

## Prolonged-fresh preservation of intact whole canine femoral condyles for the potential use as osteochondral allografts

James M. Williams<sup>a,b,c,\*</sup>, Amarjit S. Viridi<sup>a,d</sup>, Tamara K. Pylawka<sup>a</sup>,  
Ryland B. Edwards III<sup>e</sup>, Mark D. Markel<sup>f</sup>, Brian J. Cole<sup>a,d</sup>

<sup>a</sup> Department of Anatomy and Cell Biology, Rush Medical College at Rush Presbyterian St. Luke's Medical Center, Chicago, IL 60612-3864, USA

<sup>b</sup> Department of Biochemistry, Rush Medical College at Rush Presbyterian St. Luke's Medical Center, Chicago, IL 60612-3864, USA

<sup>c</sup> Department of Internal Medicine (Rheumatology), Rush Medical College at Rush Presbyterian St. Luke's Medical Center, Chicago, IL 60612-3864, USA

<sup>d</sup> Department of Orthopedic Surgery, Rush Medical College at Rush Presbyterian St. Luke's Medical Center, Chicago, IL 60612-3864, USA

<sup>e</sup> Department of Surgical Sciences, Comparative Orthopaedic Research Laboratory, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, WI 53706-1102, USA

<sup>f</sup> Department of Medical Sciences, Comparative Orthopaedic Research Laboratory, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, WI 53706-1102, USA

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### Abstract

Defects in articular cartilage are often repaired with fresh osteochondral grafts. While fresh allografts provide viable chondrocytes, logistic limitations require surgical implantation within seven days of graft harvest. Here, we provide information on cold preservation of whole intact osteochondral materials that retains cartilage cell viability and function, and histologic and biochemical integrity for 28 days. Canine femoral condyles were obtained and stored at 4°C for 14, 21 or 28 days. At the end of the storage period, cartilage was assessed for cell viability, <sup>35</sup>S uptake, proteoglycan content and histologic parameters. The most noticeable histologic change was reduced Safranin-O near the cartilage surface with 14 days of cold preservation, but had recovered with 21 and 28 days. Cartilage thicknesses did not vary significantly. Cell viability was >95% at 14 days, 75–98% at 21 days and reduced to 65–90% at 28 days. Cell function measures showed that the level of <sup>35</sup>SO<sub>4</sub> incorporation was suppressed in samples stored at 4°C. However, no significant differences were seen among groups at 14, 21 or 28 days of cold preservation. This data has implications for tissue banking protocols for osteochondral allograft material obtained for transplantation suggesting that cold preserved allograft material be implanted within 28 days.

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**Keywords:** Osteochondral; Allograft; Cartilage repair

### Introduction

An estimated 900,000 Americans suffer articular cartilage injuries each year. In patients younger than

40 years, full-thickness defects of the femur may be present in up to 5% of all arthroscopies [7]. Several options exist to treat symptomatic articular cartilage defects. Osteochondral grafting (autografts and allografts) is one option commonly utilized to treat these defects. Biologically, fresh osteochondral autografts are the standard for comparison to fresh osteochondral allograft transplants, especially in the management of large chondral or osteochondral defects (i.e., greater than 2 cm<sup>2</sup>) [4,9,10]. Historically, osteochondral allografts

\* Corresponding author. Address: Department of Anatomy and Cell Biology, Rush Medical College, 600 S. Paulina St., Chicago, IL 60612-3864, USA. Tel.: +1 312 942 3598; fax: +1 312 942 5744.

E-mail address: james\_m\_williams@rush.edu (J.M. Williams).

were commonly implanted after being frozen [17,19,26,27]. Freezing has the advantage of extending graft availability post-harvest, but it is thought that the clinical performance these grafts are diminished compared to autografts or fresh allografts. Currently, fresh osteochondral allografts are implanted following brief storage at 4°C [6,11,30]. While fresh grafts offer the distinct advantage of maintaining chondrocyte viability, there are significant logistic limitations because to maximize this viability, surgical implantation must be performed within seven days of graft harvest. In addition, appropriate serology testing implemented to minimize the chances for disease transmission often requires a minimum of 7–14 days to obtain final results.

Ideally, the ability to prolong the storage period of fresh osteochondral allografts without compromising the fitness of the graft would circumvent the limitations associated with shorter time periods typically required after graft procurement. There has been a long-standing interest in the storage of allograft materials. First attempts included storage in lactated Ringer's solution, however this storage media lacked essential nutrients (e.g. fetal calf serum) for sustained metabolic function of the chondrocytes [3,6,11,12]. Subsequently, development of storage media containing appropriate antibiotics and nutrients were developed with storage conditions utilizing temperatures at 4 and 37°C. Although early attempts at storage at 4°C were unsuccessful [5], later studies utilizing articular cartilage attached to the underlying bone demonstrated the usefulness of cold preservation of cartilage allograft materials stored in media at 4°C [1,15,22,29]. Cell viability, however, was limited largely to studies of sulfate incorporation of the chondrocytes into matrix proteoglycans (PGs).

This study reports the results of cold preservation (4°C) of whole femoral condyles to test the hypothesis that articular cartilage from prolonged cold preserved whole femoral condyles will perform similarly to articular cartilage from fresh whole femoral condyles *in vitro*. Endpoints included measurements of cell viability using a live/dead cell assay and confocal laser microscopy, assessment of cell function by quantification of sulfate incorporation into matrix proteoglycans, measurement of cartilage PG content and histologic analyses of the articular cartilage.

## Materials and methods

### *Animals, general procedures and retrieval of tissue from donor animals*

Adult male random source purpose-bred dogs (20–30 kg) were housed individually, fed Purina Dog Chow and food intake was monitored daily. Animals were assigned randomly to their respective groups. Animals were euthanized after which the distal femoral condyles were exposed using sterile technique through an arthrotomy

and removed by transecting the distal portion of the femoral shaft with a bone saw. All soft tissue was dissected away from the condyles prior to placement in the storage container for cold preservation. Although the condyles were rinsed briefly with sterile saline, no attempts were made to wash out the exposed marrow cavity. This procedure allowed the distal condyles to be placed in cold preservation while still intact. All procedures involving animal subjects were approved by the Rush Institutional Animal Care and Use Committee.

### *Groups and preservation protocols*

*Fresh intact whole femoral condyles:* These samples ( $n = 6$ ) were removed after death of the animal and immediately analyzed for end points as detailed below.

*Prolonged-fresh intact whole femoral condyles:* These femoral condyles were placed in sterile containers with 60 ml of minimal essential medium (MEM) containing 10% fetal calf serum (FCS), glutamate, non-essential amino acids and anti-microbial agents penicillin, streptomycin and fungizone. The containers were then placed in a clean cold box at 4°C for 14 ( $n = 6$ ), 21 ( $n = 6$ ) or 28 ( $n = 6$ ) days. Media was changed every 7 days and the temperature was monitored continuously. At the end of the preservation period the femoral condyles were examined grossly and then samples were removed for determination of cell viability using the live cell/dead cell technique with confocal microscopy, cell function using  $\text{Na}^{35}\text{SO}_4$  uptake, histological and biochemical analyses.

### *Cell viability using the live cell/dead cell assay*

At the end of the preservation period the medial femoral condyles were isolated using a band saw. A 1.6 mm thick wafer of cartilage and bone was then removed using an Isomet 4000 saw equipped with an irrigation system to prevent heating the samples during the sawing procedure. This procedure yielded a 1.6 mm thick sample of cartilage attached to the underlying subchondral bone from the central habitually loaded region of the medial femoral condyle (i.e. the same area from which osteochondral plugs would be removed for allo-plantation.) Care was taken to ensure that these osteochondral slices were removed from the same area of each whole femoral medial condyle. Osteochondral slices were placed in 4  $\mu\text{M}$  calcein-AM and 8  $\mu\text{M}$  ethidium homodimer [16] (in 2-ml of 0.9% saline) at room temperature for 30 min. Samples were then rinsed and examined on a confocal laser-scanning microscope (MRC-1000, BioRad, Hemel Hempstead/Cambridge, England) equipped with an argon laser and necessary filters (fluorescein and rhodamine). The calcein-AM and ethidium homodimer were supplied as part of a live cell/dead cell kit from Molecular Probes Inc. which is used to identify living cells labeled with calcein-AM (green fluorescence) and dead cells labeled with ethidium homodimer (red fluorescence). Estimates of cell viability were obtained by counting the red and green cells in a full thickness area of approximately 1 mm<sup>2</sup> and then expressing living cells (stained green) as a percentage of the total, thus percent viability.

### *Cell function using $\text{Na}^{35}\text{SO}_4$ uptake and proteoglycan (PG) content determination*

Full thickness samples of articular cartilage were removed from the medial femoral condyles for <sup>35</sup>S incorporation. All remaining articular cartilage was harvested for determination of cartilage PG content. Full thickness slices of articular cartilage from the medial femoral condyles were placed individually in sterile 12 well plates containing 3.0 ml of culturing medium (same composition as used for preservation at 4°C) and  $\text{Na}^{35}\text{SO}_4$  (5  $\mu\text{Ci/ml}$ ; 15  $\mu\text{Ci}$  total per well). The samples were incubated for 16 h at 37°C under 95% air:5% CO<sub>2</sub>, after which they were rinsed briefly in unlabelled media and weighed. Extraction of the extracellular matrix components was carried out in 2 ml of 4 M-guanidine hydrochloride/100 mM EDTA solution overnight at 4°C with continuous shaking. The extracts were dialyzed against 10 mM EDTA at 4°C to remove free nucleotides and guanidine hydrochloride. The total volume of extract from each sample was determined and 200  $\mu\text{l}$  was taken and subjected to scintillation counting. The counts were corrected for total volume and weight of sample and represented as CPM per

mg-wet weight of tissue. Cartilage PG content was determined from the remaining cartilage by the DMB method [14] and expressed per wet weight or per  $\mu\text{g}$  DNA [23].

#### Histological analyses

**Routine light microscopic analysis.** The cartilage-bone samples utilized for the live cell/dead cell assay and additional adjacent samples of cartilage and bone were subsequently fixed and processed for histologic analysis. Thus, we were able to examine the effects of live/dead cell assay technique in the histologic appearance of the samples. In addition, full thickness samples of cartilage and bone were removed for histology from the area immediately adjacent to the slice taken for the live/dead cell assay. Samples were fixed in 10% neutral buffered formalin containing 0.5% cetylpyridinium chloride and then were decalcified in aqueous formic acid/sodium citrate (22%/10%) after which they were processed for paraffin embedding. Full thickness sections ( $8\mu\text{m}$ ) through the articular cartilage and underlying subchondral bone were obtained and placed on glass slides coated with Vectabond (Vector Laboratories). Sections were stained with Safranin-O to exhibit matrix PGs [21] or with hematoxylin and eosin. Sections were processed and stained concurrently to control for any variations in uptake of the stain.

#### Data analysis

Samples were analyzed for quantification of histology parameters (articular surface, Safranin-O staining, cellularity, cluster/clone formation) according to a modification of a previously published scoring system [31], which permits comparison of groups. Digital images of the articular cartilage and underlying subchondral bone were analyzed using the MetaVue Image Analysis software. This permits lines to be drawn extending from the articular surface to the tidemark (uncalcified layer) and from the tidemark to the subchondral bone (calcified layer). The total cartilage thickness was obtained by adding the uncalcified and calcified layer thicknesses. For each sample 10 measurements of the uncalcified layer, calcified layer and total cartilage thickness were obtained. The mean and standard deviation were determined for each measurement. Statistical analysis was performed using analysis of variance (ANOVA) using Graph Pad InStat (Version 3.05, San Diego, CA). When significance was identified, appropriate post-hoc testing was performed. Significance for all analyses was set at  $p < 0.05$ .

## Results

### Histology

Using our scale for quantification of histology parameters an ideal score is  $6.0 \pm 0.5$  (mean  $\pm$  standard deviation). Fresh cartilage samples scored  $6.8 \pm 0.3$ . After 14 days the composite histology score was  $7.7 \pm 1.8$  (Fig. 1). Scores after 21 and 28 days of cold preservation were  $6.8 \pm 1.6$  and  $7.9 \pm 1.1$ , respectively. No significant differences were seen among composite histology scores of these groups. Examination of the individual parameters used to compute the composite score reveal that most noticeable change occurred with respect to Safranin-O staining. Thus, Safranin-O staining was reduced near the cartilage surface with 14 days of cold preservation, but had recovered with 21 and 28 days of cold preservation. The slight reduction in cellularity and Safranin-O staining at 14 days was confined to the most superficial layer of articular cartilage (Fig. 2). Average uncalcified and calcified cartilage layer thicknesses were compared among the 4 groups. The uncalcified cartilage layer thickness was  $567\mu\text{m} \pm 191$  for control samples. Uncalcified cartilage layer thicknesses for samples preserved for 14, 21 and 28 days were  $778\mu\text{m} \pm 253$ ,  $712\mu\text{m} \pm 122$  and  $673\mu\text{m} \pm 112$ , respectively (Fig. 3). Although higher than controls, none of these thicknesses were statistically significant. This lack of statistical significance remained even after removal of outlying values. Similarly, there was no significant variation in the calcified layer thickness as a function of the groups. Finally, there were no differences noted between samples used first for the live/dead cell confocal

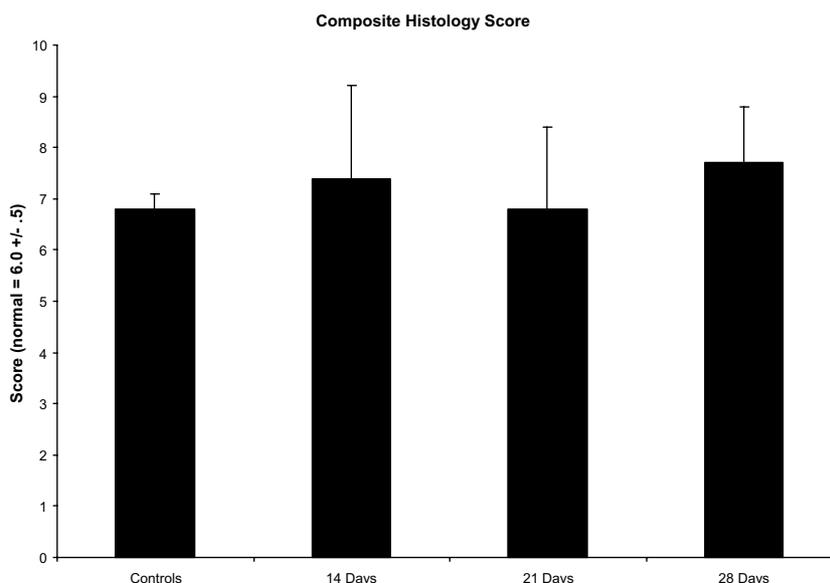


Fig. 1. Histological assessment of cold preserved condyles. Controls are fresh samples without any preservation.

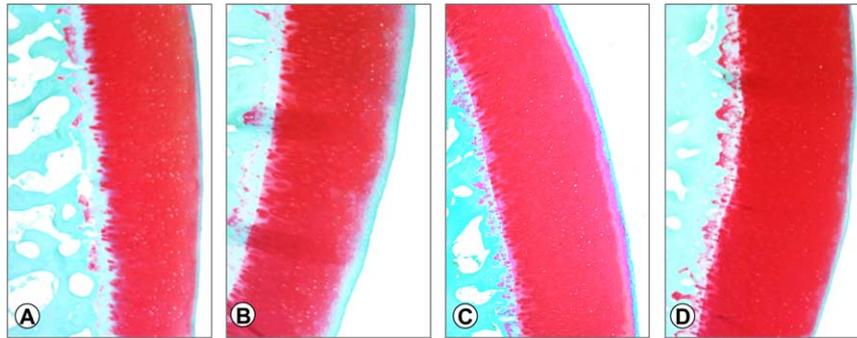


Fig. 2. Histological analysis of condyles cold preserved for various lengths of time. Control group represents fresh tissue samples without any preservation. Safranin-O (A) Fresh tissue with normal staining pattern. (B), (C) and (D) cold preserved tissues for 14, 21 and 28 days, respectively.

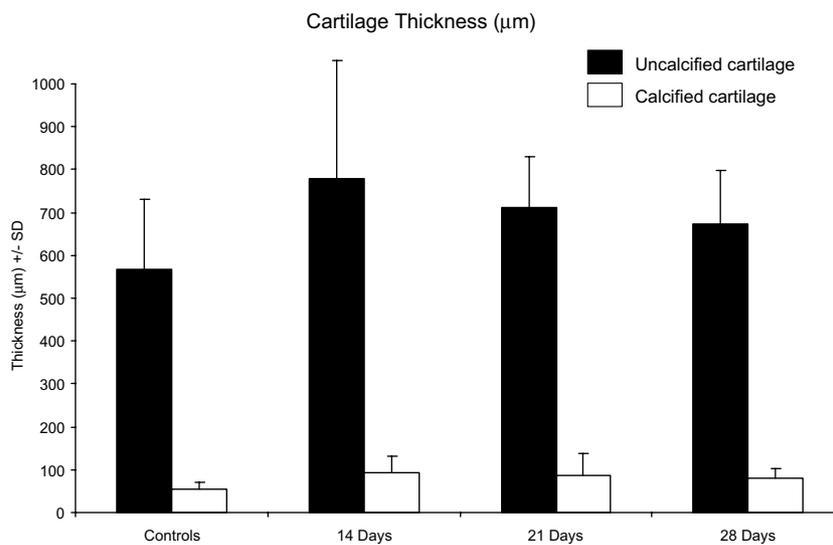


Fig. 3. Cartilage thickness measurements of cold preserved condyles. Controls are fresh samples without any preservation.

microscopy and then processed for histology and adjacent samples fixed and processed directly for histology.

*Cell viability using live/dead cell technique*

Maximum cell viability (95–100%) was noted in fresh cartilage samples not exposed to preservation media (Fig. 4). Maximum cell death (100%; not shown) was noted in cartilage samples boiled in water for 20 min. Chondrocyte viability was 94–98% ( $97 \pm 1.5$ ) in samples stored in cold preservation for 14 days and 75–98% ( $89 \pm 6.9$ ) in samples stored in cold preservation for 21 days. Chondrocyte viability was reduced to 65–90% ( $78 \pm 10.9$ ) with a wider range of values in samples stored for 28 days.

*Cell function using  $Na^{35}SO_4$  incorporation and cartilage PG content*

Cell activity was determined as a function of  $^{35}S$  incorporation by the chondrocytes (Fig. 5). The results

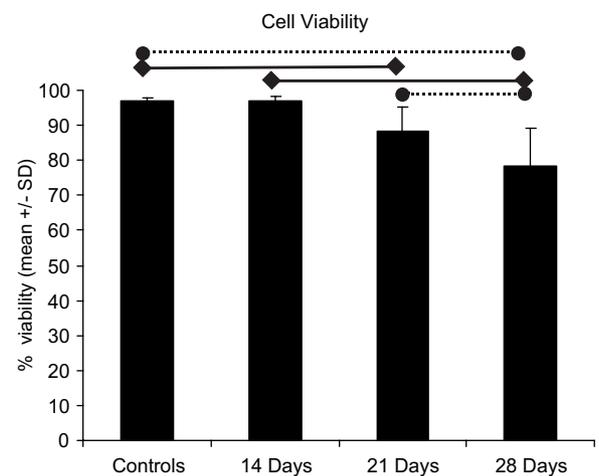


Fig. 4. Cell viability presented as a percentage of living cells [ $p < 0.05$  = solid line;  $p < 0.001$  = dashed line].

are presented as percentage incorporation compared to fresh samples. The data shows that the level of  $^{35}SO_4$

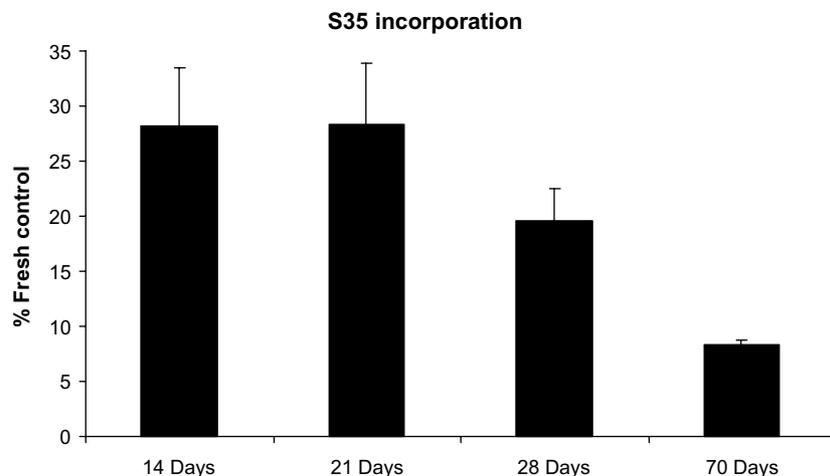


Fig. 5. Assessment of cell function, as  $\text{Na}^{35}\text{SO}_4$  uptake, in slices of cartilage removed from condyles cold preserved for various lengths of time. Data is presented as a percentage of  $^{35}\text{S}$  incorporation compared to fresh control. 70-day groups represents samples preserved in lactated Ringer's solution and was considered to be a negative control.

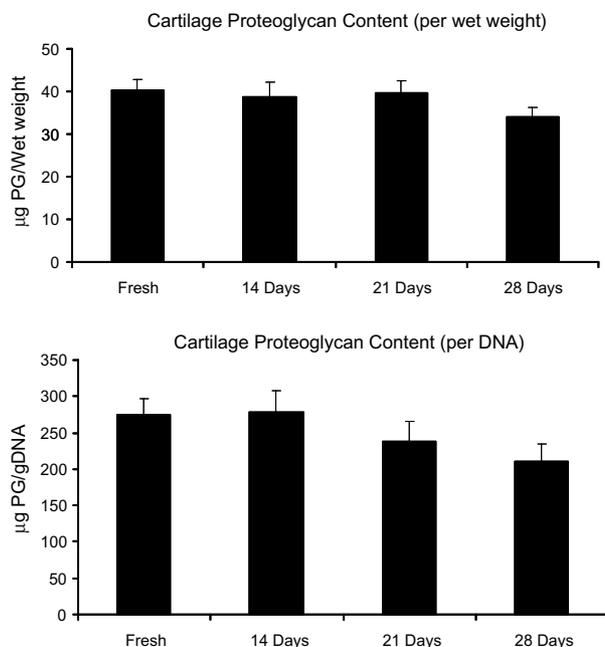


Fig. 6. Total PG content of cartilage samples obtained from condyles stored for various lengths of time. Control group represents fresh tissue samples without any preservation. Top panel depicts total PG content as a function of cartilage wet weight and the bottom panel shows total PG content as a function of cell number (DNA).

incorporation was suppressed in samples stored for 14 days (by approximately 70%;  $p < 0.0001$ ). No significant reduction was seen for the next 7 days (at 21 days), which was followed by a sharp decline after the next 7 days (at 28 days). The decline in incorporation from 21 to 28 days was significant ( $p < 0.05$ ). Total PG content in the extracellular matrix of cold preserved samples showed no significant difference from fresh control when normalized for DNA content or wet weight (Fig. 6).

There was, however, an apparent tendency for decline in the total PG content after 21 days.

## Discussion

The data obtained in this study of cold preservation of articular cartilage and subchondral bone suggests that osteochondral plugs can be obtained from intact femoral condyles that have been in cold preservation for up to 28 days. Currently, allograft material that has been cold preserved is shipped to the surgeon as an intact hemicondyle from which the osteochondral plug is prepared. While the results of the live/dead cell-staining assay are favorable, metabolic indicators such as  $\text{SO}_4$  uptake may indicate some deterioration in chondrocyte function despite excellent cell viability at 28 days. This is consistent with other similar studies where reduced  $^{35}\text{S}$  uptake has been noted [1,5,15,22,29]. Based on these results we would conclude that allograft materials obtained from tissue banks utilizing cold preservation be implanted within 28 days with optimal time being closer to 14 days. A definitive earlier study in this area suggested implantation of tibial cartilages preserved at cold condition for 28 days [15]. We agree with the assertion of this study that the best assessment for these implants will be when they are implanted in vivo. Other recent studies have success with cold preservation at 14–28 days, however the decline in metabolic state of the chondrocytes remains a concern [2].

Symptomatic articular cartilage lesions are a clinical challenge. Interest in treatment alternatives has increased rapidly with the development of newer surgical techniques. Osteochondral allografting is one such alternative with clinical experience extending over two decades. While there are many treatment alternatives to

consider, the use of fresh osteochondral allografts is particularly appealing because: (1) the allograft essentially involves a transplantation of the intact organ, i.e., mature hyaline cartilage with living chondrocytes and normal architecture, which should, in theory, provide optimal replacement tissue both biologically and mechanically; (2) the composite nature of the graft provides a mechanism to restore associated subchondral bone deficiencies commonly seen in such conditions as osteochondritis dissecans, avascular necrosis or osteochondral fracture, and (3) the ability to create anatomically appropriate grafts to restore complex articular geometry.

Osteochondral grafts have been utilized as autografts, fresh-frozen allografts, or fresh allografts that have been stored at 4°C for <7 days after retrieval [18,24]. Donor site morbidity remains a concern with autograft use. In general, long term results following repair of osteochondral defects using frozen allografts has been sub-optimal [10,26,28–30]. Despite attempts to refine the freezing process (i.e., cryopreservation), chondrocyte death remains a significant problem. While fresh allografts (minimal preservation) maintain cell and matrix viability, they must be implanted within 7 days to minimize chondrocyte death. Logistically, this causes significant scheduling difficulties required for surgical implantation and leads to supply limitations. The ideal allograft should have a prolonged shelf life with maintenance of cell and matrix viability. The development of successful cold preservation protocols has extended that shelf life from <7 to 28 days or possibly longer [16–19,30]. Based partly on these studies tissue banks now provide surgeons with osteochondral allografts which have been cold preserved for up to 28 days.

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