Human Amniotic Membrane Improves Healing In A Chronic, Massive Rotator Cuff Repair Model

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Human Amniotic Membrane Improves Healing in a Chronic, Massive Rotator Cuff Repair Model

A Thesis Submitted
to the Yale University School of Medicine
in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By
Maarouf A. Saad
2020
HUMAN AMNIOTIC MEMBRANE IMPROVES HEALING IN A CHRONIC, MASSIVE ROTATOR CUFF REPAIR MODEL.

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Rotator cuff tendon heals by fibrovascular scar that is weaker than native tissue leading to repairs that are prone to failure. To investigate the utility of an amniotic-membrane-derived human allograft in improving tendon-bone healing and skeletal muscle architecture, a rotator cuff repair model in Sprague-Dawley rats was established, tendon biomechanical testing, single muscle fiber biomechanical testing, microcomputed tomography analysis, tendon and muscle histological analysis, and gene expression analysis were performed. Our findings demonstrated single muscle-fibers with larger cross-sectional area and greater maximum isometric and specific force in the augmented experimental repair (ER) group. Additionally, microcomputed tomography revealed a larger volume of newly formed bone and histomorphometric analysis demonstrated significantly greater fibrocartilage area and collagen organization at the healing tendon-bone insertion site in the ER group compared to the control repair (CR) group. Muscle gene expression analysis revealed increased expression of COL1A1 and MMP-2 and
decreased expression of $FITM-2$ in the ER group compared to CR. These findings revealed that augmentation with human amniotic membrane was associated with positive effects on the entire muscle-tendon-bone unit. Based on these findings, we believe that further investigation is warranted to optimize local and spatial delivery of amniotic tissue-based products.
Acknowledgements

First, and most of all, I would like to thank Dr. David Kovacevic. This work, and resulting thesis, would not have been possible without your expertise, dedication, guidance, and support. I could not have asked for better mentor as I navigated my introduction to orthopaedic surgery and sincerely thank you for making me a better medical student, scientist, future surgeon, and person.

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Jeff Kepple for his assistance with the completion of single muscle fiber biomechanical testing.
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Introduction

The rotator cuff is made up of four muscles (subscapularis, supraspinatus, infraspinatus, teres minor) that are critical for maintaining glenohumeral joint stability and mobility. Rotator cuff tears (RCTs) are a very common cause of disability and discomfort in patients worldwide, with studies demonstrating a prevalence of up to 40% in middle-aged populations. RCTs have also been shown to be responsible for 67% of cases of shoulder pain and dysfunction in patients aged 40-65 years old. Massive rotator cuff tears are defined as tears with a diameter ≥ 5 millimeters or detachment of two or more tendons, with studies showing that massive tears make up to 10-40% of all RCTs. In addition to the significant disability caused by RCTs, they also represent an economic impact upwards of three billion dollars per year in the United States alone.

Management of RCTs begins with conservative measures focused on decreasing pain and improving function. Anti-inflammatory medications and physical therapy are usually employed as first-line measures; however, surgical repair remains the gold-standard for definitive management of RCTs. Though major advances have been made in surgical techniques, re-tear rates remain high, with some studies revealing rates of up to 94% for massive tears. Rotator cuff repair (RCR) failure can be defined as either clinical or mechanical failure. Clinical failure occurs when patients continue to report pain or functional impairment following repair, while mechanical failure occurs secondary to a loss of fixation, failure to heal, or a repeat tear of the repaired tendon. The high incidence of re-tear is likely due to incomplete healing of the tendon-bone insertion site secondary to the formation of a fibrovascular scar rather than recreation of the native enthesis. Additionally, poor tissue quality as well as advanced fatty
infiltration, both common attributes of chronic RCTs, are largely to blame for these high failure rates. Incomplete structural healing of the repair construct leads to deficits in rotator cuff strength and function, reducing the quality of life of afflicted individuals and limiting their ability to complete activities of daily living.

It has become clear that current surgical techniques, which have traditionally relied on maximizing footprint restoration and construct stability, fail to provide a biological environment necessary to recapitulate the processes that occur during embryonic development to promote “scarless” healing of the enthesis. Rather, an adult biologic milieu predominates, leading to inflammation and scar tissue formation, inadequate tendon-bone healing, decreased postoperative muscle strength, and poor shoulder mobility. In fact, Mirzayan et al. argue that the term “re-tear” inadequately describes the complexity of post-repair failure as this implies that adequate healing of the tendon to the tuberosity occurred when in reality, the tendon may have never actually healed. Thus, there is a critical need to develop and utilize innovative treatments which augment rotator cuff repair by recreating the fetal environment to reduce inflammation and tissue degeneration and promote “scarless” healing of the enthesis, with the ultimate goal of improving surgical outcomes.

Efforts to reduce the rates of failure following RCR have primarily focused on improving fixation constructs. The introduction of double-row and transosseous-equivalent repairs have resulted in increased mechanical strength, but these improvements fail to address the critical biological component of tendon healing. To create a more favorable local environment for healing, the use of biologics as adjunctive treatments following RCR was introduced. To date, multiple biologic therapies have been
studied including platelet-rich plasma (PRP), patches and scaffolds, mesenchymal stem cells (MSCs), and cytokines. Several studies have outlined the potential benefits of these therapies however, here we focused on the role of fetal tissue, specifically amniotic membrane, to determine its ability to aid in proper rotator cuff tendon healing.

Utilization of fetal growth factors for biological augmentation holds great promise for improving rotator cuff healing. Fetal tissues have been shown to have anti-inflammatory properties and to promote scarless healing, leading to its use for several clinical applications in ophthalmology, skin ulcers, burns, and surgical reconstructive procedures. Within orthopaedics, fetal tissue has been used as a tendon wrap to reduce inflammation and the formation of adhesions. In addition, previous work has shown that delivery of transforming growth factor β3 (TGF-β3), which has been shown to promote “scarless” healing in the fetal environment, at the repair site in a rat model of acute rotator cuff injury and repair led to greater ultimate stress when compared to surgical repair alone. Modulating the ratio of TGF-β1, which is highly expressed in adult healing, to TGF-β3 may be important with greater TGF-β3 expression promoting a fetal wound healing environment to promote tissue formation with a more favorable collagen I to collagen III ratio. The recreation of a native tendon insertion site may be best achieved with the delivery of undifferentiated stromal cells and growth factors in a coordinated fashion. For example, previous work with amniotic tissue based products has shown that local delivery of human amniotic fluid enhanced new cartilage formation from rabbit ear perichondrial flaps subjected to a cartilage defect and improved fracture healing in a rat tibia fracture model. Additionally, delivery of amniotic epithelial cells in a sheep achilles tendon defect model was shown to promote mature collagen deposition and
increased extracellular matrix remodeling. Also, the commercially available human amniotic membrane graft used in this study (XWrap, Applied Biologics), which has a relatively low cost, is easy to handle, and has acceptable suture-holding properties is currently used to prevent perineural scarring in patients with cubital tunnel syndrome. Thus, the application of a minimally processed allograft containing amniotic stromal cells and growth factors may be beneficial for tendon-bone healing following RCR.

The use of an amniotic membrane derived graft may hold an advantage over existing approaches, such as stromal cell therapy alone or precursor gene delivery of proteins implicated in tendon repair, because of the greater potential to recreate the fetal environment at the repair site and promote native regeneration. The majority of studies have focused on the use of a single compound, e.g. IGF-1, TGF-β1, or TGF-β3, but it is unlikely that single factor therapy can reliably recreate the fetal environment compared to a concentrate with amniotic derived stromal cells and growth factors. Additionally, a key innovation of the amniotic-derived graft is the standardization of processing. One challenge identified with other orthobiologics, such as the previously mentioned PRP, is the product content variability within and between patients. The biologic investigated in this study has been processed in a standardized manner providing for more product consistency. Furthermore, the amniotic-derived graft proposed in this study is FDA-approved for clinical use allowing for earlier translation to clinical trials to study safety and efficacy.
Statement of Purpose

Our objective was to investigate the ability of an amniotic membrane-derived human allograft to improve rotator cuff tendon-bone healing and skeletal muscle architecture in a chronic massive rotator cuff injury and repair model in Sprague-Dawley rats. We hypothesized that application of human amniotic membrane to the tendon-bone interface at the time of repair will result in (1) increased tendon-bone attachment strength and (2) increased rotator cuff muscle fiber force secondary to improved bone formation, increased fibrocartilage formation, improved collagen organization, less plasminogen activator inhibitor-1 (PAI-1) expression, less lipid deposition, and induced muscle repair gene expression compared to control repair.

Methods

Animal Model and Study Design

The Institutional Animal Care and Use Committee approved the protocols for animal experimentation described in this study. Eighty-five male Sprague-Dawley rats weighing approximately 350 grams were used. Twenty-five rats were allocated as uninjured controls (U). The remaining 60 animals underwent unilateral full-thickness rotator cuff injury of the supraspinatus (SSp) and infraspinatus (ISp) tendons and concomitant injection with 3 units/kg per muscle of Onabotulinumtoxin A (Botox; Allergan, Dublin, Ireland) into the SSp and ISp muscles to induce muscle atrophy. Thirty days after injury, 10 animals were euthanized as injury-only (IO) controls and the remaining 50 animals underwent rotator cuff repair utilizing a transosseous suture repair technique. Twenty-five rats received a 5-mm diameter amniotic membrane patch at the
site of tendon-bone repair (ER; experimental repair), while the remaining 25 animals received no treatment to serve as control repairs (CR), which underwent repair but did not have any biological augmentation. All repaired animals were allowed normal cage activity and euthanized 30 days following rotator cuff repair.

Immediately following necropsy, animals were weighed and the SSρ- and ISρ-muscle-tendon-bone units were retrieved for analysis. Each tendon was transected from the muscle 1 cm medial to its tendon-bone insertion site. For animals in the U, CR, and ER groups, the humerus and attached tendons underwent either biomechanical testing \( (n=15 \text{ per group}) \) followed by micro-computed tomography analysis \( (n=5 \text{ per group}) \), or tendon histological analysis \( (n=5 \text{ per group}) \). Each muscle was weighed then transected into three one-cm sections. The lateral third underwent muscle histological analysis \( (n=8 \text{ per group}) \), the middle third underwent single muscle fiber biomechanical testing \( (n=8 \text{ per group}) \), and the medial third underwent gene expression analysis \( (n=8 \text{ per group}) \). Injury only (IO) animals underwent all of the aforementioned analysis methods with the exception of tendon-bone biomechanical testing, tendon histological analysis, and muscle weighing. To maintain consistency and single-surgeon validity, Dr. David Kovacevic performed all procedures on living rats while post-mortem dissection and preparations were performed by Maarouf Saad (MS), Robert Suriani Jr (RSJ), Jacob Berman (JB) and Dr. David Kovacevic (DK).

**Surgical Technique**

*Rotator Cuff Injury* – Each animal was anesthetized with ketamine and xylazine and placed in the supine position with 90° elbow flexion, forearm supination, and slight shoulder external rotation while creating a 1.5 cm longitudinal anterior deltidoid splitting
incision. The deltoïd was retracted to allow optimal visualization of the underlying SSp and ISp tendons. A mosquito clamp was used to individually isolate each tendon, followed by sharp transection of the tendon from the superior and middle facets of the greater tuberosity bony footprint using an 11 blade. A silicone sleeve was attached using 4-0 nylon suture over the SSp and ISp tendons to reduce scarring and adhesion of the tendons. An injection of 0.3 ml of 10 Units/ml Botox (3 Units per muscle, approximately 9 Units/kg/muscle) was injected into each muscle to chemically denervate the muscle-tendon units. The deltoïd and skin were then closed using 4-0 Ethibond and 4-0 nylon sutures. Subcutaneous buprenorphine and bupivacaine were administered for analgesia during the 24-hour postoperative recovery period.

*Rotator Cuff Repair* – Thirty days following injury, animals in the CR and ER groups underwent surgical repair. The same surgical incision and approach described above were utilized to perform an anatomic rotator cuff repair utilizing a transosseous cruciate tunnel technique. Careful dissection with small dissecting scissors exposed the silicone tubes, which were removed to expose the distal muscle-tendon units. Using a 4-0 Ethibond suture, a modified Mason-Allen stitch was placed in both the SSp and ISp tendons to create anterior and posterior suture limbs for each tendon. The bony footprint of the greater tuberosity was then decorticated and three cruciate tunnels for both tendons were created with a 22-gauge needle. Using a 4-0 Nylon suture, the 4-0 Ethibond suture limbs were shuttled through the proximal tunnel aperture and out through the distal tunnel exit. For animals allocated to the ER group only, a sterile 5 mm punch biopsy was used to create a circular human amniotic membrane patch (XWrap Human Amniotic Membrane; Applied Biologics, Phoenix, AZ). The biologic graft was then applied
directly to the greater tuberosity footprint prior to fixation of the tendon to the bone. The tendons were then tensioned to ensure maximal bone footprint coverage and then tied over the proximal humeral cortical bone bridge. Deltoid closure, wound closure, and postoperative recovery occurred as described in the above section and were identical between groups. Rotator cuff injury and repair procedures were performed by DK to maintain single-surgeon consistency within and between each group under investigation.

**Tendon Biomechanical Testing**

Cross sectional area of the SSp and ISp tendons were measured at the greater tuberosity bony footprint using microCT. Humeri were potted in fast-curing epoxy to facilitate gripping of the bone. Repair-site sutures were cut and carefully removed to ensure no contribution of the suture during tension-to-failure tests. Specimens were kept moist with phosphate buffered saline throughout testing. Tests were performed on a single column, tabletop testing system (Instron 5542, Norwood, MA) with a 500 N load cell and custom fixtures. The top grip was lined with sandpaper and coated with cyanoacrylate to prevent slipping of the tendon. The bottom grip allowed for adjustment of the angle of the humerus such that the tendon was pulled in line with the angle of insertion. Tests were performed for tensile loading of the ISp and SSp first with stress relaxation (100%/s ramp followed by 5% strain hold for 300 s). Specimens were then extended to failure at a rate of 0.2%/s. Strain was measured globally as grip-to-grip displacement relative to the initial gauge length for each tendon measured with digital calipers (Starrett, Athol, MA). Stiffness and Young’s modulus were calculated by measuring the slope of the linear region of force-length and stress-strain curves,
respectively, for both ISp and SSp tendons. Failure mechanisms were assessed, and maximum load, maximum stress, and toughness were calculated. Tendon biomechanical testing was performed by Dr. Steven Tomassini and data interpretation was performed by MS, RSJ, Dr. Steven Tomassini (ST), and DK.

**Muscle Single Fiber Biomechanical Testing**

Bundles of muscle fibers approximately 5 mm in length and 0.5 mm in diameter were dissected from the middle third of each SSp muscle prior to preparation. The bundles were initially placed in a cold skinning solution with 0.5% Brij 58 for 30 minutes before being placed into a storage solution for overnight shipment from Yale University to the University of Michigan at 4°C. Upon arriving at the University of Michigan, the SSp bundles were stored at -80°C until use.

On the day of testing, bundles were thawed on ice and individual fibers were pulled from bundles using fine mirror-finished forceps. Fibers were then quickly placed in a custom-designed chamber containing relaxing solution and secured at one end to a servomotor (Model 322C, Aurora Scientific, Aurora, ON) and the other end to a force transducer (Model 403A, Aurora Scientific) using two ties of 10-0 monofilament nylon suture at each end of the fiber. Using a laser diffraction measurement system, the length of each fiber was adjusted to obtain a sarcomere length of 2.5 µm and fiber length (L_f) was then measured. The average cross-sectional area (CSA) was calculated assuming an elliptical fiber cross-section, with diameters obtained at five different equidistant positions along the fiber from high-magnification images of the top and the side views.

Relaxed fibers were then prepared for force measurement by initially immersing them in a low [Ca^{2+}] pre-activating solution for 3 minutes. The purpose of submerging
fibers in the pre-activating solution, which was weakly buffered for Ca$^{2+}$, was to allow for rapid activation of the fibers and subsequent force development once they were placed in the activating solution. Maximum isometric force ($F_0$) was then elicited by immersing the fiber in a high-[Ca$^{2+}$] activating solution. $F_0$ was determined by measuring the difference between the force produced when the fiber was stretched to $L_f$ and that obtained at the point of maximal contraction. Following activation and fiber contraction, specific force ($sF_0$) was calculated by dividing $F_0$ by the pre-measured CSA. Forty-eight fast fibers were tested from each group (6 individual muscle fibers per specimen; n=8 specimens per group). Testing and analysis were performed in a blinded fashion. All single muscle fiber experiments were completed by MS with assistance from Jeff Kepple (JK) under the guidance of Dr. Chris Mendias (CM) and Dr. Dennis Claflin (DC).

**Microcomputed Tomography Analysis**

Five animals from each group were allocated to undergo microcomputed tomography analysis. Following biomechanical testing, the humerus samples were fixed in a 1:1 solution of ethanol and sterile water and microcomputed tomography analysis of the most proximal 1 cm of the humerus was performed using a CT 35 scanner (Scanco Medical, Wangen-Bruttisellen, Switzerland) at 10 mm isotropic resolution. Using the obtained 3-dimensional renderings, the surface area of the SSp and ISp bone insertions on the greater tuberosity were measured as well as the bone volume to total volume ratio, trabecular spacing, trabecular thickness, and trabecular number. MicroCT preparation and procedures were performed by RSJ. and DK while data analysis was performed by MS, RSJ, and DK.

**Tendon Histological Analysis**
Humerus samples with attached SSp and ISp tendons (U, ER, CR, n=5 per group) were fixed in 10% neutral buffered formalin, then decalcified using Deltaform decalcifier (Delta Medical, Aurora, IL) on a shaker in the cold room for 7 days, changing the decalcification solution every other day. Once decalcified tissue was processed via routine paraffin methods. Coronal sections were cut at 5 μm and stained with Hematoxylin & Eosin, Safranin O, and Picrosirius Red. New fibrocartilage formation at the enthesis was quantified by imaging Safranin O-stained sections at 10x magnification and measuring total metachromasia area using ImageJ software (National Institutes of Health, Bethesda, MD).

Picrosirius red-stained sections were imaged under polarized light microscopy at 10x magnification using an Olympus BX40 microscope (Olympus, Tokyo, Japan) with a custom polarizing setup. The rotary polarizing filter was oriented perpendicular to the polarizing analyzer to create a dark field under which collagen birefringence was visible. The tendon enthesis of each sample was oriented within the field and rotated through 360°. The orientation of maximum birefringence at the enthesis was noted and imaged. Using ImageJ software, the images were converted to 8-bit grayscale and calibrated to a linear brightness curve on a scale of 0 (black) to 255 (white). Ten 50x50μm representative boxes were randomly selected from each image, the mean brightness of each square area was measured, and the 10 measurements were averaged for each sample.

Immunohistochemistry was performed to localize Plasminogen Activator Inhibitor 1 (PAI-1) expression in tendon sections. Prior to staining, slides were deparaffinized and rehydrated through a graded ethanol series to distilled water. Antigen retrieval was done in citrate buffer (0.3%) heated to 100°C for 20 minutes. Following
antigen retrieval, slides were washed in PBS, then quenched in a mixture of 90 ml methanol and 10 ml of 30% hydrogen peroxide to remove endogenous peroxidase. Following quenching, slides were washed three times in tris buffered saline containing .05% Triton X (TBST). Blocking was done using Biocare Rodent M (Biocare Medical, Pacheco, CA) for 20 minutes. After washing 3 times in TBST, primary antibody was applied to slides (PAI-1 rabbit polyclonal, cat # Ab217288, Abcam) and incubated overnight in a moisture chamber at 4°C. The following day, slides were rinsed 3 times in TBST and secondary antibody, Biocare rabbit on rodent HRP polymer (Biocare Medical, Pacheco, CA) for 30 minutes at room temperature. Slides were again rinsed 3 times with TBST and chromogenic staining was done using the Betazoid DAB kit (Biocare Medical, Pacheco, CA). DAB staining was developed on ice for 1-2 minutes, then slides were washed with distilled water. Counterstaining was done using hematoxylin and slides were dehydrated through graded ethanol baths, cleared in xylenes and cover-slipped. Images were graded on a semi quantitative scale from 0 (undetectable) to 3 (high) in a blinded fashion. Tendon histological analysis was performed by MS, RSJ, and DK.

**Skeletal Muscle Histological Analysis**

Muscle samples were mounted on 1” cork discs using a pyramid of 10% gum tragacanth, snap-frozen in liquid nitrogen-cooled isopentane maintained at -160°C and immediately transferred to a -24°C cryostat. Due to the need for rapid freezing to maintain muscle fiber microarchitecture, the samples were cut without additional mounting. Sections were cut at 10 mm, which was the minimum thickness necessary to maintain section integrity. Frozen sections were carefully transferred to charged slides
using a fine paintbrush, and then different sections from each sample were stained for Hematoxylin & Eosin or an immunological stain multiplex consisting of DAPI (Sigma Aldrich, St. Louis, MO), WGA Lectin (Thermo Fisher, Hampton, NH), and BODIPY (Thermo Fisher). For immunostained slides, sections were fixed with 4% paraformaldehyde and permeabilized with 0.2% TBST, then stained in succession in a dark hydration chamber with BODIPY (3 hours), WGA-Lectin Texas Red (15 minutes), and DAPI (10 minutes). Sections were washed with phosphate-buffered saline three times following each step.

Fluorescent slides were visualized and imaged with a Leica SP5 Fluorescent Microscope (Leica Microsystems, Wetzlar, Germany). Muscle cross-sectional area (CSA) of each sample was measured using ImageJ software. Quantification of fat deposition within muscle sections was performed by first taking five representative images [four quadrants (upper, lower, left, right) and center] of each section at 10x magnification. Images of the BODIPY-only channel were converted to 8-bit grayscale using ImageJ and a brightness threshold was set to measure the area of staining against the dark field background. The BODIPY staining area measurements obtained from the five images of each sample were averaged. Muscle histological analysis was performed by MS, RSJ, and DK.

**Human Amniotic Membrane-Derived Graft Histological Analysis**

A sample of the human amniotic-membrane allograph was processed via routine paraffin methods and longitudinal sections were cut and stained with Hematoxylin & Eosin, DAPI, Masson’s trichrome, and Toluidine blue. Immunostained slides were
prepared and analyzed as previously described. Graft histological analysis was performed by MS, RSJ, and DK.

**Gene Expression Analysis**

Total RNA for gene expression analysis was isolated from the supraspinatus muscle. Frozen tissue samples (~20mg) were disrupted (BR12, Omni, Kennesaw, GA) in 1ml of Tri Reagent and prepared according to the manufacturer (Molecular Research Center, Inc, Cincinnati, OH). RNA concentration and quality were assessed using a NanoDrop 2000 (Thermo Scientific, Waltham, MA). Quality of RNA was assessed using the 260/280 and 260/230 ratios. A DNA removal kit was used to treat 5µg of RNA (DNA-free, Invitrogen, Waltham, MA). Afterwards, 1µg of RNA was reverse transcribed into cDNA (iScript, BioRad, Hercules, CA). Relative mRNA quantification was completed in duplicates on a CFX Connect real time detection system (BioRad) using self-designed or pre-validated (Prime PCR, BioRad) SYBR Green assays. Values were normalized to beta-actin (ACTB) and fold change was calculated from the uninjured control [SHP1] samples using the \(2^{-\Delta\Delta C_T}\) method.\(^{15}\) Validation of the housekeeping gene (ACTB) was performed to ensure that its expression was not affected by experimental treatments. Muscle gene expression PCRs were performed by Dr. Chad Carroll (CC) and results were analyzed by MS, RSJ, CC, and DK.

**Statistical Analysis**

Statistical analysis was performed for all analysis methods using a nonparametric Wilcoxon Rank-Sum test with appropriate post hoc testing. Significance was set at \(p < 0.05\). Statistical analysis was performed by MS and RSJ.
Results

Gross Observations

At time of necropsy, tendons were atrophied, pale yellow and gelatinous in appearance. There was poorly organized, white fibrous tissue surrounding the tendon-bone insertion site in all surgical groups. These changes were more pronounced in animals sacrificed at 8 weeks (CR, ER) compared to those sacrificed at 4 weeks (IO). The amniotic membrane placed at the repair site of animals in the ER group was not visible in any sample. The repaired tendon was in continuity with the bone in all samples, signifying successful and acceptable RCR in all animals undergoing further testing.

Tendon Biomechanical Testing

Tendon biomechanical testing revealed that augmentation with human amniotic membrane did not lead to any significant difference in the physical characteristics or performance of samples from the CR or ER groups. Specifically, ultimate load-to-failure (LTF) did not differ significantly between any of the experimental groups (Uninjured (U): 22.97 ± 2.57 N, Control Repair (CR): 24.71 ± 2.73 N, Experimental Repair (ER): 24.35 ± 1.79). Stiffness also did not differ significantly between any of the experimental groups (U:14.59 ± 3.36 N/mm; CR:12.56 ± 1.54 N/mm; ER: 12.89 ± 1.24 N/mm).

Displacement to failure (DTF) was greater in the CR and ER groups compared to the U group (U: 1.42 ± 1.00 mm, CR: 3.32 ± 1.32 mm p<0.0001, ER: 3.53 ± 1.67 mm p<0.0001) but did not differ significantly between the CR and ER groups. Work to failure (WTF) of the CR and ER groups were also significantly greater than that of the U group without a significant difference between the CR and ER groups (U: 33.80 ± 26.10 mJ,
CR: 83.68 ± 37.41 mJ p<0.0001, ER: 87.00 ± 42.99 mJ p<0.0001). Strain in the CR and ER groups was significantly greater than the U group (U:18.87 ± 14.20%; CR: 37.52 ± 17.70 p<0.0001; ER: 32.45 ± 21.88% p=0.0041), again without a significant difference between the CR and ER groups.

Modulus was significantly decreased in the CR and ER groups compared to the U group (U: 4.88 ± 3.61 MPa; CR: 2.71 ± 1.74 MPa p=0.02; ER: 2.54 ± 1.16 MPa p=0.02). Tendon biomechanical testing was not performed on the injury-only (IO) group.

<table>
<thead>
<tr>
<th></th>
<th>LTF (N)</th>
<th>DTF (mm)</th>
<th>Stiffness (N/mm)</th>
<th>WTF (mJ)</th>
<th>Max Stress (MPa)</th>
<th>Strain (%)</th>
<th>Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>U</strong> (n=30)</td>
<td>22.97 ± 2.57</td>
<td>1.42 ± 1.00</td>
<td>14.59 ± 3.36</td>
<td>33.80 ± 26.10</td>
<td>0.45 ± 0.38</td>
<td>18.87 ± 14.20</td>
<td>4.88 ± 3.61</td>
</tr>
<tr>
<td><strong>CR</strong> (n=30)</td>
<td>24.71 ± 2.73</td>
<td>3.32 ± 1.32*</td>
<td>12.56 ± 1.54</td>
<td>83.68 ± 37.41*</td>
<td>0.56 ± 0.41</td>
<td>37.52 ± 17.70*</td>
<td>2.71 ± 1.74*</td>
</tr>
<tr>
<td><strong>ER</strong> (n=30)</td>
<td>24.35 ± 1.79</td>
<td>3.53 ± 1.67*</td>
<td>12.89 ± 1.24</td>
<td>87.00 ± 42.99*</td>
<td>0.50 ± 0.30</td>
<td>32.45 ± 21.88*</td>
<td>2.54 ± 1.16*</td>
</tr>
</tbody>
</table>

Table 1. Tendon biomechanical testing revealed that augmentation did not lead to any significant difference in the physical characteristics or performance of samples from the CR and ER groups. Results are reported as mean ± standard error of the mean. LTF: Load to Failure, DTF: Displacement to Failure, WTF: Work to Failure. *Statistically significant difference (P < 0.05) compared to the Uninjured group.

**Single Muscle Fiber Biomechanical Testing**
Mean fast fiber cross-sectional area (CSA) was significantly reduced in all surgical groups compared to U [U=5503 ± 843 µm², IO=3147 ± 1369 µm² (p<0.0001), CR=1728 ± 1053 µm² (p<0.0001), ER=3713 ± 2676 µm² (p=0.0002)]. CSA of the ER group was significantly greater than the CR group (p<0.0001), representing a 32.5% reduction from mean U group CSA and a 68.5% reduction in the CR group CSA compared to U group CSA. In addition, the mean CSA of the ER group was 18.0% greater than the IO group while that of the CR group was reduced by 44.7% compared to IO.

Mean specific force produced by fast fibers was greatest in the U group (140±10 kPa), with that of the IO (121±22 kPa) and CR groups (120±33 kPa) being significantly lower (p=0.0147, p=0.0098) than that of the U group. The mean specific force produced by fibers in the ER group (138±13 kPa) was significantly greater than the CR (p=0.0247) and IO (p=0.0358) groups but did not differ significantly from the U group.
Figure 1. Single muscle fiber biomechanics. A, cross sectional area was greater in the ER group compared to the CR group; The ER group also generated greater (B) maximum isometric force and (C) specific force than the CR group.

**Microcomputed Tomography (Micro-CT) Analysis**

New bone formation, quantified by the ratio of bone volume to total volume, was significantly lower for all surgical groups compared to the U group [**U**: 0.48 ± 0.053, **IO**: 0.31 ± 0.067 (p<0.001), **CR**: 0.32 ± 0.051 (p<0.001), **ER**: 0.39 ± 0.099 (p<0.001)], but greater for the ER group compared to CR group (p=0.006). The ER group, but not the CR group, had significantly more new bone formation than the IO group (p=0.045). While trabecular thickness did not differ significantly between groups, trabecular number was significantly decreased and trabecular spacing was significantly increased in all surgical groups compared to the U group.
Figure 2. A, three-dimensional reconstruction of microCT images of the proximal humerus, including the lesser tuberosity bony footprint (A), bicipital groove (B), greater tuberosity supraspinatus bony footprint (C) and greater tuberosity infraspinatus bony footprint (D). B, new bone formation quantified as bone volume/total volume (BV/TV) expressed as a percentage of maturing bone at the SSp and ISp footprint shows more new bone formation in the ER group compared to all other groups.

**Tendon Histological Analysis**

Mean collagen birefringence of picrosirius red-stained tendon sections was greatest in the ER group (82.7 ± 21.4), which was significantly greater than that of the
CR group (55.7 ± 25.8, p=0.017) and not significantly different from the U group (78.3 ± 28.6). The mean area of metachromasia measured in safranin O-stained sections, which quantified enthesis proteoglycan content, was greatest for the U group (415 ± 218 µm²/1000). While mean metachromasia measured for the CR group (72.8 ± 78.5 µm²/1000) was less than that of the ER group (200 ± 159), this difference did not achieve significance. PAI-1 immunohistochemistry semi-quantitative grading showed no significant differences between any groups.

Figure 3. A, sections for H&E (a-c), Safranin O (d-f), Picrosirius Red (g-i), and PAI-1 (j-l). B, (a) augmentation with amniotic membrane-derived graft did not improve the area of new fibrocartilage formation at the enthesis in the ER group compared with the CR group; (b) but did improve collagen fiber continuity and orientation at the enthesis in the
ER group compared to the CR group and (c) semi-quantitative grading showed no significant difference between any groups.

**Muscle Histological Analysis**

Mean muscle fiber CSA was not significantly different between any groups (U= 0.95 ± 0.05 mm$^2$, IO= 0.75 ± 0.04 mm$^2$, CR= 0.54 ± 0.12 mm$^2$, ER=0.55 ± 0.18 mm$^2$). Mean area of lipid deposition, visualized by BODIPY staining, was decreased in the ER group compared to the CR group albeit not significantly.

![Muscle histological analysis images](image)  

**Figure 4.** A, sections for H&E (a-d), WGA (e-h), and BODIPY (i-l). B, muscle fiber cross-sectional area was decreased in the CR and ER groups compared to the U group but there was no difference between the CR and ER groups. C, there was no statistically significant difference in fat deposition between the groups.

**Muscle Mass**
Combined mass of the supraspinatus and infraspinatus muscles standardized by total body mass (SS+IS/TBM), was significantly greater in the U group compared to the repaired groups \([U=0.0010 \pm 0.0002 \text{ g}, \ CR=0.0005 \pm 0.0002 \ (p<0.005), \ ER= 0.0003 \pm 0.0003 \ (p<0.001)]\). SS+IS/TBM did not differ significantly between ER and CR groups. Muscle mass analysis was not completed for the IO group.

<table>
<thead>
<tr>
<th></th>
<th>SSp + ISp Mass (g)</th>
<th>Total Mass (g)</th>
<th>SSp+ISp/Total Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjured (n=8)</td>
<td>0.46 ± 0.048</td>
<td>462 ± 18</td>
<td>0.0010 ± 0.0002</td>
</tr>
<tr>
<td>Injury Only</td>
<td>Not Reported</td>
<td>Not Reported</td>
<td>Not Reported</td>
</tr>
<tr>
<td>Control Repair (n=7)</td>
<td>0.26 ± 0.056(^b)</td>
<td>485 ± 44</td>
<td>0.0005 ± 0.0002(^a)</td>
</tr>
<tr>
<td>Experimental Repair (n=13)</td>
<td>0.23 ± 0.077(^c)</td>
<td>509 ± 30</td>
<td>0.0003 ± 0.0003(^c)</td>
</tr>
</tbody>
</table>

Table 2. Combined supraspinatus and infraspinatus mass and mass as a ratio of body weight were significantly decreased in the CR and ER groups compared to the U group but did not differ between the CR and ER groups. Statistically significant difference compared with the uninjured group: \(a:P < 0.005\), \(b:P < 0.0005\), \(c:P < 0.0001\). SSp: Supraspinatus, ISp: Infraspinatus, TBM: Total Body Mass. U, uninjured; IO, injury only; CR, control repair; ER, experimental repair

**Gene Expression Analysis**

Results of quantitative real-time PCR, reported as fold-changes relative to the U group, are reported in figure 5. Expression of \(COL1A1\) and \(MMP-2\) was greater in the ER group compared to CR \([\text{CR}: 1.66 \pm 1.41, 2.09 \pm 1.55 \ p = 0.04; \text{ER}: 5.43 \pm 3.07 \ p= 0.02, 6.14 \pm 4.00 \ p= 0.02]\). Additionally, relative expression of \(FITM-2\) was lower in the ER group compared to CR \([\text{CR}: 3.02 \pm 1.06; \text{ER}: 1.72 \pm 1.01 \ p = 0.02]\).
Figure 5. Relative expression of genes implicated in collagen formation (A) and extracellular matrix regulation (D) demonstrated increased expression in the ER group compared to CR of COL1A1 and MMP-2, respectively. Expression of genes implicated in adipogenesis (B) revealed decreased expression of FITM-2 in the ER group compared to CR. There was no difference between the CR and ER groups in genes that function as growth factors/cytokines (E), or apoptosis regulators (C).

**Human Amniotic Membrane-Derived Graft Histological Analysis**

The cellular composition of the graft was characterized by Hematoxylin & Eosin, Masson’s trichrome, DAPI, and Toluidine blue. Interpretation of the stains demonstrated the presence of cytoplasm, nuclei, and collagen and the absence of proteoglycans.
Figure 6. A, H&E stain reveals the presence of nuclei (blue/purple) and cytoplasm (red); B, Masson’s trichrome shows the presence of collagen (blue) and cytoplasm (pink); C, DAPI shows the presence of nuclei (blue); D, Toluidine blue reveals the absence of proteoglycans in the graft.

**Conclusions**

Degenerative rotator cuff tears are one of the most common pathologies seen in the orthopaedic clinic and operating room. Great strides have been made in surgical management of such tears over the past two decades, thanks in large part to the advancement of minimally invasive arthroscopic surgery and the push to continue producing innovative and highly effective orthopaedic implants. It is clear however, that a strictly mechanical approach fails to adequately address the relatively high rates of tendon re-tear or failure of primary tendon healing following repair. The goal of this
study was to establish a reliable rat model for chronic, massive rotator cuff tears which was to be used to study the possible advantages of augmenting RCR with human amniotic membrane. Specifically, we tested the hypotheses that application of human amniotic membrane to the tendon-bone interface at the time of repair can (1) strengthen the enthesis and (2) produce greater rotator cuff muscle fiber force by promoting bone formation, increase fibrocartilage formation, improve collagen organization, demonstrate lower levels of plasminogen activator inhibitor-1 (PAI-1), exhibit less lipid deposition, and induce muscle repair gene expression compared to control repair.

To determine the degree of new bone formation at the healing enthesis we performed microcomputed tomography (Micro-CT) analysis. Our results demonstrated a higher degree of newly formed bone in the repairs augmented with human amniotic membrane compared to control repairs. To fully appreciate these findings, it is important to understand the structural characteristics of the tendon-bone interface and the modulators of this process. A fibrocartilaginous transition area exists between tendon and bone, which can be divided into four distinct areas including tendon, fibrocartilage, mineralized fibrocartilage, and bone. Previous studies have demonstrated that TGF-β1 enhances the formation of fibrous tissue at the site of tendon healing through the downregulation of matrix metalloproteinase 9 and 13 (MMP-9, MMP-13).²

In addition to identifying macroscopic changes, we sought to study the effects of human amniotic membrane on the microscopic architecture of the muscle-tendon units. Several histochemical techniques exist that allow for enhanced visualization of collagen including Masson trichrome, van Gieson, Herovici, and picrosirius red. Though the interpretation of all these stains is straightforward, we chose to utilize a picrosirius red
stain as the Masson, von Gieson, and Herovici stains require reactions with proteins of which the mechanism of action remains unclear, leading to decreased selectivity for collagen fibrils. The picrosirius red stain relies on the fact that collagen is highly anisotropic and thereby birefringent. Birefringence is the difference in the refractive indices of the two rays, oriented ninety degrees apart, that are produced when light interacts with an anisotropic molecule like collagen. Picrosirius red is also a highly birefringent molecule which binds to collagen and orients parallel to the fibrils thereby allowing the collagen-picrosirius red complexes to be visualized easily under polarized light. This process allows for the determination of collagen organization at the enthesis, with more organized collagen being associated with a more robust and reliable construct. Analysis of the tendons obtained from rats having undergone augmented repairs with amniotic membrane demonstrated greater organization than those in the control repair group. These findings indicate that augmentation with human amniotic membrane may also play a role in improving repair integrity on a microscopic, organizational level.

Skeletal muscle atrophy and fatty degeneration of the rotator cuff is a common phenomenon following RCT. We found that the combined mass of the SSp and ISp muscles in the CR and ER groups was significantly lower than that of the U group, but there was no difference between the CR and ER groups. Notably, our model was designed to model chronic tears, and this was achieved in part by the denervation of the rotator cuff with botulinum toxin. Furthermore, it is believed that myotendinous retraction that occurs following RCT could lead to traction injury to the suprascapular nerve, thereby contributing to the denervation of the cuff and subsequent muscle atrophy.\textsuperscript{18}
To further characterize the skeletal muscle changes in the study groups, we performed muscle histological analysis. Hematoxylin & Eosin (H&E) staining allowed for the measurement of individual muscle fibers and showed that the average cross-sectional area was decreased in the CR and ER groups compared to the U group, but again there was no difference between the CR and ER groups. Though we were unable to identify a statistically significant difference, we observed a higher rate of fat deposition in the CR and ER groups compared to the U group. These findings demonstrate that our model for chronic, massive rotator cuff tears led to increased muscle atrophy, a characteristic feature of RCT pathophysiology. Unfortunately, our model failed to produce significantly higher rates of fatty degeneration, which may be due to the short time period between injury, repair, and sacrifice. This could also be a species-specific issue as the rat rotator cuff is a good, but clearly not perfect representation of the human rotator cuff both mechanically and biologically.

The completion of gene expression analysis also served to uncover the possible mechanisms by which human amniotic membrane serves a beneficial role following RCR. The relative degree of messenger RNA (mRNA) production of the genes of interest was used as an indicator of gene expression and ultimately protein production leading to downstream effects. The reverse transcription of mRNA, the molecules destined for translation and protein production, to complimentary DNA (cDNA) allows for the completion of quantitative real-time polymerase chain reaction (qRT-PCR) to determine relative gene expression. In completing these experiments, we were able to identify upregulation of the genes \textit{COL1A1} and \textit{MMP-2} as well as downregulation of \textit{FITM-2} in the ER group compared to the CR group.
The \textit{COL1A1} gene encodes the $\alpha 1$ chains of type-1 collagen.\textsuperscript{22} Type-1 collagen serves as the main structural component of tendons, though it is not a tendon-specific structure that is also present in large quantities in the bone, skin, and cornea.\textsuperscript{16} As previously mentioned, tendons are composed of highly coordinated, parallel collagen fibrils however, the process by which these fibrils organize remains unknown. Taken together with our histological studies that demonstrated increased collagen organization at the enthesis in the ER group, increased expression of \textit{COL1A1} further supports the finding that human amniotic membrane has a positive effect on tendon healing following repair. Additionally, MMPs are proteases that play a critical role in healing and remodeling. Previous studies have shown that MMPs, specifically \textit{MMP}-2 and \textit{MMP}-9, demonstrate low levels of expression under physiological conditions but are upregulated following tendon injury.\textsuperscript{8} Specifically, \textit{MMP}-2 has been shown to play a critical role in both the degradation and remodeling of collagen.\textsuperscript{10} Our results demonstrated a statistically significant increase in \textit{MMP}-2 expression in the ER group compared to all other study groups, uncovering a possible mechanism for the potential role of amniotic membrane in tendon healing following injury and repair.

In addition to upregulated \textit{COL1A1} and \textit{MMP}-2 expression, the ER group demonstrated decreased expression of \textit{FITM}-2 compared to the CR group. This gene codes for a transmembrane protein that may play a role in fatty degeneration of rotator cuff muscle after injury\textsuperscript{14}. The structural composition of the rotator cuff changes following injury, particularly following full thickness tears. When a tear occurs, the muscle fibers are reoriented in such a way that gaps are formed which leads to fatty infiltration as these gaps are filled with fat and fibrous tissue.\textsuperscript{4} Studies have shown that
muscle fat content (MFC) following RCT is associated with patient age and gender, but little is known regarding the underlying mechanisms guiding this phenomenon. The downregulation of FITM-2 provides a possible explanation for one of the steps involved in this process, and decreased expression following augmentation with amniotic membrane further demonstrates the positive effects of this graft on healing following RCR.

Tendon biomechanical testing failed to demonstrate a difference in the structural properties of the tendons following augmentation with human amniotic membrane. The repaired tissues under study consisted of healing scar tissue as well as the native tendon, making up a very complex tissue composite. As a result of this composition, construct failure during testing did not always occur at one particular moment in time as the scar tissue may have failed first, followed by the native tendon tissue. Despite the previously mentioned increase in bone formation and improved collagen organization identified in the ER group, differences in the composition of the tendon samples within and between each study group may have contributed to the finding of similar biomechanical properties, particularly load to failure, between the CR and ER groups.

Though there were no biomechanical differences identified through tendon biomechanical testing, single muscle fiber biomechanical testing served to further characterize the muscle atrophy previously described as well as demonstrate biomechanical differences between our study groups. H&E staining showed that average muscle fiber cross-sectional area was decreased in the CR and ER groups compared to the U group. In competing single-muscle fiber testing, we were able to measure individual fibers by isolating them from each muscle bundle. Highly magnified images
were taken of each fiber at five distinct points along its length, which were then were used to determine the CSA of each fiber. This method of determining CSA was more precise than the previously described histological analysis, and we were able to determine that there was a difference between the CR and ER groups, with the fibers in the augmented repair ER group demonstrating a higher CSA than those in the CR group. We were also able to identify increased specific force in the fibers isolated from the ER group compared to the CR group. The measurement of specific force takes into account the CSA of the muscle fibers, which was critically important as the fibers in the ER group were shown to have a higher CSA than those in the CR group. The increased specific force produced by the fibers in the ER group was likely made possible by the more favorable biological environment afforded by the amniotic membrane. Decreased inflammation and fatty degeneration may have played a key role in the demonstrated findings. Additionally, amniotic membrane has been shown to reduce fibrosis in the extra-ocular muscles following strabismus surgery therefore, amniotic membrane may play a role in decreasing the interstitial fibrosis of rotator cuff muscles following tendon injury in RCTs. These findings have provided a strong foundation for further work elucidating the underlying mechanisms associated with improved muscle fiber characteristics.

We acknowledge that modeling of chronic injury and repair in a small animal model can be challenging, especially when it comes to mimicking a clinical scenario in which there are at least equal amounts of fat and muscle in the rotator cuff. Despite unloading the tendon and chemically denervating both the supraspinatus and infraspinatus tendons for four weeks, we were able to achieve Goutallier stage 2 “fatty
atrophic” changes in this massive rotator cuff injury and repair model. We may have considered delaying the repair for either six or eight weeks after the original injury, but it is highly likely a suitable repair would have been even more difficult to achieve. Performing a repair at 4 weeks following injury, considerable dissection was required to mobilize both rotator cuff muscle tendon-units to ensure adequate tendon excursion to maximize footprint restoration. There is no doubt that the manipulation of the muscle during the repair led to additional muscle injury. A second limitation of this study is that it was powered for maximum load to failure of the healing enthesis. This outcome measure, which is a macroscale tool for evaluation of the composite tissue, may not provide an accurate estimate of the attachment strength of a complex tissue that includes fibrovascular scar. Rather, we believe that a microscale tool such as atomic force microscopy (AFM) would be ideally suited to evaluate tendon bulk samples at the micro- and nanoscale. AFM can be used to measure indentation modulus of small animal tissues by using micro- and nanoscale probes to indent the tissue, providing a measure of the elastic modulus that cells are experiencing at the cell length-scale. A third limitation is that we only augmented the tendon-bone repair site despite unloading the tendon and chemically denervating the muscle. It is possible that augmentation of the muscle with amniotic fluid would lead to less muscle fibrosis. Future work will be focused on evaluating the role of amniotic fluid injection of the injured rotator cuff muscle.

There are several strengths to this work. All of the surgical injuries and repairs were executed and completed by a fellowship-trained surgeon who has extensive experience with small and large animal models of tendon-bone healing. This quality control eliminated any variability that may be seen with surgical repair performed among
several individuals. Also, we performed a comprehensive evaluation of the entire muscle-tendon-bone unit in this pre-clinical model of chronic injury and rotator cuff repair. Third, we characterized the enthesis material properties as well as the structural properties of the tendon-bone construct.

In summary, based on our comprehensive evaluation of the entire muscle-tendon-bone unit, delivery of human amniotic membrane at the rotator cuff tendon-bone footprint can improve healing after repair in a chronic, massive rotator cuff injury model. This is evidenced by greater bone formation, a more organized tendon enthesis, induced muscle repair gene expression, and greater rotator cuff muscle fiber force.
Bibliography


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