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Abstract:
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biomechanical outcomes compared to our fresh model. Our results showed that 6 months post-implantation as an ACL replacement graft, both our tissue engineered fresh and frozen grafts demonstrated similar mechanical and histological outcomes indicating that freezing is a suitable method for preserving and storing our graft prior to ACL reconstruction. The ability to use frozen constructs significantly increases the versatility of our graft technology expanding the clinical utility of our graft.
Fresh vs. Frozen Engineered Bone-Ligament-Bone Grafts
for Sheep ACL Repair

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Keywords: tissue engineering, scaffold-free, anterior cruciate ligament (ACL) reconstruction, fresh, frozen, off-the-shelf
ABSTRACT

Surgical intervention is often required to restore knee instability in patients with anterior cruciate ligament (ACL) injury. The most commonly used grafts for ACL reconstruction are tendon autografts or allografts. These current options, however, have shown failure rates requiring revision and continued instability in the long-term. The mismatched biomechanical properties of the current tendon grafts compared to native ACL tissue are thought to contribute to these poor outcomes and potential risk of early onset osteoarthritis. As a possible solution to these issues, our laboratory has fabricated tissue engineered ligament constructs that exhibit structural and functional properties similar to those of native ACL tissue after 6 months implantation. In addition, these tissue engineered grafts achieve vascular and neural development that exceeds those of patellar tendon grafts. However, the utility of our tissue engineered grafts is limited by the labor-intensive method required to produce the constructs and the need to use the constructs fresh, directly from the cell culturing system. Ideally, these constructs would be fabricated and stored until needed. Thus, in this study we investigated the efficacy of freezing our tissue engineered constructs as a method of preservation prior to use for ACL reconstruction. We hypothesized that frozen constructs would have similar histological and biomechanical outcomes compared to our fresh model. Our results showed that 6 months post-implantation as an ACL replacement graft, both our tissue engineered fresh and frozen grafts demonstrated similar mechanical and histological outcomes indicating that freezing is a suitable method for preserving and storing our graft prior to ACL reconstruction. The ability to use frozen constructs significantly increases the versatility of our graft technology expanding the clinical utility of our graft.
INTRODUCTION

Anterior cruciate ligament (ACL) reconstruction surgeries are one of the most commonly performed orthopaedic procedures with over 100,000 patients undergoing the surgery each year in the US. Cumulative failure rates of ACL reconstruction to restore knee joint stability suggest at least 1 in 9 patients will experience re-rupture or clinical failure in the long-term. Limitations associated with current graft choices are compounded by the mismatched biomechanical properties exhibited by all tendon grafts in comparison to the native ACL.

Tissue engineering strategies are being developed to potentially address the shortcomings of the current graft options. Current tissue engineering techniques, however, focus on the development of a biological or synthetic scaffold to provide strength and an environment suitable for cellular growth either in vitro or in vivo. To date, no scaffold technology exists that is able to withstand the multidirectional mechanical forces of a native ACL and has the necessary biocompatibility and biodegradability qualities needed for ACL reconstruction.

Our laboratory has previously developed a method of tissue engineering three-dimensional multiphasic bone-ligament-bone (BLB) constructs from bone marrow stromal cells (BMSCs). During in vitro tissue engineering, the BMSCs form their own extracellular matrix eliminating the need for an exogenous scaffold. We have shown that after six months in vivo, these engineered scaffoldless BLB constructs undergo significant remodeling to develop similar mechanical and morphological properties to those of native ACLs and are capable of inducing vascular and neural development. These results show that our BLB constructs offer a promising alternative technology for ACL reconstruction.

A limitation of tissue engineering is the time necessary to fabricate engineered tissue in vitro and the task of getting freshly engineered tissues to the patient. It takes approximately 2-3 weeks to culture and expand freshly isolated BMSCs to obtain the necessary amount of cells required to fabricate a construct. An additional 2 weeks is required after 3-D formation to mature the construct in vitro in order to improve fusion between tissue interfaces and increase tensile
strength prior to implantation. The total time required from bone marrow isolation to an implantable 3-D construct is approximately 6-7 weeks. Recent studies suggest that the timing of ACL repair after injury is critical and that early intervention is most effective.\textsuperscript{2,6-8} It is therefore necessary to develop technologies for graft preservation and to have the option for an off-the-shelf allograft ligament.

Several freezing methods exist for tissue preservation including deep-freezing, cryopreservation, vitrification (ice-free cryopreservation) and freeze-drying. Although the other methods listed may have better cell preservation outcomes, deep-freezing is the simplest and most commonly used method with reported tissue storage times exceeding 3-5 years at -80°C.\textsuperscript{3,4,9,10} Thus, for this study we chose to investigate deep-freezing as a method to preserve and store our constructs.

The purpose of this study was to use our sheep ACL reconstruction model to validate the deep-freezing preservation method by comparing the histological and biomechanical outcomes of the frozen grafts to fresh grafts in a 6-month recovery study. \textit{In vitro} construct morphology and viability was also assessed at various time points post 3-D formation to document the extensive \textit{in vitro} remodeling and to identify an optimal point for freezing the graft. We hypothesized that the structural and functional outcomes of the frozen and fresh models would be similar after 6 months of implantation indicating that frozen grafts would be a viable off-the-shelf option for ACL reconstruction.
MATERIALS AND METHODS

Animal Care

BMSCs were harvested from marrow aspirations from adult female Black Suffolk sheep to fabricate our tissue engineered constructs for use as grafts in our previously described model for sheep ACL reconstruction.\textsuperscript{5,11} Animals were acclimated to the Unit for Laboratory Animal Medicine (ULAM) husbandry facilities at the University of Michigan for at least one week prior to any procedure. Sheep were given access to food and water \textit{ad libitum}. All animal care and animal surgeries were performed in accordance with The Guide for the Care and Use of Laboratory Animals\textsuperscript{12} and the experimental protocol was approved by the University Committee for the Use and Care of Animals at the University of Michigan.

Preparation of Cell Culture Supplies

The media used in this experiment have been previously described.\textsuperscript{5} All solutions and media were prepared and stored at 4°C and were warmed to 37°C in a heated bead bath prior to use. Briefly, growth medium (GM) consisted of 78\% Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA), with 20\% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 2\% antibiotic anti-mycotic (ABAM; Grand Island, NY, USA), 6ng/ml basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA), 0.13 mg/mL ascorbic acid-2-phosphotase (Sigma-Aldrich, St. Louis, MO), and 0.05 mg/mL L-proline (Sigma-Aldrich, St. Louis, MO, USA); differentiation medium (DM) consisted of 91\% DMEM, 7\% horse serum albumin (HS; Gibco, Grand Island, NY, USA), 2\% ABAM, 0.13 mg/mL asc-2-phos, 0.05 mg/mL L-proline, and 2 ng/mL transforming growth factor beta (TGF-b; Peprotech, Rocky Hill, NJ, USA). For the culture of bone cells, $10^{-8}$ M dexamethasone (DEX; Sigma-Aldrich, St. Louis, MO, USA) was added to GM and DM.\textsuperscript{13}

Construct dishes were prepared as described previously\textsuperscript{5} to house and constrain the formed three-dimensional constructs. Briefly, 100 mm diameter cell culture plates were filled
with 12 ml Sylgard (Dow Chemical Corp., Midland, MI, USA; type 184 silicon elastomer) and allowed to cure for 3 weeks at room temperature. Prior to use, plates were decontaminated with UV light (wavelength 253.7 nm) for 60 min and rinsed with 70% EtOH and DPBS.

Isolation and Expansion of BMSCs

Bone marrow was aspirated from the iliac crest of a sheep using a Monoject Illinois needle (Sherwood Medical Company, St. Louis, MO, USA) with the animal under general anesthesia. The collected marrow aspirate was filtered through a 100 mm filter to remove solid debris and combined into a total volume of 15 ml with an equivalent volume of DPBS added. A layer of 15 ml Ficoll-Paque Premium (MNC; GE Healthcare, Munich, Germany) was carefully added on top of the aspirate and the solution was centrifuged (AccuSpin FR; Beckman Coulter Inc., Fullerton, CA, USA) at room temperature at 600 g for 30 minutes to separate the aspirate components by density. The upper layer of plasma was removed and the mesenchymal cells contained in the middle mononuclear cell layer were transferred into a new conical filled with DPBS. The remaining aspirate contents of the conical were discarded. The purified isolate was then centrifuged at 500 g for 10 minutes and the supernatant removed. An equivalent volume to the pellet of ACK lysing buffer (Gibco, Grand Island, NY, USA) was added and mixed for 30 seconds to lyse any remaining red blood cells. The conical was then filled with DPBS and centrifuged at 400 g for 5 minutes to wash the cells. After the supernatant was removed, the pellet was resuspended in 20 ml GM and a cell count was taken. Cells were then plated at 40,000-60,000 cells/cm² in cell culture dishes.

Fabrication of BLBs

Using previously described methods, the BMSCs were expanded into ligament and bone lineages. Briefly, passage-3 cells in the ligament pathway and passage-4 cells in the bone pathway were seeded at a density of 21,000 cells/cm² and switched to DM after 8 days plating.
After two days in DM, the bone monolayers were rolled with sterile tweezers into a tube shape and transferred to Sylgard plates. After one to two additional days, the bone constructs were then ready to be incorporated into a ligament monolayer to create our BLB. Each confluent ligament monolayer was removed intact from the cell culture plate surface and transferred to Sylgard plates and pinned back into a single layer. Bone constructs were then pinned on each end of the ligament monolayer and subsequently surrounded by ligament tissue. The length of the BLB was adjusted with minutien pins to the desired length of approximately 60-75 mm comprised of at least a 30 mm ligament portion and two 15 mm bone ends. Four of these constructs were placed side-by-side and allowed to fuse. The DM was changed every 2-3 days. After 2 weeks of formation, two fused sets of four constructs were combined for an implantation width of approximately 4-5 mm at the ligament region. After an additional one week in culture, the fully formed BLB was ready for implantation. All tissues were constrained using minutien pins at set distances at all times during 3-D culture. Prior to implanting, the bone ends of the BLB were threaded with non-absorbable 5-0 silk suture to allow for passage into the bone tunnel and fixation onto the periosteum.

Fully formed frozen BLBs stored in DM were prepared by sealing the lid of a Sylgard dish with paraffin film. The entire dish was then placed into an -80°C freezer for a minimum of 1 hour to ensure that the construct was completely frozen. Prior to surgical implantation, plates were retrieved from the freezer and allowed to thaw in a 37°C bead bath. After complete thawing of the construct, paraffin film was removed and plates were stored in a cell culture incubator at 37°C until implantation.

**Histological Analysis of In Vitro Engineered Constructs**

Constructs were removed from culture at designated time points and fixed with paraformaldehyde for one hour at room temperature, 15% sucrose for 2-3 hours rocking at room temperature, then 30% sucrose overnight rocking at 4°C. Following fixation, constructs were
placed into tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA), frozen with dry ice and stored until needed at -80°C. Frozen samples were sliced in either cross-section or longitudinally using a Microm HM 500 cryostat system (Heidelberg, Germany) to a thickness of 14 µm and were placed onto Superfrost Plus microscopy slides. For determination of collagen, sections were stained with Picrosirius Red. Sections were also stained with hematoxylin and eosin (H&E) for general morphology characteristics.

For immunohistochemical staining, sections were rinsed 3 times for 5 minutes each with phosphate-buffered saline (PBS) containing 0.1% triton (PBST). Sections were blocked for 30 minutes with PBST containing 5% donkey serum (PBST-S) at room temperature in a humidified chamber, and incubated overnight at 4°C with the primary antibody 1:400 Rabbit anti-Caspase-3 (Abcam, Cambridge, MA, USA). After 3 washes in PBST, the slides were incubated for 2 hours with the secondary antibody 1:1000 Alexa555 Anti-rabbit (Invitrogen, Carlsbad, CA, USA), rinsed 3 times in PBST, and mounted with Prolong Gold Antifade Reagent with DAPI (Molecular Probes, Eugene, OR, USA). The sections were examined and photographed with an Olympus BX-51 fluorescent microscope.

**Surgical Procedure**

ACL reconstructions were performed arthroscopically based on a previously described procedure. Briefly, after induction of general anesthesia and preparation of the surgical site, the native ACL of the left limb was removed with remnants of the stump on both the femur and tibia used to aid in anatomical positioning of the BLB. Drill guides were used in order to position Steinmann pins for precise placement at the center of the tibial and femoral footprints. Bone tunnels (5-6 mm) were drilled using cannulated reamers over the pins. The BLB was then passed through the bone tunnels using suture until the threaded bone ends of the BLB were not visible arthroscopically in the intra-articular space. The proximal and distal ends were then secured with suture to the periosteum. Incisions were closed with staples and the entire surgical
site was sprayed with Alushield (Neogen Corp., Lansing, MI, USA). The contralateral (right) limb served as a control. Animals were monitored daily at the ULAM facility for 2 weeks after which staples were removed and the animals were sent to a large outdoor pen to allow greater mobility.

**Explantation**

After a six-month implantation, both the experimental and contralateral knees were explanted for morphological and mechanical analyses. Following euthanasia, the knee was harvested by removing the tibia and femur and taken for mechanical testing. Anterior drawer testing of the knee at 45 degrees was performed with the knee capsule intact. The knee was subsequently dissected to the BLB or contralateral ACL (C-ACL). For visualization of the entire graft during uniaxial tensile testing, the medial condyle was carefully removed. Following anterior drawer and uniaxial tensile testing, the BLB and C-ACL were resected from their tibial and femoral bone insertions and harvested for histology.

**Histological Analysis of Explanted BLB and C-ACL**

For histological preparation of explanted tissues, samples were fixed in 10% neutral buffered formalin for 8 days and stored in 70% ethanol at 4°C until processing. Each tissue was sectioned in a microtome (Leica RM 2155) at 6 µm, placed onto Superfrost Plus microscopy slides and placed in 60°C oven for one hour to dry. For morphological characteristics, sections were stained with H&E. Immunohistochemistry (IHC) staining with specific antibodies was performed to detect the presence of elastin and collagen type 1.

To deparaffinize the slides with paraffin sections for IHC, slides were washed three times in xylene for 3 minutes, twice in 100% ethanol for 2 minutes, twice in 95% ethanol for 2 minutes, and once in 70% ethanol for 2 minutes. Following deparaffinization, slides were washed in phosphate buffered saline-0.1% Triton X-100 (PBST) (Sigma-Aldrich, St. Louis, MO, USA) for
15 minutes at room temperature. Using a hydrophobic pen, sections were circled for blocking and antibody staining. Sections were covered in blocking solution containing PBST with 3% Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at room temperature. Primary antibodies were diluted in blocking solution and added to the sections and incubated overnight at 4°C in a hydration chamber. The dilutions of the primary antibodies used were as follows: 1:35 of rabbit anti-elastin (Millipore, Billerica, MA, USA, #AB2039); and 1:100 of rabbit anti-collagen type I (Abcam, Cambridge, MA, USA, #AB292). Slides were then washed for 5 minutes in PBST at room temperature three times for a total of 15 minutes. Secondary antibodies were diluted at 1:500 in blocking solution in the dark and added to the sections. The slides were kept in a hydration chamber and incubated at room temperature for 2.5 hours. The slides were then washed for 15 minutes in PBS at room temperature three times for a total of 45 minutes. Nuclei were stained using Prolong Gold with DAPI (Sigma-Aldrich, St. Louis, MO, USA) to each section. Slides were then covered with a coverslip and incubated overnight at room temperature before imaging with an Olympus BX-51 microscope.

**Knee Laxity Testing**

Knee laxity was measured using a custom designed anterior drawer tester. The bones were potted in grips using a polymer that became malleable when heated and hardened to its conformed shape when allowed to cool. The bone and hardened polymer were secured with two ¼"-20 screws in the grips and mounted onto an MTS 810 servohydraulic test system with a 25 kN load cell. Ink markings were placed at a known distance onto the femur and tibia grips for displacement tracking. The test comprised of a 0.5 mm/s extension until a 50 N force was achieved. Images were collected with a Grasshopper IEEE-1394b digital camera (Point Grey, British Columbia, CA) and analysis for displacement was determined using MetaMorph software.

**Uniaxial Testing**
Knee tissue was further dissected away leaving only the BLB or C-ACL attached at both tibial and femoral insertions. The length of the ligament as well as the width and thickness of the proximal, middle and distal regions were measured and recorded. The cross sectional areas from these three locations were averaged and used as the representative area for stress calculations. The knee was repositioned for a flexion angle of 30 degrees by fixing the tibia and femur grips at 90 and 60 degrees respectively in the sagittal plane to put the ligament in a uniaxial loading configuration. Graphite powder was blown onto the specimen to create a surface pattern for optical displacement measurement using digital image correlation (DIC) to compute full-field strain contours. Uniaxial tension tests at a strain rate of 0.05/second for a loading time of 7.5 seconds were then conducted on the BLB and ACL specimens to obtain the stiffness using previously developed testing protocols. A Photron high-speed camera was used for synchronized force and image acquisition with a custom developed LabView program. The load-unload cycle for each specimen was run in triplicate. VIC-2D Software (Correlated Solutions, Columbia, SC) was used for DIC analysis.

Statistical Analysis

Comparisons among the three groups were done using one-way ANOVA with Tukey’s post-hoc test. A p-value < 0.05 for all statistical tests was considered significant. All data were reported as mean ± standard deviation.
RESULTS

Histological Evaluation of In Vitro Engineered Ligament Tissue Over Time in Culture

To examine general morphological characteristics of the engineered ligament constructs over time \textit{in vitro}, staining with H&E was performed. H&E stained cross sections through the center of fresh ligament constructs displayed viable cells at 48 hours, 1, 2, and 3 weeks following roll up of the monolayer (Figure 1A-D). At 48 hours following roll up of the monolayer the center of the construct was not immediately fused and the nuclei were densely packed (Figure 1A). At the 1-week time point, the construct was fully fused and the nuclei were densely packed (Figure 1B). By 2 weeks in culture, the construct had reorganized into a dense 3-D structure, and the nuclei were less densely packed together (Figure 1C). At 3 weeks, extensive remodeling had occurred and the nuclei remained less densely packed together (Figure 1D). A fibrotic outer layer similar to that observed in native ligament was observed around the outer surface at the construct from 48 hours to 2 weeks \textit{in vitro} but had disappeared by 3 weeks \textit{in vitro} (Figure 1A-D).

Picrosirius red staining was performed to assess the presence of collagen. Figure 1 E-H shows stained cross sections at the center of the ligament constructs at 48 hours, 1, 2, and 3 weeks following roll up of the monolayer with collagen indicated in red. Collagen was present in the construct at 48 hours following roll-up of the monolayer however the layers of the construct remained distinct indicating that they had not yet fused (Figure 1E). By one week, extensive remodeling had occurred and the dense bands of collagen began to interweave through the cross sections of the construct (Figure 1F). By 2 weeks, the construct continued to remodel and the collagen was organized into dense, irregular bands throughout the construct that appeared to have an increase in surface area (Figure 1G), which increased in complexity by 3 weeks in culture (Figure 1H). Overall, construct remodeling was extensive with the complexity and surface area of dense collagen bands increasing with time \textit{in vitro} from 48 hours to 3 weeks in culture.
To assess cell death or apoptosis, immunohistochemical staining was performed with an antibody specific to caspase-3 at 48 hours, 1, 2, and 3 weeks following roll up of the monolayer for qualitative analysis (Figure 2). The cross sections through the center of the ligament constructs at 48 hours in culture were positive for caspase-3 in the central region of the construct (Figure 2A,C). DAPI staining of the ligament constructs showed that at 48 hours in vitro the nuclei were densely packed together (Figure 2B). At 1 week, caspase-3 was expressed not only in the central region but also along the outer regions of the construct (Figure 2D,F). By 2 weeks, there appeared to be a pronounced decrease in apoptosis as shown by a marked reduction of caspase-3 throughout the construct (Figure 2G,I), and the nuclei were less densely packed together (Figure 2H). At 3 weeks in vitro, caspase-3 expression was still observed (Figure 2I).

Analysis of Structure, Vascularization, Innervation and Elastin of the explanted BLBs

After 6 months in vivo, explanted frozen BLBs achieved an average length of 18.1±3.3 mm with explanted fresh BLBs reaching 18.5±1.2 mm (Figure 3A). The average CSAs of frozen BLBs were 33.3±9.5 mm² and fresh BLB CSAs were 29.7±10.5 mm² (Figure 3B). The average length and CSA of the C-ACLs were 21.4±3.1 mm and 39.1±9.2 mm² respectively. One-way ANOVA showed no statistically significant difference (p>0.05) between the average lengths $F(2,25)=2.3$, and CSAs $F(2,25)=1.5$ of the groups.

General morphology of the explanted frozen and fresh BLBs showed similar characteristics. H&E sections showed the formation of ligamentous crimp-like pattern with fibers arranged along the long axis of the tissue (Figure 4) as well as the formation of vasculature and innervation seen in cross section (Figure 5). Immunohistochemical staining showed similar collagen and elastin deposition compared to the C-ACL.
Knee Laxity

The knee laxity for the frozen BLB knees averaged 1.9±0.4 mm (n=6) while that of the fresh BLB knees was 1.9±0.4 mm (n=4). The C-ACL knee laxity averaged 0.5 ± 0.3 mm (n=15) (Figure 6). One-way ANOVA showed no significant difference between frozen and fresh knee laxity (p>0.05), but both groups had significantly increased laxity (p<0.001) compared to the C-ACL knees, F(2,22)=11.9.

Modulus Analysis of BLB

The tangent modulus (the slope of the stress-strain curve at a specified strain range) for the frozen BLB group averaged 42±7 MPa (n=6) whereas the tangent modulus of the fresh BLBs averaged 35±13 MPa (n=3) at a strain range of 0.04 - 0.10 (Figure 7). The tangent modulus data indicated no significant differences between the two graft types (p>0.05). Both grafts achieved approximately 23-30% of the average C-ACL modulus which was 158±32 MPa (n=12) at a strain range of 0.04 – 0.10.
DISCUSSION

Our tissue engineered BLBs are a promising solution to address the need for a better ACL replacement graft and offers advantages over currently used tendon grafts. To date, major limitations to our BLB technology are the graft’s relatively short shelf life, the requirement that the graft must be kept fresh, and the need to use the graft directly from a cell culture system. This study sought to investigate the efficacy of our grafts for ACL reconstruction following a simple deep-freezing preservation method by comparing the outcomes of the frozen grafts to our previously established fresh allografts in a 6-month recovery study. Our results showed that at 6 months post-implantation as an ACL replacement graft, both our tissue engineered fresh and frozen grafts demonstrated similar mechanical and histological outcomes indicating that deep-freezing is a suitable method for preserving and storing our graft prior to ACL reconstruction. The ability to use frozen constructs significantly increases the versatility of our graft technology expanding the clinical utility of our graft.

Fabrication of BLBs requires approximately 4-5 weeks for induction of BMSCs to ligament and bone lineages, formation of robust monolayers, and co-culture of the bone and ligament tissues into a 3-D configuration. An additional two weeks is needed in vitro for the BLB to condense and fuse into a uniform and robust 3-D construct. We have demonstrated that our BLB continues to remodel significantly in the two weeks in vitro that the constructs are left to fuse. The dense collagen bands and decreased cellularity seen at 2-week and 3-week fusion time points using Picrosirius Red and H&E/DAPI stained cross sections suggest that the constructs increase collagen deposition and advance ECM formation at these time points compared to earlier in formation. Apoptotic cells were observed at both 2 and 3 week time points suggesting that the BLB continues to remodel, possibly removing cells to accommodate the increased ECM secretion. Thus, it was decided that the optimal time for freezing would be at least 2 weeks after formation in order to allow for the achievement of favorable morphological and mechanical properties and reduce the risk of losing the construct due to breakage from the
pins. Freezing the constructs at less than 2 weeks resulted in a construct that was not sufficiently fused for implantation.

The frozen and fresh BLBs had similar outcomes as grafts for ACL reconstruction. After 6 months in vivo, frozen and fresh BLBs both increased in size to that observed in the C-ACLs. Compared to each other, frozen and fresh BLB grafts showed similar knee laxity and had similar mechanical moduli. However, compared to the C-ACL, both frozen and fresh BLB knees had increased laxity and the dissected grafts achieved approximately 30% of the mechanical modulus. Histological evaluation indicated the presence of a ligamentous crimp pattern similar to the C-ACL and the formation of vascularization and innervation in both frozen and fresh BLBs. These findings suggest that the frozen BLB is capable of mounting a regenerative response approximating those found with fresh BLBs. In agreement with our previous studies, no systemic immune responses were noted. Due to the high cost and resource demands of this project, it was necessary to gather mechanical and histological assessments on the same tissue. Mechanical tests were performed first at a conservative level to prevent damage to the tissue. As a result, there is a high variation in the selection of the strain region from the linear portion of the stress-strain curve used to calculate the tangent modulus.

Traditional methods of freezing, including the deep-freezing method used in this study, can cause ice crystal formation that can distort and possibly damage the ECM. These concerns were shown to not be as significant in tissue engineered ECM containing high densities of elastin and collagen such as our BLBs, which may explain the absence of a dramatic loss in structural or mechanical phenotype after freezing and thawing. Other preservation methods may prove superior for the maintenance of cell viability but the simplicity and success of the deep-freezing method deem this method an optimal preservation strategy for our ligament graft. Future studies will need to be done to determine the viability and efficacy of the frozen constructs after long-term storage to determine the true shelf life of the construct using this preservation method and establish criteria for product expiration.
Additional work is also needed to help elucidate the regenerative and remodeling processes that are occurring in the BLB grafts in vivo. It is evident from the results of this work along with numerous other studies utilizing acellularized or scaffold-only grafts that live cells are not necessarily required to induce these responses.\textsuperscript{16} It is possible that our tissue engineered ligament technology produces the most biomimetic structure for endogenous ACL regeneration. Viable cells were also found to have negligible contribution to tendon and ligament graft biomechanics compared to acellularized grafts in vitro.\textsuperscript{17} Therefore, lysing the cells from our BLBs after maturation to the desired level in vitro will allow us to bypass the physical conditions needed for maintaining viable cells without compromising the BLB’s structural integrity. It is important to consider that although we are lysing cells by deep-freezing, we are not necessarily removing all cellular DNA or debris. The regenerative response that we observed could in fact be due to residual cellular components within the frozen graft. An alternative explanation is that only the ECM of the graft is needed. Thus, further experiments investigating host cell interactions with the graft ECM and pathways of cellular migration including local cytokine expressions will be useful to determine the processes of cellular migration and remodeling of the graft towards native ligament. It would be advantageous if only the ECM was necessary for graft success, as this would obviate the regulatory hurdles that would otherwise be required for grafts with viable cells. Further studies should be done to determine the efficacy of various techniques to clear cell material from the graft and their responses in vivo to consider this regulatory route.

In this study, we have validated the use of deep-freezing as a means for short-term BLB graft preservation. We have shown that our constructs actively remodel in vitro after 3-D formation with collagen alignment and deposition increasing in levels after 2-3 weeks. After freezing and thawing, the BLBs maintained their structure and were implanted successfully for ACL reconstruction. Both frozen and fresh BLBs exhibited similar biomechanical and histological outcomes after 6 months implantation, remodeling in vivo to a more mature
phenotype that resembled native ACL. Thus, the use of frozen BLBs is a viable method for construct preservation and circumvents the need for viable cells. This knowledge expands our graft fabrication technology and allows for the production of a stock inventory of frozen ligament grafts available for use at time of clinical need thereby progressing the transition of the current technology towards large-scale manufacturing and clinical translation.
ACKNOWLEDGMENTS

Funding provided by the Center for Organogenesis at the University of Michigan.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.
REFERENCES


Figure Legends

Figure 1: Characterization of cross sections taken from the center of engineered ligament constructs using H&E (A-D) and Picrosirius Red (E-H) with time in vitro. At 48 hours, the center of the construct was not fused (A). By 2 weeks, the construct had reorganized into a dense 3-D structure (C). A fibrotic outer layer was observed around the outer surface at the construct from 48 hours to 2 weeks in vitro but had disappeared by 3 weeks in vitro (A-D). Picrosirius red staining revealed that collagen is present in the construct at 48 hours following roll up of the monolayer (E). The complexity and overall expression of collagen throughout the construct appeared to increase with time in vitro, with dense ribbons of collagen forming by 1 week in vitro (F). All images taken at 20x magnification with scale bars indicating 200 µm.

Figure 2: Cellularity and apoptosis of engineered ligament constructs. Immunohistochemical staining of cross sections of engineered ligament constructs at 48 hours, 1, and 2, and 3 weeks in vitro using the apoptosis marker caspase-3 (red) and DAPI (blue). Caspase-3 was expressed in the middle region of the construct at 48 hours (A,C), throughout the construct at 1 week (B,F), and decreased in expression throughout the construct by 2 and 3 weeks (G,I & J,L). 48-hour constructs had more densely packed nuclei (B). By 2 weeks in vitro, nuclei were less densely packed (H) and the trend continued at 3 weeks in vitro (K). All images taken at 20x magnification with scale bars indicating 200 µm.

FIGURE 3: Comparisons of length (A) and cross-sectional area (B) of C-ACL, explanted Fresh BLB, and Frozen BLB after 6 months in vivo. Values are mean ± standard
deviation. There were no significant differences found in the length and cross-sectional area of the C-ACL, Fresh BLB and Frozen BLB.

FIGURE 4: Longitudinal sections of C-ACL and explanted BLB (Fresh/Frozen) after 6 months in vivo. H&E staining (A-C) was used to ascertain general structure and morphology. Immunohistochemical staining for Elastin (D-F) and Type I Collagen (G-I) showed positive staining in both Fresh (E,H) and Frozen (F,I) with organization and staining showing similarities to C-ACL (D,G). All images taken at 40x magnification with scale bars indicating 100 µm.

FIGURE 5: Cross sections of C-ACL and explanted BLB (Fresh/Frozen) after 6 months in vivo. H&E images (A-C) at 40x showed pronounced vasculature confirmed by CD31 (D-F) antibody staining in both Fresh (B,E) and Frozen BLBs (C,F) indicating tissue regeneration.

FIGURE 6: Knee laxity of C-ACL and explanted BLB (Fresh/Frozen) after 6 months in vivo. No significant differences were seen between the Fresh and Frozen BLB knee laxities, however they were both significantly more lax than the C-ACL. Thick bars represent the mean and error bars indicate standard deviation. Asterisks (*) denote significance from C-ACL.

FIGURE 7: Tangent modulus of C-ACL and explanted BLB (Fresh/Frozen) grafts. No significant differences were found between Fresh and Frozen BLBs. Values are means ± standard deviation. Asterisks (*) denote significance from C-ACL.
Figure 1: Characterization of cross sections taken from the center of engineered ligament constructs using H&E (A-D) and Picrosirius Red (E-H) with time in vitro. At 48 hours, the center of the construct was not fused (A). By 2 weeks, the construct had reorganized into a dense 3-D structure (C). A fibrotic outer layer was observed around the outer surface at the construct from 48 hours to 2 weeks in vitro but had disappeared by 3 weeks in vitro (A-D). Picrosirius red staining revealed that collagen is present in the construct at 48 hours following roll up of the monolayer (E). The complexity and overall expression of collagen throughout the construct appeared to increase with time in vitro, with dense ribbons of collagen forming by 1 week in vitro (F). All images taken at 20x magnification with scale bars indicating 200 µm.

214x308mm (300 x 300 DPI)
Figure 2: Cellularity and apoptosis of engineered ligament constructs. Immunohistochemical staining of cross sections of engineered ligament constructs at 48 hours, 1, and 2, and 3 weeks in vitro using the apoptosis marker caspase-3 (red) and DAPI (blue). Caspase-3 was expressed in the middle region of the construct at 48 hours (A,C), throughout the construct at 1 week (B,F), and decreased in expression throughout the construct by 2 and 3 weeks (G,I & J,L). 48-hour constructs had more densely packed nuclei (B). By 2 weeks in vitro, nuclei were less densely packed (H) and the trend continued at 3 weeks in vitro (K). All images taken at 20x magnification with scale bars indicating 200 µm.

338x319mm (300 x 300 DPI)
FIGURE 3: Comparisons of length (A) and cross-sectional area (B) of C-ACL, explanted Fresh BLB, and Frozen BLB after 6 months in vivo. Values are mean ± standard deviation. There were no significant differences found in the length and cross-sectional area of the C-ACL, Fresh BLB and Frozen BLB.
FIGURE 4: Longitudinal sections of C-ACL and explanted BLB (Fresh/Frozen) after 6 months in vivo. H&E staining (A-C) was used to ascertain general structure and morphology. Immunohistochemical staining for Elastin (D-F) and Type I Collagen (G-I) showed positive staining in both Fresh (E,H) and Frozen (F,I) with organization and staining showing similarities to C-ACL (D,G). All images taken at 40x magnification with scale bars indicating 100 µm. 254x190mm (300 x 300 DPI)
FIGURE 5: Cross sections of C-ACL and explanted BLB (Fresh/Frozen) after 6 months in vivo. H&E images (A-C) at 40x showed pronounced vasculature confirmed by CD31 (D-F) antibody staining in both Fresh (B,E) and Frozen BLBs (C,F) indicating tissue regeneration.

FIGURE 5: Cross sections of C-ACL and explanted BLB (Fresh/Frozen) after 6 months in vivo. H&E images (A-C) at 40x showed pronounced vasculature confirmed by CD31 (D-F) antibody staining in both Fresh (B,E) and Frozen BLBs (C,F) indicating tissue regeneration.

254x190mm (300 x 300 DPI)
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FIGURE 7: Tangent modulus of C-ACL and explanted BLB (Fresh/Frozen) grafts. No significant differences were found between Fresh and Frozen BLBs. Values are means ± standard deviation. Asterisks (*) denote significance from C-ACL.

206x163mm (300 x 300 DPI)