Material Properties of Fresh Cold-stored Allografts for Osteochondral Defects at 1 Year

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Abstract Little is known about the long-term properties of fresh cold-stored osteochondral allograft tissue. We hypothesized fresh cold-stored tissue would yield superior material properties in an in vivo ovine model compared to those using freeze-thawed acellular grafts. In addition, we speculated that a long storage time would yield less successful grafts. We created 10-mm defects in medial femoral condyles of 20 sheep. Defects were reconstructed with allograft plugs stored at 4°C for 1, 14, and 42 days; control specimens were freeze-thawed or defect-only. At 52 weeks, animals were euthanized and retrieved grafts were analyzed for cell viability, gross morphology, histologic grade, and biomechanical and biochemical analysis. Explanted cold-stored tissue had superior histologic scores over freeze-thawed and defect-only grafts. Specimens stored for 1 and 42 days had higher equilibrium moduli and proteoglycan content than freeze-thawed specimens. We observed no difference among any of the cold-stored specimens for

chondrocyte viability, histology, equilibrium aggregate modulus, proteoglycan content, or hypotonic swelling. Reconstructing cartilage defects with cold-stored allograft resulted in superior histologic and biomechanical properties compared with acellular freeze-thawed specimens; however, storage time did not appear to be a critical factor in the success of the transplanted allograft.

Introduction

The repair of osteochondral and chondral defects remains a major clinical challenge to the orthopaedic surgeon [19, 26, 56]. Fresh osteochondral allograft transplantation is a well-established treatment for cartilage defects around the knee [4, 6, 8, 11, 16–18, 21–23, 27–30, 34, 35, 38, 43–45, 48, 55]. Transplantation can be performed in a single stage with reduced donor site morbidity, and fresh allografts provide clinical success in human subjects over decades of investigation [4, 9, 16–18, 34, 35, 55]. Traditionally, fresh osteochondral allograft use was limited to a few centers with the capacity to procure, store, and donor-recipient match allograft tissue [33]. In these institutionally based programs, fresh osteochondral graft transplantation has been typically performed within 7 days of specimen retrieval to ensure maximal chondrocyte viability in transplanted tissue.

By the end of the 1990s, commercially available fresh cold-stored osteochondral specimens became available for public use in the United States [33]. Unlike traditional fresh allograft, current specimens are stored in a hypothermic nutritive medium and must go through a 14-day sterility program for donor bacterial and viral testing [39, 52]. The logistics of proper sizing, specimen distribution, and

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scheduling of patients have also added to the overall storage time of the specimens before transplantation. These logistic issues led many investigators to study the effect of storage time on the cellular and biomechanical properties of stored grafts. Numerous *in vitro* and short-term *in vivo* studies demonstrate a time-dependent compromise in material properties (ie, cellular, biochemical, and biomechanical parameters) of cold-stored fresh allograft [7, 42, 53, 54, 63, 65, 66]. It is clear these changes persist at the time of allograft transplantation [1, 51, 66]. However, it is unclear whether the compromises in graft properties influence the long-term *in vivo* effects or clinical consequences of fresh cold storage and whether the deterioration compares with that of fresh-frozen grafts [24, 50].

We therefore posed two hypotheses: (1) Although fresh cold-stored osteochondral allografts have compromised material (histologic, biochemical, and biomechanical) properties at the time of transplantation, these grafts would still have superior material properties over freeze-thawed chondrocyte-free allograft specimens at 1 year *in vivo*; (2) Cold-stored allograft specimens with prolonged storage time (42 days) would have a greater deterioration in their material properties compared with fresh (Day 1) grafts. Finally we asked whether proteoglycan content or hypotonic swelling was the more critical biomechanical property in determining biomechanical function as reflected by aggregate equilibrium modulus.

Materials and Methods

Twenty adult Warhill sheep were randomly assigned to five different surgical groups. Three of these groups were fresh cold-stored osteochondral allografts (D1, D14, D42) that were stored 1, 14, and 42 days, respectively. The remaining two graft groups were surgical controls consisting of freeze-thawed (FT) and defect-only (DO) groups. After transplantation, all animals were euthanized at 1 year; harvested specimens underwent a systematic analysis that included gross morphologic, cellular, histologic, biochemical, and biomechanical testing. Prior approval of our institutional animal care committee was obtained.

Right and left distal femurs of eight adult sheep were obtained for donor allograft material under sterile conditions. Within 1 hour of euthanasia, the whole distal femoral specimens were immediately immersed in a nutrient medium as previously described [65]. The nutrient storage medium contained the following: low-glucose Dulbecco's modified eagle's medium (Gibco, Grand Island, NY), 10% fetal bovine serum (Gibco), 1% antibiotic/antimycotic (Gibco), 80 μ M MEM nonessential amino acids (Gibco), 6 mM L-glutamine (Gibco), and 20 mM HEPES (Gibco). Specimens were stored at 4°C (100%

atmospheric air); the medium was changed every 2 days under sterile conditions. Distal femoral specimens were stored for 1 day (D1), 14 days (D14), or 42 days (D42). The FT group consisted of allograft specimens that were obtained concurrently with the fresh donor specimens; FT specimens were frozen (−80°C) and thawed three times. Chondrocyte death in FT specimens was confirmed before host transplantation using a qualitative viability assay as previously described [33]. Finally, the DO control group consisted of specimens in which the surgically created defect was left unfilled.

The transplantation procedure was performed through a medial knee arthrotomy under sterile operating room conditions using general anesthesia consisting of 0.1 mg/kg valium intravenously, 3.3 mg/kg ketamine intravenously, and 1.5% to 4% isoflurane inhalation in 100% oxygen (2 L/min). In addition, we injected a 5-mL intraarticular solution of 2 mg/kg xylocaine before the arthrotomy, whereas 5 mL of bupivacaine 2 mg/kg was given intraarticularly after skin closure. One gram of cefazolin sodium was given intravenously to each sheep at induction, midway through the surgery, and during closure. Procaine penicillin (3 million units) was given subcutaneously once daily to each sheep for 3 days postoperatively.

The technique consisted of first fashioning a cylindrical osteochondral 10-mm plug in both diameter and height from the medial condyle of the stored whole femoral specimens. The Allograft OATS Instrumentation System (Arthrex, Inc, Naples, FL) was used to create both the donor osteochondral grafts and host animal defects. Donor grafts were press-fit into the 10-mm defect on the most distal aspect of the weightbearing portion of the medial femoral condyle knee of the host sheep. The size of the defect was approximately 60% of the width of the medial femoral condyle consistent with a large defect, which is suitable for allograft transplantation in humans (the width of a female sheep condyle is 17 mm, articular surface length or circumference of 50 mm) [33]. If necessary, a bone tap was used to help engage the donor specimen but was limited to only one to two blows to maximize chondrocyte viability [10].

After graft transplantation, animals were taken to a monitored holding area until their gag reflex returned. They were allowed to bear weight as tolerated. Postoperative management included a 10-mg fentanyl patch and a 5-mg fentanyl patch (150 μ g/h). One gram of phenylbutazone was given orally 1 day preoperatively, the day of surgery, and for 3 days postoperatively.

All animals were euthanized at 52 weeks posttransplantation. Euthanasia was consistent with guidelines published by the AVMA Panel of Euthanasia [5], which consisted of 88 mg/kg pentobarbitone sodium (approximately 8 g per sheep) given as an intravenous jugular

injection. All retrieved allograft cartilage specimens were analyzed for chondrocyte viability, matrix proteoglycan concentration, matrix water content, matrix response to hypotonic swelling, and matrix biomechanical properties (Fig. 1). Three of 20 sheep died before 52 weeks, including two from the D1 group and one from the DO group. Of the two D1 sheep, one died less than 1 month postoperatively because of a pulmonary infection; the other two sheep died at 6 and 10 months of mesenteric abscessation, a common cause of death in skeletally mature adult female sheep. Tissue was available for analysis for both of these two specimens but was not included in the analysis because the deaths were before the end point interval.

We used cell vital staining (fluorescein diacetate and propidium iodide) to determine the zone dependency, cell morphology, and extent of surviving cells as previously described [31, 37, 49]. Briefly, the full thickness of articular cartilage was sliced to a thin section (100–200 μm) at the perpendicular direction. The live cells with intact membranes with active esterases processed fluorescein diacetate into fluorescein and fluoresced green, whereas necrotic or dying cells with compromised membrane fluoresced red as propidium iodide bounded freely to the charged nuclear acids. Photographs were taken with either $\times 4$ or $\times 10$ objective lenses using a fluorescence microscope (Optiphot; Nikon, Melville, NY) and a CCD camera (Nikon). Multiple sections under various magnifications were obtained from the same sample. We (AR, CC) made subjective assessments on chondrocyte viability by the extent of surviving viable cell (green-fluorescent/high-power field), the location of chondrocyte loss relative to specific cartilage layers, and the growth pattern of chondrocyte repopulation such as cluster formations [13, 40].

We inspected the gross morphology of the entire femoral condyle and tibial plateau using India ink

staining. One milliliter of India ink was diluted in 1x phosphate-buffered saline (PBS). Approximately 1 mL of solution was placed on the articular surface and the specimens were digitally photographed. Gross cartilage morphology was graded using a standard visual assessment score by two independent observers (AR, JZ) (Fig. 2A–C) [2, 3, 15, 58]. In addition, the same two observers (AR, JZ) made subjective assessments of the degree of host-donor interface. The grafts were interpreted as a clock face consisting of four equal quarters. Peripheral graft to host integration was assessed as near to complete if 75% to 100% was integrated, partial (25%–75%), or little to none (0%–25%). In addition, the grafts were noted to be proud, flush, or recessed [64].

All specimens were then graded histologically to evaluate the quality and thickness of transplanted cartilage and neighboring perilesional tissue and to determine whether healing occurred between donor and host tissue. All medial femoral condyles were cut with a sagittal saw parallel through the long axis of the condyle through the center of the graft site or defect. The samples were then fixed in 10% formalin at 4°C for 3 days. Using a cooled diamond-wheel saw, the specimens were cut parallel through the long axis of the condyle and decalcified overnight at 4°C in Immunocal (Decal Chemical, Tallman, NY). The tissue was sequentially dehydrated in a tissue-processing machine and then penetrated into paraffin. The tissue was then embedded into a paraffin block and sectioned with five microtomes for each level and stained with either hematoxylin and eosin or Safranin-O red. The Osteoarthritis Research Society International (OARSI) Cartilage Histopathology and Assessment System [20] for hyaline cartilage degeneration was scored by two independent, blinded investigators (AR, JZ) and averaged. This system is designed to assess the quality of cartilage. Tissue was given a score of one point if the cartilage was intact, two if there were small discontinuities in the cartilage, three if there were vertical fissures, four if there were erosions, five if there was cartilaginous denudation, and six if the underlying bone was deformed. The same two observers (AR, JZ) also made subjective assessments of the degree of cartilage and osseous integration at the host-donor interface (ie, near to complete 75%–100%, partial 25%–75%, or little to no integration 0%–25%). Specimens designated for histologic analysis were chosen by examining the specimens that included the greatest area of graft cartilage to the host-cartilage interface as well as the graft subchondral bone to host subchondral bone interface. Typically, three specimens were picked, two focusing on the graft and one on the host cartilage and subchondral bone. The data for hematoxylin and eosin and Safranin-O were first examined separately and no differences were found so the data were combined.

Fig. 1 Graphic depiction showing sampling protocol to obtain all necessary material properties (red circle) dynamic/equilibrium tests, water content, hypertonic swelling, and proteoglycan content; blue box = 6-mm slice of bone and cartilage used for hematoxylin and eosin (H&E) and Safranin-O staining; yellow box = approximately 500- μm slices taken for paravital staining.

Fig. 2A–C (A) This Day 42 specimen displays minimal degenerative changes with good contour and integration. (B) This freeze-thawed specimen shows degenerative changes and graft failure. (C) Medial tibial plateau of a D14 specimen showing evidence of wear on the

articular surface opposing the graft consistent with early arthritis; this finding was consistently observed in all operative specimens (Stain, India ink; original magnification, $\times 1$).

The equilibrium aggregate moduli of full-thickness cartilage samples were determined by confined compression tests using a custom-designed, computer-automated soft tissue tester as previously described [12, 14, 60, 61]. Confined compression testing was used to assess the biomechanical function of the transplanted cartilage. Water content and hypotonic swelling were evaluated to assess tissue quality, knowing poorer-quality cartilage has a higher water content [31]. Matrix proteoglycan content was used to measure chondrocyte function and the status of the extracellular matrix. Equilibrium aggregate modulus of the specimens was determined using a five-step stress relaxation test after removal from the subchondral bone. Specimens were immersed in a PBS solution with protease inhibitors (Sigma Chemicals, St Louis, MO) and tested using the custom-designed biomechanical test apparatus previously described [12, 14, 61]. Specimens were compressed using a 5-mm diameter nonporous indenter with 25- μm predisplacement to ensure uniform initial contact with the indenter. Five equal compressive steps of 50 μm (total compression, 250 μm) were applied to the samples. In the beginning of each step, a compressive ramp at a rate of 50 $\mu\text{m}/\text{sec}$ was applied. Load and displacement in each step were automatically recorded. After converting load and displacement into engineering stress and strain, the equilibrium modulus in each step was determined by curve fitting to the experimental data using biphasic theory.

After the confined compression and water content determinations, analysis of matrix proteoglycan (PG) content was performed using a 1,9-dimethylmethylene blue (DMMB) dye-binding assay [60]. Cartilage specimens were rinsed in PBS and then digested using a papain solution (4%) with protease inhibitors (Sigma Chemicals) at a concentration of 0.5 mg/mL at 65°C for 4 hours. The PG concentration in the digested solution was measured using the DMMB dye-binding methods as described by Torzilli [60]. Water content within the graft as well as the change in water content in response to a hypotonic fluid bath was determined on full-thickness cartilage specimens after confined compression testing. Wet weight of the

cartilage specimens was measured before and after mechanical testing to confirm this did not alter the measurement. Plugs were subsequently placed in a hypotonic bath solution (1.5 mM NaCl in PBS) for 2 hours and the wet weight was recalculated. Dry weight was then determined after lyophilization in a freeze-dryer (Labconco, Kansas, MO) overnight. Initial water content was determined as the difference between dry weight and initial wet weight. The increase in the wet weight in response to a hypotonic bath was also recorded and reported as a percentage relative to initial wet weight.

Analysis consisted of descriptive means and standard deviations for continuous outcomes measures. Inferential analysis was performed using one-way analysis of variance (ANOVA) comparing each of the independent groups (fresh-stored, freeze-thawed, defect only, and nonoperative controls) with the dependent outcomes of interest, which included gross morphology, histologic properties, chondrocyte viability staining, and biochemical and biomechanical properties. A post hoc least squares difference test was performed if the ANOVA model was significant to determine which groups differed. In addition, the fresh-stored group was individually analyzed to compare storage time with their biomechanical properties also using the Student's t-test or one-way ANOVA. Finally, Pearson's correlations were performed to relate dependent variables such as histologic properties (OARSI score) and biomechanical properties (aggregate equilibrium modulus) with independent variables (the three cold-stored allografts: D1, D14, and D42). In addition, correlations were performed between dependent variables as well (proteoglycan content and hypotonic swelling versus aggregate equilibrium modulus). All analyses were performed using SPSS 11.0 for Windows (Chicago, IL).

A pre- and posthoc power analysis was performed to determine the power for the observed data for each of the outcome variables of interest based on our previous short-term in vivo study for equilibrium modulus and PG content, our primary dependent variables. At a sample size of three, a power of 0.961 and 0.998 was reached for

equilibrium modulus and PG content, respectively, as compared with nonviable (freeze-thawed) and fresh-stored grafts versus nonoperative controls. A sample size of four per group was therefore chosen for this study. For post hoc analysis, stored specimens versus controls were adequately powered. In addition, stored specimens versus freeze-thawed specimens were adequately powered for PG content but not equilibrium modulus. Finally, comparisons among the three stored groups were also underpowered.

Results

The fresh-stored osteochondral allografts had superior material properties compared to freeze-thawed chondrocyte-free allograft specimens at 1 year. In terms of gross morphologic properties, the fresh-stored specimens had superior properties. Of the 10 stored specimens, six showed evidence of well-maintained near-flush grafts and at least partial integration of allograft donor tissue with host tissue and only minimal degenerative changes (Fig. 2A). Although all 10

specimens had some evidence of degenerative disease near the medial tibial spine (Fig. 2C), only four specimens (one of two D1 and D42 and two of the four D14 specimens) showed evidence of moderate arthritic changes with large defects on the medial femoral condyle with recessed plugs with little to no interface integration. On the other hand, three of four of the FT specimens had medium to large defects and moderate degenerative changes (Fig. 2B), whereas the entire DO group (four of four) had evidence of only fibrous tissue fill with minimal degenerative changes. In terms of chondrocyte cell vital staining, the cold-stored groups (D1, D14, and D42) (Fig. 3A–C) had superior staining patterns over the FT specimens (Fig. 3D) with evidence of viable chondrocytes uniformly distributed in all cartilage layers. For the cold-stored specimens, most cellular attrition (ie, decreased staining) occurred in the superficial layer. The FT specimens were devoid of viable cells in all specimens and in all (superficial, middle, and deep) layers except some clustered cells sparsely seen in the superficial tangential zone. The DO group (Fig. 3E) also had a clustered cell pattern located in the repair tissue,

Fig. 3A–F The results showing cell paravital staining of osteochondral grafts at 52 weeks (see Methods and Materials; green = viable cell, red = dead cells) in the (A) Day 1 (FDA and PI cell staining; original magnification, $\times 10$), (B) Day 14 (FDA and PI cell staining; original magnification, $\times 10$), and (C) Day 42 cold-stored (FDA and PI cell staining; original magnification, $\times 10$) groups as well as the (D) freeze-thawed (FT) group (FDA and PI cell staining; original magnification, $\times 4$). This is compared with the (E) repaired tissue in the defect group and (F) the cartilage from the control group. There

was a reduction of cell population in the cold-stored groups compared with the control groups, but viable cells are still located in all zones. In the FT group, sparse and clustered “chondrocyte-like” cells (shown by the arrow) were seen in the superficial layer of the cartilage, where there were no cells in the middle and deep zones. Clustered cells were found in all the zones of the repaired tissue in the defect group (shown by the arrow) suggesting a cell repopulation in the lesion area (bar = 250 μ m).

Fig. 4A–D Histology of specimens. **(A)** This D1 graft specimen is located at the center of the allograft and shows fissures and thinning of the cartilage (Stain, hematoxylin and eosin; original magnification, $\times 4$); **(B)** D42 specimen centered at the graft-host junction with evidence of complete subchondral osseous integration and some limited cartilage integration (Stain, Safranin-O; original magnification, $\times 4$); **(C)** defect-only specimen centered near the area of the greatest pathology showing evidence of substantial fill with reparative tissue and fibrocartilage (Stain, Safranin-O; original magnification, $\times 4$); **(D)** freeze-thawed specimen centered near the area of the greatest pathology with evidence of complete loss of articular cartilage that approaches the subchondral bone (Stain, Safranin-O; original magnification, $\times 4$).

which was more numerous than the FT samples. The control group (Fig. 3F) had viable chondrocytes distributed in all cartilage layers. In terms of the OARSI histology score, all stored specimens (10) were superior to both the FT and DO specimens. The cold-stored OARSI score was 3.1 (range, 2–4) (Fig. 4A–D), whereas the FT and DO specimens were 5.1 (range, 4.5–6) and 4.5 (range, 4–5) ($p < 0.0002$ and $p < 0.02$, respectively). All stored grafts had evidence of osseous incorporation but with minimal cartilage integration. On the other hand, the FT specimens had large defects with cyst formation, loss of matrix, and disorganized reparative tissue, whereas the DO specimens demonstrated good fill of the defect tissue with reparative fibrocartilage and evidence of perilesional matrix and cartilage loss. Finally, in terms of biomechanical and biochemical material properties, fresh-stored grafts again were superior to freeze-thawed grafts. The mean equilibrium modulus (mean $\pm$ standard error of measurement) and PG content for all stored grafts were 21.4 ± 3.7 kPa and $5.2\% \pm 1\%$, respectively. Although equilibrium modulus of stored grafts was similar to ($p = 0.11$) FT grafts, PG content was superior for ($p < 0.03$) all stored grafts; the

equilibrium modulus and proteoglycan content were both similar ($p > 0.05$) for all stored grafts compared to the DO specimens. Both D1 and D42 specimens had a higher equilibrium modulus ($p < 0.02$ and $p < 0.03$, respectively) and PG content ($p < 0.04$ and $p < 0.002$, respectively) than the FT specimens (Table 1) (Figs. 5, 6). However, the equilibrium modulus and PG content for the D14 samples were not different than the FT specimens ($p = 0.06$ and $p = 0.1$, respectively). Note, when compared with the contralateral nonoperated knee, all fresh-stored grafts had inferior equilibrium aggregate modulus and PG content ($p < 0.001$ and $p < 0.001$, respectively) (Table 1) (Figs. 5, 6). However, the mean hypotonic swelling of the nonoperative controls was similar to all the other specimen groups (all $p > 0.05$) (Table 1).

We found no major differences when comparing the material properties of each cold-stored group; that is, cold-stored allograft specimens with prolonged storage time (Day 42) did not exhibit greater deterioration in their material properties compared with fresh (Day 1) grafts. In terms of morphologic and histologic assessment, three of the four D42 grafts were the most normal grafts with the

Table 1. Summary results of equilibrium aggregate modulus, proteoglycan content, and hypotonic swelling for each sample group (mean $\pm$ standard error)

Group	Equilibrium modulus (kPa)	Proteoglycan/dry weight (%)	Hypotonic swelling/dry weight (%)
Day 1	20.5 $\pm$ 0.6*, †	5.2 $\pm$ 0.7*, †	47.2 $\pm$ 12.4
Day 14	17.1 $\pm$ 6.7*	3.0 $\pm$ 0.5*	85.6 $\pm$ 38.2
Day 42	26.3 $\pm$ 8.0*, †	7.5 $\pm$ 1.8*, †	45.9 $\pm$ 11.6
Defect-only	7.0 $\pm$ 2.0*	2.9 $\pm$ 0.2*	43.3 $\pm$ 9.4
Freeze-thaw	4.7 $\pm$ 2.8*	1.4 $\pm$ 0.4*	82.8 $\pm$ 14.4
Control	74.6 $\pm$ 15.7	1.9 $\pm$ 2.0	70.4 $\pm$ 16.1

Significant difference from *control group ($p < 0.01$) and †freeze-thaw group ($p < 0.05$).

Fig. 5 Biomechanical properties (equilibrium modulus, KPa) in all operative groups are shown. D14 and D42 groups as well as defect-only and freeze-thawed groups had lower aggregate equilibrium moduli than the nonoperative control group ($p < 0.04$, $p < 0.05$,

$p < 0.02$, and $p < 0.005$, respectively) except for D1 versus control ($p = 0.09$). Both the D1 and D42 specimens had higher values than the freeze-thawed specimens ($p < 0.02$ and $p < 0.03$, respectively). There were no differences among the three cold-stored groups.

Fig. 6 Proteoglycan content per dry weight in all operative groups is shown. The D1 and D14 cold-stored groups as well as the defect-only and freeze-thawed groups had lower proteoglycan content than the nonoperative control group ($p < 0.05$, $p < 0.006$, $p < 0.006$, and $p < 0.0009$, respectively). Proteoglycan was similar ($p = 0.07$) in the D42 and control groups. The D1 and D42 groups had higher proteoglycan content values than the freeze-thawed specimens ($p < 0.04$ and $p < 0.002$, respectively). D42 was higher than the D14 group ($p < 0.01$).

excellent interface integration, while the D1 and D14 grafts were more compromised. Likewise, there was no difference or correlation between storage time and mean OARSI histology score ($r = -0.76$). All three fresh cold-stored groups had similar ($p > 0.05$) equilibrium modulus, PG

Fig. 7 Pearson correlation of equilibrium modulus versus proteoglycan content correlated with each other ($r = 0.83$, $r^2 = 0.69$, $p < 0.001$) suggesting the decrease in biomechanical properties may be related more to a decrease in proteoglycan content than collagen degradation.

content, and water content; the mean D42 PG content was higher ($p < 0.014$) than the mean D14 PG content (Table 1).

When comparing PG content and hypotonic swelling with equilibrium modulus, the equilibrium aggregate modulus correlated with ($r = 0.83$, $p < 0.001$) PG content (Fig. 7) but did not correlate ($r = 0.041$, $p = 0.45$) with hypotonic swelling.

Discussion

Our focus was to show that fresh-cold osteochondral allografts, even with compromised material properties, outperform freeze-thawed acellular grafts. In addition, we wanted to see if prolonged storage time continued to compromise material properties in vivo. Although numerous studies have described deleterious effects of prolonged cold storage on the cellular and biomechanical properties of osteochondral allografts [1, 7, 42, 51, 53, 54, 63, 65, 66], the clinical consequences of this compromise still remain unclear. Finally, we wanted to determine which biomechanical property, PG content or hypotonic swelling, is more critical in determining biomechanical function as represented by aggregate equilibrium modulus.

Our study has numerous limitations. First, we used an ovine animal model, which has limited extrapolations to humans. However, recently published clinical reports on the effect of cold storage on human osteochondral plugs appear to demonstrate a comparable effect on human tissue with cold storage [22, 45, 64]. Second, in our protocol, storage was in a nutritive medium that was changed sterily every 48 hours as previously described [65]. This method may give the chondrocytes and the extracellular matrix a survival advantage by removing potentially degradative enzymes; this protocol may be different from commercial and institutional tissue bank protocols. The described protocol may explain why there were few differences among the cold-stored groups. Third, we had a limited number for analysis as a result of numerous study groups, cost of specimens, and early attrition of specimens. A power analysis was performed for all of the combined cold-stored specimens and in addition, none of the premature deaths were believed related to the allograft or surgical procedure. Fourth, although we only had qualitative assessment of chondrocyte viability, we did not have a quantitative measurement of cell density or viability. Finally, our subjects were allowed to bear weight immediately after this procedure. It is unclear what effect this had on the health of the allograft tissue because fixation and osseous integration are linked to allograft function [36, 46, 64].

This long-term in vivo ovine study suggests that cold-stored fresh osteochondral allograft exhibits superior material properties over freeze-thawed (fresh-frozen) grafts, which are devoid of any chondrocytes. Stored grafts, even with a reduced number of chondrocytes, have superior long-term properties and outcomes over freeze-thawed chondrocyte-free grafts. All fresh-stored grafts had evidence of viable chondrocytes suggesting no increase in cell necrosis and apoptosis at 52 weeks. Thus, the initial degradation and remodeling inherent to cold storage and the transplantation process may not impair the remaining functional chondrocytes from maintaining the extracellular

matrix and long-term function and integrity of the cold-stored allograft. Therefore, grafts containing functioning viable chondrocytes, even when greatly reduced like in Day 42 specimens, do better than grafts devoid of chondrocytes, implying there may be a critical minimum of functional viable chondrocytes to maintain the extracellular cartilage framework. This is in contrast to clinical experience with large fresh-frozen osteochondral allografts, which report acceptable results [25, 59]; however, histologic analysis shows that in fresh-frozen allografts, the articular extracellular matrix often deteriorates over time, presumably as a result of insufficient chondrocyte support [24]. It still remains unclear how fresh-stored graft compares with slow-freezing cryopreserved grafts, which still have some chondrocyte viability [32, 50]. In addition, recent clinical studies with fresh-stored allograft with prolonged storage time report good outcomes, which also supports the notion that there may be a critical minimum number of functioning chondrocytes that can maintain the extracellular matrix [22, 45, 64].

In addition, we found storage time did not appear to be a critical factor in the outcome of the fresh cold-stored allografts. Our findings demonstrated no differences in histologic, biomechanical, or biochemical properties among the cold-stored specimens. In fact, in our cases, the prolonged D42 grafts were just as, if not more, effective in reconstructing defects than the fresh D1 grafts. Although numerous in vitro studies have demonstrated the stepwise deterioration in material properties with prolonged storage time [1, 42, 54, 65, 66], in vivo studies suggest similar trends but with some important distinctions. Notably, the ultimate result of a transplanted allograft depends on numerous factors besides storage time. In an ovine model, the authors reported a time-dependent deterioration in chondrocyte viability but found a sharp reduction of biomechanical properties, which were independent of storage time (Vidal A, et al. An in vivo analysis of material properties of fresh osteochondral allografts. *J Bone Joint Surg Am.* 2008; in press). Similarly, in a primate study, the authors also found a time-dependent deterioration in chondrocyte viability but found compromised grafts even in the presence of viable chondrocytes [42]. Thus, viable chondrocytes of grafts “should not be equated to its transplantability” [42]. Finally, in a recent analysis of failed osteochondral allografts, chondrocyte viability was as high as 82% [66].

The question remains what other factors, besides storage time, are critical to the long-term success of transplanted allograft. There are factors that are both intrinsic to the allograft as well as extrinsic factors. In terms of intrinsic factors, chondrocyte viability may not be the most accurate indicator of allograft performance, because many conventional fluorescent viability stains used to quantify

chondrocytes are unreliable [41, 42]. Rather, PG content and other biochemical and biomechanical parameters that represent chondrocyte function such as PG synthesis [35], SO₄ incorporation, or equilibrium modulus may be better predictors of allograft function [7, 62, 66]. We also found a correlation between equilibrium aggregate modulus and PG content versus the hypotonic swelling group, suggesting the decrease in biomechanical properties in allograft transplantation may be related more to a decrease in PG content rather than collagen degradation. Also, the exact role of the chondrocyte in maintaining the extracellular matrix, and specifically, its turnover, which is approximately 4% every 3 to 5 years, may not be as directly linked as once believed [31]. Indeed, allograft transplantation is a complicated process with a dynamic interplay of multiple tissue interactions. The predominant paradigm, allograft function is maximized by fresh grafts that optimize chondrocyte viability and maintain the extracellular matrix, is probably true but is an oversimplification of a complex process [21, 47, 49].

There are a myriad of extrinsic factors that affect long-term graft function. These include but are not limited to direct chondrocyte injury, degradation of the extracellular matrix, lack of osseous incorporation, and immune rejection [33, 67]. Surgical technique is critical in terms of chondrocyte graft survival. In one study, direct trauma caused 21% to 47% of cell death in the superficial zone at 48 hours in an in vitro cadaveric human model [10]. These results favor the notion that chondrocyte cell death from direct impaction is mediated through caspases causing apoptosis [10]. Such cell death, together with the loss of cells in the process of cold storage, may contribute to matrix metalloproteinase-mediated PG loss and extracellular matrix degradation [53]. In addition, it has been postulated adequate fixation is critical for subchondral osseous incorporation and the current press-fit technique may not provide adequate fixation, especially in an immediate weightbearing model [46]. Other technical issues such as associated instability, meniscal tears, bipolar lesions, limb malalignment, medical comorbidities, and the status of the subchondral bone influence long-term allograft function [33, 36].

Other extrinsic factors include osseous incorporation and immune rejection, which have both been recognized as important factors for well-functioning allografts [29, 57, 65]. In a prospective paper, the authors reported complete or partial trabecular incorporation based on MRI positively correlated with SF-36 scores at the time of followup [64]. They also reported longer storage time correlated with better characteristics and postulated longer storage may reduce graft antigenicity. In an MRI study, the authors [57] noted antibody-positive patients demonstrated worse MRI characteristics (greater degree of bone marrow edema

pattern and surface collapse) than antibody-negative patients. In addition, in a recent analysis of failed fresh-stored allografts, there were cases of both limited osseous incorporation and allograft rejection noted by allograft collapse, osteonecrosis, and pannus formation [67]. Therefore, there are numerous factors that ultimately determine the long-term outcome of transplanted allografts and errors in any one of these could have explained why the material properties of all implanted grafts continued to deteriorate at 52 weeks when compared with nonoperative controls [7].

Our data suggest that fresh cold-stored allografts have superior histologic and biomechanical outcomes compared with acellular freeze-thawed grafts; moreover 42-day stored grafts are effective in reconstructing an osteochondral defect of the knee. In addition, we found no differences in cellular, biomechanical, or biochemical properties among 1-day, 14-day, and 42-day cold-stored specimens. Thus, grafts with prolonged storage time did not exhibit greater deterioration in their material properties.

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