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Fresh Versus Frozen Engineered Bone–Ligament–Bone Grafts for Sheep Anterior Cruciate Ligament Repair

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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 with 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 before use for ACL reconstruction. We hypothesized that frozen constructs would have similar histological and biomechanical outcomes compared with our fresh model. Our results showed that 6 months postimplantation 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 before 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 orthopedic procedures with over 100,000 patients undergoing the surgery each year in the United States.[1] Cumulative failure rates of ACL reconstruction to restore knee joint stability suggest that at least one in nine patients will experience rerupture or clinical failure in the long term.[2] Limitations associated with current graft choices are compounded by the mismatched biomechanical properties exhibited by all tendon grafts in comparison with 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.[3,4]

Our laboratory has previously developed a method of tissue engineering three-dimensional (3D) multiphasic bone–ligament–bone (BLB) constructs from bone marrow stromal cells (BMSCs). During *in vitro* tissue engineering, the BMSCs form their own extracellular matrix (ECM) eliminating the need for an exogenous scaffold. We have shown that after 6 months *in vivo,* these engineered scaffoldless BLB constructs undergo significant remodeling to

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develop similar mechanical and morphological properties to those of native ACLs and are capable of inducing vascular and neural development.[5] 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 ~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 3D formation to mature the construct *in vitro* to improve fusion between tissue interfaces and increase tensile strength before implantation. The total time required from bone marrow isolation to an implantable 3D construct is ~6–7 weeks. Recent studies suggest that the timing of ACL repair after injury is critical and that early intervention is most effective.[2,6–8] It is therefore necessary to develop technologies for graft preservation and to have the option of 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.[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 with fresh grafts in a 6-month recovery study. *In vitro* construct morphology and viability was also assessed at various time points post 3D formation to document the extensive *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.[5,11] Animals were acclimated to the Unit for Laboratory Animal Medicine (ULAM) husbandry facilities at the University of Michigan for at least 1 week before any procedure. Sheep were given access to food and water *ad libitum*. All animal care and animal surgeries were performed in accordance with The Guide for the Care and Use of Laboratory Animals,[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.[5] All solutions and media were prepared and stored at 4°C and were warmed to 37°C in a heated bead bath before use. Briefly, the growth medium (GM) consisted of 78% Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) with 20% fetal bovine serum (Gibco, Grand Island, NY), 2% antibiotic anti-mycotic (ABAM; Gibco), 6 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), 0.13 mg/mL ascorbic acid-2-phosphotase (Sigma-Aldrich, St. Louis, MO), and 0.05 mg/mL L-proline (Sigma-Aldrich); the differentiation medium (DM) consisted of 91% DMEM, 7% horse serum albumin (Gibco), 2% ABAM, 0.13 mg/mL asc-2-phos, 0.05 mg/mL L-proline, and 2 ng/mL transforming growth factor beta (Peprotech). For the culture of bone cells, 10^{-8} M dexamethasone (Sigma-Aldrich) was added to the GM and DM.[13]

Construct dishes were prepared as described previously[5] to house and constrain the formed 3D constructs. Briefly, 100-mm-diameter cell culture plates were filled with 12 mL Sylgard (type 184 silicon elastomer; Dow Chemical Corp., Midland, MI) and allowed to cure for 3 weeks at room temperature. Before use, plates were decontaminated with UV light (wavelength 253.7 nm) for 60 min and rinsed with 70% EtOH and Dulbecco's phosphate buffered saline (DPBS; Gibco, Grand Island, NY).

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) 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) at room temperature at 600 *g* for 30 min 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 min and the supernatant removed. An equivalent volume to the pellet of the ACK lysing buffer (Gibco) was added and mixed for 30 s to lyse any remaining red blood cells. The conical was then filled with DPBS and centrifuged at 400 *g* for 5 min 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/cm^2 in cell culture dishes.

Fabrication of BLBs

Using previously described methods, the BMSCs were expanded into ligament and bone lineages.[5,11,13.] 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/cm^2 and switched to the DM after 8 days of plating. After 2 days in the DM, the bone monolayers were rolled with sterile tweezers into a tube shape and transferred to Sylgard plates. After 1 to 2 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 ~60–75 mm and comprised 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 ~4–5 mm at the ligament region. After an additional 1 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 the 3D culture. Before implanting, the bone ends of the BLB were threaded with a nonabsorbable 5-0 silk suture to allow for passage into the bone tunnel and fixation onto the periosteum.

Fully formed frozen BLBs stored in the 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 h to ensure that the construct was completely frozen. Before surgical implantation, plates were retrieved from the freezer and allowed to thaw in a 37°C bead bath. After complete thawing of the construct, the 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 the culture at designated time points and fixed with paraformaldehyde for 1 h at room temperature, 15% sucrose for 2–3 h rocking at room temperature, and then 30% sucrose overnight rocking at 4°C. Following fixation, constructs were placed into a tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), frozen with dry ice, and stored until needed at −80°C. Frozen samples were sliced in either cross sections 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 thrice for 5 min each with phosphate-buffered saline (PBS) containing PBS-0.1% Triton X-100 (PBST; Sigma-Aldrich). Sections were blocked for 30 min 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). After three washes in PBST, the slides were incubated for 2 h with the secondary antibody 1:1000 Alexa555 anti-rabbit (Invitrogen, Carlsbad, CA), rinsed thrice in PBST, and mounted with Prolong Gold Antifade reagent with DAPI (Molecular Probes, Eugene, OR). 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.[5] 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 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 sutures 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 sutures to the periosteum. Incisions were closed with staples and the entire surgical site was sprayed with Alushield (Neogen Corp., Lansing, MI). 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 6-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 a 60°C oven for 1 h 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 thrice in xylene for 3 min, twice in 100% ethanol for 2 min, twice in 95% ethanol for 2 min, and once in 70% ethanol for 2 min. Following deparaffinization, slides were washed in PBST for 15 min at room temperature. Using a hydrophobic pen, sections were circled for blocking and antibody staining. Sections were covered in a blocking solution containing PBST with 3% bovine serum albumin (Sigma-Aldrich) for 30 min at room temperature. Primary antibodies were diluted in the 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 (#AB2039; Millipore, Billerica, MA); and 1:100 of rabbit anti-collagen type I (#AB292; Abcam). Slides were then washed for 5 min in PBST at room temperature thrice for a total of 15 min. Secondary antibodies were diluted at 1:500 in the 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 h. The slides were then washed for 15 min in PBS at room temperature thrice for a total of 45 min. Nuclei were stained using Prolong Gold with DAPI (Sigma-Aldrich) 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.[14] 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 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/s for a loading time of 7.5 s were then conducted on the BLB and ACL specimens to obtain the stiffness using previously developed testing protocols.[5] 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 analysis of variance (ANOVA) with Tukey's *post-hoc* test. A p-value <0.05 for all statistical tests was considered significant. All data are 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 *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 h, 1, 2, and 3 weeks following roll up of the monolayer (Fig. 1A–D). At 48 h, following roll up of the monolayer, the center of the construct was not immediately fused and the nuclei were densely packed (Fig. 1A). At the 1-week time point, the construct was fully fused and the nuclei were densely packed (Fig. 1B). By 2 weeks in culture, the construct had reorganized into a dense 3D structure, and the nuclei were less densely packed together (Fig. 1C). At 3 weeks, extensive remodeling had occurred and the nuclei remained less densely packed together (Fig. 1D). A fibrotic outer layer similar to that observed in native ligament was observed around the outer surface at the construct from 48 h to 2 weeks *in vitro*, but had disappeared by 3 weeks *in vitro* (Fig. 1A–D).

Picrosirius red staining was performed to assess the presence of collagen. Figure 1E–H shows stained cross sections at the center of the ligament constructs at 48 h, 1, 2, and 3 weeks following roll up of the monolayer with collagen indicated in red. Collagen was present in the construct at 48 h following roll up of the monolayer; however, the layers of the construct remained distinct, indicating that they had not yet fused (Fig. 1E). By 1 week, extensive remodeling had occurred, and the dense bands of collagen began to interweave through the cross sections of the construct (Fig. 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 (Fig. 1G), which increased in complexity by 3 weeks in culture (Fig. 1H). Overall, construct remodeling was extensive with the complexity and surface area of dense collagen bands increasing with time *in vitro* from 48 h to 3 weeks in culture.

To assess cell death or apoptosis, immunohistochemical staining was performed with an antibody specific to caspase-3 at 48 h, 1, 2, and 3 weeks following roll up of the monolayer for qualitative analysis (Fig. 2). The cross sections through the center of the ligament constructs at 48 h in culture were positive for caspase-3 in the central region of the construct (Fig. 2A, C). DAPI staining of the ligament constructs showed that at 48 h *in vitro* the nuclei were densely packed together (Fig. 2B). At 1 week, caspase-3 was expressed not only in the central region but also along the outer regions of the construct (Fig. 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 (Fig. 2G, I), and the nuclei were less densely packed together (Fig. 2H). At 3 weeks *in vitro*, caspase-3 expression was still observed (Fig. 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 (Fig. 3A). The average cross-sectional areas (CSAs) of frozen BLBs were 33.3 ± 9.5 mm^2 and fresh BLB CSAs were 29.7 ± 10.5 mm^2 (Fig. 3B). The average length and CSA of the C-ACLs were 21.4 ± 3.1 mm and 39.1 ± 9.2 $mm,^2$ 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

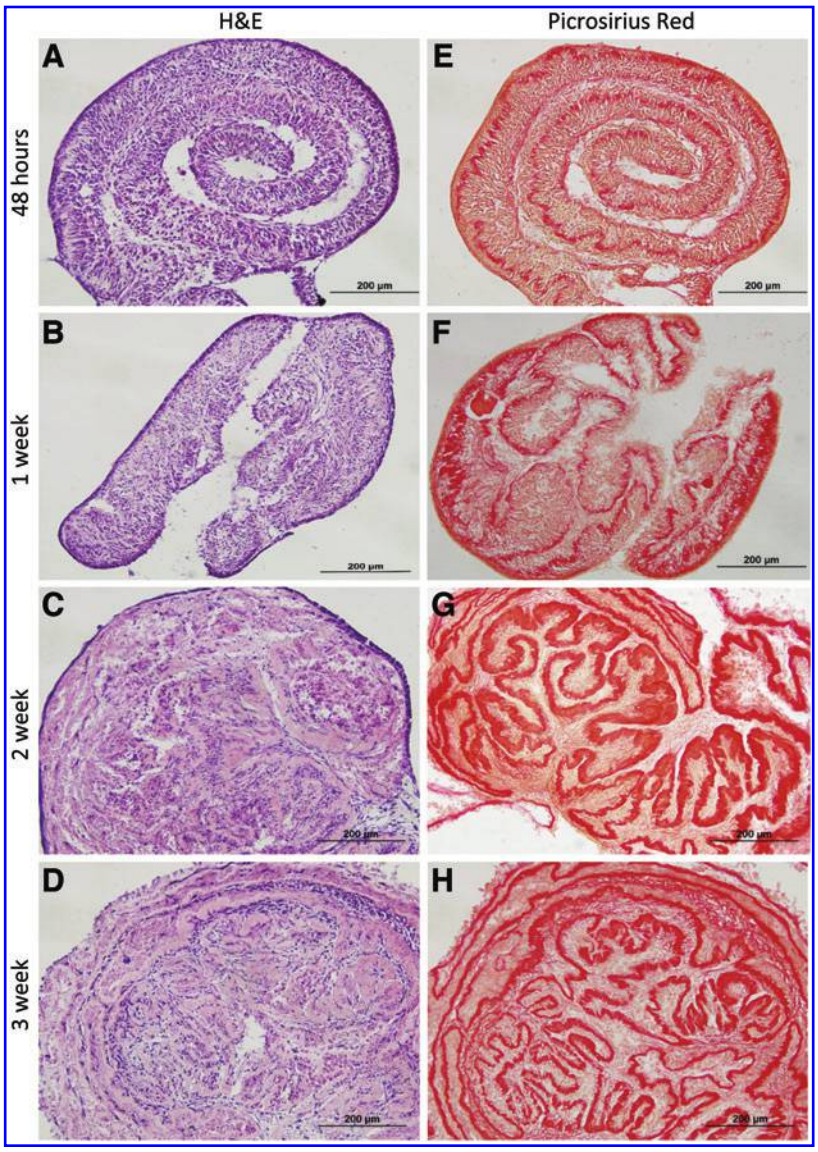

FIG. 1. Characterization of cross sections taken from the center of engineered ligament constructs using H&E **(A–D)** and Picrosirius Red **(E–H)** with time *in vitro.* At 48 h, the center of the construct was not fused **(A)**. By 2 weeks, the construct had reorganized into a dense three-dimensional structure **(C)**. A fibrotic outer layer was observed around the outer surface at the construct from 48 h 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 h 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 were taken at 20× magnification with scale bars indicating 200 μm. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tec

the formation of a ligamentous crimp-like pattern with fibers arranged along the long axis of the tissue (Fig. 4) as well as the formation of vasculature and innervation seen in the cross section (Fig. 5). Immunohistochemical staining showed similar collagen and elastin deposition compared with 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 ($n = 15$) (Fig. 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 with 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 (Fig. 7). The tangent modulus data indicated no significant differences between the two graft types ($p > 0.05$). Both grafts achieved ~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.[5] 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 postimplantation as an ACL

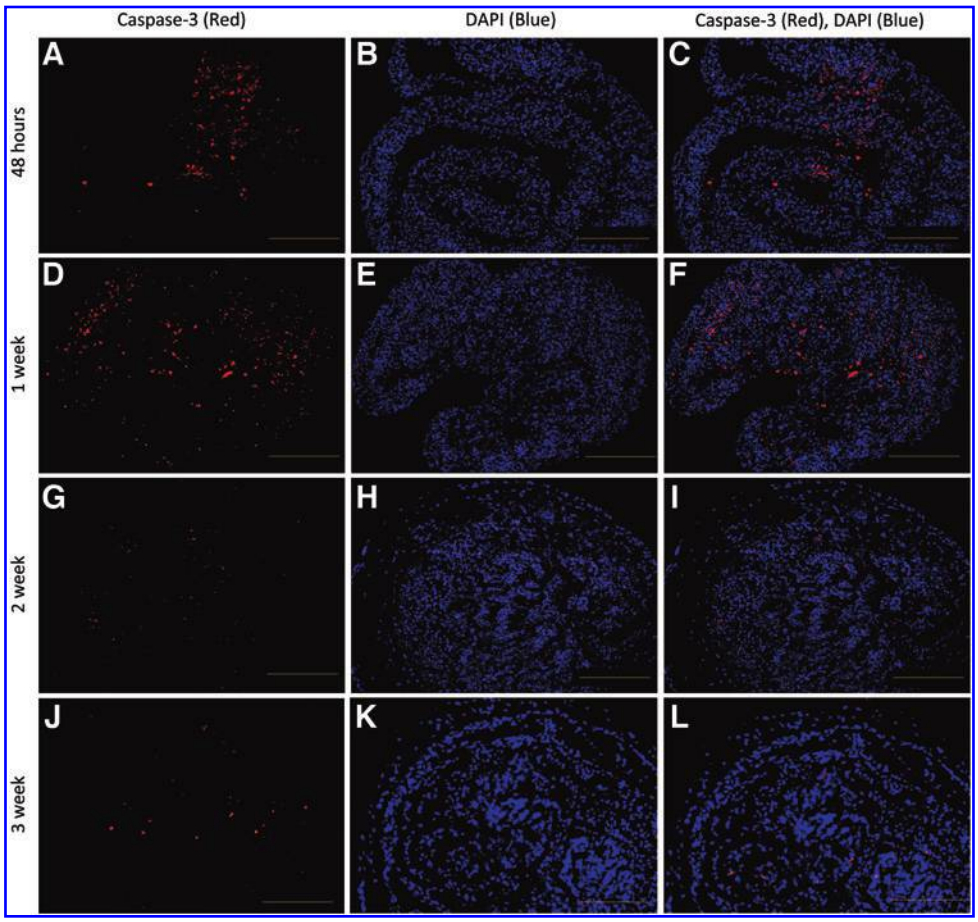

FIG. 2. Cellularity and apoptosis of engineered ligament constructs. Immunohistochemical staining of cross sections of engineered ligament constructs at 48 h, 1, 2, and 3 weeks *in vitro* using the apoptosis marker caspase-3 (*red*) and DAPI (*blue*). Caspase-3 was expressed in the middle region of the construct at 48 h **(A, C)**, throughout the construct at 1 week **(D, F)**, and decreased in expression throughout the and construct by 2 and 3 weeks **(G, I, J, L)**. Forty-eight-hour and 1 week constructs had densely packed nuclei **(B, E)**. By 2 weeks *in vitro*, nuclei were less densely packed **(H)** and the trend continued at 3 weeks *in vitro* **(K)**. All images were taken at 20× magnification with scale bars indicating 200 μm. Color images available online at www.liebertpub.com/tec

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 before 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 ~4–5 weeks for induction of BMSCs to ligament and bone lineages, formation of robust monolayers, and coculture of the bone and ligament tissues into a 3D configuration. An additional 2 weeks is needed *in vitro* for the BLB to condense and fuse into a uniform and robust 3D construct. We have demonstrated that our BLB continues to remodel significantly in the 2 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

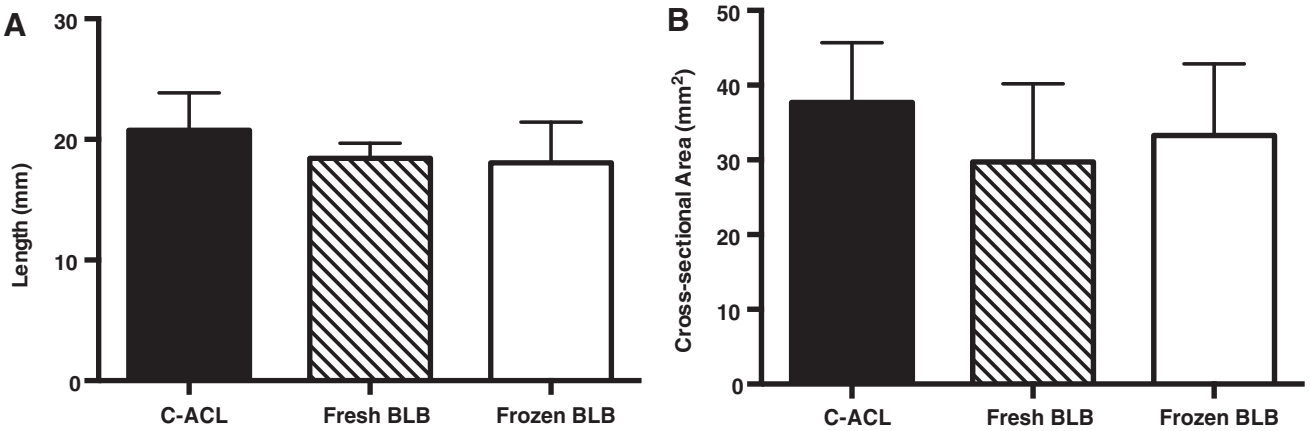

FIG. 3. Comparisons of length **(A)** and cross-sectional area **(B)** of C-ACL, explanted fresh BLB, and frozen BLB after 6 months *in vivo*. Values are mean ± standard deviation. There were no significant differences found in the length and cross-sectional area of the C-ACL, fresh BLB, and frozen BLB. BLB, bone–ligament–bone; C-ACL, contralateral anterior cruciate ligament.

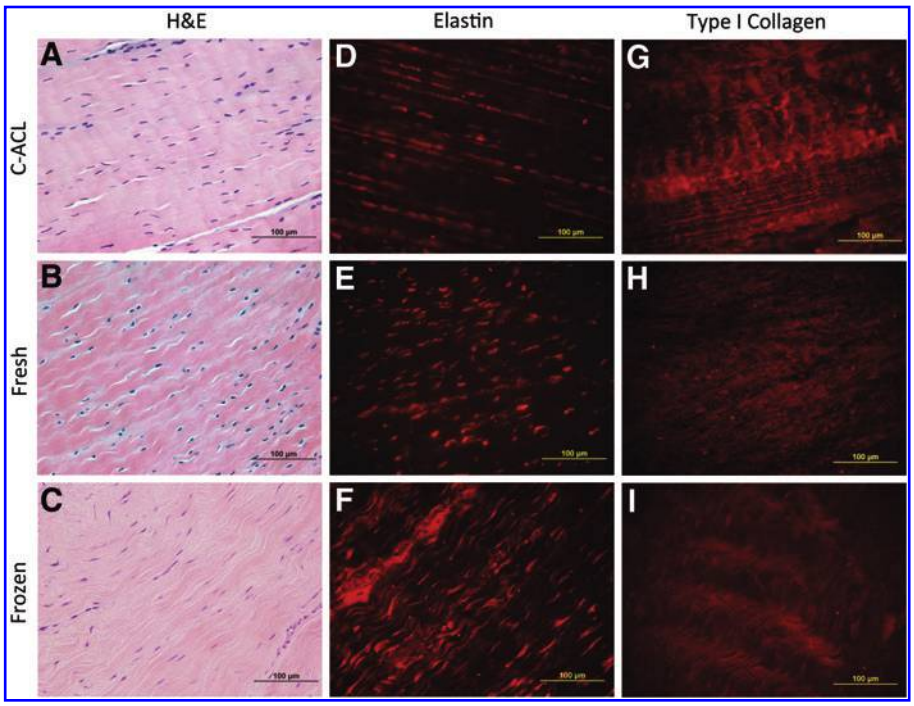

FIG. 4. Longitudinal sections of C-ACL and explanted BLB (fresh/frozen) after 6 months *in vivo*. H&E staining **(A–C)** was used to ascertain general structure and morphology. Immunohistochemical staining for Elastin **(D–F)** and type I collagen **(G–I)** showed positive staining in both fresh **(E, H)** and frozen **(F, I)** with organization and staining showing similarities to C-ACL **(D, G)**. All images were taken at 40× magnification with scale bars indicating 100 μm. Color images available online at www.liebertpub.com/tec

increase collagen deposition and advance ECM formation at these time points compared with earlier in formation. Apoptotic cells were observed at both the 2-week and 3-week time points suggesting that the BLB continues to remodel, possibly removing cells to accommodate the increased ECM secretion.

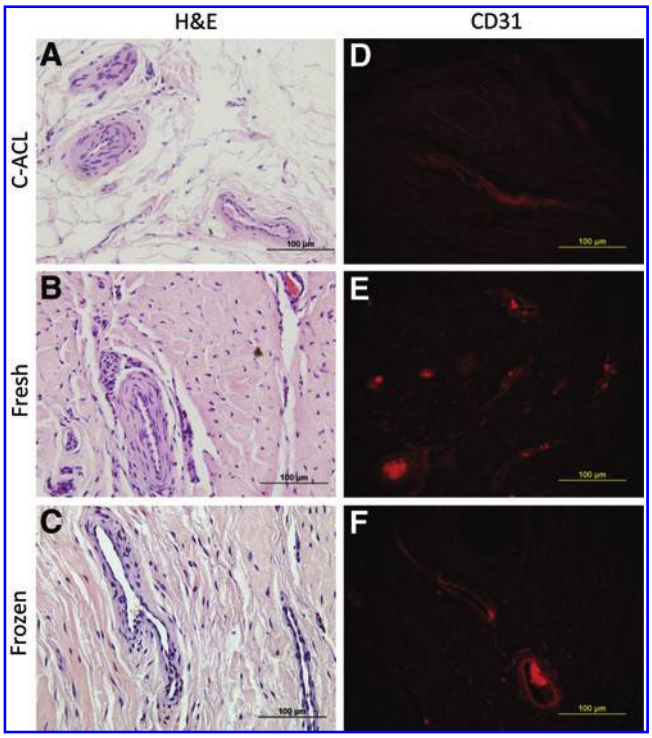

FIG. 5. Cross sections of C-ACL and explanted BLB (fresh/frozen) after 6 months *in vivo*. H&E images **(A–C)** at 40×showed pronounced vasculature confirmed by CD31 **(D–F)** antibody staining in both fresh **(B, E)** and frozen BLBs **(C, F)**, indicating tissue regeneration. Color images available online at www.liebertpub.com/tec

Thus, it was decided that the optimal time for freezing would be at least 2 weeks after formation 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 with each other, frozen and fresh BLB grafts showed similar knee laxity and had similar mechanical moduli. However, compared with the C-ACL, both frozen and fresh BLB knees had increased laxity and the

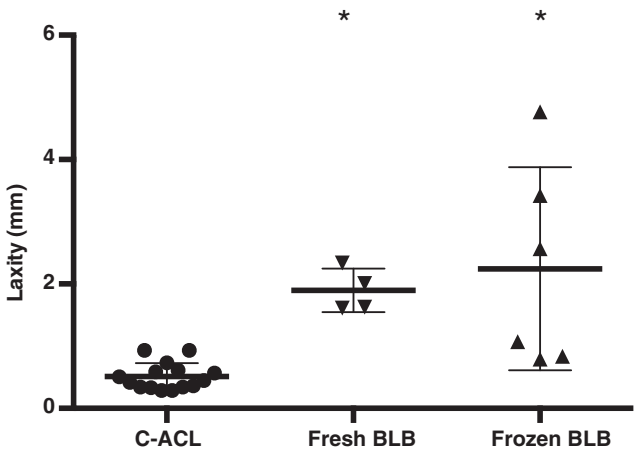

FIG. 6. Knee laxity of C-ACL and explanted BLB (fresh/frozen) after 6 months *in vivo*. No significant differences were seen between the fresh and frozen BLB knee laxities; however, they were both significantly more lax than the C-ACL. Thick bars represent the mean, and error bars indicate standard deviation.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

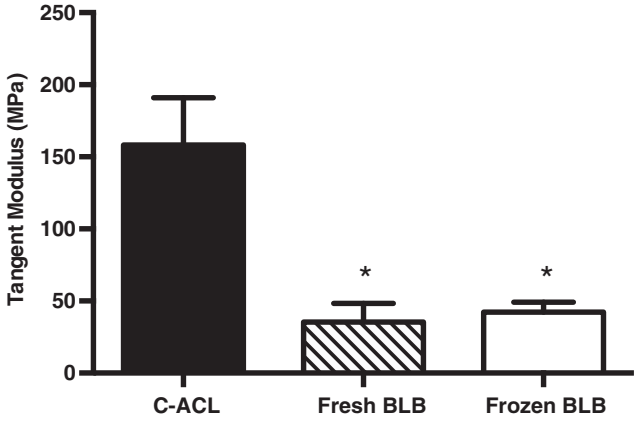

FIG. 7. Tangent modulus of C-ACL and explanted BLB (fresh/frozen) grafts. No significant differences were found between fresh and frozen BLBs. Values are mean ± standard deviation. *Asterisks* (*) denote significance from C-ACL.

dissected grafts achieved ~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,[5] 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.[10,15] These concerns were shown[10] to not be as significant in the 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.[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 with acellularized grafts *in vitro*.[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 toward 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 3D 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 of 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 times of clinical need, thereby progressing the transition of the current technology toward large-scale manufacturing and clinical translation.

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Disclosure Statement

No competing financial interests exist.

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