Long-term storage effects on canine osteochondral allografts

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We have studied long-term (to 60 days) effects of 4 °C storage in culture media on the histologic, mechanical, and chemical properties of the cartilage from osteochondral shell allografts from the dog. The structural integrity of the cartilage matrix was intact up to 60 days of storage, for the mechanical properties represented by the aggregate modulus and apparent permeability remained normal. These data are supported by normal safranin-O staining as well as normal glycosaminoglycan content and total collagen concentration. However, chondrocyte viability, as assessed by $^{35}SO_4$ uptake and hematoxylin and eosin preparations, decreased dramatically with time. We believe that the longer storage to 60 days is not indicated, unless conditions can be modified to maintain cell viability.

Articular cartilage, once injured, has a limited ability for repair (Coletti et al. 1972, Mankin 1982), and the subsequent deterioration can be progressive, leading to significant joint pain. For many afflictions, such as traumatic injury and osteoarthrosis seen in the younger population, efforts have been directed towards developing effective repair treatments. Osteochondral allograft transplantation, with the bone providing the means of securing the graft in place, has emerged in the past two decades as an alternative to artificial joint replacement (Aichroth et al. 1971, Friedlaender et al. 1983, Mankin et al. 1983, Meyers et al. 1983, Meyers 1985, Czitrom et al. 1986, Mankin et al. 1987). The replacement of articular surfaces with biologic tissue is advantageous because only damaged areas need be replaced, and the bone can revascularize as the graft is incorporated as an integral part of the joint surface. Clinical reports on the application of osteochondral allografts to joints afflicted by traumatic injuries, tumors, and osteonecrosis include Bayne et al. (1985), Czitrom et al. (1986), Mankin et al. (1987), Jofe et al. (1988), and Meyers et al. (1989). For an average follow-up time of 2–5 years, more than 70 percent of the cases were rated as good or excellent, with pain being significantly reduced and function improved from the preoperative status of the patients. The remaining 30 percent had complications such as fracture, infection, and tumor recurrence as causes for failures.

With the success of allograft treatment, the demand for tissue has risen, and the time constraints associated with coordinating a donor and a recipient create the need for banking the allografts. Although many procedures exist for graft storage, two promising methods include cryopreservation and storage in culture media at temperatures above freezing (Aston and Bentley 1986, Brighton et al. 1979, Jimenez and Brighton 1983, Sondenaa et al. 1985, Schachar and McGann 1986, Rodrigo et al. 1987, Tomford et al. 1989). The optimal freezing technique for intact cartilage that allows the chondrocytes to remain viable is still under investigation (Jiminez and Brighton 1983, Schachar et al. 1986, Tomford et al. 1989).

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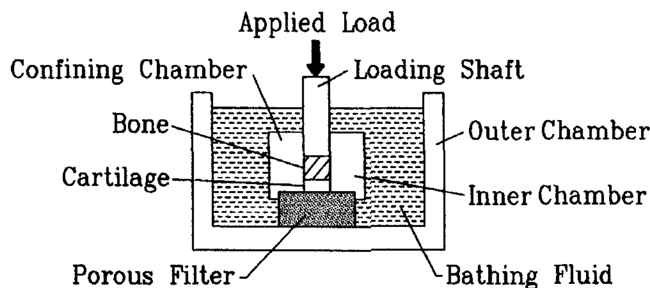

Figure 1. The confining compression chamber depicting a 4-mm-diameter, cylindrical osteochondral specimen in contact with a porous filter and bathed in Ringer's solution.

In our laboratory, 4 °C storage in culture media of osteochondral allografts was found to be satisfactory for maintaining the articular cartilage properties and the viability of the chondrocytes up to 28 days (Kwan et al. 1989, Amiel et al. 1989). However, longer storage times are desirable. We therefore investigated a prolonged period of storage of 60 days at 4 °C in culture media and evaluated the effects of storage on the histologic, mechanical, and chemical properties of the articular cartilage from osteochondral shell allografts.

Materials and methods

The knee joint of mongrel canine hind limbs was chosen as the experimental model for this study. The medial and lateral tibial plateaus, with approximately 0.5 cm of underlying bone, were separated and harvested from the knees of 6 skeletally mature dogs. The medial plateaus were evaluated mechanically and histologically, while the lateral plateaus were used for chemical analyses. The plateaus from the right joints served as controls. The left plateaus were stored submerged in individual closed containers, filled with a culture media following Brighton et al. (1979). The containers were stored at 4 °C, and the media was changed twice weekly (Kwan et al. 1989, Amiel et al. 1989. The length of time in storage was 60 days, after which the plateaus were evaluated grossly for color and surface integrity, histologically, mechanically, and chemically.

Histologic evaluation

Sections from control and stored medial plateaus were fixed in 10 percent buffered formalin, decalcified in EDTA, and embedded in paraffin. Six-micron-thick coronal sections were cut and stained with hematoxylin and eosin (H&E) for structural details/cell appearance and safranin-O for glycosaminoglycan distribution in the cartilage layer using normal light microscopy.

Mechanical analysis

For mechanical testing, cylindrical osteochondral specimens, 4 mm in diameter, were cored from the medial plateaus of both the control specimens, immediately after harvest, and the stored specimens at the end of the storage period. The specimens were equilibrated in Ringer's solution for 1 hour at room temperature, after which the cartilage thickness was measured using a cathetometer. A confined compression test was then used to determine the biphasic mechanical properties of the tissue (Kwan et al. 1989). The cylindrical specimen was placed within a confining chamber, bathed in Ringer's solution at room temperature, and in contact with a porous filter, allowing free flow of fluid in and out of the tissue (Figure 1). A step load of 0.2 N was applied and the resulting creep deformation over time recorded until equilibrium was reached (~45–60 min).

The experimental data from each creep test were curve fitted with an analytic equation derived from the biphasic theory to determine two material properties of the tissue—the aggregate modulus and the apparent permeability (Kwan et al. 1984). This theory views articular cartilage as a mixture of a solid matrix filled with a movable fluid and incorporates the contributions of both the solid and fluid phases to the behavior of the tissue. The aggregate modulus is a measure of the stiffness of the cartilage matrix at equilibrium, while the apparent permeability represents the ease with which fluid may flow in or out of the cartilage matrix. These two properties aid in describing the viscoelastic behavior of articular cartilage in compression.

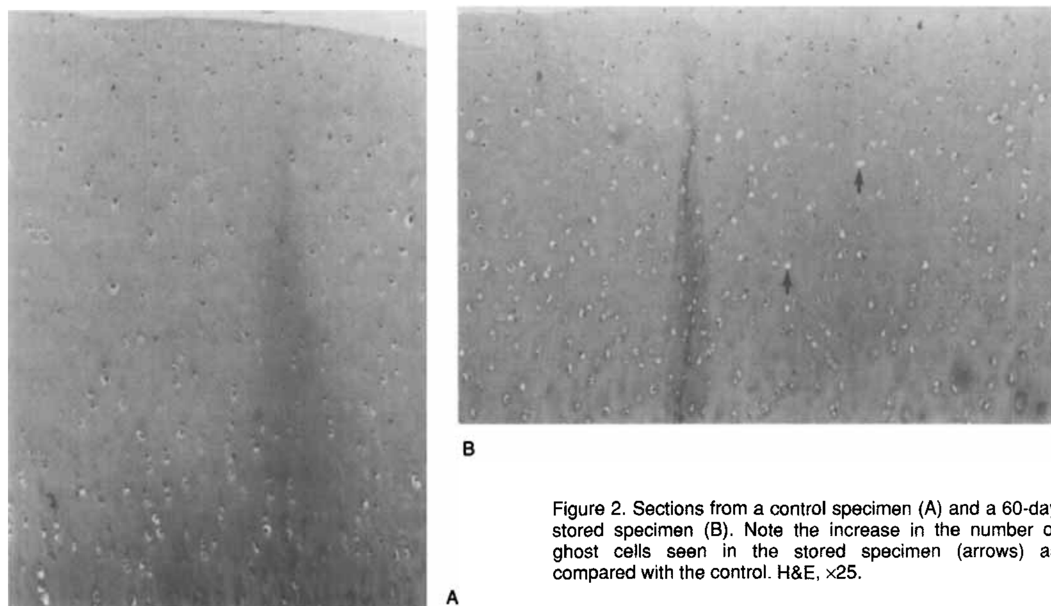

Figure 2. Sections from a control specimen (A) and a 60-day stored specimen (B). Note the increase in the number of ghost cells seen in the stored specimen (arrows) as compared with the control. H&E, ×25.

Chemical analysis

Chondrocyte viability, total glycosaminoglycan (GAG) content, and total collagen concentration were determined for the control and stored articular cartilage. Cartilage specimens were also analyzed for the presence of the major (I and II) and minor (IX and XI) collagen types to determine whether or not chondrocyte dedifferentiation – the synthesis of atypical collagen types, such as type I (characteristic of fibrocartilage) instead of type II – occurred.

For assessment of the viability and synthetic capacity of the chondrocytes, $^{35}SO_4$ incorporation into the glycosaminoglycans was measured (Curran and Gibson, 1956). The cartilage was dissected from the bone, washed, lyophilized, and finely ground. $Na^{23}SO_4$ was then added to 50mL of the media at a concentration of 4 μCi/mL (after harvest for control and after the storage period for stored specimens), and the cartilage was incubated for 48 h at 37 °C. $^{35}SO_4$ activity was measured after correction for normal radioactive decay.

The GAG concentration was determined by measuring the hexosamine content in the cartilage. The concentration of collagen was calculated from the hydroxyproline content, as measured with a Technicon autoanalyzer and converted to percent collagen by a multiplication factor of 7.46. The relative amounts of type I and type II collagen were determined by peptide analysis. The cartilage, pooled from a particular time point, was washed, lyophilized, and extracted twice with 4 M guanidine HCl to remove proteoglycans and was then digested with cyanogen bromide. Isolation and quantification of the liberated CNBr peptides were then done (Harwood and Amiel, 1986). Analysis of the minor collagen types was accomplished by differential salt precipitation of pepsin extracts. Following removal of the major collagen types, the minor collagens were analyzed by SDS-PAGE (Amiel et al. 1989). To reduce disulfide bonds, ß-mercaptoethanol (ßME) was added to the gels. Comparative densities of the peptide bands were determined by visual inspection.

Statistics

One-way analyses of variance (ANOVA) were performed on the normalized values (stored/control) to determine the effect of time in storage on the mechanical and chemical properties measured. Additionally, comparisons between control and stored values for 60 days were made using paired *t*-tests to assess whether the stored cartilage differed significantly from its own control.

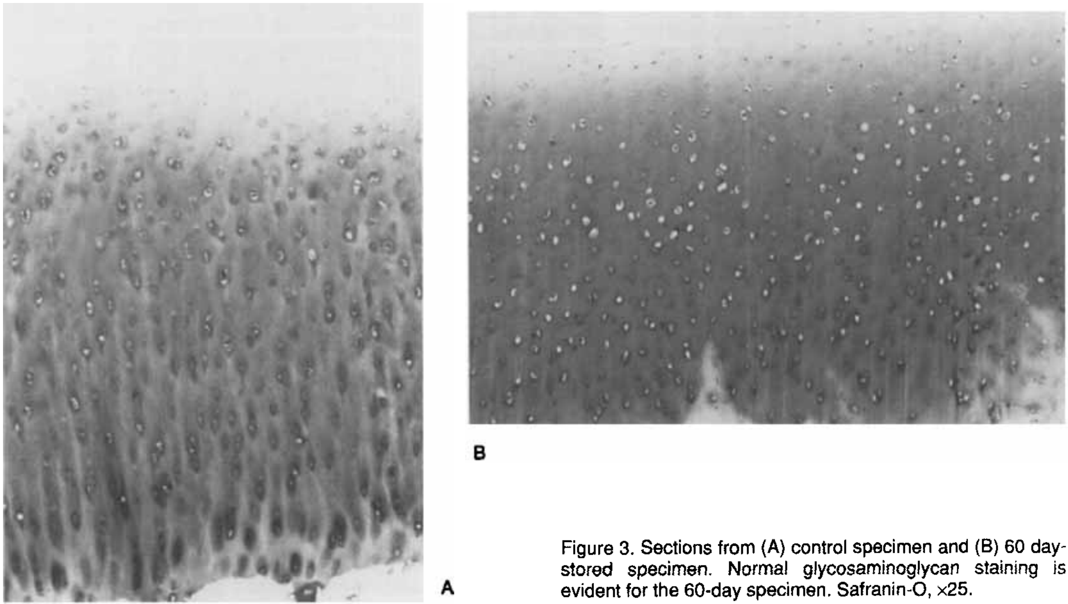

Figure 3. Sections from (A) control specimen and (B) 60 day-stored specimen. Normal glycosaminoglycan staining is evident for the 60-day specimen. Safranin-O, ×25.

Results

On gross examination, the cartilage from all the plateaus after the storage time resembled normal articular cartilage. They retained a glistening white appearance with no obvious signs of swelling, pitting, or fibrillation noted in the 60-day stored cartilage.

Histology

There appeared to be an increase in the number of ghost cells, with evidence of some degenerating chondrocytes, in the 60-day specimens as compared with control cartilage upon evaluation of H&E sections (Figure 2). There was a tendency towards cloning of cartilage cells in the 60-day stored tissue. These cellular changes were observed throughout the thickness of the cartilage layer, although the surface layer seemed relatively intact and unchanged. Safranin-O staining for the 60-day stored tissue was similar to control cartilage with the normal distribution of glycosaminoglycans evident. Staining was marked in the middle and deep zones, with little or no staining in the superficial layer (Figure 3).

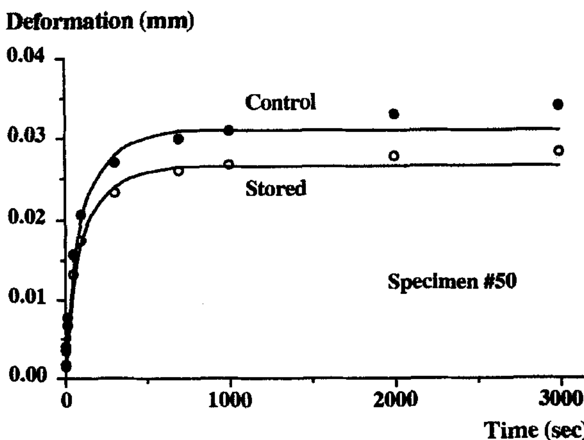

Figure 4. Typical creep curves for specimen #50, a 60 day stored specimen, and its control. The analytic curves (solid lines) show good agreement for both specimens.

Table 1. Comparison of mechanical and chemical properties measured for the control and 60-day stored cartilage specimens. Mean *SEM*, n 6

	Control		60 Days	
Aggregate modulus (MPa)	0.62	*0.04*	0.64	*0.09*
Aggregate permeability (10^{-15} m^4/N-s)	10.17	*0.73*	9.00	*1.63*
$^{35}SO_4$ incorporation (cpm/mg tissue)	12,848	*100*	2,171	*1,048**
GAG concentration (percent hexosamine/gm tissue)	4.34	*0.15*	4.23	*0.19*
Total collagen content (percent collagen/gm tissue)	51.5	*1.33*	50.0	*1.73*

* Different from the control, $P < 0.001$, n 5

Table 2. Normalized values (stored/control) for the mechanical properties at the five time points studied (mean *SEM*). All the values tended towards unity, indicating no difference between stored and control properties

Days	Aggregate modulus		Apparent permeability	
3	0.92	*0.11*	1.26	*0.20*
7	0.99	*0.06*	1.15	*0.21*
14	1.03	*0.20*	1.10	*0.18*
28	1.24	*0.24*	1.06	*0.23*
60	1.41	*0.34*	1.09	*0.29*

Table 3. Normalized values (stored/control) for the total GAG and collagen contents and $^{35}SO_4$ incorporation at the time points studied (mean *SEM*). For the $^{35}SO_4$ incorporation, which is indicative of chondrocyte viability, a decrease was noted for the 60-day specimens

Days	Total GAG		Total collagen		$^{35}SO_4$ incorporation	
3	0.98	*0.03*	1.04	*0.02*		
7	1.03	*0.03*	1.04	*0.01*	1.03	*0.08*
14	1.01	*0.04*	1.00	*0.04*	0.92	*0.07*
28	0.95	*0.02*	0.99	*0.03*	1.16	*0.19*
60	0.97	*0.02*	0.97	*0.03*	0.17	*0.10*

Mechanics

Because articular cartilage is viscoelastic, the tissue exhibits time-dependent behavior. Thus, after application of a step-load, the deformation of the surfaces increases with time (creep). Typical creep curves for a 60-day specimen and its contralateral control are depicted in Figure 4, showing similarities and differences between the stored and the control specimen. The 60-day stored cartilage was slightly stiffer than the control, having a lower equilibrium displacement value than control. The analytic curves predicted by the biphasic theory showed good agreement with the experimental data for both control and stored specimens.

Both the means for the aggregate modulus and the apparent permeability (Table 1) were found to be similar to values in the literature for bovine (Mow et al. 1980) and human (Armstrong and Mow 1982) articular cartilage. The values of the aggregate modulus and apparent permeability for the 60-day stored specimens did not differ from the control ($P > 0.40$, $P > 0.50$).

For the effects of time in storage, normalized values (stored/control) of the aggregate modulus and permeability for the time periods of 3 to 60 days were evaluated (Table 2). The aggregate modulus for the stored specimens appeared to be higher as compared with control values as the time in storage increased to 60 days (i.e., normalized value > 1.0). An opposite trend was observed for the apparent permeability—for the early time points, the stored tissue appeared to be more permeable than the control tissue, but this difference decreased with longer storage times. Despite these trends, a one-way ANOVA showed no significant effect of time in storage up to 60 days on either the normalized aggregate modulus ($P > 0.40$) or the normalized permeability ($P > 0.50$).

Chemistry

A decrease in $^{35}SO_4$ incorporation was evident at 60 days compared with the control (Table 1; $P < 0.001$), and a significant effect of storage time on the normalized (stored/control) incorporation was also seen (Table 3; $P < 0.001$). The total GAG content and collagen concentration at 60 days, however, were not statistically different from the control ($P > 0.20$, $P > 0.40$). Table 3 shows the normalized values for the time points. A one-way ANOVA revealed no statistical differences among the time periods for the normalized GAG content or collagen concentration ($P > 0.30$, $P > 0.30$).

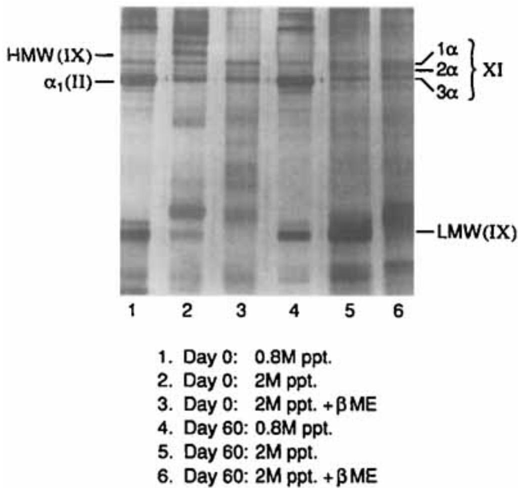

Figure 5. SDS-PAGE of collagen fractions. Note the similar marking for types IX and XI between control (lanes 1, 2, and 3) and 60-day stored specimens (lanes 4, 5, and 6).

Type II collagen remained the major collagen type synthesized. The presence of the peptide α_1(II)-CB10, characteristic of type II collagen, as well as the absence of the peptide α_2(I)-CB3,5 of type I collagen, was demonstrated for the 60-day specimens. Normal articular cartilage also contains minor types of collagen (i.e., type IX and type XI collagen). After 60 days of storage, the cartilage appeared to contain the same minor collagen types as the control (Figure 5).

Discussion

The transplantation of osteochondral allografts has emerged as a possible alternative to artificial joint replacement in the treatment of various articular joint defects. Because fresh donor tissue is used within 72 hours of harvest to insure viability of the cartilage under current storage conditions (Meyers 1985), the need for banking osteochondral allografts is pressing. Consequently, suitable banking conditions that would allow for longer storage times, while maintaining chondrocyte viability and the structural integrity of the cartilage, are required.

Storage of osteochondral tissue at temperatures above freezing has been shown to be a feasible banking method. Handley et al. (1986) stored bovine articular cartilage slices for 3 weeks in sterile culture media containing 20 percent fetal calf serum and noted no changes in the cartilage matrix. Brighton et al. (1979) demonstrated that articular cartilage slice explants could remain viable up to 60 days when stored at 37 °C in tissue culture media containing vitamin E. However in whole-organ culture, our preliminary work indicated significant bone and cartilage degradation at 37 °C. In this study, storage of osteochondral tissue up to 60 days at 4 °C revealed no changes in either glycosaminoglycan or collagen content, which is correlated with the mechanical findings of no significant changes in the aggregate modulus or apparent permeability of the cartilage. The normal GAG content is also supported by the qualitative assessment of normal safranin-O staining. This would indicate that the cartilage matrix is structurally intact. Further, chondrocyte dedifferentiation could not be demonstrated.

While we noted little cellular changes up to 28 days of storage, there was a dramatic fall in $^{35}SO_4$ incorporation after 60 days of storage, signifying a loss of functioning chondrocytes. This finding correlates with the presence of ghost cells and degenerating chondrocytes seen histologically. It is interesting to note that the chondrocytes below the surface layer seemed to suffer the most deaths, suggesting possible involvement of cartilage nutrition. Because articular cartilage lacks a vascular network as a nutrition source, the storage method used in this study may not provide adequate nutrition for the cells up to 60 days. Of interest is a recent study that reported a possible beneficial effect of compressive cyclic loading applied to the cartilage during storage (Fine et al. 1988). This may enhance current storage conditions by increasing nutrient transport throughout the tissue as fluid flows within the cartilage matrix during the loading cycle.

The results of this study are encouraging. It is apparent that osteochondral tissue can be stored in vitro at 4 °C up to 60 days without significant alterations in either the mechanical properties or the chemical concentration of matrix components (i.e., GAG and collagen) of the cartilage. Although a significant decrease in glycosaminoglycan synthesis as well as the presence of several ghost cells and some degenerating cells were noted at this time period, replenishing of degraded matrix components has not yet been significantly affected by the loss of functioning cells, and thus the integrity of the matrix has not been compromised. Because of the decrease in cell viability, however, storage to 60 days is not indicated unless current storage conditions can be modified appropriately. The data from this study provide important information for the storage and banking of osteochondral allografts. The in vivo survival of such stored allografts must be evaluated in future studies.

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References

Aichroth P M, Burwell R B, Elves M W, Ford C H J, Lawrence M. A review of recent attempts to reconstruct synovial joints with osteoarticular allografts. J West Pacific Orthop Assoc 1971; 8 (2): 1–24.

Amiel D, Harwood F L, Hoover J A, Meyers M. A histological and biochemical assessment of the cartilage matrix obtained from in vitro storage of osteochondral allografts. Connect Tissue Res 1989; 23 (1): 89–99.

Armstrong C G, Mow V C. Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. J Bone Joint Surg (Am) 1982; 64 (1): 88–94.

Aston J E, Bentley G. Repair of articular surfaces by allografts of articular and growth plate cartilage. J Bone Joint Surg (Br) 1986; 68 (1): 29–35.

Bayne O, Langer F, Pritzker K P, Houpt J, Gross A E. Osteochondral allografts in the treatment of osteonecrosis of the knee. Orthop Clin North Am 1985; 16 (4): 727–40.

Brighton C T, Shadle C A, Jimenez S A, Irwin J T, Lane J M, Lipton M. Articular cartilage preservation and storage. I. Application of tissue culture techniques to the storage of viable articular cartilage. Arthritis Rheum 1979; 22 (10): 1093–101.

Coletti J M Jr, Akeson W H, Woo S L. A comparison of the physical behavior of normal articular cartilage and the arthroplasty surface. J Bone Joint Surg (Am) 1972; 54 (1): 147–60.

Curran R C, Gibson T. The uptake of labelled sulphate by human cartilage cells and its use as a test of viability. Proc R Soc London (Biol) 1956; 144: 572–6.

Czitrom A A, Langer F, McKee N, Gross A E. Bone and cartilage allotransplantation. A review of-14 years of research and clinical studies. Clin Orthop 1986; 208: 141–5.

Fine K M, Kelly J D, Brighton C T, Jiminez S. Cyclic loading of stored rabbit medial femoral condyles and its effect upon articular cartilage when transplanted. Trans Orthop Res Soc 1988; 13: 111.

Friedlaender G E, Mankin H J, Sell K W. (Eds.). Osteochondral allograft: biology, banking and clinical applications. Little Brown & Co, Boston 1983.

Handley C J, McQuillan D J, Campbell M A, Bolis S. Steady state metabolism in cartilage explants. In: Articular Cartilage Biochemistry (Ed. Kuettner, K. E.) Raven Press, New York 1986.

Harwood F L, Amiel D A. Semiquantitavie HPLC analysis of types I and III collagen in soft tissues. LC Mag 1986; 4 (2): 122–6.

Jimenez S A, Brighton C T. Storage and preservation of viable articular cartilage. In: Osteochondral Allograft: Biology Banking and Clinical Applications (Eds. Friedlaender G E, Mankin H J, Sell K W). Little, Brown Co, Boston 1983.

Jofe M H, Gebhardt M C, Tomford W W, Mankin H J. Reconstruction for defects of the proximal part of the femur using allograft arthroplasty. J Bone Joint Surg (Am) 1988; 70 (4): 507–16.

Kwan M K, Lai W M, Mow V C. Fundamentals of fluid transport through cartilage in compression. Ann Biomed Eng 1984; 12 (6): 537–58.

Kwan M K, Wayne J S, Woo S L, Field F P, Hoover J, Meyers M. Histological and biomechanical assessment of articular cartilage from stored osteochondral shell allografts. J Orthop Res 1989; 7 (5): 637–44.

Mankin H J. The response of articular cartilage to mechanical injury. J Bone Joint Surg (Am) 1982; 64 (3): 460–6.

Mankin H J, Doppelt S, Tomford W. Clinical experience with allograft implantation. The first ten years. Clin Orthop 1983; 174: 69–86.

Mankin H J, Gebhardt M C, Tomford W W. The use of frozen cadaveric allografts in the management of patients with bone tumors of the extremities. Orthop Clin North Am 1987; 18 (2): 275–89.

Meyers M H, Jones R E, Bucholz R W, Wenger D R. Fresh autogenous grafts and osteochondral allografts for the treatment of segmental collapse in osteonecrosis of the hip. Clin Orthop 1983; 174: 107–12.

Meyers M H. Resurfacing of the femoral head with fresh osteochondral allografts. Long term results. Clin Orthop 1985; 197: 111–4.

Meyers M H, Akeson W, Convery F R. Resurfacing of the knee with fresh osteochondral allograft. J Bone Joint Surg (Am) 1989; 71 (5): 704–13.

Mow V C, Kuei S C, Lai W M, Armstrong C G. Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments. J Biomech Eng 1980; 102 (1): 73–84.

Rodrigo J J, Thompson E, Travis C. Deep freezing versus 4 degrees preservation of avascular osteocartilaginous shell allografts in rats. Clin Orthop 1987; 218: 268–75.

Sondenaa K, Alho A, Nielsen R. Cryopreservation of osteochondral grafts in rabbits. Acta Orthop Scand 1985; 56 (3): 218–22.

Schachar N S, McGann L E. Investigations of low temperature storage of articular cartilage for transplantation. Clin Orthop 1986; 208: 146–50.

Tomford W W, Hung H H, Mankin H J. Cryopreservation of articular slices. Trans Orthop Res Soc 1989; 14: 146.