral fracture. The hypothesis of this study was supported by its results: Although the histologic findings of isolated osteochondral loose bodies and repaired tissue after fixation to the osteochondral defects deteriorated in accordance with the duration of isolation periods of the fragment, some osteochondral loose bodies showed regeneration of cartilage after fixation.

CONCLUSIONS

The hypothesis of this study was supported by its results: Although the histologic findings of isolated osteochondral loose bodies and repaired tissue after fixation to the osteochondral defects deteriorated in accordance with the duration of isolation periods of the fragment, some osteochondral loose bodies showed regeneration of cartilage after fixation.

REFERENCES