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# Cryopreservation does not alter the ultrastructure of the meniscus

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**Abstract** Fresh frozen menisci have recently been shown to have an altered meniscal ultrastructure. The cause might be a deterioration of its permeability due to collagen net disarray. The purpose of this study was to evaluate the cryopreserved meniscus in terms of ultrastructure and cellularity. Ten fresh human lateral menisci were harvested. Collagen architecture was evaluated with transmission electron microscopy. The Collagen Meniscal Architecture scoring system was used to assess the degree of meniscal disarray. Cell population, was also evaluated. The fibril collagen diameters of those menisci which had been previously cryopreserved showed an average size in the longitudinal section of 12.6 ± 1.3 nm, whereas it was 13.4 ± 2.2 nm in the menisci used as controls (n.s.). In the transverse section, the cryopreserved menisci averaged 15.5 ± 2.4 and 16.7 ± 3.5 nm in the controls (n.s.). The study group scored 4.8 points ± 1.7, whereas the control group did so at 4.1 ± 1.3 (n.s.). The percentage of cell survival after the cryopreservation ranged from 4 to 54. The fibril diameters and degree of disarray showed a similar distribution in both groups. The results suggest that meniscal cryopreservation does not alter the meniscal ultrastructure. Therefore, an allograft stored in that way would not alter its biomechanical properties, although its cellular viability is highly unpredictable.

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## Introduction

When looking for a graft to be transplanted, one must wonder which properties the preserved tissue should possess if it is going to work for the patient. Does it have to contain metabolising cells that are capable of cell division or not? Does it have to maintain its architectural indemnity to function properly or not? It has been widely demonstrated that cellular repopulation occurs in the meniscal allograft after transplantation even if there is no viable cell at the moment of surgery [2, 16, 21]. Two of the most widely used meniscal allograft preservation techniques are simple freezing and cryopreservation. Freezing has been accepted as a simple way of preserving those tissues that only have to retain mechanical and some biochemical properties, for example bone tissue [12]. In the case of meniscal tissue, Fabbriciani et al. [5] have shown, in an animal model, that deep-freezing keeps its collagen net intact in a study with standard microscopy. Conversely, Gelber et al. [6] recently demonstrated that when ultrastructurally studied, this freezing process led to severe architectural disarray. According to Arnoczky et al. [2], this architectural disarray might make the menisci more susceptible to injury.

The main accepted advantage of cryopreservation over freezing is that it does not destroy cells. This ability is particularly true in cultured or isolated cells [17]. Tissues are obviously more complex than cell suspensions. Diffusion of solutes through the mass is different depending on the inherent tissue properties, the actual state of that

specific sample, its response to the cryopreservant and to the slow cooling process [13].

In answer to the two previously posed questions, it seems that preserving the architectural characteristics of the meniscal allograft should be mandatory. Cell functioning at transplant might be considered a secondary issue, although some authors have proven otherwise [18–20].

In 1999, Shibuya studied the collagen net architecture in cryopreserved menisci transplanted into rabbits [15]. He measured the fibril collagen diameters and described the characteristics of the collagen bundles and the cellular aspect at weeks 0, 4, 8 and 16. Nevertheless, he did not analyze the characteristics of the menisci before they were cryopreserved. In addition, the description of the architecture was not performed with the help of a scale so as to make possible an objective comparison with other specimens [1, 16].

The purpose of this study was to evaluate the viability of cryopreserved menisci while focusing on their cellularity and in their ultrastructure. Our hypothesis was that a cryopreserved meniscus would keep its collagen net arquitecture intact while maintaining its cellular component.

## Materials and methods

### Meniscal harvesting

Ten fresh human lateral menisci were harvested in sterile conditions during total knee replacement procedures (7 women, 3 men). Informed consent was obtained from each donor following the guidelines laid down by our local Ethics Committee. Seven of the menisci were obtained from the right side and the other three from the left. Radiographic evaluations as well as clinical intraoperative assessments were performed in order to ascertain the indemnity of the lateral femorotibial compartment. Cases with more than 50% of lateral joint space narrowing in the standing 45° posteroanterior radiographs position, macroscopic degeneration or even minimal calcification were all excluded from the study. Culture analysis was performed for each graft and if positive, they were also excluded. Five menisci did not meet these criteria, so they were excluded and another five were consecutively harvested. The study group had a mean age of 74 years (range 66–82). One square centimetre from the bodies of each single menisci was divided into four sections. Two of them were immediately embedded in PBS plus 10% dimethyl sulfoxide (DMSO) at 4°C for 30 min. Subsequently they were slowly cooled at 1°C/min to −180°C in liquid nitrogen. After 14 days, they were thawed by immersion in a 36°C sterile saline solution for 2 min. One of the two pieces was then sectioned into 1 mm$^3$ slices and preserved in a 2.0% glutaraldehyde solution. At no more than 2 h of harvesting, all the samples were fixed and prepared for analysis by a pathologist with TEM. The other piece was embedded in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (foetal bovine serum) and incubated for 48 h and then processed to evaluate cell viability. Finally, the remaining two sections of the menisci were used as controls. One was used for the TEM analysis and the other one to evaluate the cell population of each harvested meniscus.

### Transmission electron microscopy procedure

The architectural state of the menisci was analyzed with TEM. The changes observed were quantified and qualified according to the previously described scoring system [6].

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 sample of the menisci 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 a light microscope (SMZ-10A, Nikon, Japan; 40× magnification) from five different 1 μm thick toludine blue stained sections. Ninety nanometre sections from the selected zone were finally stained with metal 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.

### Cell viability assessment

The samples were washed with sterile PBS and then finely diced. For cell isolation, the diced menisci were incubated for 1 h with 0.5 mg/ml hyaluronidase (Sigma-Aldrich Química S.A.; Spain) in a shaking water bath at 37°C. The hyaluronidase was subsequently removed, and 1 mg/ml pronase (Merck, VWR International Inc.; USA) was added. After 90 min incubation in a shaking water bath at 37°C, the cartilage pieces were washed with supplemented [100 U penicillin, 100 μg streptomycin (Biological Industries Ltd; Israel) and 0.4% fungizone (Gibco-Invitrogen Corporation; USA)] DMEM (Life Technologies Inc.; USA). After removal of the medium, digestion was continued by the addition of 0.5 mg/ml of collagenase-IA (Sigma-Aldrich Química S.A.; Spain) in a shaking water bath kept at 37°C for 22–24 h. The resulting cell suspension was centrifuged and washed with DMEM supplemented with 10% FBS (Gibco-Invitrogen Corporation; USA). Tissue debris were treated

with trypsin–EDTA (Biological Industries Ltd; Israel) for 10 min at 37°C and filtered through a 70 μm pore nylon filter (BD Biosciences; Belgium) to remove it. Cells were centrifuged and washed with DMEM supplemented with 10% FBS. Finally, all cells of each meniscus were mounted on tissue culture plates with DMEM supplemented with 10% FBS. After 5 days of culture, the cells adhered to the plates were counted.

### Fibril collagen measurements and histological classification

Four hundred collagen fibrils were recorded and measured in longitudinal and transversal sections from every meniscus. Based on a recent study [6], 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) and then multiplied by 19,000 to get the corresponding measure in nn.

According to the recently published Collagen Meniscal Architecture (CMA) scoring system [6], the collagen's periodicity and degree of disruption, loss of banding, degree of collagen packing, fibril size variability and its intrafibrilar oedema were evaluated. On this scale, the meniscus scored from 0 to 7. Following the established criteria, they were classified in grades ranging from a normal state (grade I 0–2 points) to severe disarray (grade III 5–7 points). As the reliability of the CMA scoring system was unknown, the intra and extra-class correlation coefficients were calculated. Whereas intra-observer reliability was evaluated at a 30-day period interval, inter-observer reliability was calculated with four people who were pre-instructed at the same time. They then individually evaluated the menisci sections.

### Statistical analysis

Continuous variables are presented as mean ± standard deviation. Categorical variables are presented as percentages. After testing the normal distribution of the differences with the Q–Q plot, differences were analyzed with the paired Student's *t* test. Statistical analysis was performed using SPSS 12 (SPSS Inc., Chicago, Illinois, USA). Statistical significance was set at 0.05.

## Results

### Fibril collagen size

The fibril collagen diameters of those menisci which had been previously cryopreserved showed an average size in the longitudinal sections of 12.6 ± 1.3 nm, whereas the menisci used as controls averaged 13.4 ± 2.2 nm (n.s.). In the transverse sections, the cryopreserved menisci averaged 15.5 ± 2.4 nm while the control group did so at 16.7 ± 3.5 nm (n.s.).

### Architectural degree and scoring

When applying the CMA scoring system, a clear difference in both groups was not found. Five out of ten previously cryopreserved menisci were classified as grade III. Four of the samples were graded as II and the remaining meniscus as I (Fig. 1). In the control group, five out of ten samples were graded as III. Three menisci were classified as grade II and the other two as grade I (Fig 2). Similarly, the scoring aspect of scale did not show a clear difference either. The cryopreserved menisci averaged 4.8 points ± 1.7, whereas the control group did so at 4.1 ± 1.3 (n.s.).

The calculated intra-class correlation was qualified as excellent with a kappa coefficient of 0.912 (95% CI 0.795–0.964). On the other hand, the inter-class correlation between observers was considered only moderate, with a kappa coefficient of 0.51 (95% CI 0.25–0.73).

### Cell viability

The mean survival cell viability was 23%. A wide ranging rate of cell survival was observed, from 4 to 54%.

## Discussion

Several topics are still controversial in meniscal transplantation, one of them being the storage technique. The

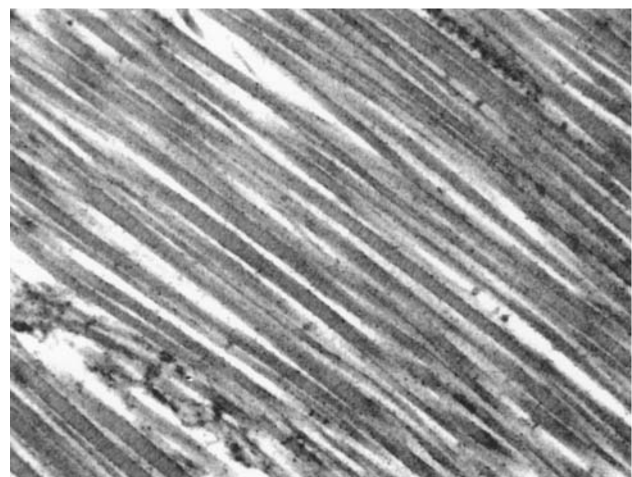

**Fig. 1** TEM photographs of a meniscus. The cryopreserved meniscus show a moderate degree of disarray (CMA scoring system: 4 points, grade II)

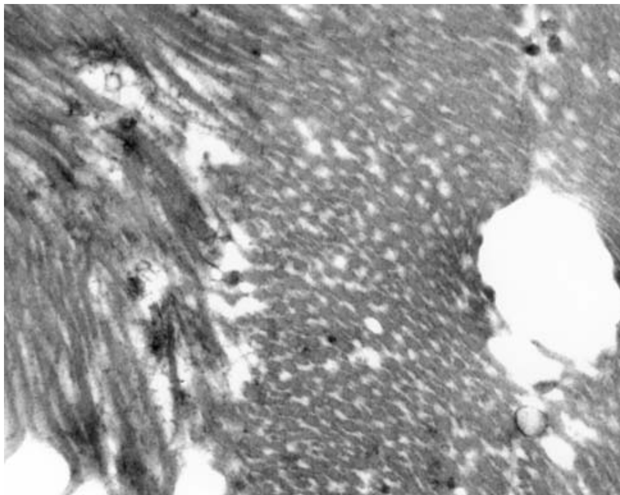

**Fig. 2** The same meniscus as in Fig. 1. Longitudinal and transversal section of the same meniscus used as control. This sample scored 3 points in the CMA scoring system, therefore it has also been graded as II

current study showed that cryopreservation does not alter the meniscal ultrastructure. The biomechanical behaviour of the meniscus depends on its particular collagen organization. Therefore, an ideal preservation method should not harm this fine structure array. Freeze-drying is not currently being performed because it has been demonstrated that the high temperatures required lead to an unwanted molecular cross linkage that has a deleterious effect on the mechanical properties. Subsequently, it results in a high graft failure rate [22]. The main difference between freezing and cryopreservation depends on the latter's ability to keep some cells viable due to the use of an anti-freezing agent. In 1997, Fabbriciani et al. [5] and Salai et al. [15] compared, in animal models, the effect of cryopreservation versus fresh freezing on meniscal grafts. In the first mentioned instance, the menisci allografts were analyzed under light and polarized microscopy. In the latter case, this was performed using TEM. They both affirmed that although the deep-freezing process completely destroys the cell components, the collagen net is kept intact.

More recently, it has been shown that the freezing process does alter the menisci's collagen net when ultrastructurally studied [6]. The authors performed their study comparing menisci from different aged donors. As the higher water content that aged menisci have may influence the cooling process, the same meniscus instead two different specimens were analyzed in each case in the present study. That prior work reported that the collagen fibrils in fresh frozen menisci have a thinner diameter. Based on a newly described scale, the menisci also showed a higher degree of disarray. The authors speculated that this phenomenon might contribute to meniscal shrinkage [6], although this was a complication mainly reported with lyophilized menisci [22].

Little evidence exists on the mechanical effects of the different storage techniques on soft tissues. In the case of tendons, it has been demonstrated that the freezing process has a tendency to alter the collagen architecture of the posterior tibialis tendons [7]. This leads to a decrease in the ultimate tensile load and ultimate tensile deformation. With regards to the meniscus, Lewis et al. [10] recently demonstrated that menisci that underwent a single freeze–thaw cycle have a significantly higher Young's modulus than those undergoing multiple freeze–thaw cycles. The authors concluded that this fact might compromise the allograft's ability to resist compression. They did not provide an explanation for the change in intrinsic compressive resistance based on its histological or biochemical findings. Perhaps, this is because no ultrastructural assessment was done.

The meniscus is mainly an avascular structure. Its mid-substance nutrition is fed by solute diffusion from the periphery on through the interfibrilar space. Ochi et al. [12] demonstrated that an abnormally higher interfibrilar space leads to a decrease in solute diffusion. Subsequently, it seems logical to look for a storage technique that produces no change or minimal changes in the menisci's collagen architecture.

Verdonk et al. [18, 20] performed allograft meniscal transplantations using fresh viable menisci. They state that not only does it contribute to cell survival but also to cell matrix production. Thus, it makes for a real functioning cell. Although they do not focus their works on the ultrastructural aspects of those grafts, we can hypothesise that it remains unchanged.

Cryopreservation is used worldwide as an allograft conservation method. Due to the fact that some time after transplantation the allograft nearly has solely host DNA [4], the advocated advantage of being a cell preservation technique might then seem to be a secondary issue. The reported percentage of cell survival after cryopreservation ranges from 10 to 30% [20]. In the present work, a wider range of cell survival ranging from 4 to 54% was observed. Therefore, keeping the collagen net architecture intact might be the main advantage of cryopreservation over the freezing procedure.

Limited clinical information comparing the different meniscal storage techniques is currently available in the same study. One of the few evidential sources comes from the Wirth et al. [21] group. These authors reported superior results with fresh frozen as opposed to lyophilised meniscal allografts, although the study was non-random and the analysis retrospective. The study of Noyes et al. [11] have also shown that gamma irradiation has a tendency to

weaken menisci viability, although 64 out of the 82 operated knees were also subjected to ACL repair which could have altered the clinical results. In most of the published long-term works [8, 9, 19], all of which have demonstrated good clinical results, different meniscal storage techniques were used. None of these different conservation techniques has shown any proven clinical advantage. The fact that these studies are not comparable does not aid in extracting any valid conclusion.

The CMA scoring system used in the present work, showed a high intra-class correlation coefficient. This means that it is a reproducible technique. Nevertheless, the moderate inter-class correlation coefficient among observers suggests that previous training would be necessary in order to extend its reliable use.

There are some limitations to the present study. One is the fact that the study was performed with meniscal tissue harvested in aged patients, which is known to have higher water content. However, the same specimens were used as experimental and control groups to thereby minimize any possible bias. The second is related to the fact that although the fibril collagen diameters were measured with the help of a precise electronic calliper, this was done manually. It could have been solved with the help of image analysis software previously described [3]. Finally, although we have obtained a very limited section of the menisci to perform all the analysis, it is known that cell viability is highly different from one location to another [14]. This could partially explain the differences founds in cell survival, even though similar results have been found by others authors [20].

Gelber et al. [6] showed that the fibril collagen in frozen menisci had a thinner diameter and a higher degree of disarray, suggesting that the freezing process alters the menisci's collagen net. In the present study, the fibril diameters and degree of disarray showed no differences in either groups. Therefore, the results suggest that, for meniscal allografts, cryopreservation might be a better storage technique than freezing.

In conclusion, cryopreservation has been shown not to alter the meniscal collagen net architecture while it does affect cellular viability with great variability (Table 1).

**Table 1** Sample data

| # | Group | Age | Trans | Long | Grade | Points | Cell/g | % Cell |
|---|---|---|---|---|---|---|---|---|
| 1 | F | 66 | 15.4 | 13.8 | 3 | 5 | 1,050,000 | |
| 2 | F | 82 | 14.3 | 10.6 | 3 | 5 | 628,930 | |
| 3 | F | 70 | 13.2 | 17.3 | 3 | 6 | 66,666 | |
| 4 | F | 78 | 20.8 | 21.8 | 3 | 5 | 264,705 | |
| 5 | F | 78 | 12.5 | 14.3 | 1 | 2 | 70,000 | |
| 6 | F | 78 | 17.3 | 20.2 | 3 | 5 | 31,250 | |
| 7 | F | 76 | 19.4 | 21.6 | 2 | 4 | 292,857 | |
| 8 | F | 73 | 19.4 | 16.4 | 1 | 1 | 25,000 | |
| 9 | F | 69 | 14.1 | 16.7 | 2 | 3 | 111,111 | |
| 10 | F | 67 | 19.6 | 17.8 | 2 | 4 | 100,000 | |
| 1 | C | | 10.6 | 12.1 | 2 | 5 | 250,000 | 23.8 |
| 2 | C | | 6.5 | 8.2 | 2 | 5 | 117,647 | 18.7 |
| 3 | C | | 13.3 | 12.9 | 3 | 6 | 16,666 | 24.9 |
| 4 | C | | 11.9 | 14.6 | 2 | 4 | 70,000 | 26.4 |
| 5 | C | | 12.1 | 14.5 | 3 | 5 | 37,500 | 53.5 |
| 6 | C | | 17.8 | 18.4 | 3 | 5 | 13,846 | 44.3 |
| 7 | C | | 14.5 | 14.9 | 3 | 5 | 45,882 | 15.7 |
| 8 | C | | 12.1 | 12.1 | 1 | 2 | 1,136 | 4.5 |
| 9 | C | | 10.4 | 16.4 | 2 | 4 | 4,444 | 3.9 |
| 10 | C | | 14.2 | 17.0 | 3 | 7 | 15,384 | 15.4 |

*F* fresh/control, *C* cryopreserved, *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, *Cell/g* cells per gram, *% Cell* percentage of cell survival

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