Multiple Freeze–Thaw Cycled Meniscal Allograft Tissue: A Biomechanical, Biochemical, and Histologic Analysis

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ABSTRACT: Meniscus allografting has provided relief of meniscal injuries that were previously thought irreparable. However, meniscus allograft tissue remains limited and a significant problem. To improve allograft tissue yield, decrease processing costs, and increase graft availability, this study investigated the biomechanical changes of meniscal allograft tissue frozen and thawed multiple times. Specifically, our study compared the intrinsic compressive resistances of meniscus undergoing four freeze–thaw cycles versus tissue undergoing a single freeze–thaw cycle. Seven menisci that were originally procured and processed for allografting were donated for the study. Each meniscus was segmented and samples independently underwent novel constant slow-rate compression testing, and histological and biochemical evaluation. The menisci that underwent a single freeze–thaw cycle demonstrated a significantly higher Young's Modulus (14 megapascals) as compared to menisci undergoing multiple freeze–thaw cycles (10 megapascals, $p = 0.03$). These results were maintained when medial and lateral menisci were compared independently. Histological and biochemical analyses supported, but did not provide an explanation for the change in intrinsic compressive resistance. From these results, transplantation of meniscal allograft tissue frozen and thawed four times may be compromised in its ability to resist compression; and thus may undermine its role in replacing native meniscal tissue. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: meniscus; allograft; transplant; graft preparation

INTRODUCTION

Since 1948,[1] the treatment strategy for meniscus pathology has been evolving from one of resection to preservation. Despite this, there is still a large population of patients that have undergone total- and subtotal-meniscectomy; additionally, there are many instances when preservation is impossible.[2] In such cases, meniscal allografting is the treatment of choice.[3]

Unfortunately, tissue availability and cost for meniscal allografting is a growing concern. In 2004, there were 1,400 meniscus transplants performed with an estimated waiting period of 4 weeks [personal communication: Musculoskeletal Transplantation Foundation (MTF), January 2005]. This waiting period will only increase as more orthopedic surgeons gain experience and competence in meniscus allografting. Moreover, in comparison with figures cited by Vangsness et al.[4] in 1996, the cost of a meniscal allograft has increased an astonishing 490%. This increase is a reflection of the necessary costs to maintain the successful screening procedures for excluding diseased and contaminated tissue[5,6] under a strict and tightly organized timeline. This process (i.e., donor recruitment, tissue procurement, anatomic manipulation, infectious disease screening, and sterilization techniques) is further complicated by the physical distance and time between the donor, the procurement organization, and the recipient.

The ability to repeatedly freeze–thaw meniscal tissue without compromising its structural or functional integrity may exponentially decrease the loss of tissue due to these concerns. The timeline in which tissue is currently processed can be

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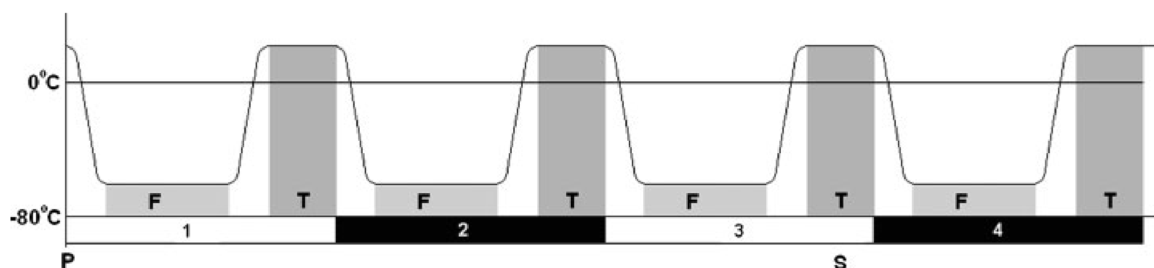

Figure 1. Multiple freeze–thaw cycle scheduling. The figure represents the schedule for the repeated freeze–thaw cycled menisci with procurement (P), segmentation (S), cycle number (1–4), the 1-week −80°C frozen stage (F), and the 8-h room temperature thaw stage (T).

broadened. This will allow more time to transport, coordinate, and process the tissue, as well as staged or repeat testing. In this same respect, tissue processing could be centralized to streamline the screening and procurement process thus decreasing costs. Finally, tissue could be re-frozen should the surgery be cancelled between thawing and implanting the allograft.

Scientific considerations for cell viability are minimized in advancing today's single freeze–thaw meniscal allograft tissue process to a multiple freeze–thaw process.[7] A greater concern is to maintain the ability of the allograph to mimic native meniscal tissue as the host cells repopulate the scaffold.[8] To date, there is little known about the biomechanics of a graft tissue that has undergone multiple freeze–thaw cycles, as compared to the current standard of a single cycle. We undertook this experiment to survey the impact multiple freeze–thaw cycles have on meniscal allograft tissue. Specifically, we hypothesized that four freeze–thaw cycles will not affect (1) the intrinsic compressive resistance, (2) biochemical composition, and (3) histological structure of allograft tissue.

MATERIALS AND METHODS

Sample Tissue

A total of seven human menisci were received fresh-frozen from the MTF. The menisci were originally conditioned and processed for transplantation but unused because of their "out" sizes. The donor profile included three males and three females, two menisci coming from the same female subject. The male subjects were aged 36–47 years old (mean, 44.25 years), and the females were 17–46 years old (mean, 36.0 years). The menisci were tracked with an identification number and randomly assigned to either the multiple freeze–thaw cycle group ($n = 4$) or the single freeze–thaw cycle group ($n = 3$).

Freeze–Thaw Cycle

A freeze–thaw cycle consisted of a 1-week frozen stage (−80°C) and an 8-h thaw stage (25°C) (Fig. 1). The tissue

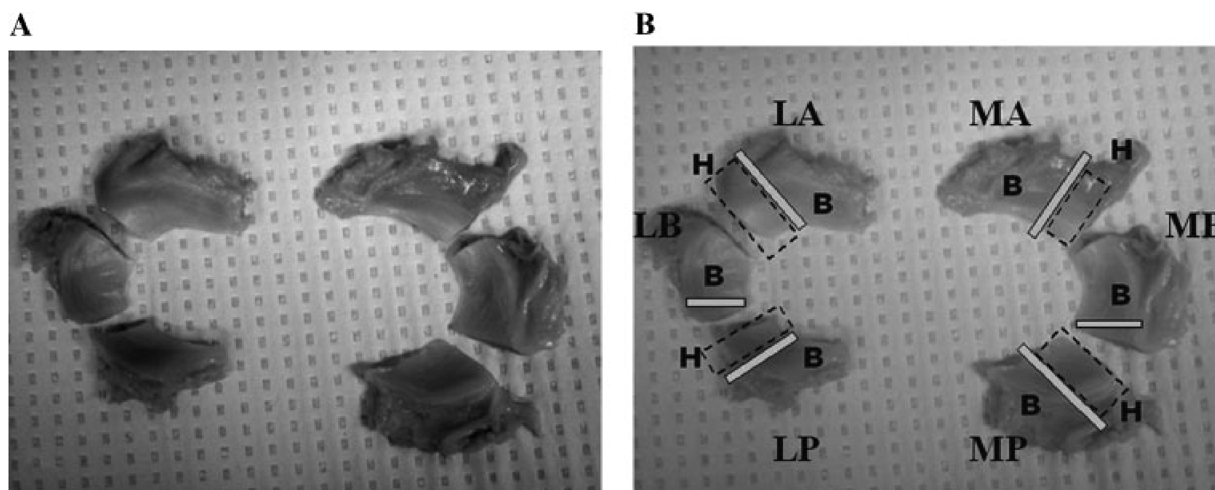

Figure 2. Meniscus sample segmentation. (A) Meniscus sample (medial on right) after cutting with No. 15 blade (segmentation). (B) Segmented meniscus with overlay of testing assignments per area (medial on right). B, biomechanical testing sample; white bar, biochemistry testing sample; H, histologic sample; LA, lateral anterior; LB, lateral body; LP, lateral posterior; MA, medial anterior; MB, medial body; MP, medial posterior.

was placed in a 25°C water bath incubation for 2 min prior to each thaw stage. The freezing (uncontrolled fresh-freezing) and thawing procedures were designed to mimic actual tissue processing and operating room times.

Sample Segmentation

Medial and lateral menisci were trisected into anterior, middle, and posterior segments, in accord with a previous study by Fabbriciani et al.[9] As in that study,[9] the menisci were regionally sectioned and sent for histological ($n = 28$) and biochemical ($n = 42$) testing. Our study additionally included biomechanical testing ($n = 42$) of each regional section, as depicted in Figure 2.

Biomechanical Compressive Testing

After gross assessment of the meniscus, the height of each sample was measured with a digital caliper (Precision Graphic Instruments, Inc., Spokane, WA) at the center of the sample. After orientation of the meniscus, the sample was resected perpendicular to the inferomedially directed, weight bearing axis of the meniscus (Fig. 3). This was done to provide bulk compression of the tissue. Samples were then potted in an acrylic mix (Isocryl, Lang Dental Manufacturing Co., Wheeling, IL) within a plastic tray (Crate and Barrel, Chicago, IL) for a partial-closed compression test of the tissue.

Specimen strain was measured using the change in position of the compression rod loaded in the Instron mechanical testing machine (Instron Model 8871 m, Canton, MA). Each sample was pre-loaded with a 5 Newton force and returned to 1 Newton force before testing. The compressive load was then applied to each specimen at a constant displacement rate of 0.75 mm/min, and the test was allowed to continue until the final displacement equaled half of the sample height. The specimen strain and modulus were determined by the recorded load and compressive rod displacement. All results were exported from the Instron software to a spreadsheet (Microsoft Excel, Microsoft Corporation, Richmond, VA) in order to determine the point of mechanical failure and tissue modulus. Calculations based on studies of meniscal area,[10,11] allowed analysis to include only data relevant in physiological loads (408.2–1043.2 N).

Biochemistry

Independent of biomechanical testing, samples were weighed for a wet index weight and placed in sterile tubes; the dry weights were then recorded after lyophilization. Samples were then each digested in a papain enzyme digestion solution (20 μg/ml papain in 0.1 M sodium acetate, 0.05 EDTA, pH 5.53) overnight at 56°–60°C.

Samples were then pipetted at 150 μl in the first well of each row of a 96-well cytoplate; 75 μl of dilution buffer was then added to each following well. Serial dilutions of one-half were made across the row and then guanidine hydrochloride was added to the sample wells. DMB reagent (200 μl) was added to each well before the plate was shaken for 30 s to insure thorough mixing. Absorbance was made immediately at 530 nm and 595 nm, and the ratio of 530 nm/595 nm was calculated for standards and samples. The plate was read using the Kineticalc Program on the Bio-Tek Microplate Reader (Model EL-311, Winooska, VT) and data analysis performed through Microsoft Excel.[12]

Histology

The sampled segments were fixed in 10% neutral buffered formalin and stained for routine histologic

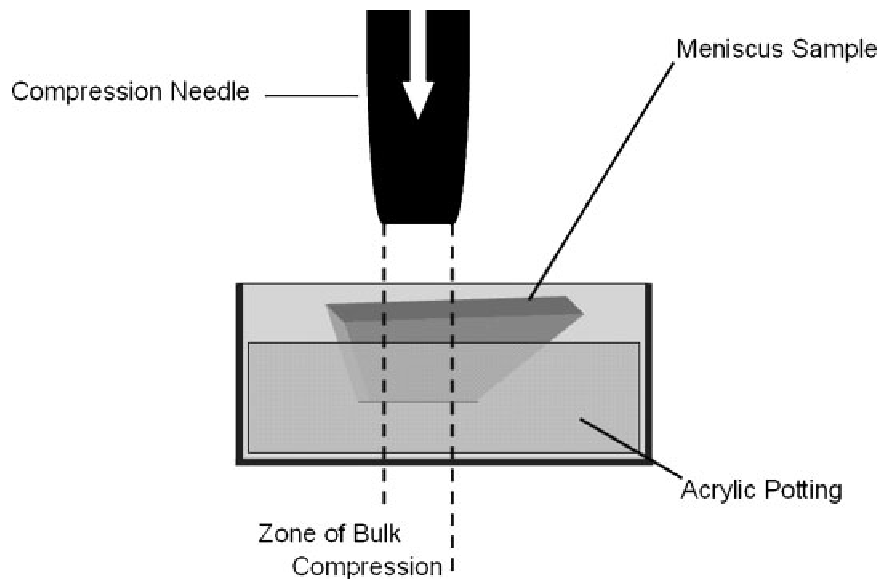

Figure 3. Meniscus potting for zone of bulk compression. To achieve a partial-closed, zone of bulk compression, the meniscus samples were set in acrylic potting and compressed perpendicular to the femoral articulating surface.

analysis with hematoxylin and eosin (H&E) and safranin-O and evaluated under plane light with a binocular phase contrast microscope (Model BH-2, Olympus Nikon Instruments, Inc., Melville, NY) with 20×, 40×, and 100× magnification. Additional picrosirius-red staining was done to assess the collagen orientation under plane polarized light. Sections stained with picrosirius red were observed with a Nikon polarization microscope equipped with a lambda/4 compensator plate and interference filter (lambda = 589 nm). The presence or absence of birefringence indicated orientation of the collagen fibers.

Statistical Analysis

Data was stratified on three levels, collective (all medial and lateral samples of each group), medial (all medial segments of each group), and lateral (all lateral segments of each group). Comparisons were then made with a single-tailed, independent student t-test, with significance at $p = 0.05$ for all data.

RESULTS

Biomechanics

All but one full single freeze–thaw meniscus successfully underwent and produced data for the biomechanical testing. The one meniscus was lost in devising a reproducible, reliable compressive test. Additionally, one single freeze–thaw segment slipped from the acrylic casting material during compression and therefore was omitted from the following results ($n = 11$). All samples of the multiple freeze–thaw menisci were successful in producing data ($n = 24$).

Tissue failure was not reached in either group; however, enough data was collected to determine the Young's Modulus for 11 single freeze–thaw, and 24 multiple freeze–thaw meniscal segments. The mean Young's Modulus for the single freeze–thaw segments were calculated from the respective trend lines (mean $r^2 = 0.992$, range: 0.978–0.999) composed of a mean 301 data points (range: 169–433). The Young's Modulus for the multiple freeze–thaw group were based on similar data sets (435 data points (range: 226–24) and an r-squared value of 0.994 (range: 0.980–0.999).

Multiple freeze–thawing decreased the intrinsic compressive resistance of meniscal segments and was significant when data were compared as full ($p = 0.03$), medial ($p = 0.03$), and lateral ($p = 0.01$) meniscal samples. The representative data are demonstrated in Table 1.

When anatomic regions were compared within each group, the medial menisci were universally less resistant to compression as compared to the lateral menisci samples. While in the multiple

Table 1. Biomechanical Results for Single Freeze–Thaw and Multiple Freeze–Thaw Segments

	Single Freeze–Thaw Samples			Multiple Freeze–Thaw Samples		
Mean Percent	All Segments ($n = 18$)	Medial Segments ($n = 9$)	Lateral Segments ($n = 9$)	All Segments ($n = 24$)	Medial Segments ($n = 12$)	Lateral Segments ($n = 12$)
Water (range)	58.6 (37.1–83.6)	59.1 (37.7–83.6)	58.1 (37.1–75.3)	67.0 (51.4–77.6)	65.0 (51.4–74.4)	69.0 (61.8–77.6)
PG per wet weight (range)	0.25 (0.10–0.58)	0.27 (0.10–0.58)	0.22 (0.10–0.36)	0.31 (0.10–0.53)	0.33 (0.15–0.51)	0.29 (0.10–0.53)

PG, proteoglycan.

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Table 2. Biochemical Results for Single Freeze–Thaw and Multiple Freeze–Thaw Segments

Young's Modulus (Pascals)	Single Freeze–Thaw Samples			Multiple Freeze–Thaw Samples		
	All Segments ($n = 11$)	Medial Segments ($n = 5$)	Lateral Segments ($n = 6$)	All Segments ($n = 24$)	Medial Segments ($n = 12$)	Lateral Segments ($n = 12$)
Mean	1.4×10^7	1.2×10^7	1.6×10^7	1.0×10^7	8.5×10^6	1.2×10^7
Maximum	2.4×10^7	1.9×10^7	2.4×10^7	1.7×10^7	1.3×10^7	1.7×10^7
Minimum	9.2×10^6	9.2×10^6	9.9×10^6	3.3×10^6	3.3×10^6	5.5×10^6

freeze–thaw group, this difference was significant ($p = 0.01$), the difference in the single freeze–thaw group was not ($p = 0.05$).

Biochemistry

All meniscus samples were included in the biochemical analysis (single freeze–thaw, $n = 18$; multiple freeze–thaw, $n = 24$). The results were evaluated on three levels: (1) all segments of each group, (2) the medial segments of each group, and (3) the lateral segments of each group.

The multiple freeze–thaw meniscal tissue demonstrated significantly greater percent water than the single freeze–thaw group at the level of the full meniscus ($p = 0.01$) and lateral meniscus ($p = 0.025$). The medial menisci did not demonstrate a significant difference between groups. These results are reviewed in Table 2.

Multiple freeze–thaw cycles were associated with higher mean percent proteoglycan (PG) per weight than single freeze–thaw cycled menisci. This difference, however, was not significant. These results are also summarized in Table 2.

Histology

Histologic staining with H&E demonstrated that both groups of menisci had an even distribution of collagen throughout the body of the meniscus in transverse sections. All samples demonstrated similarities in the amount of cellularity and matrix organization. In longitudinal section, both groups demonstrated the expected longitudinally directed collagen bundles with occasional radial tie fibers.

Safranin-O staining showed an even distribution of PG content in the body of the menisci of both groups. A subjective rating from 0 (little to no PG) to 2 (maximum PG staining) was used to rate the PG content of a meniscus. The single freeze–thaw group had a rated PG content mean of 0.9 (0–2). The mean PG content of the multiple freeze–thaw group had a rated PG content of 1.2 (0.5–2).

There were no differences in collagen organization between the single and multiple freeze–thaw groups stained with picrosirius red stain on polarized light microscopy. In both groups, the collagen fibers near the articulating surface were organized into both longitudinal and circumferential directions, thus providing an interlocking array of fibers.

DISCUSSION

The primary question of this investigation was whether multiple freeze–thaw cycles would affect the biomechanical integrity of meniscal allograft tissue. Using the Young's Modulus as a measure of

compressive resistance, this study showed that the multiple freeze–thaw cycles decreased the intrinsic compressive resistance of meniscal samples undergoing constant, slow-rate compression.

The Young's Modulus is the ratio of the applied pressure to the change in height of the compressed material. This provides a measurement of compressive resistance and carries a direct correlate to the ability of the meniscus to reduce the pressures on the articulating surfaces. Given the results of this study, the multiple frozen–thawed menisci might allow increased contact pressures on the articular cartilage.

A *"softer"* meniscus can be compressed more easily and this compression will occur until one of two scenarios occur; either full articular contact is achieved (as seen in meniscectomy) or the meniscus simply has less ability to tolerate compression with a proportional decrease in chondroprotection. In the former case, like the meniscectomized knee, the transmitted load between the femur and tibia are completely focused to a more localized area of articular cartilage. While the loss of the meniscus allows increased articular cartilage contact, the increased forces transmitted over that area grossly overshadows the increases in area. This will cause greater tensile strain to the articular cartilage and possibly predispose it to fissuring, cracking, or tearing of the articular cartilage.

Biochemical studies supported that multiple freeze–thaw cycles damaged meniscal allograft tissue. As understood from studies of articular cartilage,[12,13] increased water content is associated with collagen network damage. In this study, there was significantly increased water content in the multiple freeze–thaw group. The increased percent water may be a result of the damage as much as it is a cause; this relationship was not elucidated in this experiment. Histological analysis did not demonstrate sufficient evidence for a cause of this damage; it is our hypothesis that the damage occurred not at the histological level, but the macro-molecular level. The use of electron microscopy would likely be helpful in obtaining this information. Rather unexpectedly, the proteoglycan content was higher in the multiple freeze–thaw group as well. A hypothesis for this finding is that the viable cells in the meniscus samples were stimulated to produce proteoglycan.

A strength of this study is that it provides a novel biomechanical test that allowed for parallel biochemical and histologic analyses. The intrinsic compressive properties, as well as other biomechanical properties of the meniscus, are predicated upon its gross morphology, histological organization, and biochemical composition. This study reasonably considered each of these aspects in the context of changing biomechanics. To our knowledge there has not been any other work that combined the study of these properties into one investigation. At the same time, this study design required the mechanical testing of menisci that were removed from the tibial plateau and segmented. This effectively removes the translation of compressive forces to hoop stresses. It is important to note, therefore, that our results represent a worst case scenario (i.e., no contribution of intact meniscus to the mechanical integrity), and that the resistance to compression is by the bulk of the tissue specimen itself, not the full meniscus.

A strength of the slow-rate indentation test used in this work minimizes the fluid phase properties of the meniscus and elicits the intrinsic compressive properties of the tissue. Therefore, our results represent two important physiological instances of meniscus loading: The first is immediate loading of the meniscus, before the fluid phase reacts to resist compression; and the second is at the time of long continuous loading after the fluid phase has equilibrated.[13] Acute tears of the meniscus are seen in younger active patients through the first instance, while older patients often suffer degenerative tears through the second. Additionally, the indentation test proved to be an expedient, reliable, and consistent test for a large number of samples. The authors, at the same time, recognize that the exclusion of the fluid phase limits the application of this data to the true physiologic properties of meniscal load bearing.

Limitations to the study design included sample size, relatively extreme end points, and variably aged menisci. The privilege of working with human tissue comes with the cost of limited availability. The biological sample size of this study was four multiple freeze–thaw menisci and three single freeze–thaw menisci; we understand and recognize this limitation. The quantity of available tissue for the study, however, was overshadowed by the quality of tissue available.

The end points used in this experiment were minimized to one freeze–thaw cycle and maximized to four freeze–thaw cycles. No fresh meniscal tissue was used for the work due to costs and availability; those involved understand and recognize the importance of what that could provide to the results. That said, the aim of the study was to advance the current allografting process and so we used today's clinical standard as the minimum. The maximum of four freeze–thaw cycles was designated as the most extreme need by a survey

of the major tissue banks. Future investigations can now take a more appropriate, step-wise approach to determine at what repeated freeze–thaw cycle number meniscal tissue loses its biomechanical integrity.

Finally, the meniscus used in this experiment came from a wide range of ages (17–47 years old). This can be considered a limitation to the study due to possible changes in composition and integrity of the menisci at the respective ages. However, the impact of multiple freeze–thaw cycles should be considered in multiple age groups as a commensurate age range is participating in the tissue allografting process.

While the results of this work demonstrate biochemical and biomechanical alterations of menisci frozen and thawed multiple times, future in vitro animal experiments for investigating the impact of multiple freeze–thaw cycles on meniscal allograft tissue will be important in developing the allografting process. Additionally, the model of this study can be modified to investigate other freezing methods. For example, perhaps cryopreservation can allow for multiple freeze–thaw cycles—an advantage that will overshadow the current problems of technical demand and cost.

The results of this work indicate that four freeze–thaw cycles significantly decrease the intrinsic resistance of meniscal allograft tissue to a constant, slow-rate compression. The current study represents an important first step in the mechanical characterization of the multiple frozen–thawed meniscus. However, the clinical ramifications of any alterations in biomechanical properties are not currently known, and whether the changes observed in this study contribute or detract from the long-term performance of the allograft remains unknown.

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