

THESIS

EVALUATION OF MENISCAL CHANGES IN TWO MODELS OF KNEE
OSTEOARTHRITIS: TRAUMATIC LOADING AND MODIFIED TRANSECTION

Submitted by

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ABSTRACT

EVALUATION OF MENISCAL CHANGES IN TWO MODELS OF KNEE OSTEOARTHRITIS: TRAUMATIC LOADING AND MODIFIED TRANSECTION

Osteoarthritis (OA) is a debilitating joint disease characterized by the erosion of articular cartilage on the ends of long bones, causing painful bone on bone contact. OA can affect any joint but is commonly seen in the knee and is a major cause of disability. Normally thought of as a degenerative disease, the early onset of OA can be triggered by a number of factors including injury to the joint. With the number of younger athletes increasing as well as the incidence of knee injury increasing it is important to understand the development and progression of post traumatic OA (PTOA). Only then can measures be taken to prevent, or slow the progression.

The most common method to study PTOA of the knee involves using an animal model where the anterior cruciate ligament is transected (ACLT). The ACLT model fails to account for a number of factors that are commonly seen in clinical cases. The compressive forces experienced by the joint as well as damage to other joint structures are not accounted for in the traditional ACLT model. Furthermore, despite the well documented role of the meniscus in joint stability and joint kinematics other tissues such as the articular cartilage and subchondral bone have received more attention.

PTOA is a “whole organ” disease where damage to one structure influences other structures, and in order to fully understand the progression the entire joint must be studied. There is a lack of knowledge as to how the meniscus is both affected and influences the development of OA. To

better understand its role there have been two PTOA models developed for this study. The first is a modified ACLT model (mACLT), where meniscal damage is surgically induced at the time of ACL transection. The second model is a traumatic tibiofemoral compressive impact model (ACLF) where the ACL is ruptured due to a blunt force trauma to the joint.

The objective of this thesis was to monitor meniscal changes twelve weeks following impact for both the mACLT and ACLF model. Meniscal damage was monitored over time with the use of magnetic resonance imaging MRI. At dissection gross morphology was graded and compared to the acute and chronic MRI notes. Each meniscus was then sectioned into regions and mechanically tested. Indentation relaxation testing allowed for the instantaneous as well as equilibrium elastic moduli to be calculated. Following mechanical testing meniscal tissue was fixed and stained for glycosaminoglycan (GAG) content. Using semi qualitative analysis, the GAG intensity and coverage was analyzed. Acute and chronic damage, elastic moduli, and GAG content from the injured limbs was then compared to the contralateral controls.

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LIST OF DEFINITIONS

Osteoarthritis (OA) – A form of arthritis characterized by cartilage degradation, joint space narrowing, and pain

Post-traumatic Osteoarthritis (PTOA) – a form of secondary osteoarthritis that occurs as a result of injury to a joint

Meniscus – Crescent shaped fibrocartilaginous tissue in the knee

in vitro – performed in controlled laboratory environment

in vivo – performed in whole, living organism

Chapter 1 - Introduction

Osteoarthritis (OA), the most common form of arthritis, can simply be defined as the degradation of a joint. OA normally occurs as a result of aging; however genetics, obesity, and altered mechanical loading conditions such as repeated motions and trauma can all increase the risk of development of OA. ^{Fleson2004, Lohmander2000, Lohmander2007} As of 2004, OA was the cause for moderate to severe disability in as many as 43.4 million people worldwide. ^{Brown2006} Posttraumatic OA, or OA resulting from trauma, is thought to account for 12% of all patients exhibiting OA. The financial burden associated with posttraumatic OA was projected at \$3.06 billion annually in 2006. ^{Brown2006} A number of injuries can lead to posttraumatic OA of the knee, but is most commonly seen following sports injuries when the anterior cruciate ligament (ACL) is torn.

^{Daniel1994, Kannus1989, Lohmander2004, Maletius1999, vonPorat2004}

The Knee

The knee is a complex six degree of freedom joint and is usually described in two parts, the patellofemoral joint and the tibiofemoral joint. The patellofemoral joint consists of a sesamoid bone, the patella, which glides along the femoral groove to protect the articular surfaces throughout knee flexion. The patella also functions to increase the leverage that the patella tendon can exert on the femur. The tibiofemoral joint is composed of four major ligaments and two menisci (Figure 1.1). The main function of the ligaments is to provide stability and prevent excessive flexion/extension, internal/external rotation, and varus/valgus rotation.

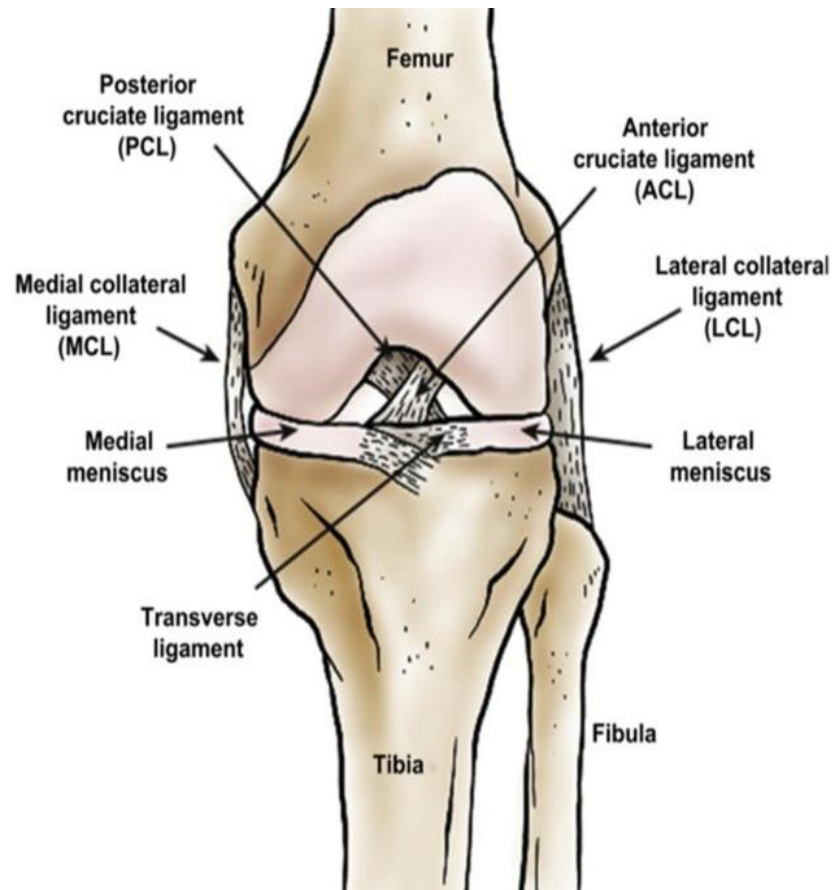


Figure 1.1. Knee anatomy (with permission from Makris E. a, Hadidi P., and Athanasiou K. a, 2011, “The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration.” Biomaterials)

The anterior cruciate ligament (ACL) resists anterior tibial translation while the posterior cruciate ligament (PCL) acts to prevent excessive posterior tibial translation. Both collateral ligaments, the lateral collateral ligament (LCL) and the medial collateral ligament (MCL) provide restraint against varus and valgus movement respectively. Damage to any of the ligaments can cause knee instability and joint laxity. Improper alignment creates altered joint kinematics placing an individual at a higher risk for future joint damage and accelerated joint degradation. Chaudhari2008

The Meniscus

Menisci are C shaped biphasic tissues composed of approximately 70% fluid and 30% organic matter ^{Mow1992} found in both the medial and lateral compartments of the knee (Figure 2.1). The wedge like profile of the meniscus, with the concave proximal surface, functions to distribute load from the round femoral condyles to the relatively flat tibial plateau. Each meniscus has an anterior and posterior attachment, which prevent meniscal extrusion. Originally thought to be an artifact of evolution ^{Sutton1897}, the menisci have been shown to bear 45-75% of the loading experienced in the knee joint. ^{Shrive1978, Walker&Erkman1975}

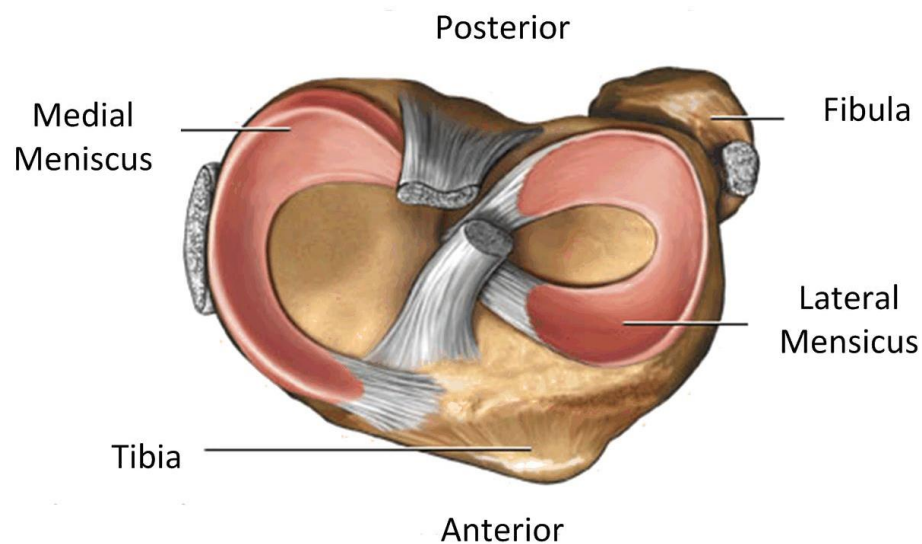


Figure 1.2. Superior view of the left knee (modified from <http://www.precisionnutrition.com/all-about-the-knee>)

Not only does the meniscus distribute load, but it also helps with joint lubrication and protects the articular cartilage of the knee. ^{Krause1976, Walker&Erkman1975} The medial meniscus covers approximately 50% of the articular cartilage contact area in the medial hemijoint, and the lateral meniscus covers approximately 70% of the contact area in the lateral hemijoint. ^{Ahmed&Burke1983,}
^{Walker&Erkman1975} The anterior horn and posterior horn of the medial meniscus are dissimilar in size

with the posterior horn being slightly larger. The horns of the lateral meniscus are more similar in size (Figure 1.2). The two menisci are connected to each other through a transverse ligament that spans between the anterior horns. The medial meniscus is less mobile than the lateral as the outer rim of the medial meniscus is connected to the joint capsule.

Organic components of the meniscus include collagen, proteoglycans, elastin, and other glycoproteins. ^{Mow1992} Meniscal matrix is primarily type I collagen fibrils with only small amounts of the other collagens (types II, III, V, and VI). When collagen is elongated in the same direction as its fibrils, it has a high strength allowing it to bear and transmit high loads. Collagen fibrils are orientated primarily circumferentially with radial tie fibers and an interwoven surface layer (Figure 1.3). Structurally the circumferential fibers transfer compressive loads into tensile hoop stresses. ^{Upton2006} These hoop stresses are then transmitted to meniscal attachments and down into the underlying bone.

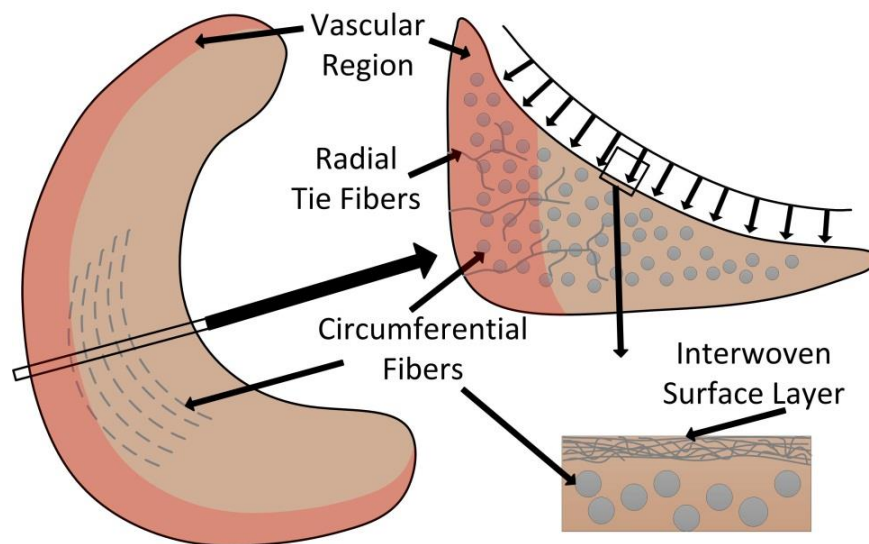


Figure 1.3. Meniscus with collagen fiber orientation and vascularization (area of vascularization shown in red)

Proteoglycans help to retain the interstitial fluid within the tissue, contributing to the biphasic nature of the tissue.^{MacConaill1950} Proteoglycans are composed of a core protein with side sugar chains, or glycosaminoglycans (GAG) (Figure 1.4). There are a number of different proteoglycans in the menisci,^{Esko1991} but the most common is aggrecan.^{Sandy1997} GAG chains are negatively charged thus attracting the positively charged interstitial fluid.^{McNicol1980} Sanchez-Adams et. al. has reported that in meniscal tissue the depletion of GAG reduces the coefficient of viscosity and reduces the equilibrium modulus in some regions.^{Sanchez-Adams2011}

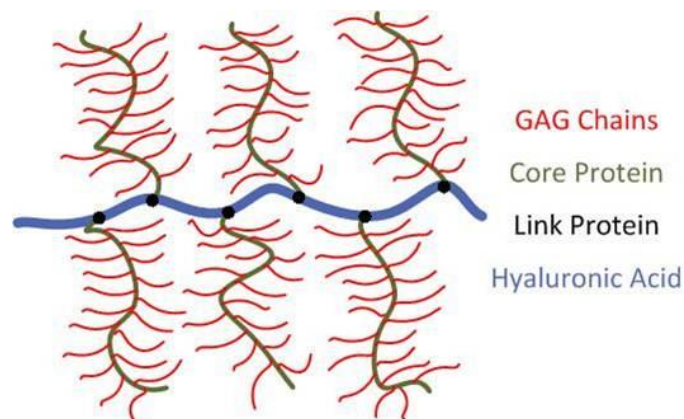


Figure 1.4. Aggrecan

In a fully developed meniscus, vascularization is only present in the outer most region of the tissue accounting for 10-30% of the total meniscus^{Clark1983} (Figure 1.3). This lack of vascularization means tears or damage in the avascular regions cannot heal. Prior to 1982 the removal of the meniscus, or a meniscectomy, was the common treatment for a damaged meniscus. However, it was later discovered that a meniscectomy results in the deterioration of articular cartilage and the gradual development of arthritis.^{Fairbank1948} The development of arthritis is thought to be mainly due to altered biomechanics and the increase in instability.

Macnicol2000, McNicholas2000 An alternative to a full meniscectomy is a partial meniscectomy, where only the damaged tissue is removed. Partial meniscectomies have shown better results than full meniscectomies but still have degenerative long-term effects. McDermott2006

Meniscal tears have been well described and documented. Cerabona1988, Greis2002, Kidron2002 Tears typically occur in three orientations; longitudinally (circumferentially), horizontally, and radially. Tears can then displace leading to bucket handle, flap, and parrot beak tears (Figure 1.5). The cause of meniscal tearing is typically described in two ways; a result of increased loading on the tissue, or due to a degenerative meniscus. Tears resulting from the former are typically longitudinal or radial tears, while horizontal tearing is more common in degenerative cases.

Hough1990

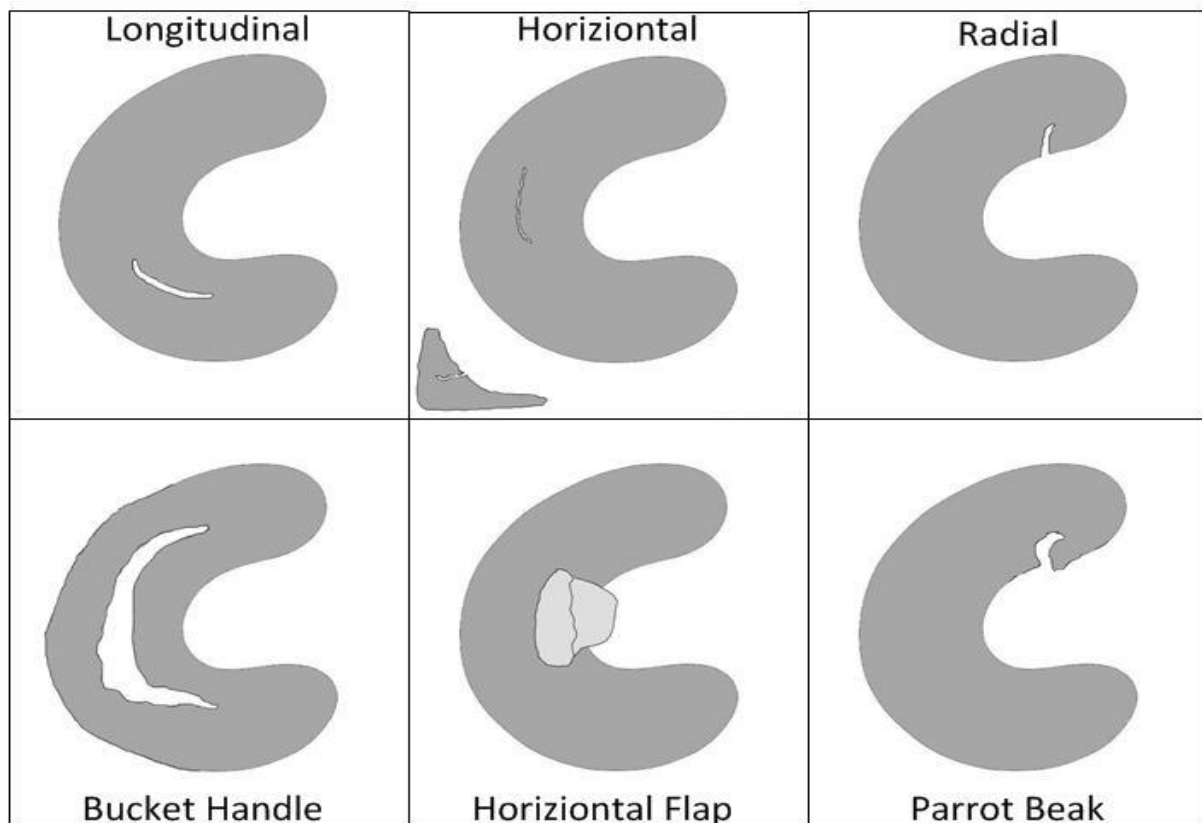


Figure 1.5. Meniscal tear patterns

Longitudinal tears can be located anywhere along the length or width of the meniscus and usually reach 50–65% of the whole length of the tissue. Horizontal tears divide the meniscus into separate tibial and femoral halves. The most probable zone for horizontal tears is the junction of the middle and posterior thirds. Radial tears are also typically located at the intersection of the middle and posterior thirds of the meniscus.^{Hulet2001} Clinically, about 81% of the injuries are parrot beak (displaced radial tears) or longitudinal (circumferential) tears affecting more often the medial meniscus.^{Greis2002}

A number of researchers have found meniscal mechanical properties to be dependent on hemijoint (medial and lateral) as well as region (anterior, central, and posterior).^{Fithian1990, Lechner2000, Sweigart2004, Tissakht1995} These studies found the anterior region to have a higher tensile stiffness in both the medial and lateral menisci. Tissakht et al. also observed much higher tensile stiffness in the circumferential direction as opposed to the radial direction, which was expected given collagen orientation.^{Tissakht1995} However, much of this research focused on the medial meniscus and a full understanding of both menisci is needed.

Models for investigating OA

The development of OA is a complex problem and the inability to isolate causes makes it difficult to study in humans. Therefore animal models are often used to study the disease. The most widely used model to investigate the role of altered kinematics on the chronic advancement of OA has thus far been the ACL transection (ACLT) model.^{Adams1983, Batiste2004, Chang1997, Killian2010, HelioLeGraverand2001, McDevitt1976, Sniekers2008, Yoshioka1996} In the ACLT model the ACL is transected with a clean scalpel incision. Animals are then euthanized at various points to allow for morphological,

mechanical, and histological testing to be done on the various tissues. Some of the more commonly studied tissues include the articular cartilage^{Batiste2004, Chang1997, McDevitt1976, Yoshioka1996} and subchondral bone.^{Batiste2004, Sniekers2008} Based on these studies it is clear that the joint undergoes osteoarthritic changes including cartilage fibrillation, erosion, subchondral bone exposure, and osteophyte formation. However, very few studies^{Adams1983, Killian2010, HelioLeGraverand2001} have studied or recorded changes to the menisci despite it playing a major role in knee kinematics.

Additionally, only a few studies have been conducted where an ACLT plus a full or partial meniscectomy have been performed to induce the onset of OA.^{Hayami2006, Intema2010, Pickarski2011} Again these studies have primarily focused on the cartilage and underlying bone. Nevertheless, as expected from clinical studies following meniscectomies^{Dandy1975, Jackson1968, Tapper1969}, the meniscectomy animal models resulted in severe cartilage damage in a canine model at 12 weeks.^{LeRoux2000} The rapid onset of OA following meniscectomy provides some insight into the important load distribution role it plays in the joint and how if absent cartilage degradation is quickened.

Ligaments, such as the ACL in the knee, are traumatically torn during dynamic movements such as jump landings and pivot motions.^{Arnold1979, Boden2000, Levine2012, Meyer2005, Yeow2009} While ACLT models have been shown to result in the onset of OA, occult damage to surrounding structures is not addressed. The knee does not experience force-induced trauma in an ACLT model and, therefore, no acute damage to surrounding tissues. Both meniscal tearing and bone microcracks

are commonly seen clinically in injuries involving the ACL. McDaniel et al. reported damage to the menisci occurs in as high as 82% of knees with acute ligament tears.^{McDaniel1980}

A number of impact models are being developed to better recapitulate damage seen during a traumatic injury.^{Borrelli2010, Brophy2012, Isaac2010, Killian2010, Tochigi2013, Yeow2008} These models range from *in vitro* and *in vivo* testing on pigs^{Tochigi2013, Yeow2008}, combined impact and meniscectomy models^{Brophy2012}, and tibiofemoral impact models using rabbits.^{Borrelli2010, Isaac2010, Killian2010} The objective of these current studies was to induce an ACL rupture by a single or repeated compressive load to the tibiofemoral joint. However like the surgical models, much of the focus of these studies has been cartilage and bone changes with little attention paid to the meniscus.

Problem Statement

There exists a lack of information on the role of the meniscus in the progression of knee OA. A better understanding of the morphological, mechanical, and histological changes of osteoarthritic meniscal tissue could help researchers better understand the complex progression of knee OA. Furthermore improving on the ACLT animal model to include meniscal and occult damage will provide a modeling scenario that more closely mimics that of a real trauma case.

The research effort will require the use of mechanical testing, histology, and magnetic resonance imaging. The large majority of the ACLT animal models in the literature have used a lapine model. To maximize the amount of tissue available for analysis, Flemish Giant rabbits will be used in this study. One group of animals will be subjected to traumatic injury of the ACL and meniscus using the newly developed tibio-femoral impaction device^{Issac2008} while the second

group will receive surgical transections of the ACL and both menisci. Contralateral limbs will serve as inter-animal controls for both groups.

Twelve weeks following injury all animals will be euthanized and indentation relaxation mechanical testing and histological staining for GAG will be performed. All testing will be performed on individual regions (anterior, central, and posterior) on both hemijoints (lateral and medial) to account for any differences between locations. Magnetic resonance imaging (MRI) will be used just following trauma and just prior to euthanasia to help document the acute and chronic condition of the joint.

Research Aims

Aim 1: To determine the instantaneous and equilibrium compressive elastic moduli and GAG content of Flemish Giant rabbit menisci 12 weeks after a single blunt force tibiofemoral impact.

Aim 2: To determine the instantaneous and equilibrium compressive elastic moduli and GAG content of Flemish Giant rabbit menisci 12 weeks after a combined ACL transection and meniscal transections.

Aim 3: To compare the acute and chronic meniscal damage seen using the tibiofemoral impact model and the combined ACL and meniscal transection model.

Chapter 2 - Evaluation of Meniscal Mechanics and Proteoglycan Content in a Modified ACL Transection Model

The material in this chapter has been submitted for publication in the Journal of Biomechanical Engineering

Introduction

Posttraumatic osteoarthritis (PTOA) is a debilitating joint disease resulting from joint trauma. A number of injuries can lead to PTOA of the knee, but it is most commonly seen following sports injuries where anterior cruciate ligament (ACL) and meniscal injuries have occurred. ^{Felson2004} Because the menisci have been shown to bear 45-75% of the load in the knee joint, ^{Shrive1978} meniscal damage is thought to be associated with the development of OA and merits further investigation. ^{McGonagle2010}

In order to study the chronic advancement of PTOA, numerous *in vivo* animal models have used a surgical transection (ACLT) model to mimic damage to injured joints. ^{Batiste2004, Chang1997, Intema2010, Killian2010, HelioLeGraverand2001, McDevitt1976, Sniekers2008, Yoshioka1996} These models destabilized the knee joint surgically by transecting the ACL, and then monitor joint degradation over time. While the ACLT model has shown injuries to the ACL can lead to the classical characteristics associated with human OA, it fails to account for accompanying acute meniscal damages that are often associated with the human trauma. With meniscal damage occurring in as high as 82% of knees with acute ligament tears ^{McDaniel1980}, it is important to understand how this damage affects the mechanical and morphological properties of menisci in the subsequent chronic setting.

To better study the combined injury mode of acute ACL and meniscal tearing a modified ACL transection (mACLT) model has been developed. In the mACLT model the ACL is fully transected, and medial meniscus and lateral meniscus are both partially transected. To our knowledge this is the first combined ACL and meniscal transection study that investigates chronic changes in the menisci. There have been previous studies that combine an ACL transection with a partial or total meniscectomy ^{Hayami2006, Intema2010, Picharski2011}, but the focus of these studies was on articular cartilage and bone changes. Partial transections will be performed in both the medial and lateral hemijoints as literature has shown them to occur almost equally (44% medially and 56% laterally) ^{Bellabarba1997} in acute ACL injuries.

Because of the important load distribution properties of the menisci, meniscal tissue is likely not benign in the development of OA. ^{McGonagle2010, Fairbank1948} This is supported clinically by cartilage degeneration following meniscectomies. ^{Dandy1975, Jackson1968, Tapper1969} Water flow through meniscal tissue is regulated in part by aggrecan, a proteoglycan with GAG side chains, which contributes to the viscoelastic nature of the tissue. ^{Fithian1990}. GAG depletion in meniscal tissue has been shown to reduce the coefficient of viscosity and reduce the equilibrium modulus of the tissue. ^{Sanchez-Adams2011} Additionally, the tissue is limited in its healing capacity due to the primary avascular nature of the meniscus. Clinically it has been shown that when left untreated, a combined ACL and meniscal tear will become more severe over time. ^{Bellabarba1997, Smith2001, Wickiewicz1990}

The objective of this study was to investigate changes to meniscal tissue twelve weeks after joint trauma in the form of ACL and meniscal transections. This mACLT model will characterize meniscal damage that has been previously unaccounted for in the traditional ACLT model. An

indentation relaxation test will be used to determine the instantaneous and equilibrium compressive elastic moduli of the menisci. Histological analysis will be performed to monitor changes in glycosaminoglycan (GAG) coverage. It is hypothesized that both mechanical properties and GAG coverage will be decreased in the mACLT joint compared to the contralateral control 12 weeks post trauma.

Methods

Modified ACL Transection Model

Six skeletally mature Flemish Giant rabbits (5.3 ± 0.6 kg) were used in the study. All animals were housed in individual cages for the duration of the study, which was approved by Michigan State University and Colorado State University All-University Committees on Animal Use and Care. Animals were placed under anesthesia and the right limb of each animal underwent an ACL transection, as well as meniscal transections to both the medial and lateral menisci. The left limb was left unaffected and served as a control. A licensed veterinary technician monitored the rabbits for pain and buprenorphine (0.3mL/kg BW) was given every 8 hours for 72 hours following surgery. All animals were housed in individual cages (60 x 60 x 14 in), and twelve weeks post injury the animals were euthanized.

The right limb of each animal was shaved and prepared using a 70% povidone-iodine scrub and 70% alcohol. Using a medial parapatellar arthrotomy the knee joint was exposed and the ACL was transected. The medial meniscus received a radial transection in the white zone of the central region with a longitudinal transection extending through the main body. The lateral meniscus was transected radially in the white zone of the central region and with a minor longitudinal cut

extending anteriorly (Figure 1). The joint capsule was sutured immediately after transection using 3/0 PDS. The subcuticular layer and skin was closed in sequence using 4-0 PDS and SQ-CED, respectively.

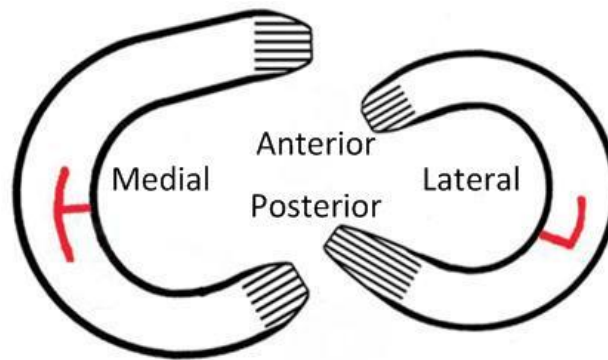


Figure 2.1. Diagram of meniscal transections

Meniscus Harvesting and Preparation

The menisci were harvested immediately following sacrifice and kept refrigerated (1.7 to 3.3°C) until mechanically tested. The menisci were photographed and damage was quantified using a combination of previously established grading systems.^{Matyas2004, Pauli2011} A morphological score from 0-4 was assigned to each region of both hemijoints with 0 = normal, 1 = surface damage, 2 = un-displaced tears, 3 = displaced tears, 4 = tissue maceration. Blind scoring was performed by four separate individuals and average numbers reported.

Because mechanical and histological properties have been seen to be regionally dependent,^{Chia2008, Fithian1989, Killian2010, Sweigart2004} each meniscus was sectioned into anterior, central, and posterior regions for testing. Specimens were kept hydrated with 0.9% phosphate buffered saline (PBS) solution before and during mechanical testing. Following mechanical testing meniscal sections were fixed in 10% formalin for 14 days. Sections were then embedded in optimum

cutting temperature medium (OCT, Pelco; Redding, CA) and flash frozen using liquid nitrogen. The face parallel to the cut surface of these regional sections was then cryosectioned using 6 μ m slices and stained for GAG using Safranin-O and Fast Green.

Mechanical Evaluation

Meniscal tissue was subjected to indentation, relaxation tests (Bionic Model 370.02 MTS Corp, Eden Prairie, MN) in the anterior, central, and posterior regions, when enough intact tissue was present. The samples were tested in a PBS bath to prevent dehydration. The bath was attached to a two degree of freedom camera mount, and an x-y plate allowed for the indentation surface to be oriented normal to the indenter. A spherical tip with a diameter of 1.59 mm was used as an indenter, and loads were read using an 8.9 N load cell (Futek LSB200, Irvine, California).

Specimens were subjected to a preload of 20 mN before being indented 0.25 mm for 900 seconds. Similar to previous indentation testing on meniscal tissue of rabbits,^{Li2006} a Hertzian contact equation was applied and used to determine both the instantaneous and equilibrium moduli. The contact equation assumed contact between an elastic half space and a sphere. The elastic modulus and Poisson's ratio of the indenter tip were 210GPa and 0.3, respectively. Based on a previous study^{Sweigart2004} Poisson's ratio of the menisci was assumed to be 0.01 for all regions. A paired sample t-test was performed on the data and significance identified when $p < 0.05$. Data was evaluated instantaneously and at equilibrium.

GAG Staining

GAG content was determined histologically using Hematoxylin, Safranin-O (Saf-O), and Fast Green (FCF) staining.^{Rosenberg1971} This process stains GAG red, nuclei black, and cytoplasm blue/green. Slides were imaged using an Olympus BH2 Microscope (Center Valley, PA) and MicroPublisher 5.0 RTV camera (QImaging, Surrey, BC Canada). Staining intensity was graded similar to previous studies^{Pauli2011} with no Saf-O staining represented by a score of 0, slight staining = 1, moderate staining = 2, and strong staining = 3. (Figure 2) Reported intensity grades were averaged from four separate graders.

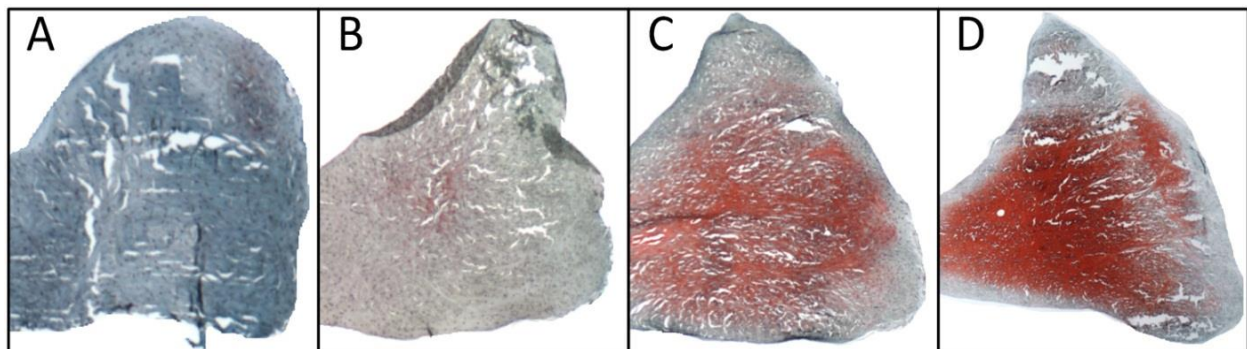


Figure 2.2. Safranin-O-Fast Green staining intensity: (A) no stain = 0 (B) slight staining = 1 (C) moderate staining = 2 (D) strong staining = 3

Coverage was analyzed using Image J (NIH, Bethesda, MD) with FIJI package. Images were trimmed and converted to 8 bit images. Total area of the image was calculated using the Analyze Particles tool and summing particles. The area stained red, or the area associated with GAG coverage, was separated from the image using the Colour Deconvolution tool. Once separated the area of GAG coverage was calculated by thresholding and analyzing particles. Paired t-tests were performed on the control and transected limbs with significance determined when $p < 0.05$.

Results

Morphology

Rabbits favored the contralateral limb for the first 1-3 days, but showed no signs of gait abnormality for the duration of the study. At time of euthanasia, all mACLT joints demonstrated osteoarthritic changes characterized by cartilage fibrillation, erosion, subchondral bone exposure, and osteophyte formation. Inflammation of the synovium and increased synovial fluid was present in all mACLT joints (Figure 3 for mACLT and Figure 4 for control images). Morphological scoring was highest in the lateral posterior region followed by the lateral central and medial central regions. (Table 1)

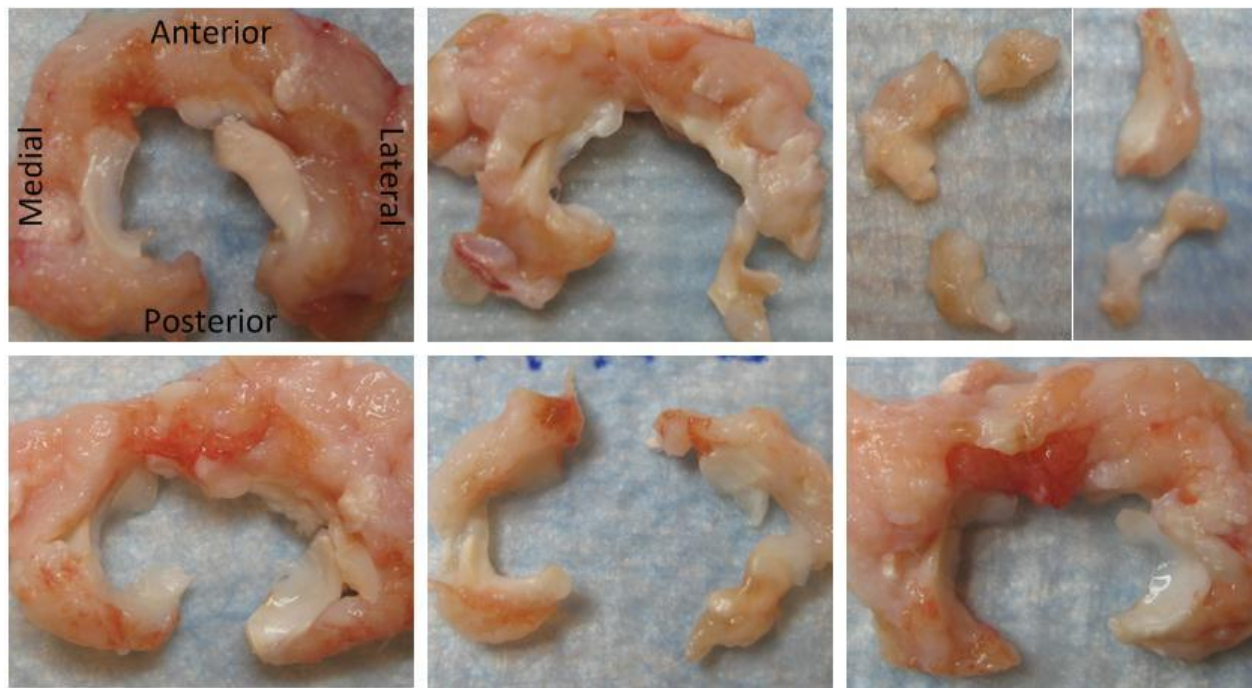


Figure 2.3. Menisci 12 weeks post-surgery. (animals 1-6 left to right and top to bottom, all specimens are oriented identical to the first image)



Figure 2.4. Control menisci

Table 2.1. Morphological scores at 12 weeks for mACLT menisci

Animal	Medial			Lateral		
	A	C	P	A	C	P
1	1	0	2	2	3	4
2	1	2	1	1	4	4
3	4	4	4	1	4	4
4	2	1	1	1	3	0
5	0	4	4	0	4	4
6	2	4	1	4	2	0

Mechanics

Due to the state of tissue damage (Table 1), various regions of the menisci were unable to be mechanically tested, leading to varying sample sizes. Lateral meniscus had a sample size of $n=5$ in the anterior region, with the more damaged central and posterior regions having sample sizes of $n=1$ and $n=2$, respectively. The medial meniscus had enough testable tissue for $n=5$ in both the anterior and posterior regions while the central region had $n=2$. All regions of the contralateral (control) limb had a sample size of $n=6$. Because mechanical data was lacking for specific regions, data from the anterior, central, and posterior regions were averaged for each animal and evaluations were made at the hemijoint level to allow for statistical analysis (Figures 5 A and B).

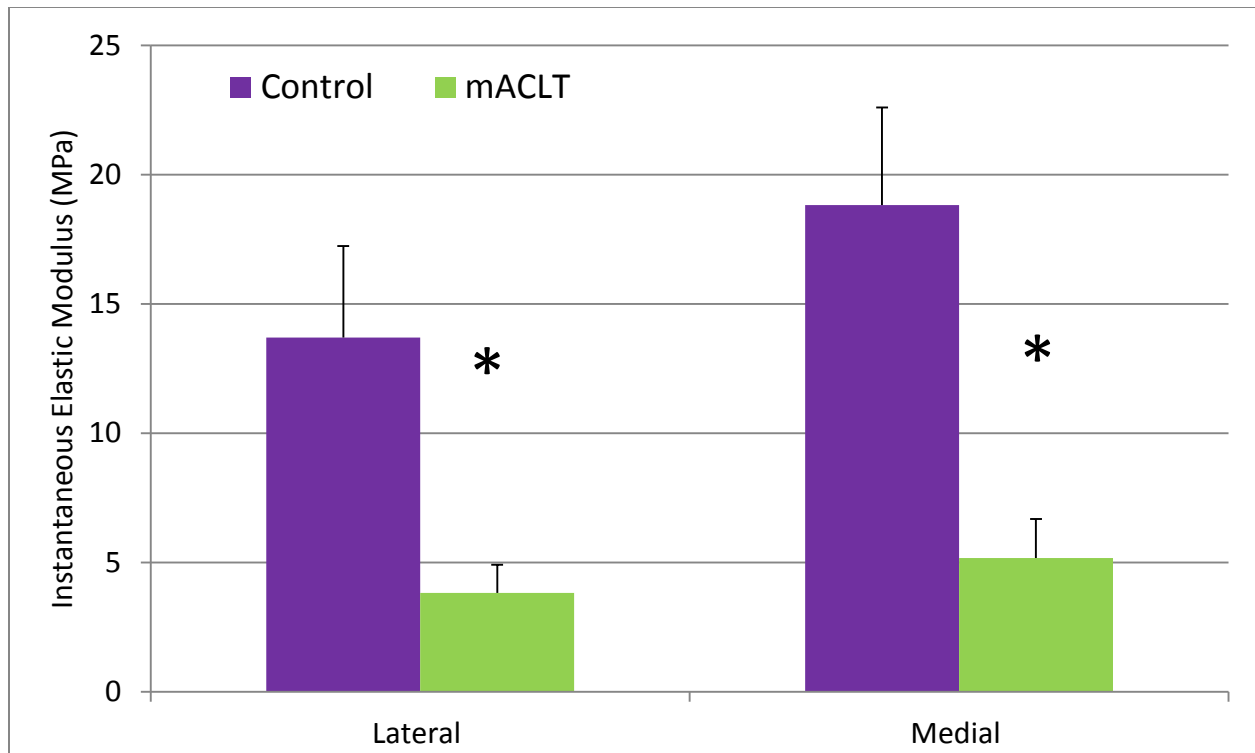


Figure 2.5A. Instantaneous elastic modulus by hemijoint (mean with standard error) *denotes significant difference between control and mACLT.

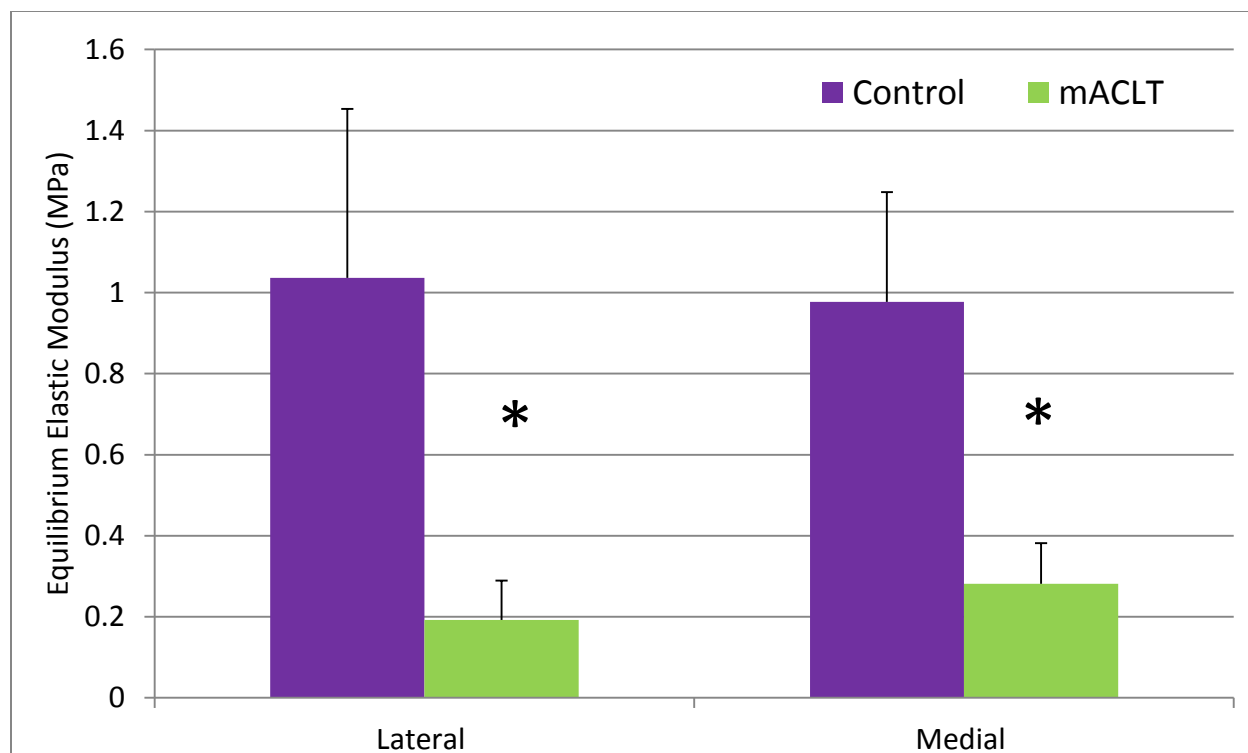


Figure 2.5B. Equilibrium elastic moduli by hemijoint (mean with standard error) *denotes significant difference between control and mACLT

Mechanical results showed significant decreases ($p < 0.05$) in both the instantaneous and equilibrium elastic moduli between the control and transected limbs in both the medial and lateral hemijoints. Instantaneous elastic moduli decreased $72.1 \pm 11.1\%$ from control to transected limb in the lateral hemijoint and $72.5 \pm 17\%$ in the medial hemijoint. The equilibrium elastic moduli decreased $81.4 \pm 12.4\%$ in the lateral hemijoint and $71.2 \pm 35.8\%$ in the medial hemijoint.

GAG

Enough tissue was present in all regions for histological analysis allowing for an $n=6$ in all regions. Saf-O staining intensity was evaluated at the regional level and tended to be less intense in the injured limb compared to the control limb, indicating a decrease in GAG concentration (Figure 6). Significant differences ($p < 0.05$) between the control and mACLT group for GAG

concentration were found in the lateral anterior, lateral central, lateral posterior, and medial anterior regions.

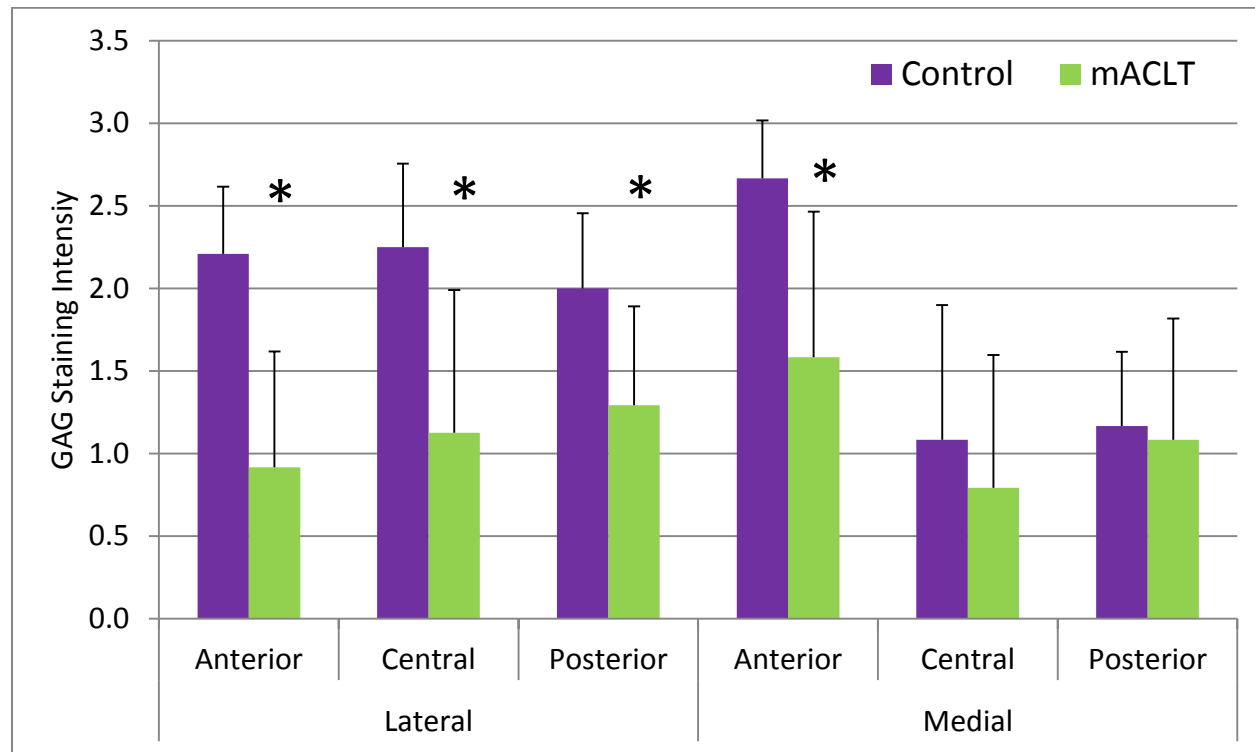


Figure 2.6. GAG staining intensity (mean with standard error) *denotes significant difference between control and mACLT

GAG coverage decreased from the control to transected limbs (Figure 2.7). Percent GAG coverage was reduced in all regions in the mACLT model, and was significantly lower in all 3 lateral regions ($p < 0.01$) and in the medial anterior region ($p < 0.05$). When averaging regions, the lateral control menisci showed 30 ± 5.2 % GAG coverage compared to only 10.1 ± 4 % GAG coverage in the mACLT menisci. Similarly in the medial menisci GAG coverage was 20.2 ± 7.9 % compared to only 10 ± 5.1 % GAG coverage in the mACLT menisci.

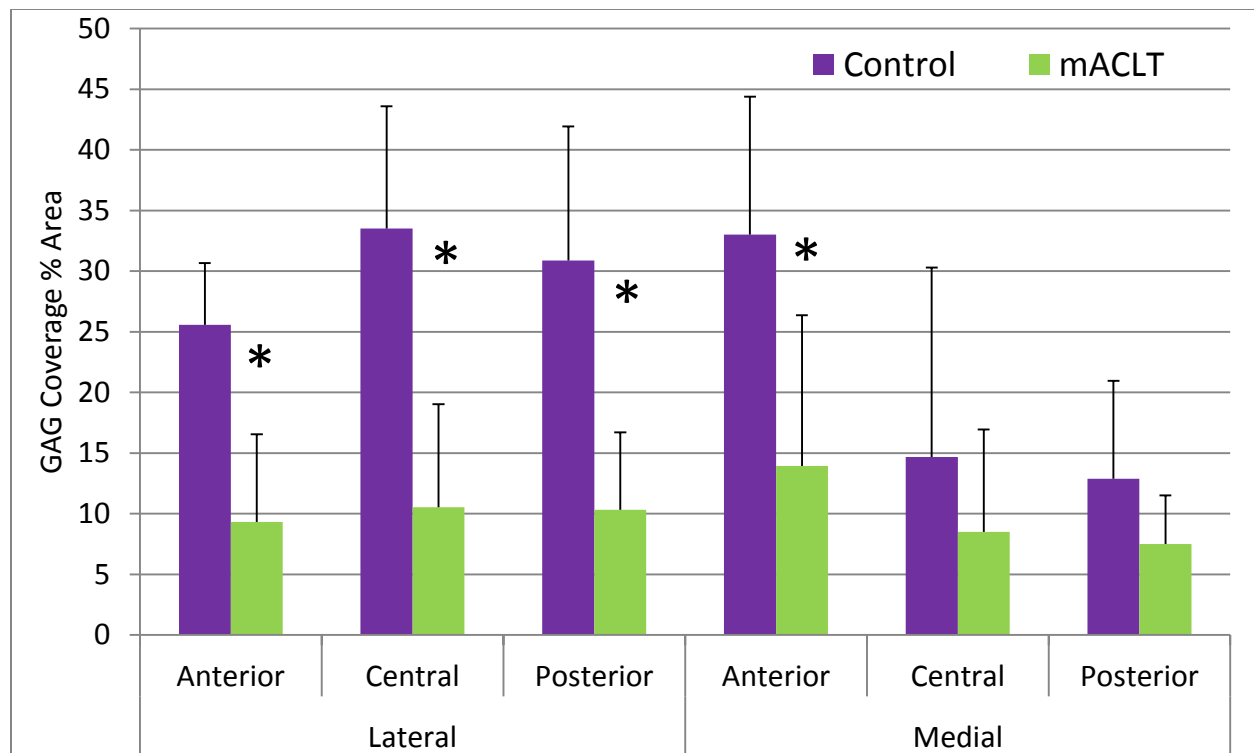


Figure 2.7. Percent area of GAG coverage (mean with standard error) *denotes significant difference between control and mACLT

Discussion

The findings of this study showed that ACL injury combined with meniscal damage results in a significant decrease in meniscal elastic modulus and significant decreases in GAG coverage over time. In general, surgical transection of the meniscus introduced un-displaced tears that eventually became displaced or lead to tissue degradation and loss. Damage occurred most often in the medial and lateral central regions and the lateral posterior region. This localization of damage was expected as these were the regions where initial damage was induced acutely, and chronic damage has been seen clinically. ^{Chan1991, Greis2002, Smith2001}

Tissue maceration limited sample sizes in the central and posterior regions of the lateral meniscus and the central region of the medial meniscus. However, the remaining regions (medial

anterior, medial posterior, and lateral anterior) showed significant decreases ($p < 0.05$) in both the instantaneous and equilibrium moduli between the control and mACLT menisci. Despite the inability to run statistical analysis on the lateral central, lateral posterior and medial posterior; these regions were trending a decrease between control and transected limb for both the instantaneous elastic moduli and the equilibrium elastic moduli. It is hypothesized that this decrease would have been significant if a larger sample size was used.

The majority of previous PTOA models have used the traditional ACLT model and often times focus on cartilage and bone changes, without documenting changes in the material properties of the menisci. One previous study ^{Sweigart2004} did evaluate equilibrium moduli in the medial meniscus of healthy rabbits. A direct comparison of this data and our control data is difficult as sectioning and testing methods were different. However, the trending pattern of a higher modulus in the anterior region of the medial meniscus seen in the current study was consistent with this earlier study.

Histological analyses indicated GAG coverage decreased 12 weeks after joint injury. Staining intensity tended to decrease from control to transected limb as well, with significant decrease in the lateral anterior, lateral central, lateral posterior, and medial anterior regions. All regions with significant decrease in staining intensity also showed significant decreases in GAG coverage. Data from the control limb is in agreement with a previous study of healthy lapine menisci ^{Killan2010} as well as to GAG coverage in human studies. ^{Bursac2009} GAG coverage was found to be relatively uniform in the lateral hemijoint, while the medial anterior region had significantly more GAG than the medial central and posterior regions. This variation in GAG coverage may

be responsible for differences in mechanical properties seen in the medial meniscus in the current and previous lapine studies.^{Sweigart2004}

There are conflicting reports as to whether or not GAG presence decreases in osteoarthritic knees. Some studies have shown an increase in GAG in osteoarthritic human knees.^{Pauli2011, Sun2012} However, a canine study using an ACLT model showed an initial decrease in GAG (1 week) with levels returning to normal or above normal over an extended period of time (3-18 months).^{Adams1983} Based on this it is hypothesized that GAG decreases due to initial trauma, but if altered loading persists GAG production may rebound and increase in an attempt to compensate for damaged tissue. Developing a longitudinal study to look at time points before and after 12 weeks would help to better understand these post trauma changes in GAG content of the meniscal tissue.

Both elastic modulus and GAG coverage significantly decreased from the control to transected menisci at the hemijoint level. Looking at overall decreases from mean values the lateral hemijoint experienced a larger decrease compared to the medial hemijoint. Unfortunately due to tissue damage, regional comparisons between GAG and elastic moduli were not possible in the current study. It is believed that the instantaneous elastic modulus is more closely related to the collagen content while equilibrium modulus is likely more closely associated with GAG content. There were some limitations in the current study including the semi-qualitative nature of determining Saf-O coverage and intensity. Furthermore, some studies have indicated Saf-O staining for proteoglycan content is less accurate in diseased tissues.^{Camplejohn1988} The possibility also exists that animal variability caused differences in transection location and size as the

surgeries were performed by eyesight. However, this may have been limited by the use of a single surgeon (CD) for the surgeries. Finally, the effect of the isolated meniscal transections, without damaging the ACL, is yet unknown. For this reason future studies looking at just the meniscal transections may prove helpful in this regard.

When compared to traditional transection models, the mACLT model showed a number of notable differences. Numerous studies of articular cartilage in ALCT lapine studies have shown more extensive damage in the medial hemijoint.^{Batiste2004, Chang1997, Yoshioka1996} Similarly, ACLT models that have reported gross meniscal damage^{Adams1983, Brophy2012, Burger2007, HelioLeGraverand2001, Smith2002, Yoshioka1996} have also shown damage to be more extensive in the medial meniscus. The theory behind the more highly worn medial compartment is that without the stability offered by an intact ACL, the medial meniscus acts to prevent tibial translation.^{Allen2000, Shoemaker1986, Thopson1993, Warren1983} This in turn causes more wear in the medial as opposed to lateral hemijoint. With the mACLT model, chronic damage did not favor the medial compartment. In fact the lateral compartment showed more gross damage, a greater decrease in equilibrium elastic moduli, and a greater decrease in GAG coverage. Most importantly, based on tear type and occurrence, the mACLT model also showed more extensive meniscal damage to both hemijoints than previously reported in studies using the ACLT model. For example, a 12 week ACLT study by Smith et. al.^{Smith2002} resulted in medial severe tearing in 20% of the samples, mild tearing in 30%, and normal menisci in 50% of the samples. Using the same metrics our mACLT model demonstrated severe tearing in 67% of the medial samples and mild tearing in 33% of our samples. This difference between the two models is even more evident in the lateral hemijoint. Smith et. al. reported only 10% of their samples having a tear in the lateral meniscus meanwhile

100% of the mACLT lateral menisci showed severe tearing. While this comparison is slightly biased since damage was introduced initially in the mACLT model, the current study suggested that the combined ACL and meniscal trauma in the mACLT model resulted in more severe degradation of meniscal tissue.

Current studies are underway to document changes in articular cartilage, synovial fluid and subchondral bone associated with this mACLT model. Combining all these data will allow correlations to be made between damage to the menisci, articular cartilage, and bone. Additionally, more time points will help to develop a longitudinal study that will document the changes in mechanical and histological properties of the menisci over time in order to more fully correlate property changes with the degree of meniscal tearing.

Chapter 3 - Meniscal Mechanical and Glycosaminoglycan Changes in a Traumatic Impact Model

This work presented in this chapter will be submitted for publication in a biomechanical journal

Introduction

Osteoarthritis (OA) has been considered by the World Health Organization to be among the top 10 conditions representing a global disease burden.^{Lopez2006} While OA is often a result of general joint degradation over time, early onset can be triggered by a number of factors^{Felson1998, Felson2000, Lawrence2008, Srikanth2005, Zhang2001} including joint trauma.^{Gelber2000, Lohmander2007} With high incidence of knee injury^{Hootman2002} and knee OA,^{Zhang2008, Oliveria1995, Felson1988} posttraumatic osteoarthritis (PTOA) of the knee has been of particular interest to researchers and clinicians.

It has been reported that as high as 78% of knee injuries involving anterior cruciate ligament (ACL) tears are due to “non-contact” type of injuries.^{Noyes1983} High compressive tibiofemoral loading, often experienced during jump landings^{Boden2000, Felson2004, Yeow2008, Meyer2005, Speer1995, Hewett1996, Ettlinger1995} is one mode of non-contact ACL rupture. These compressive loads have been shown to cause acute damage to articular cartilage, bone, and the menisci.^{Killian2010, Meyer2005, Levine2012, Mankin1982, Meyer2008} both clinically and in the laboratory settings. Current models of knee PTOA have primarily used *in vivo* animal models with surgical transection of the ACL to model damage to joints.^{Intema2010, Yoshioka1996, LeGraverand2001, Batiste2004, McDevitt1976, Pond1973} However, these ACL transection (ACLT) models do not account for occult and acute damage to the surrounding structures which is often present in ACL injuries.^{Rosen1991, Atkinson2001, Issac2010}

One structure often injured in conjunction with the ACL is the meniscus.^{McDaniel1980, Felson2004}

The menisci are fibrocartilaginous structures found between the femoral condyles and tibial plateau, taking a semilunar shape with a wedge profile. The main role of the menisci is to distribute load and stabilize the knee joint.^{Shrive1978, Mow1992, Fithian1990, Arnocxky1996} Their unique

wedge like shape and composition allow them to bear up to 75% of loads seen in the knee joint,

^{Shrive1978} Meniscal tissue is biphasic with fluid flow regulated in part by aggrecan, a proteoglycan with glycosaminoglycan (GAG) side chains. In mature meniscal tissue, vascularization is only present in the outer most region of the tissue accounting for 10-30% of the total meniscus.

^{Clark1983} This lack of blood circulation limits the potential healing capacity of this tissue. Meniscal damage that disrupts this normal distribution increases the force translated to the articular cartilage, which may lead to increased cartilage damage and osteophyte development^{Crema2010,}

^{McCann2009}. While particular focus in OA research has been given to articular cartilage, the role the menisci play in load distribution suggests they are likely involved in the development of OA^{McGonagle2010, Fairbank1948} and should be further investigated.

To better recapitulate what is seen in non-contact ACL rupture scenarios, an *in vivo* tibiofemoral impact lapine model has been developed. Previous work with this tibiofemoral impact (ACLF) model^{Killain2010, Issac2010} has shown that in addition to ACL rupture the impact produces acute meniscal, cartilage, and bone damage. The objective of this study was to assess the gross, mechanical, and histological changes to the menisci 12 weeks following impact. It was hypothesized that any untreated acute meniscal damage would result in a decrease in both the instantaneous and equilibrium moduli. Furthermore from previous reports of GAG increases in

osteoarthritic patients^{Pauli2011, Sun2012} it is hypothesized that this ACLF model will result in an increase in GAG content in the impacted limb.

Methods

ACLF Model

This study was approved by Michigan State University and Colorado State University All-University Committees on Animal Use and Care. Six skeletally mature Flemish Giant rabbits ($5.7 \pm .2$ kg) were anesthetized (2% isoflurane and oxygen) and subjected to an impact of the right tibiofemoral joint. Animals were housed in individual cages (60 x 60 x 14 in), and were euthanized twelve weeks post injury.

Impact was delivered similar to previous studies.^{Issac2008} In brief rabbits were placed in the supine position with the right tibiofemoral joint at 90° flexion and a 1.75 kg mass attached to a pre-crushed Hexcel (Hexcel, 3.76 MPa crush strength) head was used to impact the distal femur (Figure 3.1). The impact interface was mounted in front of a 4.45 kN (1000 lb) load transducer (model AL311CV, 1000 lb capacity, Sensotec, Columbus, OH). Following impact right limbs were subjected to an anterior drawer test to evaluate ACL rupture. Within 72 hours magnetic resonance imaging (MRI) was performed to verify ACL tearing and document meniscal damage. Left limbs of all animals were un-impacted and acted as paired controls. The rabbits favored the contralateral limb for the first 3-5 days, but showed no signs of gait abnormality for the duration of the study. A licensed veterinary technician monitored all rabbits and buprenorphine (0.3mL/kg BW) was given every 8 hours for 72 hours following impact for pain.

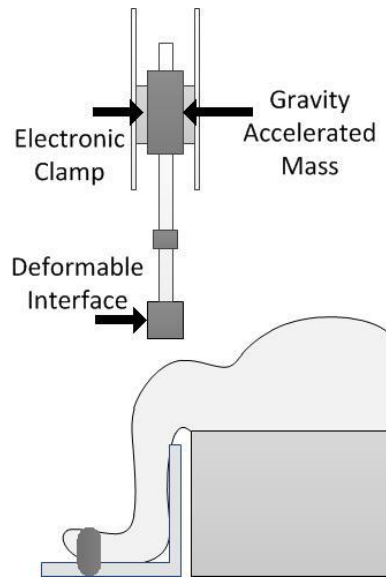


Figure 3.1. Diagram of impact

Tissue Harvesting and Morphological Assessment

Both the lateral and medial menisci were harvested immediately following sacrifice and were refrigerated (1.7 to 3.3°C) until mechanically tested (within 18 hours). The menisci were assessed for morphological damage and photographed. Any visible damage was recorded and morphologically scored similar to previous grading systems.^{Matyas2004, Pauli2011} Scoring was performed by four individuals and blinded to prevent bias. The data were then averaged for each subject limb. Blinded graders were asked to assign a score from 0-4 to three different regions (anterior, central, and posterior)^{Killian2010} for both the medial and lateral menisci, with 0 = normal, 1= surface damage, 2 = un-displaced tears, 3 = displaced tears, and 4 = tissue maceration. Grading, as well as mechanical and histomorphological assessments, were performed regionally to account for previously identified regional variations.^{Fithian1989, Chia2008,}

Sweigart2004, Killian2010

Mechanical Analysis

An indentation, relaxation test (Bionic Model 370.02 MTS Corp, Eden Prairie, MN) was performed on meniscal tissue to determine instantaneous and equilibrium elastic moduli. Specimens with enough intact tissue in a given region were mounted on a wedge and placed in a 0.9% phosphate buffered saline bath (PBS). The set up was similar to that done previously for articular cartilage and menisci.^{Hoch1983, Li2006, Sweigart2004} The bath was attached to a camera mount and an x-y plate such that specimen position could be adjusted to allow the indenter to act normal to the surface of the tissue. The indenter chosen was a cylindrical steel tip with a diameter of 1.59mm, and an 8.9 N load cell (Futek LSB200, Irvine, California) was used to record load.

Specimens were subjected to a preload of 20 mN to establish contact between indenter and sample. Based on the average specimen size and indenter tip diameter, an indentation depth of 0.25 mm was applied, and held for 900 seconds. Similar to previous studies^{Li2006} Hertzian contact was assumed between an elastic half space, the meniscus, and a ridged sphere, the indenter. A Poisson's ratio of .01 was assigned to all regions of the menisci based on an average from a previous study.^{Sweigart2004} The elastic modulus and Poisson's ratio of the indenter were 210 GPa and 0.3 respectively. A paired sample t-test was performed on both the instantaneous and equilibrium data and significance determined when $p < 0.05$.

Histological Analysis

After mechanically testing each specimen, tissue was fixed in 10% formalin. After 14 days each specimen was embedded in optimum cutting temperature medium (OCT, Pelco; Redding, CA)

and flash frozen using liquid nitrogen. The cut face of each specimen was then cryosectioned into 6µm slices. Hematoxylin, Safranin-O (Saf-O) and Fast Green (FCF) were used to stain for GAG. This staining method resulted in nuclei staining black, GAG red, and cytoplasm green.

Once stained, slides were imaged using an Olympus BH2 Microscope (Center Valley, PA) and MicroPublisher 5.0 RTV camera. As done previously ^{Pauli2011} GAG staining intensity was evaluated as follows: 0 = no staining, slight staining = 1, moderate staining = 2, and strong staining = 3. (Figure 3.2) Staining intensity evaluations were performed by 4 separate individuals and data from each specimen and location was averaged across individuals.

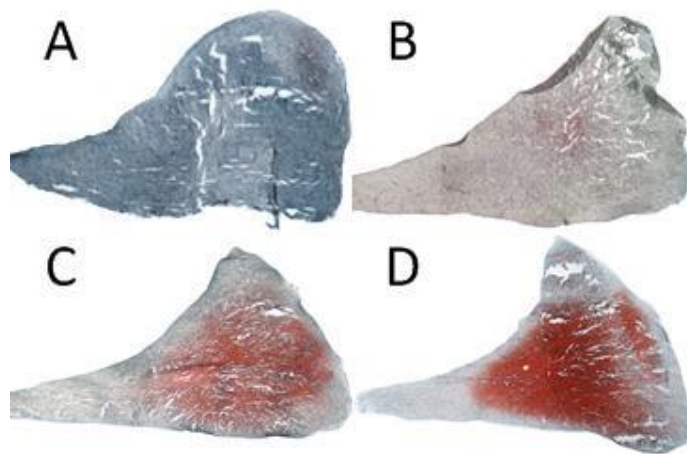


Figure 3.2. Safranin-O-Fast Green staining intensity: (A) no stain = 0 (B) slight staining = 1 (C) moderate staining = 2 (D) strong staining = 3

GAG coverage was analyzed semi-quantitatively using Image J (NIH, Bethesda, MD) with FIJI package. First the total area of tissue was calculated. Isolating the red associated with GAG from the blue/green of the cytoplasm, total area of GAG coverage was determined. Percent GAG coverage was calculated from these two measures. Paired t-tests were performed between the

impacted and control limbs with significance determined when $p < 0.05$ for both staining intensity and GAG coverage.

Results

Morphology

Acutely, all impacted joints experienced ACL failure. Acute meniscal damage was present in 5 of the 6 medial menisci and 3 of the 6 lateral menisci. Acute damage was almost exclusively located in the posterior horn, and most commonly identified as longitudinal vertical tearing. All contralateral controls showed no gross morphological damage at the time of dissection. At twelve weeks post impact all impacted joints demonstrated some level of osteoarthritic changes including osteophyte formation, fibrillation, and synovial inflammation (ACLF menisci Figure 3.3 and control menisci Figure 3.4). Chronically the medial meniscus sustained more damage than the lateral (a score ≥ 3 in 11/18 medial regions and only 6/18 lateral regions). However, in both hemijoints damage was most severe in the posterior and central regions. Tearing was seen chronically in all 6 medial menisci and 5 of the lateral menisci. (Table 3.1)

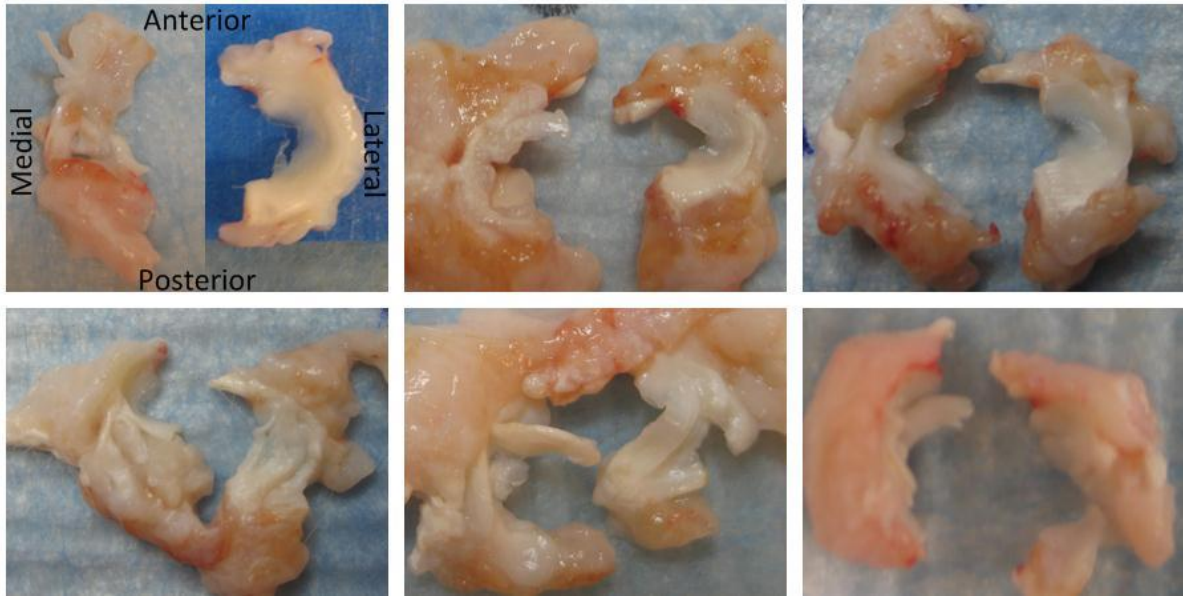


Figure 3.3. Menisci 12 weeks post-impact. (animals 1-6 left to right and top to bottom, all specimens are oriented identical to the first image)



Figure 3.4. Representative control menisci

Table 3.1. Morphological scores at 12 weeks for ACLF menisci

Animal	Medial			Lateral		
	A	C	P	A	C	P
1	1	3	3	0	0	2
2	2	3	4	0	0	0
3	0	4	0	0	0	0
4	2	3	3	2	3	3
5	0	3	4	0	3	4
6	1	3	4	2	4	4

Elastic Modulus

Tissue damage limited mechanical testing in certain regions. The lateral anterior region was the only full sample set at n=6. The remaining regions were as follows: lateral central and posterior n=5, medial anterior n=4, medial central n=2, and medial posterior n=1. Because statistical analysis could not be performed on the medial central and medial posterior regions due to small sample sizes, the collected regional data was averaged for a given hemijoint and specimen. (Figure 3.5A and 3.5B)

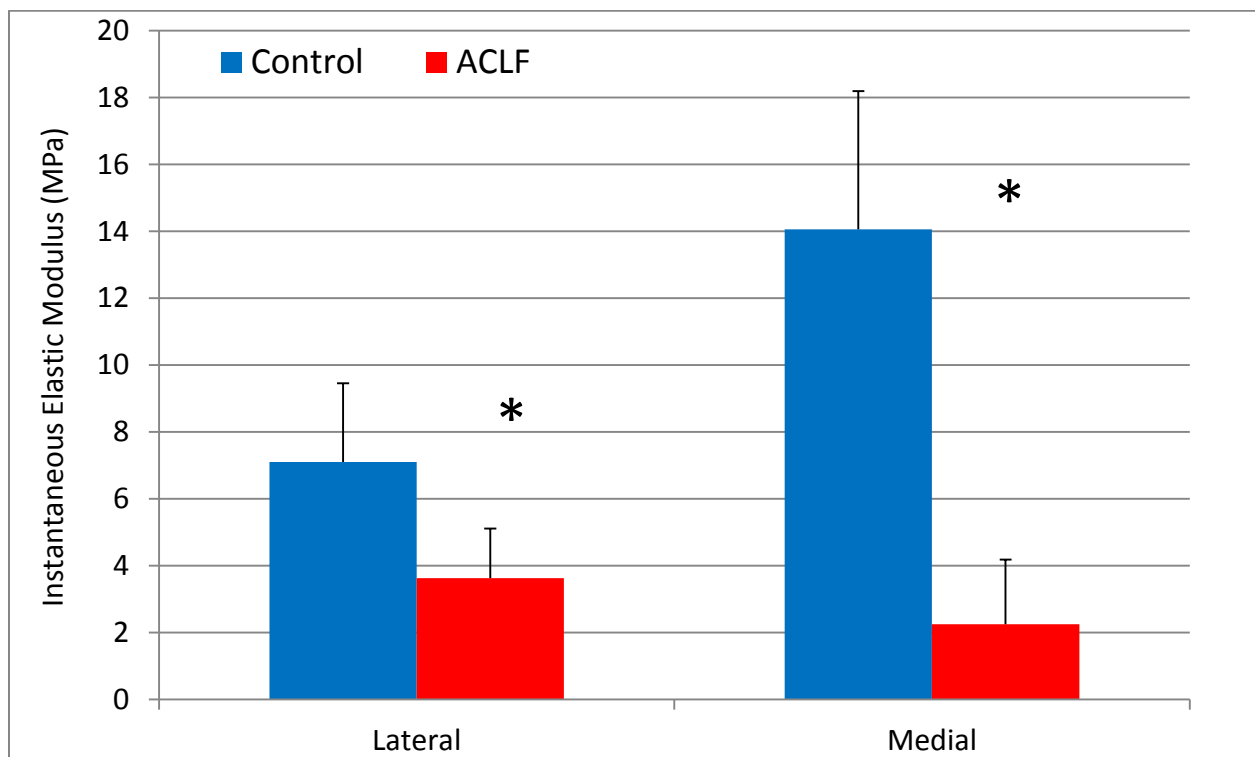


Figure 3.5A. Instantaneous elastic modulus by hemijoint (mean with standard error) *denotes significant difference between control and ACLF.

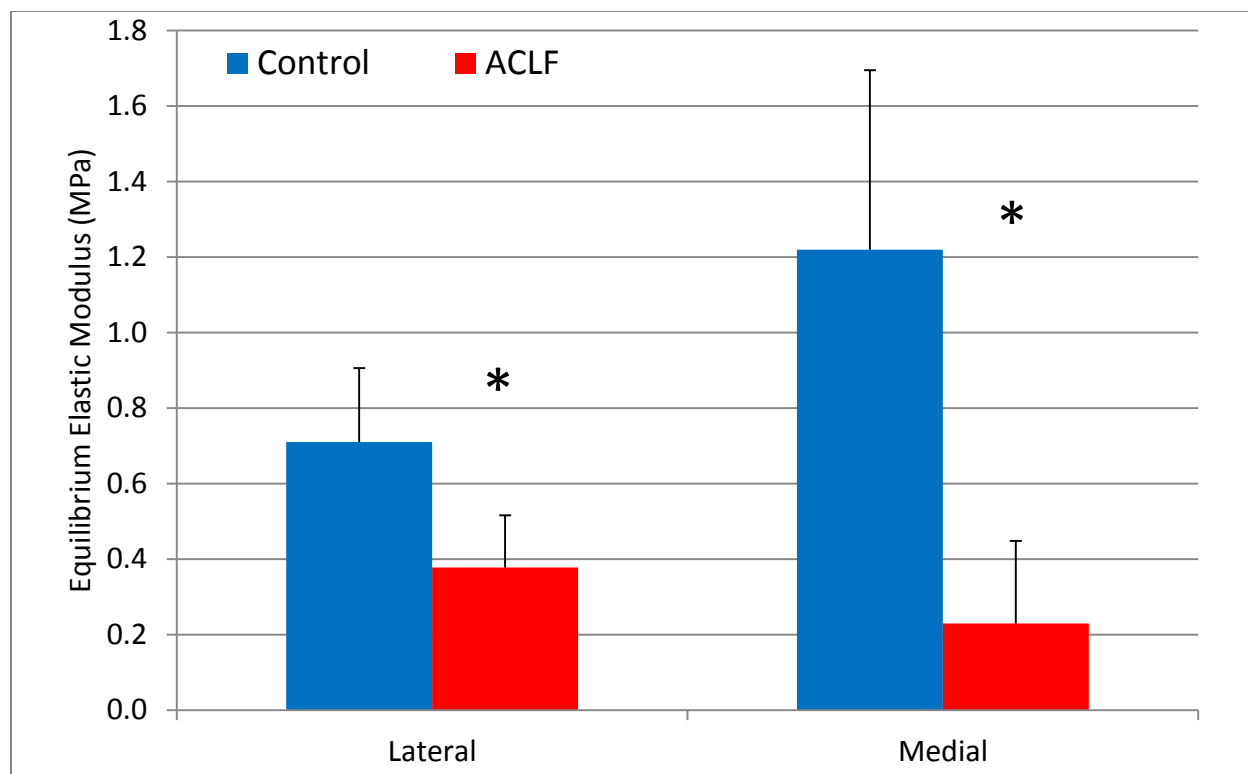


Figure 3.5B. Equilibrium elastic modulus by hemijoint (mean with standard error) *denotes significant difference between control and ACLF

Significant decreases ($p < 0.05$) in the instantaneous and equilibrium elastic moduli were documented in both hemijoints. The medial meniscus indicated the largest decrease from un-impacted to impacted limb in both the instantaneous elastic modulus and the equilibrium elastic modulus ($78.4 \pm 26.9\%$ and $75.4 \pm 24.3\%$ respectively). The lateral meniscus experienced an instantaneous elastic moduli decrease of $48 \pm 24\%$ and an equilibrium elastic moduli decrease of $46.1 \pm 23.5\%$ from un-impacted to impacted limb. Damage to the medial central and medial posterior regions reduced the number of mechanically testable samples in those regions. However of the remaining four regions (lateral anterior, central, and posterior and medial anterior) significant decreases were seen in the lateral anterior, lateral central, and medial anterior both instantaneously and at equilibrium.

GAG

All samples were sliced and stained for GAG (n=6 in all regions). Staining intensity decreased in both anterior regions, remained relatively the same in the central regions, and slightly increased in the posterior regions (Figure 3.6). The only significant was observed in the anterior regions where GAG intensity decreased from the control to ACLF meniscus.

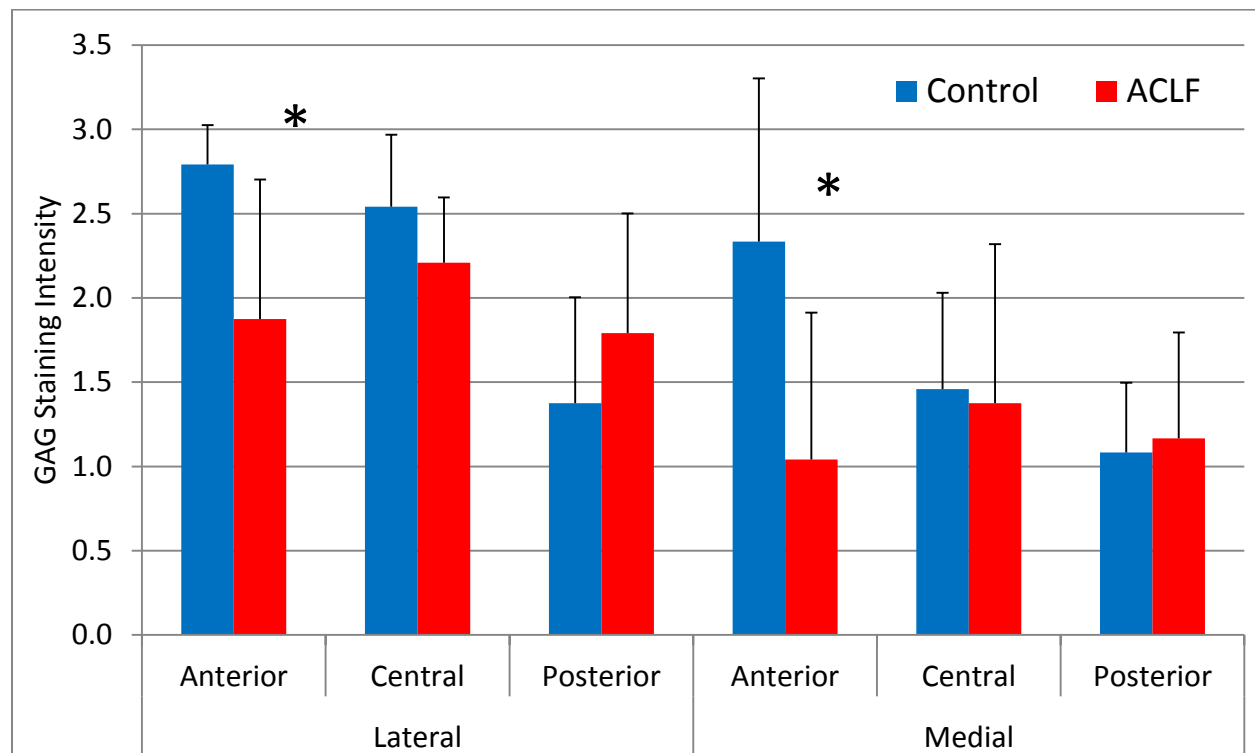


Figure 3.6. GAG staining intensity (mean with standard error) *denotes significant difference between control and ACLF

GAG coverage trended similar to GAG intensity with the largest decrease seen in the anterior regions. Significant decreases in GAG coverage between impacted and contralateral limb were found in both hemijoints with the lateral meniscus decreasing from 33±16.4% to 22±17.1% ($p<0.05$) while the medial hemijoint decreased from 23±24% to 11±12% ($p<0.05$). However,

regional evaluations offer a better representation of the changes. (Figure 3.7) Significant decreases in GAG coverage were seen in the lateral anterior ($p<0.05$), lateral central region ($p<0.05$) and the medial anterior regions ($p<0.02$). While not significant, both the lateral posterior and medial posterior increased slightly in GAG coverage in the impacted limb compared to the un-impacted.

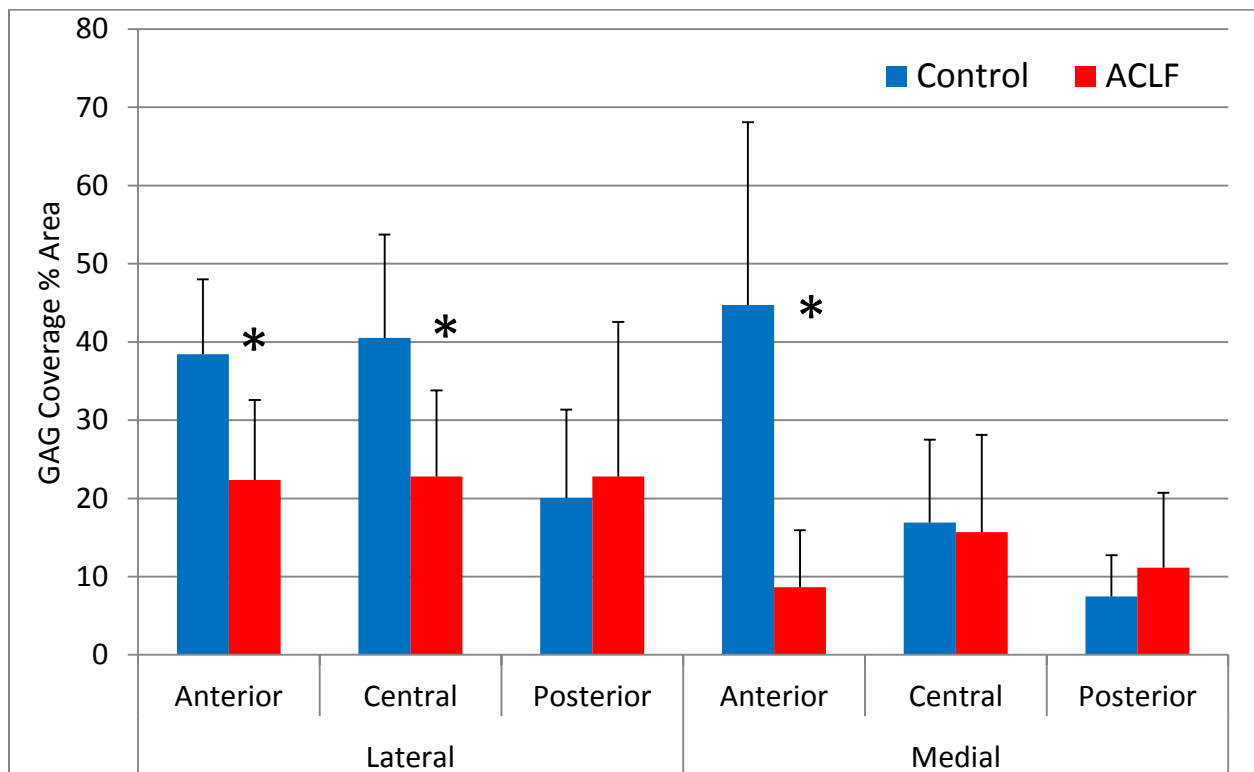


Figure 3.7. Percent area of GAG coverage (mean with standard error) *denotes significant difference between control and ACLF

Discussion

Using this *in vivo* closed joint traumatic impact model we were able to create meniscal damage and ACL rupture following a single tibiofemoral impact. This is in agreement with other studies documenting meniscal damage following compression loading in both human and animal

studies.^{Dumont2012, Killian2010, Levine2012} Chronically more damage was seen in the medial hemijoint as opposed to the lateral, which is consistent clinically in OA patients.^{Bellabarba1997, McDaniel1980, Smith2001, Thompson1993, Wickiewicz1990}

This is one of the first studies to look at whole body meniscal mechanical properties in both healthy and OA tissue of rabbit. Sweigart et. al. did perform creep indentation testing on both the tibial and femoral surfaces of the medial meniscus of healthy rabbit and found that there was a decrease in equilibrium elastic moduli from anterior to posterior regions.^{Sweigart2004} While equilibrium elastic moduli values between the Sweigart et. al. study and this current ACLF study cannot be compared due to differences in testing protocols, the trend of decreasing moduli in the medial hemijoint is similar. This same trend has been seen shown in studies of healthy bovine meniscal tissue as well.^{Proctor1989}

The irregular changes in GAG coverage and staining intensity of the impacted limb are of particular interest. GAG coverage and staining intensity were shown to decrease in the regions of lesser damage while they remained constant or slightly elevated in the more heavily damaged regions. A previous canine ACL transection study^{Adams1983} documented initial decreases in GAG with levels returning to normal or above normal over time. This initial decrease in GAG could be due to an inflammatory response, as numerous studies^{Lemke2010, McNutty2010, Voight2009, Wilusz2008} have demonstrated GAG degradation in inflammatory conditions. Additionally there may be a biochemical response to altered loading due to meniscal and ACL damage. Immobilization has been shown to decrease GAG content in the meniscus^{Djurasovic1998}, and dynamic overloading under specific conditions has been shown to up-regulate aggrecan production.^{Zielinska2009} We are

limited in our assessment of GAG changes, as they may be temporal and this study is presenting results from a single time point of 12 weeks.

The main tearing orientation of the acute damage created in the ACLF model was longitudinal vertical tears, which have been reported as the most common in clinical injury scenarios.

^{Lewandrowski1997, Tandoğan2004} Meniscal damage in the ACLF model was most commonly seen in the posterior region which is also in agreement with clinical findings. ^{Smith2001, Chan1991, Greise2002} When

compared to previous ACLT models ^{Smith2001, Brophy2012, Yoshioki1996, HelioLeGraverand2001, Adams1983,}

^{Burger2007} the chronic gross meniscal damage observed in the ACLF model is more extensive with a higher incidence of tearing and more severe tearing patterns. While chronically past ACLT models have recorded primarily incomplete tears and the occasional bucket handle tears ^{Adams1983,}

^{Smith2001, LeGraverand2001}, the ACLF model produced full thickness, displaced, and complex tearing of meniscal tissue. The differences between models suggests that when acute damage is present the pattern and progression of meniscal damage is different and more rapid than in a purely ACL deficient model.

Some limitations of this study include limited sample sizes for mechanical testing due to extensive damage. A more complete data set would have allowed for more regional comparisons. However, the anterior regions of the impacted limb (both medial and lateral) provided the most testable tissue, and given that the anterior regions have been shown to have higher moduli values, the presented data is a “worse case” scenario. By averaging regional values for a given meniscus, the impacted data may represent a higher value due to the higher incidence of anterior regions. Had all regions been able to be mechanically tested it is likely that the decreases in

moduli between impacted and non-impacted limbs would have been greater. Another limitation is the semi-quantitative nature of determining Safranin-O coverage. However, one individual (KMF) processed all images to limit viewer to viewer discrepancies. There have been reports suggesting that Saf-O/FCF staining of diseased tissue does not yield accurate proteoglycan measurements, ^{Camplejohn1988} yet it remains as a standard for qualitative GAG analysis. ^{HelioLeGraverand2001, Chevrier2009, Brophy2012, Killian2010} Lastly, some of the findings in this study make it impossible to identify if they are progressive or temporal. Therefore to better understand the process a longitudinal study is underway to look at time points of 4 and 8 weeks post-trauma.

Traditional ACLT models have shown that destabilization of the joint can lead to the early development of OA, but fail to address damage to surrounding structures often seen following injuries. The ACLF model may, in this regard, provide a more thorough understanding of whole joint changes following a compressive tibiofemoral impact. This is one of the first studies to document changes to the menisci following an impact loading. While focus is often placed on articular cartilage and bone changes, it is clear from these results that significant changes are occurring in the menisci and should be considered in future knee PTOA studies. Correlating this work with parallel studies on the articular cartilage, bone, and synovial fluid from these ACLF animals will provide one of the first comprehensive investigations of the whole joint.

Chapter 4 – Comparison of Acute and Traumatic MRI Readings in Two Models of Knee Osteoarthritis

The material contained in this chapter will be submitted for publication in an imaging journal.

Introduction

Once thought to be vestigial structures ^{Setton1897}, in the past when menisci were injured they were simply removed. A better understanding of the function of the menisci in load distribution, joint stabilization, and joint lubrication has resulted in more interest and investigation. ^{Chevrier2009, Fithian1990, Shoemaker1986, Walker1975} The menisci are now thought to play a role in the development of knee osteoarthritis, ^{McGonagle2010} a debilitating joint disease affecting an estimated 37% of individuals under 60 years of age. ^{Dillon2006}

Posttraumatic osteoarthritis (PTOA) is a form of secondary osteoarthritis resulting from joint trauma. A number of models have been used to recapitulate PTOA for the purpose of investigating the pathogenesis of the disease. One of the most widely used models is an anterior cruciate ligament transection (ACLT) model, where the ACL is transected and degradation of the joint structures is then monitored over time. However this model does not take into account acute meniscal damage that is often seen in conjunction with ACL tears, ^{Felson2004, McDaniel1980} nor does it account for common compressive tibiofemoral forces through the joint at the time of injury. ^{Boden2000, Ettlinger1995, Felson2004, Hewett1996, Meyer2005, Speer1995, Yeow2008} For this reason two new lapine models have been developed: a modified ACLT (mACLT) model ^{Fischenich2013} and a traumatic impact (ACLF) model. ^{Issac2008} Similar to the ACLT model the mACLT model destabilizes the knee by transecting the ACL; however, in the mACLT model partial meniscal

transections are also introduced. The ACLF model induces ACL rupture and meniscal damage from a single blunt force impact to tibiofemoral joint.

Magnetic resonance imaging (MRI) of the knee is a common method of diagnosing damage to the menisci. Surgeons and researchers use information from MR images in diagnosing the severity of damage and determining the appropriate course of action following injury. Depending on location and extent of meniscal damage a surgeon may choose to proceed or not proceed with surgical measures. While noninvasive, the sensitivity of MR imaging at detecting various meniscal tears is highly dependent on location and tear type.^{Fox2007} Often times when damage is detected from MRI, an arthroscopy is then performed to verify damage. However, even an arthroscopy has a limited accuracy in detecting meniscal damage due to the difficulty of observing the tissue.^{Minns1988}

Combined ACL and meniscal injuries are most common in athletes, with many athletes returning to their sports after ACL reconstruction. However studies have showed that even with ACL reconstruction there is a much higher rate of OA development following trauma.^{Gillquist1999,}
^{Lohmander2007} For this reason it is important to monitor changes to other joint structures to help better understand the pathogenesis of OA. The objective of this study was to use MR imaging to document meniscal damage immediately following trauma (acute) and 12 weeks post injury (chronic) in both the mACLT model and ACLF model. Additionally visual inspection of meniscal damage was recorded following euthanasia and used for comparison. The hypothesis being acute meniscal damage will propagate and become more severe over time, and MR images provide an accurate indication of damage seen at dissection.

Methods

Animal Care

All animals were housed in individual cages (60 x 60 x 14 in) for the duration of the study which was approved by Michigan State University and Colorado State University All-University Committees on Animal Use and Care. Twelve skeletally mature Flemish Giant rabbits ($5.5 \pm .5$ kg) were used in this study. Animals were placed under anesthesia (2% isoflurane and oxygen) and the right limb of each animal was subjected to a trauma. The contralateral was unaffected and served as a control. Six animals received an impact of the tibiofemoral joint (ACLF), similar to previous studies.^{Issac2008, Killian2010} The remaining animals underwent a surgical transection of the ACL and a partial transection of both menisci (mACLT), which has been previously described^{Fischenich2013} All animals received buprenorphine every 8 hours for 72 hours for post-trauma pain, and were monitored by a licensed veterinary technician.

ACLF Model

Animals in the ACLF group were subjected to a closed-joint tibiofemoral impact, with the limb positioned at 90° flexion and unconstrained to allow for anterior tibial translation. Force was applied using a gravity accelerated mass of 1.75 kg attached to a pre-crushed Hexcel head (Hexcel, 3.76 MPa crush strength). To insure a single insult the impact sled was electronically arrested following impact. To insure ACL rupture and anterior drawer test was performed on all impact limbs. ACL tearing was also verified in a post impact MRI (Figure 4.1).



Figure 4.1. MRI image of ACL rupture (red arrows pointing to the two ends of the ACL)

mACLT Model

Animals in the mACLT group received an ACL transection followed by: a radial transection in the white zone of the central region of the medial meniscus with a longitudinal transection extending through the main body and a radial transection of the lateral meniscus in the white zone of the central region with a minor longitudinal tear extending anteriorly (Figure 4.2).

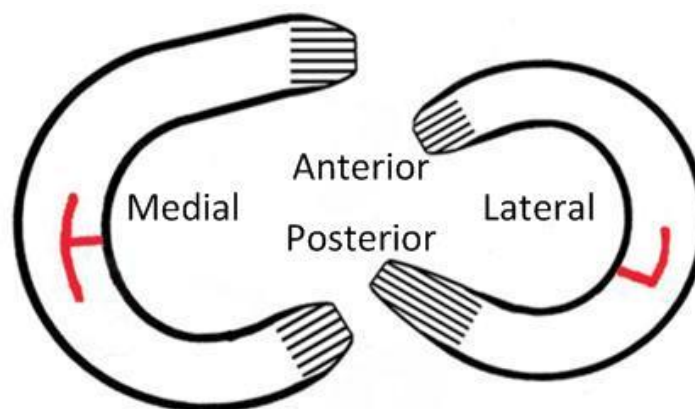


Figure 4.2. mACLT meniscal transections

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was used to document the condition of each joint following initial trauma as well as just prior to euthanasia, or 12 weeks post trauma. Damage was identified as being in the anterior horn, anterior junction, body, posterior junction, or posterior horn. (Figure 4.3) Imaging was performed on a GE (Waukesha, WI) HD x t 3.0 T magnet utilizing an 8 channel HD wrist coil. Sequences included sagittal and coronal proton density, 3000-5000 ms repetition time, 32-34 ms time to echo, 62.5 kHz receiver bandwidth, 2 excitations, 1.5 mm slice thickness with 0 interstice gap, 512 x 384 matrix size, and an 8 cm field of view, as well as sagittal and coronal fat suppressed proton density, 3000-5000 ms repetition time, 32-34 ms time to echo, 50 kHz receiver bandwidth, 2 excitations, 1.5 mm slice thickness with 0 interstice gap, 416 x 256 matrix size, and an 8 cm field of view.

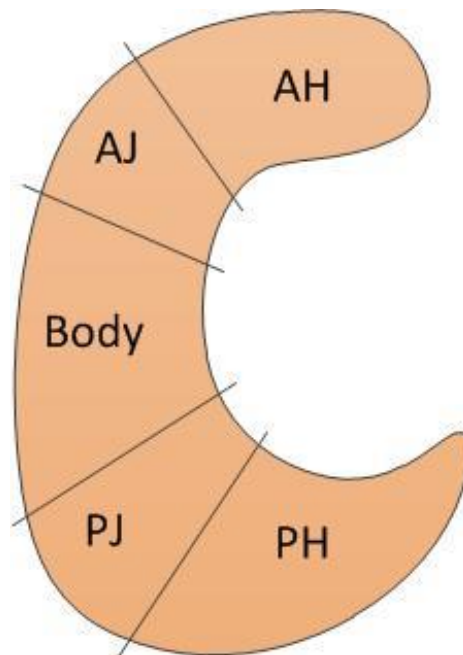


Figure 4.3. Regions of the meniscus as used for MRI and dissection notes (AH = anterior horn, AJ = anterior junction, PJ = posterior junction, PH = posterior horn)

Results

All ACLF joints experienced ACL failures. Rabbits favored the contralateral limb for the first 3-5 days, but returned to normal gait thereafter. Rabbits in the mACLT group favored the contralateral limb for 1-3 days post-surgery, but also returned to normal gait. All ACLF animals experienced ACL tearing. The ACLF model resulted in a variety of tearing patterns, most commonly longitudinal vertical tears acutely that developed into complex tears chronically. (Table 4.1) With the mACLT model the most common chronic advancement was tissue maceration. (Table 4.2) Representative MR images of three types of meniscal tears are shown in Figure 4.4

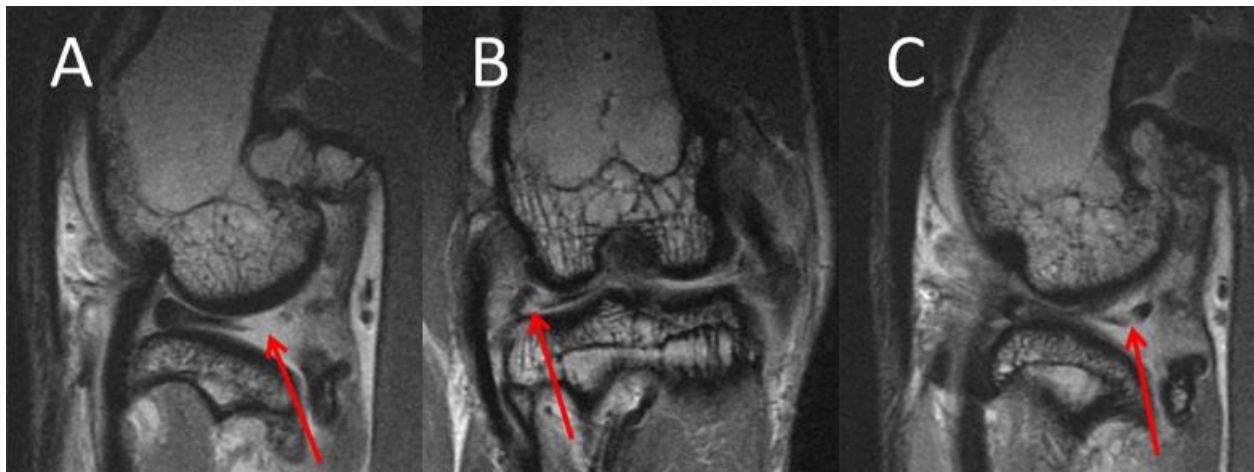


Figure 4.4. MRI images of meniscal damage A) red arrow pointing to posterior horn maceration B) red arrow identifying a vertical tear to the body C) red arrow pointing to a vertical tear to the posterior horn.

Table 4.1. ACLF Meniscal Damage (PH= posterior horn, AH = anterior horn, PJ = posterior junction, AJ = anterior junction, CXT= complex tear) Damage identified as new or worsened is in bold text in the chronic column. Italicized text in the dissection notes or acute notes represents damage not seen on the chronic MRI. Italicized text in the chronic notes identifies damage not seen at dissection.

Rabbit	Acute MRI Notes		Chronic MRI Notes		Dissection Notes	
	Medial	Lateral	Medial	Lateral	Medial	Lateral
1	Longitudinal vertical tear from PH	No Damage Observed	CXT in body from PH	Horizontal tear in PH	CXT in body from PH	Horizontal tear in PH
2	CXT in body from PH	No Damage Observed	CXT in body from PH, tissue macerated	<i>Oblique undersurface tear in PJ to PH</i>	CXT in body from PH, PH tissue macerated	<i>Surface damage to PH</i>
3	Free edge radial tear in PH	<i>Small horizontal tear in PH</i>	<i>Full thickness radial tear in PH</i>	Indeterminate for tear.	<i>Tissue maceration to body</i>	No Damage Observed
4	No Damage Observed	No Damage Observed	Bucket handle tear in body to PH	<i>Horizontal tear at AJ. CXT in body from PH</i>	Bucket handle in body from PH	CXT in body from PH
5	Peripheral vertical tear in PJ	CXT in body from PJ	CXT with PH macerated	CXT in body with PH macerated	CXT in body, PH tissue macerated	CXT in body, PH tissue macerated
6	Longitudinal, vertical tear extending from the body into the posterior horn	Full thickness radial tear in PJ, PH macerated	Full thickness radial tear in PJ, PH maceration, free edge tearing in the body.	Full thickness radial tear in PJ, free edge tearing in PH.	CXT in body from PH, PH maceration	<i>Radial tears in AH, Full thickness radial tear to PJ, free edge tearing in PH</i>

Table 4.2. mACLT Meniscal Damage (PH= posterior horn, AH = anterior horn, PJ = posterior junction, AJ = anterior junction, CXT= complex tear) Damage identified as new or worsened is in bold text in the chronic column. Italicized text in the dissection notes or acute notes represents damage not seen on the chronic MRI. Italicized text in the chronic notes identifies damage not seen at dissection.

Rabbit	Acute MRI Notes		Chronic MRI Notes		Dissection Notes	
	Medial	Lateral	Medial	Lateral	Medial	Lateral
1	Full thickness radial tear in PH	Full thickness radial tear at PJ	Full thickness radial tear in PH	CXT to body from PH with PH macerated	Full thickness radial tear to PH	CXT to body from PH, PH macerated
2	Full thickness radial tear in PH	Full thickness radial tear in PH	Radial tear in PJ with maceration of PH	Full thickness radial tear in PJ, Free edge fraying of PH	<i>CXT in body</i> , maceration of PH	CXT to PJ, free edge fraying in PH
3	Full thickness radial tear in PH. <i>Undersurface fraying extending into PJ and body segments.</i>	Full thickness radial tear in PJ, Free edge tearing of PH	Full thickness radial tear in the body. Free edge tearing in AH	PH maceration , free edge tearing near of posterior root	Full thickness radial tear to body, free edge tearing to AH	CXT to body from PH, free edge tearing near posterior root
4	Full thickness radial tear in PH	CXT in body from AH and PH	Full thickness radial tear in PJ	CXT in body from AH and PH	Full thickness radial tear in PJ	CXT to body, <i>surface damage to AH</i>
5	Full thickness radial tear in AJ	Full thickness radial tear in the body, with suggestion of a short segment horizontal component extending into PJ	High grade radial tear to body, maceration of PH	Full thickness radial tear in PJ, contiguous free edge fraying extending into PH	Radial tear to body, maceration of PH	<i>CXT to body</i> , free edge fraying in PH
6	<i>Incomplete radial tear in PH</i>	Full thickness radial tear to body	CXT in the AJ	CXT to body	CXT to AJ	CXT to body

Based on the MRI, acute damage was present in five of the six ACLF animals while all six experienced chronic damage. Acute damage was more common in the medial hemijoint with 5 of the 6 animals experiencing tearing, and all 5 sustaining damage to the posterior horn or posterior junction. Only 3 of the six lateral menisci showed signs of acute meniscal tearing; however, similar to the medial hemijoint all tearing occurred in the posterior horn and posterior junction. Chronically, the medial meniscus saw tears propagate, or new tears develop, in all 6 animals. In the lateral hemijoint, 5 of the 6 menisci showed tearing worsen over time. There was one case, (animal 3) where an initial tear was detected in the acute MRI acute but not seen on the chronic images (italicized in Table 4.1). Neither the chronic notes nor the dissection notes indicated any tear suggesting a limitation or misinterpretation on the initial acute MRI. Comparing the dissection notes to the chronic MRI notes it can be seen between both hemijoints there were only three instances where the MRI showed damage not seen at dissection and three instances where damage was seen at dissection that did not appear on the MRI.

Since meniscal transections were introduced during surgery all animals in the mACLT group had similar acute damage. MRIs of the mACLT animals show 4 of the 6 medial menisci having progressive damage 12 weeks post-surgery. The lateral experienced slightly worse chronic damage with 5 of the 6 menisci having worsened over the 12 week time period. The mACLT group had two cases where damage was seen on the acute MRI and not seen on the chronic MRI. These included undersurface fraying (animal 1) and an incomplete radial tear to the PH (animal 6). However in both these cases the chronic MRI notes and dissection notes matched, suggesting the tears were missed in the initial acute MRI. Only three instances were found where there was damage seen at dissection that was not found in the MRI readings. Two of these cases (animals 2

and 5) the dissection notes assigned a complex tear label when the chronic MRI notes showed full thickness radial tearing in conjunction with free edge fraying. This combination of tearing can be referred to as complex tear due to the multiple tearing orientations present, and was likely missed due to slice thickness.

Discussion

These findings show that in both models, meniscal damage worsened in the trauma inflicted menisci with time. The ACLF model showed damage worsening in 83% of the menisci while only 75% of the menisci in the mACLT model worsened with time. Clinically the medial meniscus has been shown to be injured less frequently acutely with ACL rupture but does make up 70% of chronic meniscal injuries.^{Bellabarba1997} Furthermore chronic damage is most commonly seen in the posterior regions,^{Chan1991, Greise2002, Smith2001} which MRI readings from both models are in agreement with.

In a comprehensive review of human meniscal tearing patterns, Bellabarba et. al. and reported acute tears occur slightly more often in the lateral meniscus (56%) as opposed to the medial meniscus (44%).^{Bellabarba1997} In our ACLF model acute tearing in the medial meniscus was seen in 83% of our samples while lateral tearing was only present in 50% of the animals. In a previous pilot study^{Killian2010} using the same ACLF model on 4 rabbits, gross acute damage was more common in the lateral meniscus (100%) as opposed to the medial (25%). In the same pilot study, Killian et. al. observed that all medial and lateral menisci were severely damaged (displaced, flap, and degenerative tears) after 12 weeks post impact. These preliminary chronic results were

consistent with our findings as 100% of the medial menisci and 83% of the lateral menisci were damaged at dissection.

MRI data from this study suggests that acute damage serves as a location for damage to propagate and become worse over time. Longitudinal, bucket-handle, and complex tears have been shown to strongly correlate with articular cartilage damage as opposed to radial, flap, and horizontal cleavage tears.^{Lewandrowski1997, Tandogan2004} The most common tear type for the impacted animals was longitudinal vertical tearing. Hough et al. also suggested longitudinal and radial tears are the most common tearing orientations when excessive force was applied to the meniscus.^{Hough1990} Horizontal tears are more common in degenerative cases.^{Hough1990} Chronic tears in the mACLT model menisci generally followed the tear orientation of the surgical transections, but there were two incidences of chronic horizontal tears in the ACLF animals where acutely no damage was seen. Since no acute damage was seen these tears are more likely degenerative tears that resulted from the change in joint kinematics due to ACL rupture.

Given the limitations the MR imaging used, the dissection notes were assumed to match sufficiently. There were limited instances where chronic MRI readings did not match damage seen during dissection. Of these instances most can be explained by limitations with the imaging. The largest limitation was due to tissue size. With the entire meniscus being on the order of 3x5x12 mm identifying specific regions, for example anterior junction vs. anterior horn, was difficult. Additionally with a slice thickness of 1.5mm there was a limited number of slices available making the sensitivity of the imaging low as compared to that of a traditional human MRI image of the menisci. With a limited number of images available it was possible certain

damage was missed or unable to be clearly identified. Working with a smaller tissue size also made damage defined as “surface damage” at time of dissection impossible to identify on the MRI. In the future imaging with a smaller slice thickness would increase the sensitivity and reliability of the MRI readings.

Future studies are underway to document changes at 4 and 8 weeks post-trauma to develop a more complete timeline of damage propagation. Additionally, changes in the material properties of the menisci are being investigated in order to determine if the properties change before or after the increase in degree of meniscal tearing. Finally in addition to studying the menisci, other soft and hard tissue structures including articular cartilage and the underlying bone are being investigated for both models.

Chapter 5 – Conclusions

Significance of Research

It has been shown that removal of meniscal tissue, either in full or as a partial meniscectomy, results in damage to the underlying articular cartilage over time. ^{Krause1976, Kurosawa1980} This articular cartilage damage can lead to an early onset of OA. Additionally meniscal tears have been shown to correlate to articular cartilage degradation. ^{Lewandrowski1997} Despite only a few studies of meniscal damage as a feature of OA ^{Englund2009} little is understood about the progression of untreated meniscal tears as they relate to trauma and the development of PTOA.

In an attempt to better understand the progression of PTOA a number of animal models have been developed. These models range from ACL transection, ^{Batiste2004, Chang1997, HelioLeGraverand2001, MeDevill1976, Yoshioka1996} impact, ^{Brophy2012, Borrelli2010, Isaac2010, Killian2010, Yeow2008} meniscectomy, ^{Arunakul2013, Beveridge2013} and even combination models. ^{Han1999, Hayami2006, Intema2010, Sniekers2008, Picharski2011}

While all the aforementioned models have provided much needed information, there still exists a lack of understanding as to what changes occur in individual soft tissue structures and the interplay between structures. The menisci, despite playing a critical role in joint mechanics, have often been overlooked in these past studies. With the incidence of combined meniscal and ACL injury being reported as high as 82% ^{McDaniel980}, it is important that these structures are studied as they likely play an important role in the development of OA. ^{Fairbank1948, McGonagle2010}

Our study provided much needed data for acute damage, chronic damage, mechanical data, and histological data for meniscal tissue. Within this study a new model of PTOA was developed which accounted for combined ACL and meniscal damage (mACLT). Additionally we looked at

changes to the menisci following a compressive tibiofemoral impact (ACLF). Both of these models showed that when left untreated meniscal damage present in an ACL deficient knee will progress with time. Tears tended to propagate in the direction of initial damage. Because the mACLT model introduced radial tears which were not commonly seen acutely in the ACLF model the chronic meniscal damage between the two models was not identical. The mACLT model primarily showed full thickness radial tearing, while the ACLF model complex and displaced tears.

One of the aims of this study was to not only document acute and chronic meniscal tearing but also verify that MR imaging could accurately identify meniscal damage in these models. While there were a few discrepancies between the dissection findings and the chronic MRI results, it was determined to be a valid method for identifying meniscal damage in these models. MRI has been used extensively to diagnose human meniscal damage but have been used much less frequently in animal models. The main reason being the limitations associated with diagnosing damage on such a small scale. The slice thickness needs to be small relative to tissue size, so imaging capabilities are a limiting factor when dealing with smaller animal models. Ideally a smaller slice thickness would be used in future studies to increase the confidence and reproducibility of the MRI findings. However, even with a slice thickness of 1.5mm used in this study we were able to fairly successfully identify if damage was present.

Both models showed a decrease in elastic moduli both instantaneously and at equilibrium on the hemijoint level. Greater decreases were seen in the mACLT model compared to the ACLF model. With the mACLT model a decrease of 72% was seen in the medial hemijoint, and the

lateral hemijoint decreased 73% instantaneously and 81% at equilibrium. In the ACLF model the lateral meniscus decreased around 47% both instantaneously and at equilibrium, while the medial meniscus had a much higher decrease at 78% instantaneously and 75% at equilibrium. While all were significant this shows there is potentially differing affects between the two models as a result of the different trauma conditions. Unfortunately, the more heavily damaged regions of the meniscus were unable to be mechanically tested limiting sample sizes in those regions.

GAG coverage differences between the two models was perhaps one of the most interesting findings from this study. GAG coverage tended to decrease in all regions of the mACLT model and was significant in the lateral anterior, lateral central, lateral posterior, and medial anterior regions. Overall GAG decrease in the mACLT model was slightly higher in the lateral hemijoint (66%) as compared to the medial (51%). In the ACLF model GAG coverage decreased in the lesser damaged regions, but remained constant or increased in the lateral posterior, medial central, and medial posterior regions. In regions where GAG was seen to decrease the medial anterior showed the largest decrease at 74%. The other regions that decreased included the lateral anterior and the central regions with an average decrease of 33%. The regions with no change in GAG coverage or slightly elevated coverage were the more heavily damaged regions. This increase could be a result of altered loading sending a biochemical response to increase GAG production or could be attributed to some inflammatory response associated with the impacts.

As compared to other ACLT models where meniscal damage was reported,^{Adams1983, Smith2001,}

^{HelioLeGraverand2001} the meniscal damage seen in both the mACLT and ACLF model is more severe.

Damage is more prevalent as well as more pronounced in tear type. As one of the first PTOA

models to evaluate both the instantaneous and equilibrium elastic moduli it is difficult to conclude if the mechanics are more affected in the traditional ACLT model or one of the models in this study. However between the two models there are some interesting differences in mechanics and histology that merit further investigation.

Future Work

In addition to determining instantaneous and equilibrium elastic moduli, GAG staining intensity, GAG coverage, and acute and chronic damage; we will be determining permeability, staining for calcium deposits, and looking at cell viability for all menisci 12 weeks post trauma. Furthermore, a longitudinal study is underway looking at 4 and 8 weeks post trauma for both models. Characterizing temporal changes will help in identifying a timeline of meniscal degradation within each model. Identifying a time point where changes rapidly occur or plateau could be critical in devising potential interventions.

In addition to monitoring meniscal changes in these models, changes to the articular cartilage, subchondral bone, trabecular bone, and synovial fluid are also being analyzed to develop a comprehensive whole joint study. Hopefully by evaluating all the soft and hard tissue components of the joint, we will be able to correlate changes between structures. Lastly, determining the biochemistry of the synovial fluid may provide insight into the inflammatory pathway.

Beyond the scope of the current study and the longitudinal component, it would be valuable for a future study to look at a meniscus transection only model. Such a study would be beneficial in

determining the effects of the un-stabilized joint verse solely acute meniscal damage. Another direction for a future study would be looking at an impacted then repaired model. In the human condition often following impact injuries where the ACL is torn, an ACL replacement surgery takes place. Unfortunately this is typically the only tissue to undergo any repair. Having a better understanding how the joint changes after an impact, ACL rupture, and subsequent ACL replacement could provide much needed insight for surgeons. It would also potentially help in determining why regardless of ACL repair; the incidence of OA is still higher than in an uninjured limb. ^{Daniel1994, McDaniel1980, Noyes1983}

Once the changes to the meniscus following trauma are better understood, it will be important to investigate therapeutic strategies to help prevent or mitigate damage. One intervention method that has been studied in preventing cell death in articular cartilage has been a non-ionic surfactant, Poloxamer 188 (P188). ^{Isaac2010, Lee1999, Natoli2008, Phillips2004} P188 works in part by entering into damaged fibrochondrocytes and sealing the cell to prevent necrosis and apoptosis. ^{Issac2010, Serbest2006} However these finding have been from testing of the articular cartilage and the effects of P188 on meniscal tissue are largely unknown. Another potential avenue for degradation prevention would be further investigating the effects of Interleukin 1 (IL-1). Osteoarthritic joints have been found have increased levels of IL-1 which is a pro-inflammatory cytokine. ^{Schlaak1996} In a recent bovine explant study, the treatment of IL-1 α resulted in a decrease in GAG content, and affected the compressive tissue properties. ^{Lemke2010} Interleukin 1 receptor antagonist (IL-1ra) is a protein in the human body inhibits the inflammatory response and helps prevent the expression of IL-1 α . Therefore IL-1ra may also be a method to help prevent meniscal degradation. ^{McNulty2007} An alternative method for pain and inflammation relief is the application

of bee venom. Lee et. al. was able to show a decrease in arthritis index of the knee following injections of bee venom in a rat model. ^{Lee2004}

There are a number of methods to investigate in preventing the progression of knee OA. The work presented in this thesis is the first step in better characterizing the damage to the meniscal tissue following trauma. Fully understanding the progression of damage and the interplay between various structures will help future research in the prevention of damage, and potentially help to avoid early onset post traumatic osteoarthritis.

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Appendix A: Standard Operating Procedures

Meniscus Indentation Testing Protocol

Equipment/Supplies:

Item	Location
Specimens (Meniscus)	Will receive in shipment
Specimens (Synovial Fluid)	
Water Bath	In tooling cabinet
2 and 10 lb. Adapter Plate	Attached to actuator or on fixture rack
X-y plate	On fixture rack or in tooling cabinet
Camera Mount	In Electronics cabinet (top shelf) or on camera tripod
2 lb. Futek Load Cell	In load cell cabinet
Flea Camera	In container next to MTS – CCD Kit
Firewire Cable	Next to MTS computer
X-Y plate Adapter Plate and Screws	In Tooling box – on fixture rack or in tooling cabinet
Camera Mount Adapter Plate and Screws	
Water Bath Adapter Plate and Screw	
Adapter Block for 2lb. Load Cell	
Load Cell to Indenter Adapter	
Indenter Tip	
Specimen Wedge and Screws	
Superglue	
Razor Blade	Dissection Tools Drawer
Scalpel	
Tweezers	
0.9% Saline Solution	On counter next to sink
12 Test Tubes	Test Tube Drawer
Test Tube Rack	Shelf above and to the left of drying rack
Drop cloth (cut into halves)	Under dissection table
Gauze	Shelf above PBS
Disposable pipets 1.5mL	Shelf above PBS
Low Temp Freeze Vials	Vials Drawer
Tape	Shelf above office supplies
Marker – Fine Point	Office Supplies Drawer
Camera	Portable Hard Drive and Digital Camera Drawer
Portable Drive	

Steps for Setup – Usually set up a day prior to testing

1. Attach X-Y plate adapter to lower load cell
2. Attach X-Y plate to X-Y plate adapter (smaller bolts 4)

3. Attach Camera Mount to Camera Mount Adapter Plate
4. Attach Camera Mount Adapter Plate to X-Y Plate (larger bolts 4)
5. Attach Water Bath Adapter Plate to Camera Mount
6. Attach Water Bath to Water Bath Adapter Plate



Figure A.1. Lower Set Up for Indentation Testing

7. Attach 2 and 10 lb. Adapter Plate to the actuator
 - a. Follow tightening instructions on coupler
8. Attach Adapter Block for 2lb. Load Cell
9. Attach 2lb. Load Cell
 - a. Take note of how the load cell cable is attached to MTS
 - i. When removing take special care
 1. Hold square part of cable adjustments steady

2. Turn cylindrical part clockwise
10. Attach Load Cell to Indenter Adapter
11. Attach Indenter Tip (Just leave sitting beside if setting up a day early)
 - a. NOTE: Do NOT run warm-up with indenter tip attached

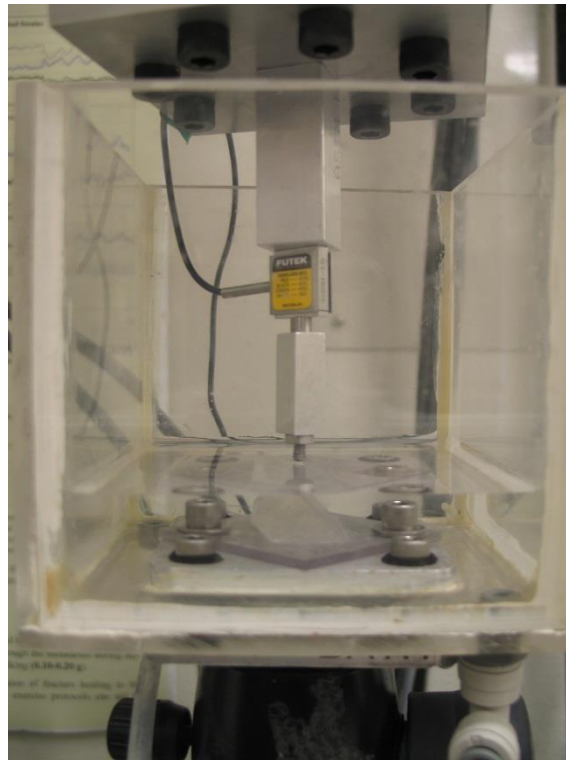


Figure A.2. Upper Set Up for Indentation Testing (note this image was during testing and thus includes the wedge mount with specimen attached)

Testing

1. Follow “MTS_Bionix370.02Landmark_Setup_KMF.docx” Set up instructions
2. Start Warm-up Cycle (See end of “MTS_Bionix370.02Landmark_Setup_KMF.docx”)

Steps for Specimen Preparation

1. Lay out ½ Drop Cloth, Gauze, Test Tubes, Test Tube Rack, Disposable Pipets, Low Temp Freeze Vials, Tape, Marker, Camera on dissection table
2. Remove specimens from packaging

3. Using Disposable pipets remove synovial fluid from vials and transfer into Rubber Sealed Vials
4. Label Rubber Sealed Vials appropriately and store in -80C in appropriate container (as of now freezer on back wall third row down in pink container labeled Tammy Donahue)
5. Label Drop cloth with specimen name, limb, and hemijoint
6. Take photos of meniscal tissue as received laid by label
7. Using tweezers and scalpel remove excess synovial tissue
8. Take another photo of specimen
 - a. Be sure to highlight any damage
 - b. May need to take a photo of back few if damage is extensive
 - i. Be sure to re-label if necessary
9. Return to PBS soaked gauze and re-bag specimen
10. Repeat for both joints
11. Place specimens in fridge until testing

Steps for Other Preparation

1. Fill all test tubes with 4mL of PBS
2. Using tape and marker, label each tube accordingly EX. BBGB4 LLA, BBGB4 LLC.....
3. Place all test tubes in test tube rack
4. Place rack in fridge
5. Place ½ drop cloth around water bath to prevent leakage

Camera Set Up

1. Attach Flea Camera to Camera Mount
2. Connect Flea Camera Via Fire Wire Card

- a. Be careful with plugging in as the firewire card was added to machine

Testing

1. Turn Warm-up off if still going
2. Section menisci
 - a. Can do all sectioning at once or as you go
 - b. Will need anterior, central, and posterior regions
 - c. Lateral posterior is a more ligamentous attachment so be sure to leave meniscal tissue when sectioning

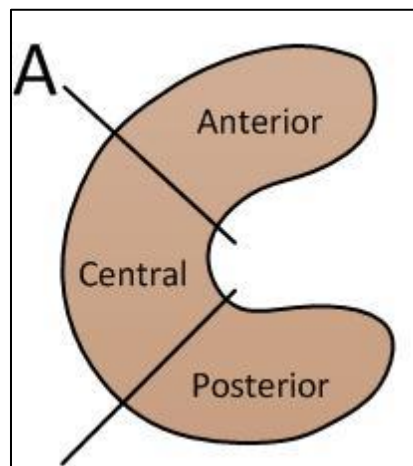


Figure A.3. Meniscal Sectioning

3. Clean wedge using razor blade to scrape off left over glue remnants
4. Place small amount of super glue on wedge mount
5. Place Specimen near top edge trying to get specimen face as “flat” as possible
6. Attach wedge to water bath
7. Fill water bath with PBS
 - a. Be sure valve is closed
 - b. Fill slightly over wedge but not far enough over that PBS will come in contact with nut on indenter when testing

8. Adjust crosshead so specimen will not bottom out if hydraulics are lost
9. Create new Specimen
 - a. Label Ex. “BBGB4_LLA” , HIT ENTER

****Very important to label this way when using my Matlab code to process
10. Be sure Procedure is set to “Indentation2” can be found in p188 folder under procedures
11. Using Fly Cap (can be found on desktop or in computer programs) take an image of the specimen
 - a. File -> Save As
 - i. Create file Ex: “BBGB4 PICS”
 - ii. Save as Ex: “BBGB4_LLA.tiff” ***Note please save as tiff images
12. Lower indenter tip till just in water
13. Adjust X-Y plate and camera mount to get specimen perpendicular to inventor tip and indenter tip is located over thickest part of specimen

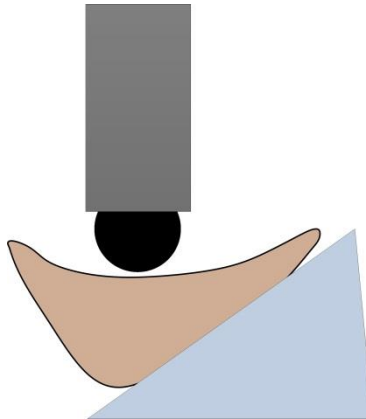


Figure A.4. Indentation Alignment

14. Lower indenter tip till just above specimen
15. Zero out Axial Force 2
16. Apply 20mN force to specimen

17. Disable manual controls

18. Run (“Play”)

Test Done – Specimen Removal

1. Create new Specimen
2. Manually enable controls
3. Raise up actuator
4. Drain PBS
5. Remove wedge mount
6. Using razorblade carefully remove specimen
 - a. Will need to remove all superglue
 - i. If care is taken you can “pop” off specimen with razor bade
 - ii. Alternatively once off wedge, can use scalpel to scrape off leftover superglue
7. Place specimen in corresponding PBS filled test tube
8. Repeat Testing for all samples

All Testing Done – Shut Down

1. Be sure when hydraulics are turned off and actuator drops to -50mm it would hit anything
 - a. Adjust crosshead if necessary
 - i. (See “MTS_Bionix370.02Landmark_Setup_KMF.docx”)
2. Disable Hydraulics
 - a. HSM1 low, HPU low, Off
3. Close Station Manager
4. Turn off controller and record Pump Time

5. Create Folder in “Specimens” file found on desktop
 - a. Label Ex. “BBGB4”
6. Place all test files and PICS file in overarching folder
7. Move folder from “Specimens” into “Kristine -> Rabbits”
8. Copy From “Kristine -> Rabbits” to Red Drive “Kristine -> Rabbits -> Rabbit Testing - New MTS Raw”

Meniscus Fixation Protocol

Equipment/Supplies:

Item	Location
Specimens	Varies
10% Neutral Buffered Formalin	On countertop
Tubes/Cassettes	In drawer marked tubes
Label Tape	On countertop

Fixation

1. Place each sample in labeled cassettes or individual centrifuge tubes; samples can be kept in them through the whole preservation series.
2. Immerse in 10% Neutral Buffered Formalin (NBF) solution for at least 7 days. The NBF volume to tissue ratio should be about 10:1 for proper fixation.

Cryo-Embedding of Soft Tissue

Equipment/Supplies:

Item	Amount	Location
Phosphate Buffered Saline (PBS)	NA	PBS Storage Tank
Chemistry Grade Crystalline Sucrose (or store bought cane sugar if contaminants not an issue)	Various	In cabinet under scale
Tissue Tek OCT	1 Tube	Shelf above scale
Pella Disposable Base Molds (Tissue Tek Cryomolds)	NA	Cabinet next to left of fume hood
Liquid Nitrogen	1 Dewar	Tank in room
Styrofoam bowl	1	Dissection Bench
Forceps/Tweezers	1 pair	Drawer Beside Dissection Bench
Plastic Wrap	1 box	Drawer Beside Dissection Bench
Weigh Boat	1	Drawer Under Scale
Beakers	3-4	Glassware Shelf

Prepared Solutions:

- 10% sucrose solution:
 1. Dissolve 10g crystalline sucrose in 100ml PBS.
- 20% sucrose solution:
 1. Dissolve 20g crystalline sucrose in 100ml PBS.
- 30% sucrose solution:
 1. Dissolve 30g crystalline sucrose in 100ml PBS.

Methods:

1. Fill 100 mL glass beakers approximately half or $\frac{3}{4}$ full with *10% 20% and 30% sucrose solutions* respectively.
2. Place specimen into, cover with parafilm; the tissue should float.

3. Once the tissue drops to the bottom of the vial tissue has been infiltrated, move tissue to 20% beaker and repeat.
4. Once the tissue drops to the bottom of the vial, transfer tissue to 30% sucrose solution
5. Once the tissue drops to the bottom of the vial, remove tissue from vial and blot with Kimwipes.
6. Put small amount of OCT into a weight boat – this will be used to apply a small amount of OCT to the cutting face of each specimen to aid in position retainment in cryomold during the freeze cycle.
7. Cut tissue if necessary to attain a flat cross section and orient appropriately in cryomold with the cut face lying flat on the bottom of the mold
8. Cover fill mold with OCT.
9. Pour liquid nitrogen into Styrofoam dish.
10. Carefully submerge tissue/mold in liquid nitrogen so as not to disrupt orientation of tissue in the mold.
11. Once the sample sinks to the bottom of the dish, remove and place on absorbent towel (slows down thawing of frozen specimen/OCT). Either let rest at room temperature for a minute to bring up to a temperature that is safe to handle and then remove from mold and wrap immediately with plastic wrap, label and store at -20°C.

Notes (as per MLK)

Not sure what will happen with the butanol/liquid nitrogen slurry. I have only ever used straight LN₂ and didn't have any issues with tissue cracking/matrix disruption compared to paraffin embedding.

Puck should “pop” out of sample. Be careful not to crack tissue/medium by forcing the puck to pop out.

Options: Warm up with hand by holding/rubbing base of the mold to melt periphery of frozen OCT

Cryo-Sectioning of Soft Tissue

Equipment/Supplies:

Item	Amount	Location
Cryostat	1	
Tissue Tek OCT	1 Tube	On shelf above scale
Subbed Slides	NA	In drawer beside scale
dH ₂ O in Spray Bottle	1 Sprayer	On countertop

Methods:

1. Inside the microtome, place the chuck (grooved platform) in one of the first four holes in the “fast freeze rail” (2 columns of 6 holes each located on the far left inside the microtome). These chucks can be left in the cryostat during cool-down time as well.
 - a. The slots are NOT numbered – make sure to label the order of your samples if you are prepping more than one sample at a time.
2. Remove a blade from the blade container, or reuse one designated for your tissue, and grip it by the flat edge. Raise the clamp on the right hand side of the blade holder and slide the blade in along the left hand side of the blade holder. If it won’t go in, push gently on the bottom edge of the blade holder (the side closest to you). Push the clamp back up to lock the blade in place.
 - a. Do not over-tighten blade
 - b. Inspect the blade before putting it into the blade holder. Do not reuse the blade if there are any cosmetic defects, cracks, or chips taken from the blade.
3. Wearing gloves remove the chuck and hold the post in your hand, letting it warm up slightly. Spread embedding medium on the face of the chuck (over the grooved surface, making sure it goes down into the grooves). NOTE: if the chuck is too cold when you put the medium on the grooved face, the medium will not go down into the grooves. You

will have to pop off the frozen medium, wait for the chuck to warm up a bit more, and try again.

4. As soon as crystals start to appear on the outside edges of the embedding medium on the chuck face (this will happen quickly, and the medium will turn white near the chuck), push your embedded specimen into the center. The cutting surface (flat surface) should be exposed and parallel with the chuck surface.
5. Allow the entire chuck/specimen combination to freeze inside the cryostat.
6. Insert the post of the chuck into the chuck holder. The small metal knob behind the chuck holder secures the chuck in the holder, and the black lever to the right of the chuck allows the chuck to rotate once it has been loosened.
7. Adjust the vertical position of the sample with the knob on top of chuck so that it is centered vertically with respect to the blade. Adjust side to side alignment with knob on left side.
8. Use the retract/advance buttons on main screen of the cryostat to move chuck and holder to/away from blade. Make sure the notch needle on the displacement wheel is up when this is done.
9. Using the hand wheel on the right hand side of the cryostat, raise and lower the embedded sample to trim through the frozen medium until you start to see the sample appear. Continue trimming until a good cross-section appears. Adjust the angle of the chuck if the sample appears to be cutting unevenly (i.e., if sections appear to be thicker on one side, if they appear angled, etc.). There is no blade guard, so **MAKE SURE TO BE CAREFUL TO NOT CUT YOURSELF WITH THE BLADE** when you do this.

10. Flip the “glass anti-curl plate” down so that any sections cut will slide into it underneath the glass; this will prevent sections from curling.
11. Start with 6um sections and increase to 8um if 6um does not yield good sections. Raise the glass plate periodically to clean out any “junk sections” with the brush kept inside the microtome
12. When your first good (even thickness, longitudinal appearance) section is cut, transfer it to a piece of slide glass by raising the anti-curl plate and pressing a piece of slide glass against the specimen.
13. Cut at least 2 more sections that you’ll dispose of before the next section you plan to transfer to a slide (so that they’ll be separated through the depth of the core). Do not place more than 3-4 sections per slide or the cover glass will not fit. Be careful of their placement when transferring to the slide glass – you won’t be able to move them once they’ve been stuck to the slide, so make sure they’re close enough together so that one cover glass will cover all 3. Obtain a total of 9 sections (2-3 per slide; 3-4 slides) per sample.
14. The remaining specimen can be refrozen by carefully melting the OCT medium from the underside the chuck with your fingers. DO NOT let the sample melt. Refold the sample in saran wrap and place in -20°C non-frost free freezer.
15. Remove the blade from the cryostat; raise the blade clamp to unlock it and use forceps to gently push along the right hand side of the blade, pushing the blade out the left side of the holder. Place the used blade in the disposal side of the blade container.

16. Pull up the notch needle on the displacement wheel and push the “RETRACT” button to the left of the cryostat menu panel to retract the chuck and holder away from the blade holder.
17. Clean out any remaining junk by pushing it down to the side/bottom of the cryostat, but wipe out the junk while it is frozen before turning off the cryostat for an extended period of time.
18. Lay slides on a flat surface.
19. Using a spray bottle, spray 60°C (warm in microwave in northeast lab by liquid nitrogen tank) distilled water on slides to remove bubbles and promote the section adherence to the slide. Do not dip slides in water or saturate the slides. Allow to dry overnight.

Troubleshooting:

1. If sectioning becomes inconsistent and slices appear shredded, one of these methods may alleviate the problem:
 - a. Adjust roll plate
 - b. Change blade angle
 - c. Slice using another section of the blade
 - d. Flip blade over or try new blade
 - e. Try the sample later! – seriously it works
 - f. The sample may not have been embedded correctly
 - g. The blade may not be cold enough! Allow the cryostat to drop in temperature.
 - h. Try a thinner or thicker sectioning depth.

Microscopy of Saf-O Stained Specimens

(Using Olympus BH2 Microscope)

Start Up:

1. Remove the dust cover from the microscope
2. Plug in firewire cable to either port of the Q Capture camera (mounted on the top of the microscope).
3. Turn on the microscope (power button on front of base).
4. Log on the computer
5. Open the Q Capture software by selecting the icon on the desktop

Basic Operation:

NOTE: The Olympus BH2 is only capable of bright field transmitted light imaging. Therefore, the specimens that can be imaged using transmitted light are limited to stained or colored samples mounted on microscope slides almost exclusively.

1. Make sure that light is visible from base of the microscope, and the swinging condenser is in the out position. This indicates that light is, indeed, being transmitted to the sample.
2. Set the path to the camera and eyepiece by positioning the light path selector knob to the middle position.
3. Use the light intensity adjustment slider on the right side of the microscope base to control the brightness of the image (A power level of 2 -3 is generally sufficient).
4. Make sure the filter turret is turned to the Carl Ziess objective (Has best field of view for rabbit meniscal sections)
5. Make sure the light source, slides to be imaged and condenser are free of dust.

6. Before each imaging session, you will need to check/align the condenser.
 - a. Close the diaphragm until you see the outline of a circle.
 - b. Use the condenser height adjustment knob to bring the circle into focus.
 - c. Use the centering screws to bring the circle of light to the middle of view.
 - d. Open the diaphragm until the border of the circle just surpasses the field of view.
7. Use manual X and Y stage control knobs to move to an area of interest.
8. Use the coarse and fine focus adjustment to create an image that is in focus.
9. Place a slide onto the stage and center the best specimen on the slide in the view field
10. In the software, select a square of empty space around the specimen. In the Camera Settings window select the white balance button.
11. Manually adjust the exposure of the image by adjusting the slider in the Camera Settings window (this setting will be different depending on the exact positioning of the power intensity slider on the microscope.) Once the exposure is set, the setting is generally left the same for the whole imaging session.
12. Select the camera icon to capture the image. A new window will appear with the captured image. When multiple images are necessary try to start at one end of the section and move methodically for each subsequent image to achieve complete coverage of the section with the least amount of images
13. Once the image collection is complete, save your image as a TIF file with notation RabbitName_Section_2x_#ofImage (ex ZBF1_RMA_2x_1).

Meniscus Safranin-O Imaging Protocol

Equipment/Supplies:

Item	Location
Image J with FIJI package	Must download
Microsoft Excel	Must download
Portable Drive	Attached to actuator or on fixture rack

Image J

1. Open Image J
2. Calibrate Image
 - a. Drag and drop bright field image with scale
 - b. Using straight line tool draw line on calibration image
 - c. Set scale to known distance & unit length
 - d. Set globally
3. Import Image
 - a. May need to stitch images (see next step)
 - b. Drag and drop in image
4. Stitch Image (use Mosaic J whenever possible if blurry then pairwise stitching)
 - a. Mosaic J: Plugins -> Stitching -> Mosaic J
 - b. Pairwise stitching: Drag and drop two images into Fiji (see “Import Image”)
5. Trim Image
 - a. Import image if not already open
 - b. Using freehand selection tool outline image (remove outer synovium and background)
 - c. Clear Outside

6. Color Deconvolution

- a. Import trimmed image if not already open
- b. Use Color Deconvolution tool to select the Saf-O stain, the FCF stain, and the background

7. Thresholding Saf-O image

- a. Import deconvoluted Saf-O image if not already open
- b. Adjust threshold appropriately and apply

8. Analyzing Saf-O image particles

- a. Use the analyze particles tool to analyze the area identified as Saf-O coverage
- b. Save results as “SpecimenName_Results_SafO”

9. Thresholding Full image

- a. Import trimmed image if not already open
- b. Change image type to 8 bit
- c. Adjust threshold to cover entire image and apply

10. Analyzing Full image particles

- a. Use analyze particles tool to analyze total area of the specimen
- b. Save results as “SpecimenName_Results”

Excel

1. Open result files in excel (...Results andResults_SafO)
2. Sum all areas (column B)
3. Calculate percent area of Saf-O coverage.

Appendix B: Code

Indentation Relaxation MTS Code

Acquire:

Continuous Sampling

Sampling Rate 102.4 Hz

Signals – Axial displacement, axial force 2, running time (SI units small)

Linear Buffer size 10000

Start with procedure

Ramp Stroke (1):

Time based ramp for 1 sec

Relative axial displacement -.25mm

Start with procedure

Hold:

Time based hold for 15 min

Axial displacement

Start when Ramp Stroke 1 is done

Ramp Stroke:

Time based ramp for 5 sec

Relative axial displacement 5mm

Start when Hold is done

End procedure

Indentation Relaxation Data Processing Matlab Code

```
%Program to determine instantaneous and equilibrium elastic moduli

clear all

close all

clc

%% given

R = 1/32*25.4; %Radius of Indenter Tip - mm i.e. ~1.59mm diameter

d = 0.25; %Displacement - mm

v1 = 0.01; %Poisson Ratio of Rabbit Menisci (Sweigert 2004 paper)

v2 = 0.3; %Poisson Ratio of Indenter Tip (AISI E 52100 Steel)

E2 = 210*1E3; %Elastic Modulus of Indenter Tip (AISI E 52100 Steel) - MPa

%% retrieve data

mode = 0; % Processing mode [0 - Single, 1 - Batch]

%manually choose file

if mode == 0

    [FileName,folder_name,FilterIndex] = uigetfile('*.dat','indentation file');

    files = {FileName};

else

    folder_name = uigetdir;

    files_char = ls(folder_name);

    files_char(1:2,:) = [];

    files = cellstr(files_char);

end
```

```

%% process data

for i = 1 : size(files,1)

    IndentationData = importdata(fullfile(folder_name,files{i}),'t', 5);

    % [Column 1 - Displacement, Column 2 - Force, Column 3 - Time]

    TimeOfTest = 15*60; %true run time only 15 minutes. Data set includes extra time for
indenter tip to come off specimen

    Time = IndentationData.data(:, 3); %Time= all rows of third column of file

    Index = find(Time < TimeOfTest,1,'last'); %Find all time points before end of 15 minutes
(cuts out last 7 seconds when indenter is rising off sample)

    Force = -1.*IndentationData.data(1:Index, 2); %Convert Force to positive value for all rows
where time points are "index" values in column 2

    Displ = -1.*(IndentationData.data(1:Index, 1) - IndentationData.data(1, 1)); %Convert
Displacement to positive value for all rows where time points are "index" values in column 1 and
then subtract first location to get original displacement equal to zero

    Time = IndentationData.data(1:Index, 3); %Rename time to so non index numbers are cut
off

    %figure(1)

    plot(Time,Force); title('Indentation');

    xlabel('Time [sec]'); ylabel('Force [N]'); grid on; axis tight

```

% Equation from Elastic Compression of Spheres and Cylinders at Point and Line Contact By

M. J. Puttock and E. G. Thwaite National Standards Laboratory Technical Paper No. 25

% Estar solved for every time point

$$Estar = 3 * Force ./ (4 * d.^{(3/2)} * R.^{(1/2)});$$

% Rearrange to solve for unknown elastic moduli of meniscal material

% E1 solved for at every Estar value ie every time point

$$E1 = (-E2 * Estar * v1.^2 + E2 * Estar) ./ (Estar * v2.^2 + E2 - Estar);$$

% Determine specimen information

% Files labeled as ex. BBGF_LLA.dat

% Remove .dat from file name i.e. take off last four characters from file name

$$Specimen = files\{i\}(:, 1:end-4);$$

% Break apart label/file name

% Last letter in specimen name gives region

switch Specimen(end)

case 'A'

Region = 'Anterior';

case 'C'

Region = 'Central';

case 'P'

Region = 'Posterior';

end

% Second to last letter in specimen name gives location

switch Specimen(end-1)

```

    case 'L'

        Location = 'Lateral';

    case 'M'

        Location = 'Medial';

end

% Third to last letter in specimen name gives location

switch Specimen(end-2)

    case 'L'

        Limb = 'Left';

    case 'R'

        Limb = 'Right';

end

%Present results in individual columns

Results{i,1} = Specimen(:,1:end-4); % In First Column - File name minus last four
characters ie specimen name

Results{i,2} = Limb; % Second Column

Results{i,3} = Location; % Third Column

Results{i,4} = Region; % Fourth Column

Results{i,5} = max(E1); % Fith Column - Instantaneous Modulus i.e. max E1 value found-
in MPa

Results{i,6} = E1(end); % Sixth Column - Equilibrium Modulus i.e. E1 value at end of 15
minute test - in MPa

End

```

LIST OF ABBREVIATIONS

ACL – anterior cruciate ligament

ACLT – anterior cruciate ligament transection

mACLT – modified anterior cruciate ligament transection

ACLF – anterior cruciate ligament traumatic failure

OA – osteoarthritis

PTOA – post traumatic osteoarthritis

Saf-O – Safranin-O Fast Green

GAG – glycosaminoglycans

MRI – magnetic resonance imaging