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Freezing causes changes in the meniscus collagen net: a new ultrastructural meniscus disarray scale

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Abstract Alterations in meniscal permeability leading to nutritional deficit have been suggested as a cause of shrinkage in meniscal transplantation. The purpose of this study was to ascertain how freezing, one of the most common procedures used to preserve meniscal allografts, alters the collagen's architecture. Twenty-six fresh human external menisci were analyzed with transmission electron microscopy. Thirteen of them were previously frozen at −80°C while the rest were used as controls. A new scale of the collagen meniscal architecture was proposed according to the collagen's periodicity and degree of disruption, loss of banding, degree of collagen packing, fibril size variability and its intrafibrilar oedema. Each meniscus was scored from 0 to 7. Subsequently they were classified in grades ranging from a normal state (grade I; 0–2 points) to severe disarray (grade III; 5–7 points). The fibril collagen diameters of those menisci which had been previously frozen showed an average size in the longitudinal section of 14.26 nm, whereas it was 17.28 nm in the menisci used as controls ($p = 0.019$). In the transverse section, the frozen menisci averaged 13.14 and 16.93 nm in the controls ($p = 0.003$). Samples of the 13 previously frozen menisci were classified as grade III in 61.54% of the cases. In the control group, all the menisci were classified either as grade I or II. The frozen menisci averaged 4.85 points, whereas the control group did so at 2.46 ($p < 0.001$). The fibril diameters in frozen menisci showed a thinner diameter and had a higher degree of disarray. Therefore, the results suggest that the freezing process alters the menisci's collagen net. This could partially explain the pathological changes found in shrunken menisci after transplantation.

Keywords Meniscus allograft · Shrinkage · Meniscal nutrition · Transmission electron microscopy

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Introduction

Allograft meniscal transplantation (AMTX) was first introduced in the early 1980s by Carl Wirth's team in Germany [13]. It was initially used as an added surgical procedure in ligament-deficient knees. Some 20 years later, AMTX is even more widespread as a procedure thought as a potential solution for patients with early degenerative osteoarthritis and pain due to a previous meniscectomy [28]. Due to the lack of scientific evidence, its indications have not yet been expanded to prophylaxis for articular degenerative changes. A few long-term results of AMTX have recently been published [7, 28, 30]. These reports demonstrated good results

with different types of meniscal preservation. While Wirth et al. [30] transplanted most of the menisci after deep freezing and Noyes et al. [15] using irradiated meniscal allografts, Verdonk et al. [27, 28] performed the AMTX using viable menisci to preserve meniscal allografts. Other authors have shown good mid-term results transplanting cryopreserved meniscal allografts [17, 25].

Shrinkage of the allograft is one of the most common complications seen after AMTX [17, 23]. It has been hypothesized that this phenomenon could lead to insufficient protection of the articular cartilage in transplanted knees [4, 10, 12]. Although the final causes of shrinkage have not been clearly defined, some degree of immune rejection [19] as well as alterations in meniscal permeability have been proposed [6]. According to Rodeo et al., subtle immune reactions, which might influence the healing process and structural remodeling of the graft, are present in most cases. However, there is no evidence that this affect the clinical outcomes.

On the other hand, alterations in meniscal permeability as a consequence of collagen net disarray can lead to a nutritional deficit. Changes in the meniscus collagen ultrastructure have been observed after immobilization in animal studies [16]. More recently, the meniscal architecture has been studied under the effects of different conditions, such as cryopreservation [3, 22], deep-frozen in liquid nitrogen [4], and lyophilization [13]. While lyophilization has demonstrated weakening menisci viability, no proven advantage of the other graft preservation modality has been shown over others [5, 9, 26]. Most studies have focused on the cellular viability of the grafts. Nevertheless, none have been centralized on the isolated effects of freezing at −80°C in the human meniscal collagen net. The purpose of the present study was to ascertain how freezing, one of the most common procedures used in European countries to preserve allografts, alters the collagen's architecture.

Materials and methods

To determine the meniscus architectural state, a transmission electron microscopy (TEM) study was designed. The changes seen, which had previously shown capital importance in meniscus function [4, 8, 16, 19, 22], were qualified and quantified and then recorded.

Meniscal harvesting

Twenty-six fresh human external menisci were harvested in sterile conditions during total knee replacement procedures (15 women, 11 men). Informed consent was obtained from each donor following the rules of our local Ethical Committee. Seventeen of the menisci were obtained from the left side and the other nine from the right. Radiographic evaluations as well as clinical intraoperative assessments were performed in order to ascertain indemnity of the external femorotibial compartment. Cases with more than 50% of external joint space narrowing in the standing X-ray 45° posteroanterior position, macroscopic degeneration or even minimal calcification were all excluded from the study. Culture analysis was performed for each graft, and if positive, was also excluded. Thirteen out of 26 menisci were immediately frozen at −80°C (Forma Scientific Inc., Freezer, USA). After 7 days, they were thawed by immersion for 2 min in a 36°C sterile saline solution and then processed. The other 13 menisci were used as controls. Both groups were comparable in age and gender. The study group had a mean age of 75.69 ± 6.96 years (range 65–84) and the control group 71.38 ± 6.8 (range 59–83). One square centimeter from the inner part of the meniscus body was then sectioned into 1 mm^3 slices. Subsequently, they were embedded and preserved in a 2.0% glutaraldehyde solution. Within the succeeding 2 h of harvesting, all the samples were fixed and prepared to be analyzed by the pathologist.

Transmission electron microscopy procedure

Due to the fact that the parties obtaining the initial menisci samples were also executing the final analysis of the histological sections, a double-blinded study design was implemented so as to minimize possible biases.

Forty sections of 1 mm^3 from each meniscus were immediately fixed in a 2.0% glutaraldehyde cacodylate buffer solution. Postfixation in osmium tetroxide was done before dehydration in increasing concentrations of ethanol. Next, the menisci sections were treated with propylene oxide and included in progressive concentrations of epon. The most representative zone was chosen with the help of the light microscope from five different toludine blue stained 1 μm thickness sections. Ninety nanometer sections from the selected zone were finally stained with metals salts (uranyl acetate and lead citrate) and were analyzed with a transmission electron microscope (Philips, model #CM100, Holland). For each cross-section, four TEM photos were randomly taken.

Fibril collagen measurements and histological classification

Four hundred collagen fibrils were recorded and measured in longitudinal and transversal sections from every meniscus. The analyzed photographs were set at 19,000× magnification. All measurements were determined with the help of an electronic digital calliper (ProMax, Fowler; USA, range 0–150 mm, resolution 0.02 mm).

A new Collagen Meniscal Architecture (CMA) scoring system was set based on the collagen's periodicity or cross-banding frequency and degree of disruption, loss of banding, degree of collagen packing, fibril size variability and its intrafibrilar oedema. On this scale, the meniscus scored from 0 to 7 following the established criteria (Table 1). The loss of banding and intrafibrilar oedema was a common finding in most samples. However, these phenomena were considered abnormal (positive), when present in more than 20% of the fibers. Nevertheless, packing and degree of disruption were considered the two variants that held the most influence due to their known role in meniscal nutrition [6, 16].

By adding up all items, when samples scored 0–2, they were classified as normal or grade I. Three or four points meant that the meniscus had moderate ultrastructural changes and was qualified as a grade II (Fig. 1). Finally, the menisci that showed a higher degree of disarray, scoring 5, 6 or 7, were classified as grade III.

Table 1 Collagen meniscal architecture scoring system

	0 Point	1 Point	2 Points
Disrup/periodicity	Mild	Moderate	Severe
Intrafibrilar oedema	No	Yes	–
Packing	High density	Intermediate	Low density
Banding	Yes	No	–
Fibril size variability	Low	High	–

Each section was scored depending on five variables. Intrafibrilar oedema as well as absence of banding was considered positive when seen in more than 20% of the fibers. Grade I: 0–2 points, grade II: 3–4 points, grade III: 5–7 points

Statistical analysis

Continuous variables are presented as mean ± standard deviation. Categorical variables are presented as percentages. Differences between normally distributed group data were analyzed by the unpaired Student's *t*-test. Either the chi-square Pearson or Fisher Exact test was used to compare categorical variables among groups. Statistical analysis was performed using SPSS 12 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at 0.05.

Results

Fibril collagen size

The fibril collagen diameters of those menisci which had been previously frozen showed an average size in the longitudinal section of 14.26 ± 2.59 nm, whereas the menisci used as controls averaged 17.28 ± 3.46 nm ($p = 0.019$). Similarly, in the transverse section, the frozen menisci looked thinner. They averaged 13.14 ± 2.99 nm while the control group did so at 16.93 ± 2.9 nm ($p = 0.003$) (Fig. 2).

Architectural degree and scoring

There was a clear difference in both groups. Eight of the 13 previously frozen menisci (61.54% of the cases) were classified as grade III (Fig. 3). The remaining five (38.46% of the cases) were graded as II. The control group showed either a normal collagen net structure or a lower degree of disarray (Fig. 4). They were classified as grade I in six cases (46.15%) and grade II in seven cases (53.85%). When applying the scoring aspect of the scale, the frozen menisci averaged 4.85 points, whereas the control group did so at 2.46 ($p < 0.001$) (Fig. 5).

Discussion

Currently, several subjects are controversial in AMTX. Surgical techniques, indications, isolated versus associated lesions, effect in cartilage protection and method of graft

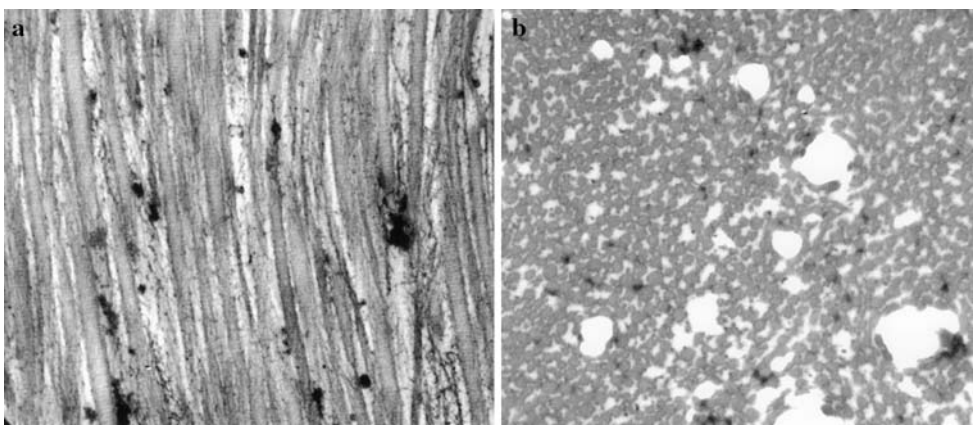

Fig. 1 TEM photographs of a meniscus which scored 4 points and therefore was classified as a grade II in the CMA scoring system. **a** Longitudinal section: moderate disruption of the architecture (1 point); both intrafibrilar oedema, and loss of banding, in more than 20% of the fibers (1 point each); **b** Transverse section: intermediate degree of packing (1 point) and low variability in the fibril size (0 point)

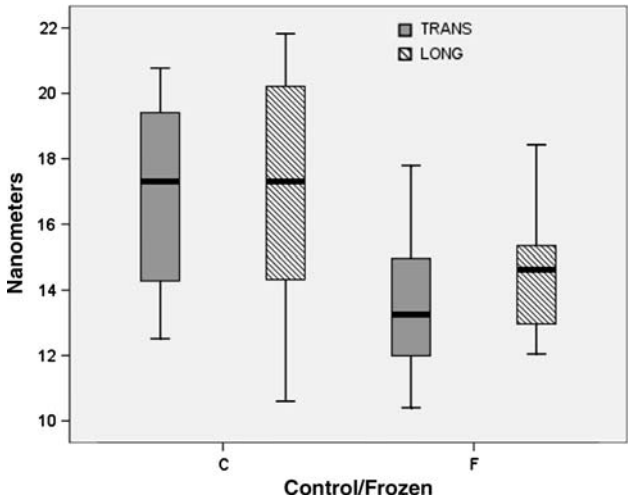

Fig. 2 Median diameter in nanometers both in transverse and longitudinal sections comparing the control versus the frozen group. The freezing process decreased the fibril collagen diameters in the transverse as well as in the longitudinal sections. *TRANS* transversal sections, *LONG* longitudinal sections

conservation are some of the most discordant topics. It is still not clearly demonstrated that AMTX prevents joint degeneration [27]. Nevertheless, in recent experimental works performed in a sheep model, meniscal transplant have been shown to have some chondral protective effect on the joint cartilage [1, 9, 14]. Clinical and pathological changes occurring when a meniscus retracts are not clearly defined [21]. Therefore, a shrunken meniscus should reduce its protective effect [18].

Little information is found in literature regarding the causes of meniscus synovialization. Freeze-drying (lyophilization) is one of the few causes that have certainly proven to increase the risk of meniscal size reduction [30]. A subtle immune rejection in the transplanted graft has also been proposed [11, 19]. The presence of HLA II and ABO antigens from dead synovial and endothelial cells found in meniscal tissue at the time of transplantation [19], and sensitization to class I and II HLA recipients of cryopreserved allografts [24] may support this theory. However, no clinical evidence of frank immune rejection has been reported in medical literature.

A meniscal nutritional deficit might also be a possible cause of shrinkage. The meniscus is mainly an avascular structure, with perimeniscal vessels supplying only 10–30% of its periphery in adult human beings. The fibrocartilage's mid-substance nutrition is brought on by solute diffusion from the periphery through the interfibrilar space [2, 4, 6, 16]. Ochi et al. [16] studied the effect of knee immobilization on the meniscus collagen net in an animal experiment. They suggested that an increased interfibrilar space might lead to a decreased solute diffusion. This enlarged space between the collagen fibers was also observed in the current study. Arnoczky et al. [4] analyzed transplanted meniscal allografts previously frozen at −80°C at 6 months follow-up in a canine model. Those menisci showed the central substance to be relatively acellular, suggesting a nutritional deficit in the inner layer of the fibrocartilage. This also was sustained by Gershuni et al. [6]. The same findings suggesting a possible cause of shrinkage were observed in the present work.

There are differences in the description of the same graft conservation technique as seen in various studies. Some of them described the deep freezing process as a sudden temperature drop; brought down within in 1 min with the help of liquid nitrogen either to −80° [4] or to −196°C [29]. Others simply freeze the samples without processing either at −70°C [11] or at −80°C [5, 21, 26]. Deep freezing of the meniscal allograft at −80°C is one of the most common conservation methods used in orthopaedics. The main difference between fresh frozen and cryopreserved tissue is that the latter is able to keep some cells viable due to the use of an anti-freezing agent. In terms of biomechanics, the cryopreservation technique seems not to alter the microarchitecture or the material properties of the meniscus [3]. Some other investigations compared, in animal models, the effect of cryopreservation and direct freezing at −80°C. The menisci allografts were analyzed under light and polarized light microscopy [5] or TEM [20]. Their authors affirmed that although deep freezing

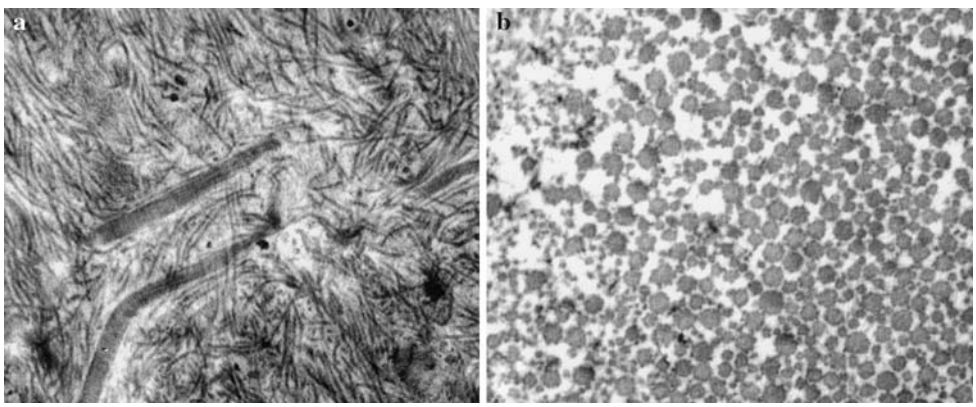

Fig. 3 TEM photographs of a meniscus which scored 7 points and therefore was classified as a grade III in the CMA scoring system. **a** Longitudinal section: severe disruption of the architecture (2 points), intrafibrilar oedema as well as loss of banding in most of the fibers (1 point each one); **b** Transverse section: low density packing (2 point) and high size fibril variability (1 point)

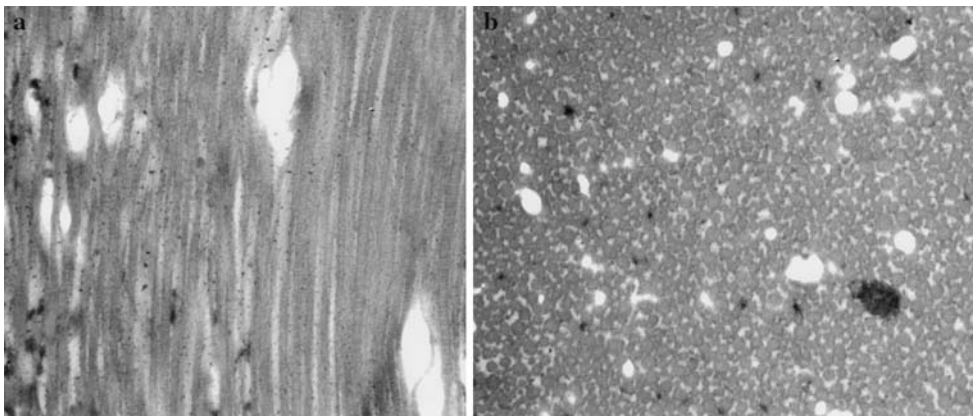

Fig. 4 TEM photographs of a meniscus which scored 0 points and therefore was classified as a grade I in the CMA scoring system. **a** Longitudinal section: normal or mild disruption of the architecture (0 points), intrafibrilar oedema rarely observed (0 points) and banding of the fibers is preserved (0 points); **b** Transverse section: high density fibril packing (2 points) and the fibril size is homogeneous (0 points)

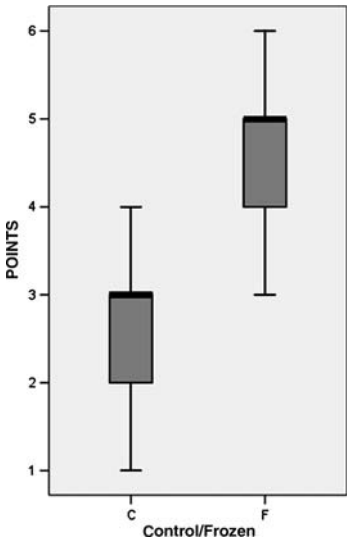

Fig. 5 Median score in nanometers when applying the collagen meniscal architecture (CMA) scoring system. Comparison between the control versus the frozen group. The menisci which had been previously frozen scored higher. Thus, the freezing process seemed to disarray the meniscal collagen net

completely destroys the cell components during the freezing process, the collagen net is kept intact. Some other researchers were in agreement with this observation [25]. Interestingly enough, there is a lack of ultrastructural studies on the effect of these aforementioned procedures on the collagen network. The ultrastructural findings observed in the present investigation are clearly in contrast with currently accepted knowledge.

There are some limitations to the present study. Besides the small sample number, the second and most important limitation is related to the fact that although performed in a blind manner, we did not assess the agreement between observers to further validate the CMA scoring system accuracy. The third limitation perceived was that although the fibril collagen diameters were measured with the help of a precise electronic calliper, this was done manually. Fourth, even though the study and the control group were statistically comparable it would have been better to compare the freezing effect on the same meniscus instead of two different specimens. Finally, the specimens studied were all harvested from aged patients. Although it might have been interesting to include results from younger population, the possible bias was minimized by comparing an age-matched population

Despite these limitations, this new qualitative and quantitative scale system for assessing the collagen architecture state (CMA scoring system) seems to be a promising tool in evaluating the meniscal ultrastructure. This might also help predict a different risk of shrinkage depending on the allograft's ultrastructural grading before transplantation.

In conclusion, the present study showed how fibril diameters in frozen menisci had a thinner diameter and a higher degree of disarray. Therefore, the results suggest that the freezing process alters the menisci's collagen net in most cases. This could partially explain the pathological changes found in shrunken menisci.

Further studies are needed to ascertain if these changes are also present after different meniscal preserving procedures.

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Appendix

Table 2.

Table 2 Sample data

No.	Group	Age	Gender	Side	Trans	Long	Grade	Points
1	C	70	M	L	15,385	13,807	2	3
2	C	60	F	R	14,281	10,599	1	2
3	C	77	F	L	13,155	17,305	2	4
4	C	59	F	R	20,777	21,829	2	3
5	C	73	M	L	12,519	14,323	2	4
6	C	68	F	R	17,300	20,214	2	3
7	C	71	F	L	19,409	21,576	1	1
8	C	80	M	R	19,367	16,411	1	2
9	C	71	F	L	14,128	16,469	1	2
10	C	69	F	R	19,625	17,831	2	3
11	C	83	M	R	20,256	18,620	1	1
12	C	74	M	L	15,407	14,176	2	3
13	C	73	F	L	18,552	21,476	1	1
14	F	84	F	L	10,599	12,056	3	6
15	F	79	F	L	6,548	8,232	3	7
16	F	65	M	L	13,255	12,966	2	3
17	F	74	M	L	11,993	14,623	3	5
18	F	72	F	R	12,082	14,512	2	4
19	F	79	F	L	17,791	18,421	2	4
20	F	82	F	L	14,481	14,907	3	5
21	F	82	M	L	12,145	12,051	3	5
22	F	75	M	R	10,401	16,378	3	5
23	F	84	M	L	14,226	17,006	3	6
24	F	65	M	R	16,264	15,287	2	3
25	F	66	F	L	16,139	13,549	3	5
26	F	77	F	L	14,965	15,342	2	5

C control, *F* frozen, *Age* age in years, *L* left, *R* right, *Trans* median diameter in nanometers in transverse sections, *Long* median diameter in nanometers in longitudinal sections, *Grade* grade in the CMA scoring system, *Points* points in the CMA scoring system

References

1. Aagaard H, Jörgensen U, Bojsen-Möller F (1999) Reduced degenerative articular cartilage changes after meniscal allograft transplantation in sheep. Knee Surg Sports Traumatol Arthrosc 7:184–191
2. Arnoczky SP, Warren RF (1982) Microvasculature of the human meniscus. Am J Sports Med 10:90–95
3. Arnoczky SP, McDevitt CA, Schmidt MB, Mow VC, Warren RF (1988) The effect of cryopreservation on canine menisci: a biochemical, morphologic, and biomechanical evaluation. J Orthop Res 6:1–12
4. Arnoczky SP, Dicarlo EF, O'Brien SJ, Warren RF (1992) Cellular repopulation of deep-frozen meniscal autografts: an experimental study in the dog. Arthroscopy 8:428–436
5. Fabbriciani C, Lucania L, Milano G, Schiavone Panni A, Evangelisti M (1997) Meniscal allografts: cryopreservation vs deep-frozen technique. An experimental study in goats. Knee Surg Sports Traumatol Arthrosc 5:124–134
6. Gershuni DH, Hargens AR, Danzig LA (1988) Regional nutrition and cellularity of the meniscus. Implications for tear and repair. Sports Med 5:322–327
7. Graf KW Jr, Sekiya JK, Wojtys EM (2004) Long-term results after combined medial meniscal allograft transplantation and anterior cruciate ligament reconstruction: minimum 8.5–year follow-up study. Arthroscopy 20:129–140
8. Gulisano M (1991) Scanning electron microscopy study of the meniscus of the knee in patients with anterior cruciate ligament lesions. Arch Ital Anat Embriol 96:55–65
9. Jackson DW, McDevitt CA, Simon TM, Arnoczky SP, Atwell EA, Silvino NJ (1992) Meniscal transplantation using fresh and cryopreserved allografts. An experimental study in goats. Am J Sports Med 20:644–656
10. Kelly BT, Potter HG, Deng X, Pearle AD, Turner AS, Warren SF, Rodeo SA (2006) Meniscal Allograft Transplantation in the Sheep Knee. Evaluation of the Chondroprotective Effects. Am J Sport Med 24 (Epub ahead)
11. Khoury MA, Goldberg VM, Stevenson S (1994) Demonstration of HLA and ABH antigens in fresh and frozen human menisci by immunohistochemistry. J Orthop Res 12:751–757
12. Mikic ZD, Brankov MZ, Tubic MV, Lazetic AB (1993) Allograft meniscus transplantation in the dog. Acta Orthop Scand 64:329–332

13. Milachowski KA, Weismeier K, Wirth CJ (1989) Homologous meniscus transplantation. Experimental and clinical results. Int Orthop 13:1–11
14. Mora D, Alvarez E, Ripalda P, Forriol F (2003) Articular cartilage degeneration after frozen meniscus and Achilles tendon allograft transplantation: experimental study in sheep. Arthroscopy 19:833–341
15. Noyes FR, Barber-Westin SD (1995) Irradiated meniscus allografts in the human knee. A two to five year follow-up study. Orthop Trans 19:417
16. Ochi M, Kanda T, Sumen Y, Ikuta Y (1997) Changes in the permeability and histologic findings of rabbit menisci after immobilization. Changes in the permeability and histologic findings of rabbit menisci after immobilization. Clin Orthop Relat Res 334:305–315
17. Rath E, Richmond JC, Yassir W, Albright JD, Gundogan F (2001) Meniscal allograft transplantation. Two- to eight-year results. Am J Sports Med 29:410–414
18. Rijk PC, Van Noorden CJ (2002) Structural analysis of meniscal allograft after immediate and delayed transplantation in rabbits. Arthroscopy 18:995–1001
19. Rodeo SA, Seneviratne A, Suzuki K, Felker K, Wickiewicz TL, Warren RF (2000) Histological analysis of human meniscal allografts, a preliminary report. J Bone Joint Surg Am 82-A:1071–1082
20. Salai M, Givon U, Messer Y, von Versen R (1997) Electron microscopic study on the effects of different preservation methods for meniscal cartilage. Ann Transplant 2:52–44
21. Sekiya JK, Ellingson CI (2006) Meniscal allograft transplantation. J Am Acad Orthop Surg 14:164–174
22. Shibuya S (1999) Meniscus transplantation using a cryopreserved allograft. Histological and ultrastructural study of the transplanted meniscus. J Orthop Sci 4:135–141
23. Stollsteimer GT, Shelton WR, Dukes A, Bomboy AL (2000) Meniscal allograft transplantation: a 1- to 5-year follow-up of 22 patients. Arthroscopy 16:343–347
24. van Arkel ER, van den Berg-Loonen EM, van Wersh JW, de Boer HH (1997) Human leukocyte antigen sensitization after cryopreserved human meniscal transplantations. Transplantation 64:531–533
25. van Arkel ER, de Boer HH (2002) Survival analysis of human meniscal transplantations. J Bone Joint Surg Br 84:227–231
26. Verdonk R, Khon D (1999) Harvest and conservation of meniscal allografts. Scand J Med Sci Sports 9:158–159
27. Verdonk PCM, Demurie A, Almqvist KF, Veys EM, Verbruggen G, Verdonk R (2005) Transplantation of viable meniscal allograft. survivorship analysis and clinical outcome of one hundred cases. J Bone Joint Surg Am 87:715–724
28. Verdonk PCM, Verstraete KL, Almqvist KF, De Cuyper K, Veys EM, Verbruggen G, Verdonk R (2006) Meniscal allograft transplantation: long term clinical results with radiological and magnetic imaging correlations. Knee Surg Sports Traumatol Arthrosc 14:694–706
29. Wada Y, Amiel M, Harwood F, Moriya H, Amiel D (1998) Architectural remodeling in deep frozen meniscal allografts after total meniscectomy. Arthroscopy 14:250–257
30. Wirth CJ, Peters G, Milachowski KA, Weismeier KG, Kohn D (2002) Long-term results of meniscal allograft transplantation. Am J Sports Med 30:174–81