Chondrocyte Death and Cartilage Degradation After Autologous Osteochondral Transplantation Surgery in a Rabbit Model

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Background: Autologous osteochondral transplantation surgery requires an impact force on the graft that may cause chondrocyte death and matrix degradation. This study attempted to determine the degree to which this occurs in a rabbit model shortly after the procedure.

Hypothesis: Impaction of a press-fit autologous osteochondral graft in vivo results in chondrocyte necrosis, apoptosis, and matrix degradation at early time points.

Study Design: Controlled laboratory study.

Methods: Twenty New Zealand White rabbits underwent unilateral osteochondral transplantation (OT) surgeries, and 10, bilateral sham surgeries. Fifteen animals were sacrificed at time zero (10 sham-0 limbs, 10 OT-0 limbs), and 15, 4 days after surgery (10 sham-4 limbs, 10 OT-4 limbs). Chondrocyte viability/necrosis was determined with cell vital staining. Chondrocyte apoptosis was determined by TUNEL, Bcl-2, and M30 assays. Cartilage matrix degradation was determined by routine light and polarized light microscopy and COL2-3/4C$_{short}$ immunohistochemistry. Statistical analysis was performed with a 2-way analysis of variance ($P < .05$).

Results: There were significantly fewer viable cells in OT-4 than in sham-4. A similar difference in cell viability was found in OT-0 versus sham-0. There were more TUNEL-positive cells in OT-4 as compared with OT-0, sham-0, and sham-4; however, there was little or no staining of Bcl-2 and M30. Mankin scores were higher in both OT groups versus both sham groups at time zero and day 4. The OT-4 group had positive staining for COL2-3/4C$_{short}$ that corresponded with a loss of collagen birefringence at the superficial zone.

Conclusion: Osteochondral transplantation procedures performed by tamping a press-fit graft induce chondrocyte necrosis and matrix metalloproteinase–mediated cartilage matrix degradation. However, apoptosis was not found to a major contributor to cell death in this model.

Clinical Relevance: Results of osteochondral transplantation procedures may be improved by atraumatic insertion and fixation techniques or by pharmacologic agents that can block these degradative processes.

Keywords: osteochondral transplantation; chondrocyte death; apoptosis; cartilage degradation

Hyaline cartilage lacks the capacity to regenerate, and few therapeutic options are available for patients with full-thickness cartilage defects. Nonoperative measures can be effective in controlling symptoms of pain and swelling, but they are unable to cure the underlying cause. There are no disease-modification treatments to date that reverse the course of cartilage degradation. For that reason, the treatment of isolated full-thickness cartilage defects in relatively young patients requires surgical manipulation of the local environment to either induce cartilage growth or transplant healthy cartilage.

Autologous osteochondral transplantation surgery is a procedure that results in the filling of a full-thickness cartilage defect with normal articular cartilage.[37] The procedure involves transferring an osteochondral graft from a nonweightbearing region of the joint to the area with a chondral defect. Although this procedure may result in donor site morbidity, it is the only procedure that can

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reliably fill the defect with the patient's own articular cartilage. Several studies have documented the efficacy of this technique in animal and human models.[20,26,36] Longer-term data evaluating the clinical outcomes of this technique are emerging.[7,20,33] Although these studies are encouraging, little is known about the long-term viability of these grafts.

To obtain an optimal press fit, the graft must be tamped into the host bed. This process requires successive loads applied to the articular surface with a mallet and specially designed tamp. Several studies in the trauma literature have demonstrated that chondrocyte necrosis, apoptosis, and cartilage degradation are induced after a single acute traumatic load and after repetitive impact loads similar to those used during autologous osteochondral transplantation procedures.[3,5,6,9-11,26,27] The results of impaction on osteochondral grafts have also been studied in an in vitro human explant model. This study showed similar findings of chondrocyte death and apoptosis.[4] However, it is unclear if this occurs in vivo, given that the complex mechanical and biological environment of the synovial knee joint may influence these processes. An understanding of the cellular and molecular events that occur after osteochondral transplantation procedures may be useful to improve surgical techniques and to guide the development of pharmaceuticals that can preserve cartilage to optimize long-term results.

The aim of this project was to determine the extent and type of chondrocyte death (necrosis or apoptosis) and the extent of cartilage degradation after impaction of a press-fit autologous osteochondral graft in vivo—namely, in a rabbit model—at early time points. Our hypothesis was that this procedure would induce chondrocyte necrosis, chondrocyte apoptosis, and cartilage degradation.

MATERIALS AND METHODS

Determination of Impact Stresses for Graft Insertion

Approval for this study was obtained from our Institutional Animal Care and Use Committee. In a preliminary experiment, 5 lower limbs from New Zealand White rabbits were disarticulated from the hip and used to determine the peak stresses, peak forces, and number of tamps necessary for insertion of the osteochondral grafts. Five grafts were harvested from the medial femoral condyles and transplanted into a defect created in the lateral condyles of the same knee. A piezoelectric load transducer (22.2 kN, model 9212, Kistler, Amherst, New York) was rigidly attached to the hammer, and it was used to measure the insertion force applied to the graft. The cylindrical tamp (diameter, 2.7 mm) was inserted to the articular surface of the graft as it sat in the harvester, and the insertion force was applied with a hammer by a single surgeon, in a manner identical to that performed during the rabbit surgery. A high-speed data acquisition system was used to record the impact load and time at a 5-kHz sampling rate per channel. Insertion force was normalized to the graft area, and for each knee the stress versus time was plotted. The number of distinct peak stresses were counted and averaged. The data were reported as average stress and number of tamps before and after the graft was contacting the bottom of the defect, as well as total average stress and number of tamps.

Animal Model

Twenty New Zealand White rabbits underwent unilateral autologous osteochondral transplantation procedures (OT group), and 10 animals underwent bilateral sham surgeries (sham group). This study used an established model of autologous osteochondral transplantation surgery in the knees of skeletally mature male New Zealand White rabbits.[36] A rabbit model was employed for this study because the rabbit's knees are large enough to facilitate this procedure, the anatomy is well defined, and they have been used in previous studies.[5,28,31,34,36]

The rabbits were housed in individual cages and allowed ad libitum cage activity for 1 week before surgery. All rabbits received no food or water 12 hours before surgery. Anesthesia was induced with ketamine (40 mg/kg) and acetylpromazine (0.5 mg/kg) delivered subcutaneously in a single syringe. Anesthesia was then maintained via isoflurane inhalation through an endotracheal tube throughout the surgery. Animals received ampicillin (25 mg/kg) 30 minutes before surgery for infection prophylaxis. Bilateral lower extremities were shaved and sterilely prepared. A 4-cm medial parapatellar arthrotomy was created in each knee, exposing the medial femoral condyles. The fat pad was excised in all animals to improve exposure. At this point, the 10 animals assigned to the sham group underwent irrigation of their wounds, followed by closure in layers. Because these animals underwent bilateral procedures, this yielded 20 specimens.

The 20 animals assigned to the experimental group underwent osteochondral transplantation procedures. An osteochondral graft was harvested from the right medial femoral condyle with a mosaicplasty harvester (Smith & Nephew, Memphis, Tennessee). The graft was 2.7 mm in diameter and 4.0 mm deep. A full-thickness defect was created in the medial femoral condyle of the left knee with a drill, to the same dimensions of the harvested osteochondral graft, using standard disposable surgical instrumentation for osteochondral autograft procedures in humans. The defect was carefully debrided of any remaining cartilaginous remnants; the osteochondral graft from the right knee was tamped into the defect in the left knee; and the number of tamps were recorded to ensure that the technique was standardized across all specimens. Care was taken to ensure that the graft was tamped to a level in which the articular surface was the same height as that of the surrounding host cartilage (Figure 1). The goal was to seat the graft evenly with the host cartilage—that is, not deliver a standardized number of tamps or load. We thought that this objective best reflects the goals of surgery in patients. The defect in the right knee was not backfilled. The wounds were then irrigated and closed in layers.

Fifteen animals were sacrificed immediately after surgery using pentobarbital (100-150 mg/kg, intravenously) and thus served as time zero controls. The animals in the OT group underwent unilateral procedures, thus resulting in 10 OT-0 specimens (*0,* for time zero). Animals in the sham

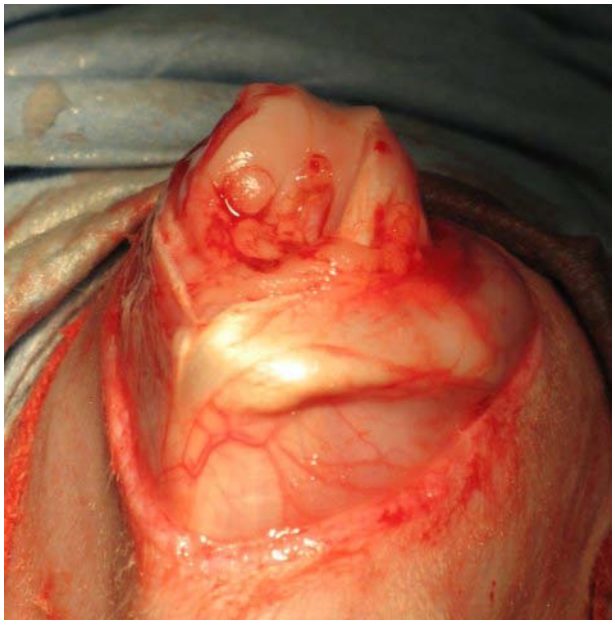

Figure 1. Surgical technique. Osteochondral grafts were harvested from the medial femoral condyle of the right knee with a 2.4-mm harvester to a depth of 4 mm. These grafts were then tamped into a defect of the same size that was created on the contralateral medial femoral condyle such that the articular cartilage of the graft was the same height as that of the surrounding host cartilage.

group underwent bilateral procedures; therefore, 5 animals yielded 10 sham-0 specimens (again, *0* for time zero). The remaining 15 animals were assigned to the 4-day sacrifice group (10 OT-4 and 10 sham-4 limbs). These animals were given buprenorphine (0.05 mg/kg, subcutaneously, every 8-12 hours as needed) for pain control for 3 days and allowed ad libitum cage activity. They were sacrificed at 4 days, as described above.

Histology

Specimen Harvest and Preparation. At the time of necropsy, sections of fresh cartilage approximately 20 µm thick were harvested from the osteochondral graft (or control cartilage from the medial femoral condyle) with a custom-made double-razor-blade device. The specimens included the entire depth of the articular cartilage down to the tidemark. These specimens were used for cell vital staining immediately upon harvest (see below). The remainder of the osteochondral plug was then removed en bloc from the femur, with an oscillating saw through the normal host cartilage down to the cancellous bone beneath the subchondral bone. The resulting block was 1 cm wide × 1 cm high × 1 cm deep. The specimen was fixed in 10% neutral buffered formalin at 4°C for 7 days before being decalcified in a sodium citrate–formic acid solution for another 7 days. These specimens were then embedded in paraffin. This is a well-established method of preparing osteochondral specimens in our laboratory.[34] No nondecalcified fragments were noted during sectioning. The specimens were cut into 5-µm-thick sections through the center of the osteochondral plug and mounted on Superfrost Plus coated slides (Fisher Scientific, St. Louis, Missouri). For all specimens, the 300 µm at the edge of the graft were omitted from analysis to minimize the possibility of attributing injury from the graft harvest to impaction.

Detection of Chondrocyte Necrosis

Cell Vital Staining. Cell viability was determined by a combination of propidium iodide, permeable only to cells with compromised membranes, and fluorescein diacetate, a metabolic dye for live cells. Sections of fresh cartilage were immediately immersed in 40 µg/ml of propidium iodide (Sigma-Aldrich, St. Louis, Missouri) and 1 µmol of fluorescein diacetate (Molecular Probes, Invitrogen, Carlsbad, California) for 20 minutes. The sections were then washed with phosphate-buffered saline and imaged with the use of a fluorescence microscope (Eclipse E800, Nikon, Melville, New York). Two observers manually counted the number of cells in the cartilage region (alive, dead, total) (L.G. and D.K.) and averaged them. The results were represented as a percentage of viable cells present (alive cells/total cells). The observers were blinded to the time point but not to whether the specimens underwent OT or sham surgeries, because they could easily obtain this information when looking at the slide.

Detection of Chondrocyte Apoptosis

Terminal dUTP Nick-end Labeling. Chondrocyte apoptosis was determined with terminal dUTP nick-end labeling (TUNEL), Bcl-2, and M30 assays. TUNEL assay was performed according to the manufacturer's protocol (In Situ Cell Death Detection Kit, Roche Applied Science, Indianapolis, Indiana). Fluorescein TUNEL labels DNA fragmentation that occurs in apoptotic and late-stage necrotic cells, and it emits a bright green signal when viewed with fluorescent microscopy. TUNEL-positive cells and total cells were counted to determine the percentage of TUNEL-positive cells. The slides were analyzed by 2 independent observers, and the results were averaged. Again, these observers were blinded to time point but not to the surgical group. Apoptotic cells in the bone marrow served as a positive control. Negative controls were performed using nonspecific antibodies. Because TUNEL can overestimate the amount of apoptosis, Bcl-2 and M30 immunostaining were performed to confirm the apoptosis.[41,47]

Bcl-2 and M30 Assays. Bcl-2 is an anti-apoptotic protein (Sigma-Aldrich) that is often overexpressed in company with apoptosis-related proteins, such as Bax and Fas, in the apoptotic cells or the cells adjacent to them; as such, it is commonly used to identify apoptosis-related events.[45] The M30 antibody (M30 CytoDEATH-FITC, Axxora, San Diego, California) was used for the early detection of apoptosis via its detection of a specific epitope of cytokeratin 18 that is present after cleavage by caspases. In both assays, specimens were deparaffinized with xylene and ethanol. The antigen was retrieved in 10 mmol/L heated citric acid buffer, then blocked with 1% bovine serum albumin. The specimens were then incubated with either Bcl-2 or M30 primary antibody working solution for 1 hour at room temperature,

incubated with fluorescein isothiocyanate–linked anti-mouse secondary antibody with DAPI (4,'6-diamidino-2-phenylindole) nucleus counterstaining. Slides were analyzed by 2 observers under fluorescent microscopy and subjectively graded as *strong*, *moderate*, *minimal*, or *no positive staining*. Again, the bone marrow with abundant apoptotic hematopoietic cells served as positive controls, and nonspecific antibodies were used for negative controls.

Determination of Cartilage Degradation

Conventional Light Microscopy (Mankin Score). Alternate slides were stained with hematoxylin and eosin (H&E) and safranin-O/fast green. The H&E and safranin-O slides were evaluated at magnification ×40 and ×100 (Eclipse E800) for cartilage degradation based on the Mankin score.[32] This score is based on a scale from 0 (normal) to 14 (total cartilage destruction). The H&E slides were used to determine the structure and cellularity of the cartilage, whereas the safranin-O slides were used to semiquantitatively evaluate proteoglycan content. Each slide was scored by 2 independent observers.

COL2-3/4C$_{short}$ Immunohistochemistry. Cleavage of type II collagen, as mediated by matrix metalloproteinase (MMP), was detected using the COL2-3/4C$_{short}$ antibody (Ibex Technologies, Montreal, Quebec, Canada).[29] The specimens were deparaffinized with xylene and washed in ethanol. The antigen was retrieved with incubation in 0.1% hyaluronidase and chondroitinase ABC (1 unit) for 30 minutes, then blocked with 10% normal goat serum and incubated with the primary COL2-3/4C$_{short}$ antibody overnight. The specimens were then incubated with a goat anti-swine IgG–fluorescein isothiocyanate secondary antibody for 30 minutes and mounted with DAPI mounting medium. The specimens were analyzed by 2 observers and subjectively graded as *none*, *minimal*, *moderate*, or *strong staining*.

Cartilage Surface Birefringence. The collagen organization of the cartilage surface was determined by examining safranin-O-stained slides under polarized light microscopy. The more organized the collagen fibrils, the more birefringence they exhibit under polarized light. When the organization of the cartilage collagen is disrupted, there is less birefringence. Analysis was made by rotating the polarization plane until maximum contrast was obtained, to control for variations in specimen orientation on the slide. To facilitate comparisons between groups, all tissues were embedded and cut in exactly the same orientation, with sections cut to a uniform thickness. The polarized images were captured with an Olympus BH-2 light microscope (Olympus Opticals, Lake Success, New York), interfaced to a charge-coupled device video camera mounted on the eyepiece tube. The images were then analyzed by 2 observers for birefringence and subjectively graded as *none*, *minimal*, *moderate*, or *strong*.

Justification for a 4-Day Time Point

A time point of 4 days was chosen because this study aimed to determine the metabolic pathway of cartilage deterioration shortly after osteochondral transplantation. For biologically programmed events, such as apoptosis and MMP-mediated collagen breakdown, a period of 4 days was deemed adequate, given that these events cannot often be seen immediately after the procedure. Likewise, these processes cannot be determined at longer time points, because the body in question will have cleared the evidence of such events. D'Lima et al[14,16] found that human osteochondral disks subjected to 30% compression in vitro show signs of apoptosis as early as 6 hours after injury and that the process can continue for as many as 7 days. The researchers found that apoptosis, as measured by internucleosomal DNA fragmentation, was present in 34% of chondrocytes 96 hours (or 4 days) after mechanical loading at 14 MPa. In vivo experiments in a rabbit cartilage impaction model have shown that apoptotic chondrocytes remain present up to 7 days after injury.[5] Type II collagen breakdown, as evidenced by positive COL2-3/4C$_{short}$ staining, has also been shown to be present in bovine osteochondral explants that have been cyclically loaded in vitro at 24 hours.[29] Based on this evidence, a 4-day period was determined to be optimal to capture apoptosis and MMP-mediated cartilage degradation.

Statistical Analysis

The study was powered for the primary outcome of chondrocyte viability based on cell vital staining. Previous studies have demonstrated a 10% standard deviation for nonviable chondrocytes in osteochondral grafts after impaction in transplantation models.[4] Therefore, 6 specimens were needed to detect a 20% difference at a significance of $\alpha = .05$ and $\beta = .20$. We allocated 10 specimens in each group to ensure adequate power in the event that some of the specimens were unusable.

Data are expressed as mean and standard deviation. For the preliminary biomechanical testing data, the average stress required for impaction before the graft was fully seated was compared with the average stress that the graft received after being fully seated, using a 2-tailed unpaired Student *t* test. Cell vital and TUNEL results were compared between all groups (sham-0, sham-4, OT-0, OT-4) via a 2-way analysis of variance and post hoc testing (SPSS, Chicago, Illinois). Mankin scores were also compared between all groups using the Kruskal-Wallis test, given that these data were nonparametric. A difference between groups was considered statistically significant at $P < .05$. No statistical analyses were performed for Bcl-2, M30, COL2-3/4C$_{short}$, or surface birefringence, because these data were qualitative.

RESULTS

Impact Stresses for Graft Insertion

The average duration of a single impact was 5.23 ± 0.43 milliseconds, which did not significantly differ between the samples ($P = .60$). The average stress required to impact an osteochondral graft into a defect was 14.80 ± 3.22 MPa; the

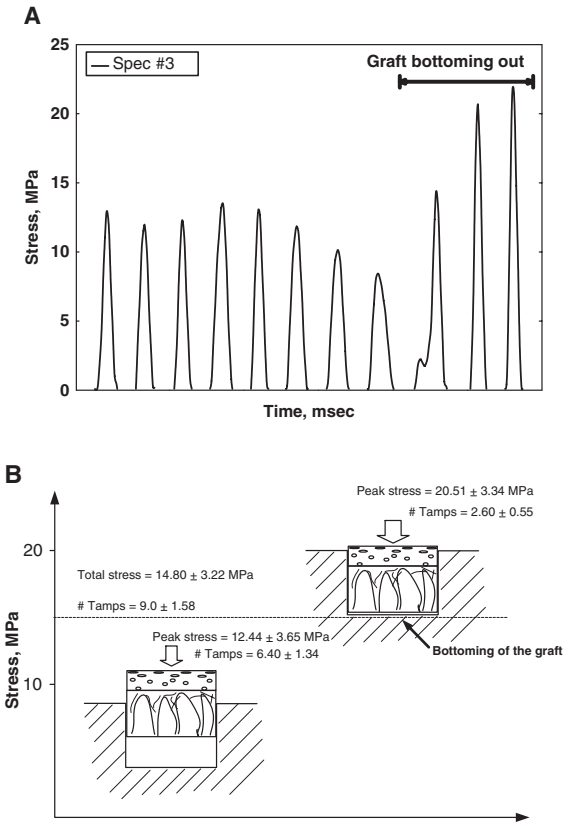

Figure 2. A, Typical stress-time response for graft insertion into the defect. Significantly higher stresses were recorded when the grafts were fully seated in the defect. B, Two distinct peaks of impact stresses were recorded during the insertion of the graft. The dashed line represents total average stress magnitude and number of tamps for graft insertion.

average force was 84.7 ± 18.4 N; and the average number of tamps required was 9.00 ± 1.58. However, 2 distinct peaks of stresses were measured during the graft insertion (Figure 2). The initial set of impact stresses were recorded before the osseous portion of the graft reached the bottom of the defect: these impact stresses averaged 12.44 ± 3.65 MPa; the force averaged 71.2 ± 20.9 N; and an average of 6.40 ± 1.34 tamps were required. The second set of impact stresses occurred when the graft had fully seated in the defect: these impact stresses averaged 20.50 ± 3.34 MPa; the force averaged 117.3 ± 19.1 N; and an average of 2.60 ± 0.55 tamps were required. The stress that occurred after the graft had fully seated was significantly higher than those achieved during the first peak ($P = .007$).

Gross Observations

All animals reached their anticipated necropsy date without any postoperative complications. Upon specimen harvest and preparation, a few samples were deemed unfit for analysis because the osteochondral plug had either dislodged from the host bed or was obliquely oriented in the host bed, thus suggesting that the tamp was not flush with the surface cartilage at the time of implantation. As such, 2 samples in the OT-4 group and 2 samples in the OT-0 group were excluded, which left 8 specimens in each group for final analysis.

TABLE 1
Mankin Score Results

Group	Structure	Cells	Stain	Tidemark	Total
OT-0	0.6 ± 0.5[a]	0.0 ± 0.0	1.0 ± 0.7[a]	0.0 ± 0.0	1.6 ± 1.1[a]
Sham-0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
OT-4	0.9 ± 0.8[b]	0.0 ± 0.0	1.9 ± 0.7[b]	0.0 ± 0.0	2.8 ± 1.1[b]
Sham-4	0.0 ± 0.0	0.0 ± 0.0	1.0 ± .06	0.0 ± 0.0	1.0 ± 0.6

[a] $P < .05$ compared with Sham-0.
[b] $P < .05$ compared with Sham-4.

Chondrocyte Necrosis

Chondrocyte Viability. The OT-4 group had the least percentage of viable chondrocytes (51.6% ± 11.6%). This finding was statistically significant when compared with the sham-0 group (81.0% ± 5.0%, $P < .001$) and the sham-4 group (74.2% ± 5.1%, $P < .001$) but not when compared with the OT-0 group (63.3% ± 7.2%, $P < .07$). There were also significantly more viable chondrocytes in the sham-0 group when compared with the OT-0 group ($P < .001$). Qualitatively, chondrocyte necrosis was concentrated in the superficial zone of the articular cartilage; very little necrosis was seen in the middle and deep zones (Figure 3).

Chondrocyte Apoptosis

There was a significant increase in TUNEL-positive cells in the OT-4 group (27.8% ± 9.6%) as compared with the OT-0 group and both sham groups (all $P < .001$). However, there was very little positive staining for Bcl-2 and M30 across all groups such that no differences were seen. These results indicate that the majority of TUNEL-positive cells were necrotic or postapoptotic.

Cartilage Degradation

There were higher total Mankin scores in the OT groups as compared with the sham groups at 4 days and time zero: OT-4 (2.8 ± 1.1), sham-4 (1.0 ± 0.6), $P = .03$; OT-0 (1.6 ± 1.1), sham-0 (0.0 ± 0.0), $P = .02$ (see Table 1). No significant differences were found between the OT-4 and OT-0 groups ($P = .07$). The extent of tissue damage in the OT-0 group was higher than that in the sham-0 group, whereas the OT-4 group had more tissue damage than that of the sham-4 group. The OT groups were significantly higher than their controls at both time points in regard to structure (time zero, $P = .01$; 4 days, $P = .04$) and staining (time zero, $P = .01$; 4 days, $P = .04$). Two specimens in the OT-4 group had small fissures isolated to the superficial zone, which corresponded with the higher score for structure.

Evidence of type II collagen cleavage was present in all specimens from the OT-4 group, which showed moderate or strong staining for COL2-3/4C_{short}. Intense staining was

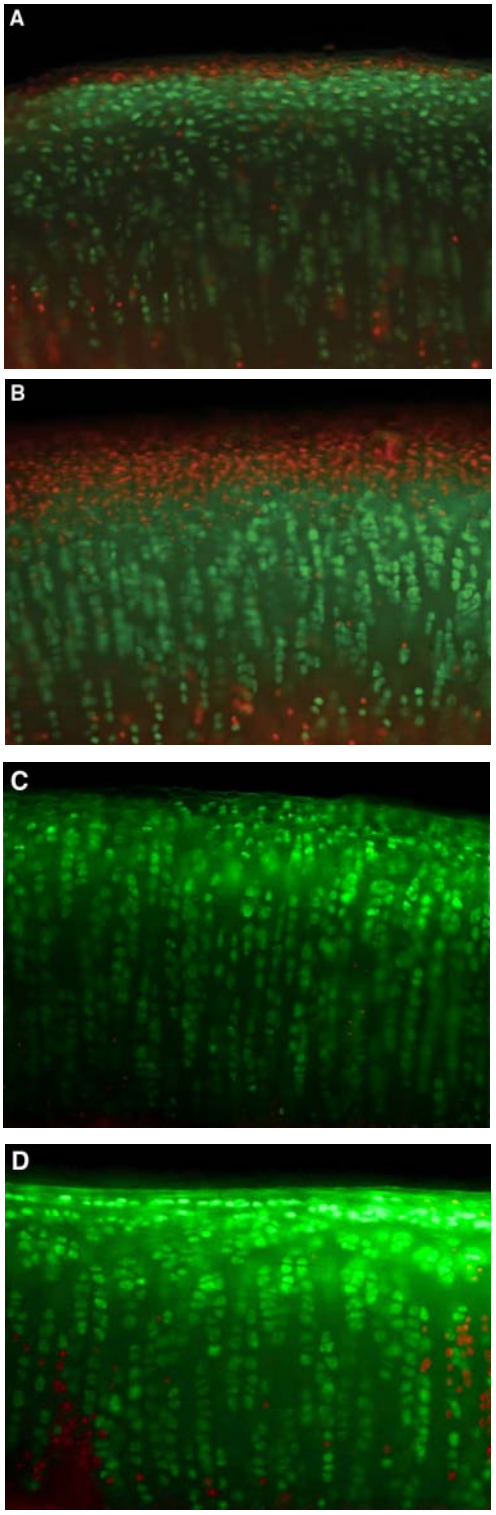

Figure 3. Cell vital histology. A, Representative histology of a specimen in the OT-0 group, with some cell necrosis in the superficial zone of the osteochondral graft. B, Specimen from the OT-4 group, which shows a moderate amount of cell necrosis; again, much of the necrosis is localized to the superficial zone. Very little necrosis is seen in the sham-0 (C) and sham-4 (D) groups. Original magnification for all panels ×100. Green, viable chondrocytes; red, necrotic chondrocytes. OT-0 and OT-4 groups, osteochondral transplantation, time zero and 4 days; sham-0 and sham-4 groups, bilateral sham surgery, time zero and 4 days.

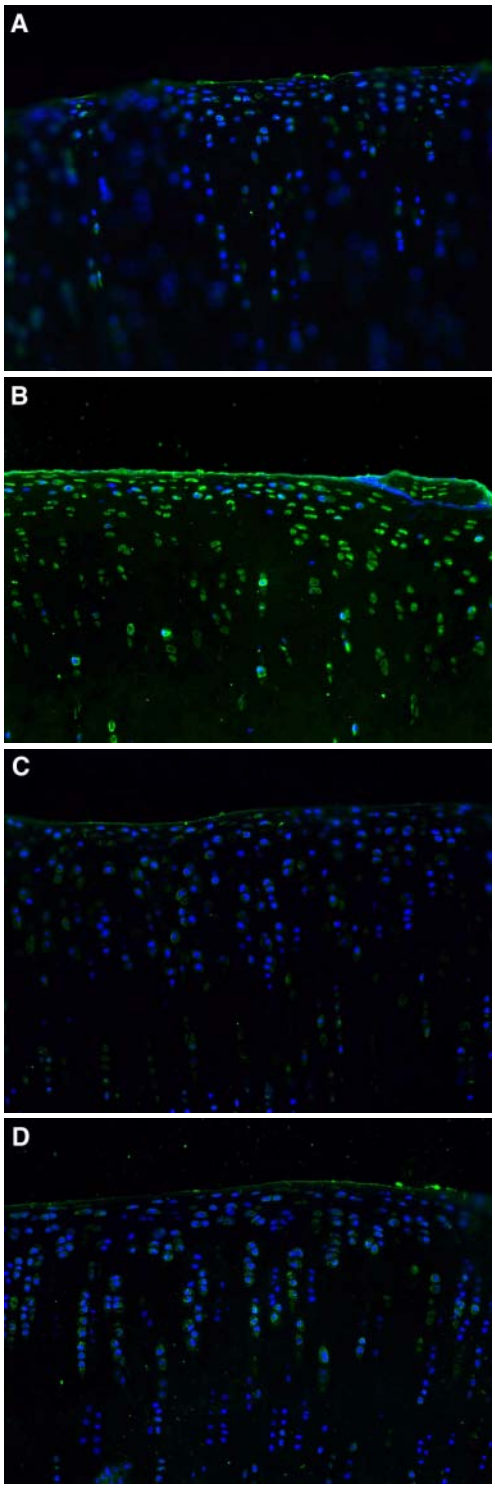

Figure 4. Matrix metalloproteinase–mediated type II collagen cleavage as shown by COL2-3/4C$_{short}$ immunohistochemistry. There was no positive staining in the OT-0 (A), sham-0 (C), and sham-4 (D) groups; the OT-4 group (B) had at least moderate staining in all specimens. Original magnification for all panels ×100. Green stain, COL2-3/4C$_{short}$-positive; blue counterstain, normal cells. OT-0 and OT-4 groups, osteochondral transplantation, time zero and 4 days; sham-0 and sham-4 groups, bilateral sham surgery, time zero and 4 days.

seen around the cells of the superficial and middle zones where chondrocyte death occurred, as well as in the interface between host and donor plugs, as compared with no staining seen in all specimens from the OT-0 group and both sham control groups (Figure 4). This finding suggests that MMP-mediated remodeling of the collagen network occurs after the osteochondral transplantation procedure. This finding also correlates with a loss of birefringence in the superficial zone of the cartilage in both OT groups (the OT-4 group more than the OT-0 group). None of the specimens in the OT-4 group exhibited positive birefringence under polarized light microscopy. In the OT-0 group, 5 specimens had minimal birefringence and 1 had no birefringence. All specimens in the sham-0 and sham-4 groups had strong birefringence. These findings indicate that the procedure disrupts the organization of the collagen network, especially in the superficial zone.

DISCUSSION

Autologous osteochondral transplantation surgery is a popular procedure in the care of patients with full-thickness cartilage defects, because it replaces the defect with hyaline cartilage. Whereas short- to midterm clinical results of this procedure are encouraging, there is concern over the long-term durability of the grafts.[7,8,17-20,33,37,40] A technical aspect of the surgery that may affect long-term survival is the need to tamp the grafts into the defect for a press fit, thereby placing a load to the articular surface. The trauma literature has well established in both in vivo and in vitro studies that the delivery of an impaction load on articular cartilage causes cell death, matrix degradation, and apoptosis.[§] Previous work with in vitro models for osteochondral transplantation revealed that chondrocyte necrosis and apoptosis occur after a series of impaction forces necessary to seat a graft into a defect.[4,38] However, what is unknown is whether these processes continue to occur in vivo, where the osteochondral graft is subjected to the shear forces from joint motion and to the potentially toxic environment of synovial fluid. The findings of the present study support our hypothesis that osteochondral transplantation procedures induce chondrocyte death and matrix degradation within the osteochondral graft of an in vivo model at an early time point. However, we did not find an increase in chondrocyte apoptosis in this model at the time points examined.

Our preliminary work in this study showed that there were 2 peaks of stress magnitude during insertion of the osteochondral graft into the host bed. The first peak occurred before the graft reached the bottom of the defect, and it averaged 12.44 ± 3.65 MPa. Once the graft was fully seated, it was tamped 1 to 3 more times, thereby resulting in peak stresses that averaged 20.5 ± 3.34 MPa. These stress values are similar to those reported by Borazjani et al,[4] who found that a mean stress of 14.0 ± 7.3 MPa, with a mean maximum stress of 22.1 ± 10.8 MPa, was required to fully seat human osteochondral grafts in vitro. These stresses are above the threshold that can cause cartilage injury, and they are a reasonable explanation for the chondrocyte necrosis and matrix degradation seen in our study. Milentijevic et al[34] have reported that the stress threshold for chondrocyte necrosis is above 25 MPa. However, a recent study by Patil et al[38] showed that the threshold may be even lower, with chondrocyte necrosis occurring with stresses between 8 and 13 MPa. In addition, cartilage injury may have occurred because of the accumulative trauma of repetitive tamps. Chen et al[10] have reported that chondrocyte necrosis can occur as a result of repetitive trauma, even if each impact is below the threshold for cartilage injury. As such, these findings serve as a reminder to surgeons to pay special attention to match the depth of the harvested osteochondral plug to the depth of the prepared defect to avoid excessive impaction once the graft is fully seated. This is especially important when considering that the goal of surgery is to place the graft even with the surrounding host cartilage (or slightly recessed), given that protruding grafts result in high shear stresses, with resulting graft degeneration.[22,39]

Several studies have shown that impaction of osteochondral grafts in vitro results in chondrocyte necrosis and apoptosis shortly after the procedure. Whiteside et al[46] and Kang et al[24] showed in a bovine explant model that the force of the impact applied to cartilage predicted the percentage of chondrocyte death. Borazjani et al[4] confirmed these findings of chondrocyte necrosis in human explants but showed that apoptosis also occurred. In a follow-up study, Patil et al[38] found that impaction forces usually remain below the threshold for chondrocyte necrosis unless the graft is 2 mm longer than the defect is deep. The main limitation of these studies is that they were all performed in vitro, with explant models; therefore, they may not represent the complex mechanical and biologic environment of the knee joint.

Our study confirms that chondrocyte necrosis and cartilage degradation occur shortly after impaction in vivo, but our results do not confirm the presence of apoptotic chondrocytes at the time points that we examined.[4,24,38] We found that only 63.3% of chondrocytes remain viable immediately after the procedure and that only 51.6% are viable at 4 days. This finding is consistent with prior in vitro studies on the topic.[4,24,38] In light of the negative Bcl-2 and M30 staining, the positive TUNEL staining seen in the OT-4 group must be interpreted as a false-positive staining of necrotic chondrocytes. TUNEL is a sensitive test for apoptosis but not a specific test; it has been shown to also detect a significant number of necrotic cells.[41,47] It is possible that apoptosis was present at other time points after the procedure, which were not detected with our study design.

Cartilage degradation was also observed immediately after the procedure. We theorize that this occurs through at least 2 mechanisms. First, it appears that a portion of cartilage degradation occurs at time zero as a direct mechanical result of impaction; this is evidenced by our findings that the OT-0 group had less cartilage surface birefringence under polarized light microscopy and less safranin-O staining when compared with the sham-0

[§]References 4-6, 9-11, 14-16, 29, 30, 34, 38, 43, 44.

group. These findings suggest that collagen fiber organization had been disrupted, thereby resulting in less birefringence. The other mechanism of degradation appears to be MMP-mediated breakdown of type II collagen, as initiated from the biologic mechanotransduction of impaction. This is evidenced by the positive COL2-3/4C$_{short}$ staining seen in the OT-4 group. Positive staining was not seen in the OT-0 group, because it takes time for this biological process to take place. These findings correlate with a previous in vitro study that showed that the application of load on articular cartilage causes the release of MMP-3, proteoglycan degradation, and collagen damage.[29] Our finding of MMP-mediated cartilage degradation may provide a possible target for therapeutic agents. Inhibitors of MMPs, such as doxycycline and alpha2-macroglobulin, may be useful agents to prevent graft degeneration after implantation.[12,13]

Although our study and others have shown significant chondrocyte death and cartilage degeneration at early time points, there is controversy in the literature regarding the long-term fate of these grafts. Clinical studies have shown encouraging results of this procedure within 3 to 7 years of follow-up. Hangody et al[20] reported good to excellent results in 91% of their 113 patients with 3 to 6 years of follow-up, which corresponded with normal histologic findings on arthroscopic biopsies performed on 12 patients. Marcacci et al[33] reported good or excellent results in 76% of their patients with at least 7 years of follow-up. Barber and Chow[1] performed arthroscopic biopsies up to 12 months after osteochondral transfer and found that all grafts retained viable chondrocytes with no obvious disruption of the cartilage architecture. In contrast, Evans et al[17] published a case report of 2 patients who underwent knee arthroplasty approximately 12 months after osteochondral transplantation. Histologic evaluation of the grafts revealed a loss of proteoglycan content, as evidenced by safranin-O staining in the superficial zone of one graft and by complete loss in the other. The chondrocytes in both grafts were randomly oriented and did not represent normal hyaline cartilage.

Animal studies have also shown conflicting long-term results of osteochondral graft survival. In a goat model, Lane et al[27] showed that 95% of the graft chondrocytes remained viable 12 weeks after implantation, although the grafts had a higher stiffness than that of the adjacent host cartilage. The same group found a chondrocyte survival rate of 86%, 6 months after surgery (again, in an ovine model).[26] In addition, Harman et al[21] found in a porcine model that 75% to 100% of the transplanted chondrocytes remained viable at 6 months. In a rabbit model, Makino et al[31] and Nam et al[36] reported that the transplanted cartilage had a histologic appearance that was different from the intact cartilage at 24 weeks. However, they also showed that the grafts provided smooth restoration of the cartilage surface with integration of the bone plug,[31] as well as improvement of the mechanical stability of the graft, from 6 to 12 weeks, without evidence of progression of degenerative changes.[36]

Other animal studies have shown that osteochondral grafts deteriorate with time. Tibesku et al[42] used an ovine model and reported that the osteochondral grafts exhibited more severe signs of degeneration than that of the adjacent host cartilage, based on Mankin scores at 3 months. Specifically, they found more chondrocyte cloning and irregularities of the articular surface. Baumbach et al[2] also showed deterioration of the osteochondral grafts at 52 weeks via a minipig model. The researchers reported a wide variation of graft survival, ranging from vital tissue that resembled native cartilage at 52 weeks to severe degenerative signs beginning as early as 2 weeks after the procedure. Using an ovine model, Kleemann et al[25] showed that 4 of 6 specimens had signs of cartilage degeneration, as evidenced by hypercellularity and chondrocyte clustering at 3 months. The authors concluded that although the grafts resulted in good surface congruity, their histologic results raise doubts regarding the long-term durability of the osteochondral repair.

Based on these studies, the probability is that the majority of patients who undergo this procedure experience immediate chondrocyte necrosis after surgery, followed by a reincorporation of their grafts with time. However, there appears to be instances when reincorporation does not take place and the graft deteriorates with time. This latter group of patients would benefit from changes in the surgical technique that decrease trauma on the graft's articular surface, or from biological therapies that can inhibit the degenerative process. Unfortunately, it is difficult to determine which group patients fall into preoperatively—that is, when treatments would be most fruitful.

We acknowledge several limitations to this study. First, rabbit cartilage is relatively thinner than adult human cartilage and so may distribute stress differently across the tissue. As such, the effects of impaction may be magnified in this model relative to a human model. Also, we harvested the osteochondral grafts from the weightbearing surface of the contralateral femoral condyle, as opposed to the nonweightbearing area of the trochlea, as is routine in clinical practice. We did so because of the size constraints of the rabbit knee. It is uncertain what differences, if any, this has on our results; nonetheless, the factor must be taken into consideration when extrapolating our results to humans. However, several authors have used rabbit knee cartilage to study its response to injury.[5,28,31,34-36] There is precedent in the literature for the model chosen in the present study.

Second, our study did not separate the effects of graft harvest, manipulation, and implantation in a controlled fashion, given that all are inherent in the performance of this procedure. Huntley et al[23] showed that harvesting an osteochondral graft results in superficial chondrocyte death an average of 382.0 ± 68.2 μm from the edge of the graft. In an effort to eliminate this confounding variable, we excluded the peripheral 300 μm of the graft from histologic analysis. Therefore, it is possible that a small amount of necrosis that we attributed to the impaction could have come as a result of graft harvest. The sham control group in this study served to offset any effects from surgical manipulation of the cartilage. Although surgical times were not recorded, the experimental procedure lasted approximately 5 to 10 more minutes than the sham

surgeries. It is possible, although unlikely, that this additional exposure resulted in the cartilage damage that we observed. Cartilage in the sham groups was also subjected to a postoperative hemarthrosis because the fat pad was excised in all limbs. Given the extensive literature regarding the response of cartilage to load, as well as the precautions outlined in this study to avoid confounding variables, we believe that our results of chondrocyte necrosis and cartilage degradation can be mostly attributed to the impaction phase of the procedure.

Third, this study examined only early time points, to characterize the catabolic events that are induced by this procedure. It is unclear whether these findings are permanent or transient. Further studies are needed at longer time points to determine this.

Finally, it is certainly possible, even probable, that apoptosis occurs after this procedure. However, in the present model and at the time points of time zero and 4 days, significant apoptosis was not observed. We chose a 4-day time point because previous studies have shown that apoptosis can be observed from 6 hours to 7 days after injury.[5,14,16] One possible explanation for why no apoptosis was detected in our model is that the humeral (eg, cytokines, complement) and cellular immune response to an intra-articular injury and the resulting hemarthrosis may have pushed chondrocytes toward necrosis over apoptosis. It is also possible that this immune response may have cleared the evidence of apoptosis that occurred at earlier time points such that we could not detect it at 4 days. Further studies are needed to determine the extent of apoptosis after this procedure. These findings may also represent a type II error, given that the study was powered for chondrocyte necrosis only and not apoptosis. However, given that there were no signs of apoptosis seen in the specimens examined, we would have not likely uncovered a significant difference with more specimens.

This in vivo study confirms previous authors' in vitro observations that cartilage degradation and chondrocyte death occur after autologous osteochondral transplantation procedures at early time points. Furthermore, this study demonstrated MMP-mediated cartilage degradation after the procedure. By understanding the mechanism by which cartilage is degraded in response to load, we may be able to develop methods to impede or avoid this phenomenon. Potential interventions include devising an atraumatic method to insert and secure the graft, as well as pharmaceuticals that can block MMP activity. Further study is needed to better characterize the pathophysiology after autologous osteochondral transplantation procedures. Specifically, long-term studies are needed to determine whether the results seen in this study are permanent or transient.

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