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Characterization of the Meniscus for Future Tissue Engineering Efforts

by

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ABSTRACT

Characterization of the Meniscus for Future Tissue Engineering Efforts

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Mark Sweigart

The meniscus, a fibrocartilagenous tissue found between the femur and tibia, is responsible for shock absorption, load transmission, and stability within the knee joint. Damage to this tissue can lead to osteoarthritic changes, suggesting that the meniscus protects the knee joint from degenerative joint disease. Historically, repair techniques consisted of excision or suturing of the damaged tissue. Unfortunately, neither of these techniques successfully repairs the damaged tissue; tissue engineering is one possible solution. When attempting to tissue engineer a tissue, it is ideal to start in a small animal model, such as a rabbit, before attempting the repair process in a larger animal model. The objective of this work was to characterize the medial rabbit meniscus ultrastructurally, biomechanically, biochemically, and cellulary and to perform biomechanical characterization on larger animal models for future scale up efforts. The medial rabbit meniscus was found to have a higher hydration level, greater amount of sulfated glycosaminoglycans, and lower level of hydroxyproline at the inner 1/3 of the tissue, which confirmed the more chondrocytic nature of this region. It was also found that the anterior portion of the tissue, particularly in the inner 1/3, had a higher hydration level, sulfated glycosaminoglycan level, aggregate modulus, permeability, shear modulus, and a lower hydroxyproline level than the central and posterior locations. It is believed that this topographical variation is due to the bent-knee resting stance of the
rabbit and its propensity to jump. It was also determined that significant variations exist in the compressive creep properties, both intraspecies and interspecies, in a variety of animal models, indicating caution when comparing animal models and determining which animal model to use in future tissue engineering efforts. The characterization in this study can serve as a "gold standard" reference for future meniscal tissue engineering efforts and be used as a baseline for future large animal tissue engineering efforts.
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Chapter 1: Introduction

The general approach to tissue engineering (Fig. 1) consists of two phases: analysis and synthesis. In the analysis phase, topographical and spatial properties of the tissue are determined using biochemical, biomechanical, ultrastructural, and cell culture techniques. The objective is to characterize the tissue at the cellular and tissue levels. In the synthesis phase, cells are seeded onto scaffolds that are exposed to a variety of mechanical and chemical stimuli to assist in the development of new tissue. The construct is then implanted in an animal model to repair/replace the damaged tissue. In tissue engineering efforts, small animal models are originally used to test individual concepts, and, if the concepts are sound, larger animal models are then used before human trials. One tissue that has the potential to be tissue engineered is the knee meniscus, and an excellent small animal model for initial studies is the lapine model.

The objective of this project was to perform the analysis stage, specifically, to characterize topographically the ultrastructural, biomechanical, biochemical, and cellular properties of the native medial rabbit meniscus and to compare the compressive properties of the meniscus among several animal models. This characterization allowed us to create a gold standard reference for all future repair and regeneration studies using the lapine model and gave us a mechanical baseline for future large animal model studies. *The study's hypothesis is that the native medial rabbit meniscus can be characterized via ultrastructural, biomechanical, biochemical, and cellular techniques to create a gold standard reference for future tissue engineering studies and that the relationship of the*
Compressive properties among the rabbit animal model and other animal models can be determined. The following specific aims were used to test this hypothesis:

1) To evaluate the ultrastructural properties of the medial rabbit meniscus. Histological processing was performed and the meniscal tissue stained with Hematoxylin & Eosin, Masson’s trichrome, and Safranin O/Fast Green. Furthermore, the orientation and size of the collagen fiber bundles was determined by scanning electron microscopy. *It was hypothesized that the inner portion (white zone) of the meniscus would exhibit different staining than the outer portion (red zone) and that the collagen fibers would be oriented circumferentially within the meniscus.*

2) To quantify the biomechanical properties of the medial rabbit meniscus. Biomechanical characterization of the rabbit meniscus was accomplished through uniaxial tensile testing and compressive creep indentation testing. Circumferential tensile testing was performed on the medial meniscus and compressive creep testing was performed at the anterior, central, and posterior portions of the femoral and tibial sides of the meniscus. *It was hypothesized that the posterior portion of the tissue would have significantly different mechanical properties than the anterior and central portions of the tissue.*

3) To obtain the biochemical properties of the medial rabbit meniscus. Collagen content, collagen type, sulfated glycosaminoglycan content, and water content were quantified through the use of hydroxyproline assays, protein immunoblots, dimethylmethylen blue assays, and wet to dry weight comparisons. The medial rabbit meniscus was tested at both the “red” and “white” zones in the anterior, central, and
posterior portions of the meniscus. *It was hypothesized that the inner portion* (white zone) *of the meniscus would have significantly different biochemical properties than the outer portion* (red zone) *of the tissue.*

4) **To characterize the cellular properties of rabbit fibrochondrocytes on a P(PF-co-EG)-co-GRGDS hydrogel.** The effects of the GRGDS peptide on fibrochondrocyte attachment and proliferation on a P(PF-co-EG)-co-GRGDS hydrogel was tested by seeding fibrochondrocytes in monolayers on the hydrogel. Three different concentrations of the peptide were tested in the attempt to find an optimal concentration level. *It was hypothesized that the P(PF-co-EG) hydrogel, with the addition of the GRGDS peptide, would aid in the attachment and proliferation of fibrochondrocytes on this biomaterial.*

5) **To obtain the compressive creep properties of the meniscus in several animal models.** Compressive creep indentation testing was performed to determine the creep indentation properties of the baboon, bovine, canine, human, lapine, and porcine medial meniscus. Creep testing was also performed on the lateral porcine meniscus. *It was hypothesized that significant variation in the compressive properties would occur among the different animal models and between the medial and lateral meniscus.*

The completion of these specific aims results in a fully characterized lapine model that serves as a reference and guide for future meniscal tissue engineering studies and a mechanical baseline that can be used for future large scale animal model regeneration studies. Details on the studies performed for the completion of these aims can be found in the following chapters. Chapter 2 provides an overall examination of the meniscus and the current efforts to tissue engineer this unique tissue. Chapter 3 provides a more in-
depth examination of natural and synthetic scaffolds that have been used in meniscal tissue engineering efforts. Chapter 4 provides an examination of fibrochondrocytes, including their reaction to different isolation methods, culturing conditions, reaction to peptides and growth factors, and response on different biomaterials. Chapter 5 details studies performed in specific aims 1 and 2, specifically the compressive and tensile properties of the lapine medial meniscus and the collagen orientation within the tissue. Chapter 6 illustrates the biochemical and histological studies examined in specific aims 1 and 3, including topographical variations of sulfated glycosaminoglycans, collagen amount and type, and degree of hydration. Chapter 7 depicts the study from aim 4, the fibrochondrocyte reaction to P(PF-co-EG)-co-GRGDS. Chapter 8 describes comparison of the compressive properties of the medial and lateral meniscus in the porcine model, part of specific aim 5. Chapter 9 depicts the remainder of specific aim 5, specifically the variation in compressive properties between six different animal models. An overview of all the studies and their results can be found in Chapter 10.
Fig. 1. Strategy for tissue engineering the knee meniscus.
Chapter 2: Toward Tissue Engineering of the Knee

Meniscus*

Abstract

This review details current efforts to successfully tissue engineer the knee meniscus. The meniscus is a fibrocartilaginous tissue found within the knee joint that is responsible for shock absorption, load transmission, and stability within the knee joint. If this tissue is damaged, either through tears or degenerative processes, then deterioration of the articular cartilage can occur. Unfortunately, there is a dearth in the amount of work done to tissue engineer the meniscus when compared to other musculoskeletal tissues, such as bone. This review gives a brief overview of meniscal anatomy, biochemical properties, biomechanical properties, and wound repair techniques. The discussion centers primarily on the different components of attempting to tissue engineer the meniscus, such as scaffold materials, growth factors, animal models, and culturing conditions. Our approach for tissue engineering the meniscus is also discussed.

Introduction

The meniscus, fibrocartilaginous tissue found within the knee joint, is responsible for shock absorption, load transmission, and stability within the knee joint.[1-8] According to the National Center for Health Statistics over 600,000 surgeries each year are the result of complications with the meniscus.[9] The meniscus has the intrinsic ability to heal itself; unfortunately this property is limited only to the vascular portions of the tissue.[10] For damage outside of these areas and overall degeneration of the tissue, methods need to be developed that will assist the meniscus in healing itself; tissue engineering is a potential solution. Figure 1 depicts our philosophy for addressing cogently the notoriously complex problem of successful regeneration of the knee meniscus.

Meniscus Anatomy

The meniscus is a tissue consisting of two wedge-shaped semilunar sections of fibrocartilaginous tissue between the tibial and femoral bearing surfaces of the knee joint (Fig. 2). On gross inspection the meniscus is a white, glossy, and smooth tissue; this smoothness is also present at the microscopic level.[11] The peripheral portion of the meniscus is covered by the synovial membrane and consists of vascularized tissue, whereas the inner portion is avascular.[12] The amount of tissue with blood supply varies with age; an infant’s meniscus is fully vascularized whereas an adult’s is usually only vascularized in the outer 1/3 to 2/3 of the tissue.[13, 14] The blood is supplied by the lateral, medial, and middle genicular arteries and through the perimeniscal capillary
plexus at the peripheral attachment to the joint capsule.[13] The other attachment points are the medial collateral ligament, the meniscofemoral ligaments, the transverse ligament, and the anterior and posterior horns. There are two meniscofemoral ligaments: the Humphry ligament and the Wrisberg ligament. Both ligaments transverse between the posterior horn of the lateral meniscus to a location near the posterior cruciate ligament insertion site on the medial femoral condyle.[15] People have been shown to have either one of these ligaments or both.[15] The transverse ligament attaches the anterior lateral meniscus to the anterior medial meniscus and the anterior and posterior horns are the locations where the meniscus joins with the tibial plate.[16-20] The anterior insertion site consists of four zones: the ligamentous zone, uncalcified fibrocartilage, calcified fibrocartilage, and bone.[19] The posterior site consists of the same three deeper zones found in the anterior horn.[19] It has also been shown that there is variation in the exact location of these attachment sites between people, specifically the anterior horn.[20] These horns are also the site of the highest amount of innervation within the meniscus.[21-23] The rest of the innervation occurs in the outer 1/3 of the meniscus, with large nerve fibers being oriented circumferentially and smaller nerve fibers oriented radially; no innervation is found in the inner 1/3 of the meniscus.[22, 23]

*Cells in the Meniscus*

There are two main zones that the meniscus can be divided into, the superficial zone and the deep zone. The cell type present in each of these zones is different. The superficial zone contains cells that are oval or fusiform, have few processes, and a scant amount of
cytoplasm, resulting in the nucleus of the cell looking abnormally large.[11, 12] The deep zone cells are rounded or polygonal, are usually alone, but occasionally groups of two or three can be found, and have a large amount of rough endoplasmic reticulum.[11, 12] What exactly these cells are is a matter of some debate: are they fibroblasts or chondrocytes? They have properties that are found in both cell types: the tissue is fibrous, yet they more closely resemble chondrocytes and there is a territorial matrix present around the cells.[12, 24] In 1985 Webber and coworkers[25] proposed the term fibrochondrocytes to describe these unique cells. Microvascular endothelial cells are another cell type found in the meniscus.[26] Myofibroblasts are also occasionally detected in the meniscus, but usually only around injured tissue.[12]

**Extracellular Matrix of the Meniscus**

The extracellular matrix can be separated into four different categories: water, fibrillar components, proteoglycans, and adhesion glycoproteins. Human meniscal tissue has been shown to be 72% water, 22% collagen, 0.8% glycosaminoglycans, and the rest is made up of DNA and adhesion molecules.[27] These numbers can vary depending on age, animal, and location within the tissue.[28, 29]

**Fibrillar Components**

There are two main types of fibrillar components found in the meniscus: collagen and elastin. A combination of both mature and immature elastin fibers have been found within the adult meniscus, though they exist in small quantities (0.6% dry weight) and their exact function is still not known; more research needs to be done in this area.[12,
More is known about the collagen structure within the meniscus due to the volume of studies done on this component of meniscal tissue. Collagen types I, II, III, V, and VI have been found within meniscal tissue, which account for 60-70% of the dry weight. Type I collagen is by far the most predominant, accounting for >90% of the collagen within the tissue. A study done by Cheung has shown that in bovine menisci the outer 2/3 of the menisci is predominantly type I collagen, whereas the inner 1/3 is 60% type II collagen and 40% type I collagen. He also found that the concentration of collagen was greater in the outer section, 80.1% dry weight vs. 69.3% dry weight. The meniscus has a unique collagen structure orientation that is related to its function and consists of three different layers (Fig. 3). The superficial layer consists of a thin layer of randomly oriented fibers. The lamellar layer, situated just inside the superficial layer, also consists of randomly oriented fibers, with the exception of the peripheral portions at the anterior and posterior sections; here the fibers are oriented radially. The deep zone consists of circumferentially oriented fibers with a small amount of radially oriented fibers, also referred to as tie fibers. It has also been shown that the amount of collagen synthesized by the fibrochondrocytes decreases with age. The unique fiber orientation found in the knee meniscus leads to the tissue’s exceptional properties, which will be described later.

**Proteoglycans**

Proteoglycans are responsible for hydration within the meniscus and the compressive properties of the tissue. The concentration of proteoglycans in meniscal tissue is 8 fold less than the concentration found in articular cartilage. There are two main
classes of proteoglycans: large and small. Large proteoglycans are responsible for tissue hydration and various studies have been performed on this component.[35] The inner 2/3 of the meniscus produces more proteoglycans than the outer third and the lateral side produces more proteoglycans than the medial side, though the glycosaminoglycan makeup of the proteoglycans stays the same at all of these locations.[33, 38] Normal human meniscal tissue consists of 40% chondroitin-4-sulfate, 10-20% chondroitin-6-sulfate, 20-30% dermatan sulfate, and 15% keratan sulfate.[39] The distribution of small proteoglycans within pigs has been tested by Scott and coworkers[34], who found that the highest concentration of biglycan was in the inner 1/3 of the meniscus whereas the highest concentration of decorin was in the outer 1/3. One theory on the function of biglycan is to protect cells during loading, and this study seems to support that theory.[34] Decorin might help with collagen fibril organization and this theory is also supported by this study.[34]

**Adhesion Glycoproteins**

As the name suggests, adhesion molecules are partly responsible for binding with other matrix molecules and cells. There are three of these molecules that have been identified within the meniscus: Type VI collagen, fibronectin, and thrombospondin.[28, 40] While the exact nature of these three glycoproteins has not yet been discovered, the RGD sequence, which plays a central role in cell adhesion, has been found in Type VI collagen, fibronectin, and thrombospondin.[28, 41]
Meniscal Biomechanics

Functionally, the meniscus is a shock absorber, helps with load bearing and transmission in the knee joint, improves stability in the knee, and helps with lubrication.[1-8] Because of all these different functions and the geometry of the tissue, the meniscus is subjected to compressive, tensile and shear stress (Fig. 4). Whenever a load is applied to the knee joint the meniscus is compressed, but due to its wedge-shaped architecture it is also displaced away from the center of the femoral condyles, resulting in tensile stress because of the anterior and posterior attachment to the tibial plate.[8, 42-44] If these attachments are not present then the biomechanical function of the meniscus is altered and degeneration of the tissues in the area can result.[45-49] In terms of shear properties of the meniscus, it is known that they depend heavily on the collagen orientation of the meniscus and the low circumferential shear strength is thought to be partly responsible for the occurrence of longitudinal tears.[50, 51]

Movement of the Meniscus and Force Transmission

The menisci partially cover the articular cartilage on the tibial plate and are responsible for absorbing some of the load transmitted through the knee. Application of these loads causes the meniscus to be displaced, and different flexion angles also cause displacement of the tissue.[6, 8, 42-44] In general, the lateral meniscus is displaced more than the medial meniscus during compression and greater displacements also occur at angles of 15° and 30° flexion when compared to a straight joint.[42] The contact area is greatest when the knee is in 0° flexion and when the knee is flexed the contact area decreases and
moves posteriorly on the tibial plate.[6] Where the load is applied also varies depending on the total force being transmitted through the knee; when a small force is applied the meniscus absorbs most of the load, whereas if 150kg is applied the lateral meniscus absorbs most of the load and the medial meniscus splits the load 50/50 with the exposed articular cartilage.[8] If the meniscus is cut or removed then the pressure seen by the tibial plate increases. Hoshino and coworkers[2] discovered that if a radial cut is put into the meniscus, but the tissue is left in place, the force on the tibial plate is 12.8% greater; if the meniscus is removed, then the force is 20.8% greater.

**Mechanical Properties of Meniscal Tissue**

Tensile, compressive, and shear tests have been run on meniscal tissue, but there has been variability in testing methods, including differences in specimen size, specimen harvest location, test conditions, and animal models.[7, 29, 50-58]

The tensile test has been the most widely used testing modality. Tension has been employed to test meniscal tissue from different animal models: human, bovine, dog, rabbit, and ovine.[7, 29, 52-56, 58] Fithian and coworkers[58] have done a study where the tensile moduli from circumferential specimens harvested from different locations of the human meniscus was found. Their results are shown in Table I. Their theory on why the medial posterior 2/3 of the tissue is weaker is due to the capsular attachments in this area of the tissue disrupting the collagen network. They also tested water and proteoglycans concentrations in these different regions and both values were similar among all locations.[58] Tissackt and coworkers[55] measured the tensile modulus in
both the radial and circumferential directions at eighteen different locations within the human meniscus. Rectangular tensile specimens were taken from the anterior, central, and posterior portions of the lateral and medial menisci at three different depths: the proximal surface, the deep zone, and the distal surface. The elastic modulus, the maximum stress, and the maximum strain were obtained at each of these locations in both the radial and circumferential directions. Their study showed that the meniscus is much stronger and stiffer in the circumferential direction than the radial direction.[55] Another animal model that lends itself well to mechanical testing is the bovine model due to the ease of specimen creation and the ability to harvest from specific locations within the meniscus.[29, 52, 54, 55] Tests run by Proctor and coworkers[29] showed the following:

1. Tensile specimens taken from the surface layer, where collagen orientation is random, are isotropic.
2. Tensile specimens taken from the deep zone, where the majority of the collagen fibers are oriented circumferentially, are anisotropic, and are the stiffest in the circumferential direction.
3. Circumferential specimens from the posterior section are stiffer than specimens from the anterior section.

The effect of the radial tie fibers was quantified in a study by Skaggs and coworkers[52] using bovine menisci. They found that radial tie fibers are more frequent in the posterior section of the tissue; the presence of these tie fibers in the posterior section increases the tensile modulus 392% when compared to specimens with no tie fibers present.[52] Newman and coworkers[7], who performed their study on the canine model, found that after a longitudinal tear is healed mechanical function is restored. If a radial cut is
present, where the circumferential collagen fibers are transected, then normal mechanical function is not restored.[7] Tensile tests of the attachments have also been performed, showing that on a rabbit model the anterior attachment is stronger than the posterior attachment and both of these attachments are vital to keep mechanical function.[49, 53]

Compression tests of the meniscus, although not performed as frequently as tensile tests, have yielded a wealth of information. Proctor and coworkers[29] have done a confined compression test on different locations in the medial bovine meniscus. They found that samples taken from the deep zone in the posterior section of the meniscus are stiffer than similar specimens taken from the anterior section, but articular cartilage is stiffer than specimens taken from either meniscal location.[29, 58] They also discovered that the meniscus is 1/10 as permeable as articular cartilage.[29] The combination of the lower compressive stiffness and lower permeability suggest that the meniscus is an excellent shock absorber.[58] A study by Joshi and coworkers[57] compared the confined compression characteristics of the medial menisci of six animals: human, bovine, ovine, monkey, porcine, and canine. They found that the ovine model is the most similar to human tissue, and the wide differences in animal models should suggest caution when comparing mechanical properties of meniscal tissue between different models.[57] Our lab has done a study using a creep indentation apparatus[59, 60] to find the aggregate modulus ($H_A$), permeability ($k$), and Poisson’s ratio ($v_S$) at different locations of the human meniscus; the results are shown in Table II.
The shear properties of the meniscus are theorized to contribute to the larger occurrence of longitudinal tears.[58, 61] It has been shown that shear properties are anisotropic, with the stiffness being the lowest in the planes that run parallel to the major collagen orientation.[58] These properties are thought to come from the interactions between the collagen fiber extensions and the proteoglycan molecules within the meniscus.[50, 51]

**Injuries of the Meniscus**

Injuries to the meniscus usually consist of tears in the tissue, though separation from the tibial attachments and degeneration of the tissue also occur.[7, 10, 14, 49, 62, 63] Different styles of tears can occur, such as longitudinal and bucket-handle tears (most common), radial tears, and complex tears.[62, 63] In 1936, King[10] was the first to show that tears in the vascularized portion of the meniscus would naturally heal, whereas tears in the avascular portion would not. It has also been shown that longitudinal tears, if they heal, restore native mechanical function, but radial tears, where the collagen structure is disrupted, do not have restored mechanical function after healing.[7] There is a wide variety of repair techniques that have been attempted to help the meniscus heal; they are reviewed in the next section.

**Meniscal Repair Techniques**

The first major type of repair technique attempted for meniscal defects was meniscectomy, or removal of the meniscus. Due to the poor long-term results of meniscectomy efforts have been made to seal tears that occur in the meniscus.
Replacement of the meniscus is the third major repair technique that has been attempted.[62]

*Menisectomy*

The meniscus was originally thought to have little importance in knee function and was believed to be a functionless remain of leg muscle.[62, 64] These beliefs led to the practice of removing the meniscus whenever there was a complication. In the 1930’s, King[10] showed that degenerative effects in the articular cartilage are a side effect of menisectomy, though widespread attention to this complication did not occur until a series of studies in the 1960’s and 1970’s.[62] Directly after removal of the meniscus, the mechanical function of the knee joint alters, leading to greater stress applied to the articular cartilage.[65-68] Over time a new tissue is grown by the body to fill the defect, though it is usually smaller and narrower when compared to the native meniscus; also the cellular distribution within the tissue shows discrepancy.[24] Partial menisectomy, which is removal of a small portion of the meniscus, usually in the avascular zone, has been shown to cause less degenerative changes in the articular cartilage than a full menisectomy.[69] A partial menisectomy has the advantage of leaving the majority of the collagen structure of the meniscus in the knee, allowing the partial meniscus to still absorb some of the shock.[69] After a partial menisectomy, a fibrin clot forms in the area of the removed tissue, which then acts as a scaffold for cellular ingrowth, though this does not always occur.[70]
Repair of Meniscal Tears

After the realization that meniscectomy causes degeneration of articular cartilage, the healing method of choice started to be repair of the meniscal tear. A variety of different methods have been tried: suture, meniscal arrows, fibrin sealant, laser welding, abrasion therapy, and induced vascularization.[71-80] Sutures are used to reconnect the torn tissue until it heals together. There are several different suturing methods that have been attempted, the goal being to increase the strength of the wound site.[73, 74, 77] Meniscal arrows are biodegradable devices that are marketed to replace sutures because they are easier to use.[80, 81] Fibrin sealant and laser welding tests have been done to test their effectiveness in sealing tears; in general they are inferior to suture but can be used in conjunction with suture for better results.[71, 77, 79] Abrasion therapy, which roughens up the tissue surrounding the tear and removes necrotic tissue, will usually also be used to aid the healing process.[72, 79] Suture, meniscal arrows, fibrin sealant, and laser welding are all techniques that can be used alone to help healing in the vascularized zone, but if the tear is in the avascular zone it is considered necessary to induce vascularization.

Methods of inducing vascularization include vascular access channels and trephination.[75, 78, 79] Vascular access channels are large channels that are made from the periphery of the meniscus to the defect site; unfortunately this procedure does disrupt some of the collagen structure.[79] Trephination is a procedure with the same basic concept, needles are used to create the channels for blood flow, but due to their small size damage of the collagen structure is minimal.[75, 78, 79] Trephination tests in both dog and goat models have been shown to help the healing process in the avascular zone.[75, 78]
Meniscal Replacement

The third major technique that has been attempted for meniscus healing is replacement of the meniscus, with either natural meniscal tissue or synthetic replacements. Meniscal replacement has the distinct advantage over the previous method in that replacement can help repair degenerative changes in the meniscus and be used to replace menisci with radial or complex tears.

Meniscal Allografts

Meniscal allograft research has been rather extensive, though the effectiveness of this procedure is still under debate.[46, 47, 82-84] Results seem to be promising, though there are no long term data.[85] The effects of different preservation techniques and attachment methods have been tested. Studies so far have shown that, at one year, there is little difference between cryopreserved and deep-frozen meniscal tissue in the goat model.[86] The deep-frozen technique kills all of the cells, leaving the allograft to act as a scaffold; whereas the cryopreservation technique leave 10-30% of the fibrochondrocytes viable.[87] The deep-frozen grafts have been shown to still have no viable cells in the deep zone at 6 months in a dog model.[88] Cryopreserved allografts have been shown to hold a higher water content and a decreased proteoglycan concentration when compared to native tissue.[89] One of the difficulties with full meniscal replacement is attachment to the tibial plate. It has been shown that bone plugs offer the best mechanical results, but these plugs do not simulate the native tissue and long term results are unknown.[46-48] Overall, while meniscal transplantation offers the
best results for certain meniscal conditions, such as radial tears, complex tears, and degenerative tissue, the current research shows that degeneration of the articular cartilage still occurs and a better alternative needs to be developed.

**Meniscal Substitutes**

Another possible solution that has been attempted is to replace the meniscus with a graft from a tendon. This graft would have the advantage of having a circumferential collagen structure and over time the graft could possibly convert to fibrocartilage and in the process preserve the joint compartment.[85, 90] Unfortunately, this has not been found to occur.[85, 90]

**Meniscal Prostheses**

The development of meniscal prostheses has seen very little research. Several materials have been tried, such as poly(tetrafluoroethylene) (PTFE), Dacron, and polyurethane-coated versions of the previous two materials.[91-93] While the polyurethane coated PTFE gave the best results, the prosthesis was still mechanically inferior to native meniscal tissue.[92] Tissue engineering of the meniscus seems to be the preferred method for healing the meniscus.

**Tissue Engineering of the Meniscus**

When compared to other musculoskeletal tissues such as bone or articular cartilage, there is a dearth in the number of studies done to-date in the attempt to tissue engineer the
meniscus. A large amount of work has been done on a variety of scaffold materials. Knowledge on how fibrochondrocytes react to a variety of growth factors has been discovered through a variety of tests, both from tissue engineering studies and meniscal repair enhancement studies. A variety of animal models has been used, though an ideal model is still under debate. Some studies have been done on culturing conditions for fibrochondrocytes, though there is still plenty of room for development. Non-invasive imaging has been developed to observe the state of the meniscus, and these methods could be used to determine the status of a tissue engineered meniscus. The complex nature of the cells (two different types) and extracellular matrix (both components and organization) make the meniscus a difficult tissue to successfully engineer.

_Scaffolds_

Scaffolds can be separated into two major categories, natural and synthetic. Table III gives a summary of different scaffold materials that have been attempted and their effectiveness in tissue engineering the meniscus.

_Natural Scaffolds_

Four natural scaffolds have been attempted for tissue engineering of the meniscus: collagen, small intestine submucosa (SIS), periosteal tissue, and perichondral tissue. Of these four, periosteal tissue had the least acceptable results. A study done by Walsh and coworkers[94], using periosteal tissue in a rabbit model, showed both hyaline cartilage and bone growing in the repair tissue at the end of the 24 week trial. The results from perichondral tissue were not much better; these 12 month sheep tests gave repair tissue
that resembled the meniscus grossly but the tensile modulus of the repair tissue was much lower than native menisci.\textsuperscript{[95]} SIS studies done in dogs by Cook and coworkers\textsuperscript{[96]} have shown some promising results, but the study was only for a 12 week period and no mechanical testing of the scaffold was performed. By far the most promising results have come from using a collagen scaffold. Tests done by Walsh and coworkers\textsuperscript{[94]} and Mueller and coworkers\textsuperscript{[97]} yielded encouraging results, though no mechanical tests were performed on the repair tissue. The best results appear to have come from using a collagen-GAG scaffold developed by Stone, Rodkey and coworkers. This scaffold, made from collagen retrieved from bovine tendons and then molded into a circumferential orientation, is already in phase II clinical trials.\textsuperscript{[98]} The major animal trial consisted of removing 80\% of the medial meniscus of the dog and replacing it with this scaffold.\textsuperscript{[99]} The phase I and II clinical trials consisted of removing the damaged portion of the meniscus, trimming the scaffold to the appropriate size, and then suturing it in place, with the replaced section varying between 35\%-85\% of the total meniscus.\textsuperscript{[98, 100]} After three years, the results were promising, with no degeneration of the articular cartilage noted.\textsuperscript{[98, 100]}

**Synthetic Scaffolds**

There is a wider variety of synthetic scaffolds that have been tried, though none of them have yet reached the state that collagen scaffolds have in the development cycle. There is a series of earlier scaffolds containing carbon fibers that were attempted, but mediocre results halted research into the use of these scaffolds. Veth and coworkers\textsuperscript{[101, 102]} attempted just plain carbon fibers and carbon fibers embedded in a polyurethane-poly(L-
lactide) matrix. The gross results for the short term were promising, though no biochemical or biomechanical studies were performed.[101, 102] The same group also did a study where they used PLLA fibers instead of carbon fibers; the results were better, but again no biochemical or biomechanical tests were performed.[103] One other group, Wood and coworkers[104], also tried using carbon fibers, though their embedding matrix was polyester. Their results were mediocre overall, with degeneration of the articular cartilage and little ingrowth of tissue occurring.[104] Another scaffold material that was attempted but later discarded was the use of a Teflon-net.[1] The net was sutured in place after a total meniscectomy and the tissue was allowed to infiltrate the scaffold over a 12 month period.[1] There was less degeneration of the dog’s articular cartilage with the use of the Teflon-net when compared to a meniscectomy, though the results still were not very promising.[1] The next major synthetic material tried was a porous polyurethane.[105-107] This material was first used in the attempt to improve healing in a longitudinal tear of a canine’s meniscus; gross and biochemical results were promising.[105] The polyurethane scaffold was used to replace the lateral canine meniscus in a later study; they noted better results than what occurred after a meniscectomy, but there was still degeneration of the articular cartilage.[106] The third study consisted of a 50 week canine study where the polyurethane was used to replace a portion of the meniscus.[107] They found that vascularization approached the defect, and then when the wound was healed it retracted.[107] Types I and II collagen were found, though no biomechanical tests were performed and degeneration of the articular cartilage was noted.[107] Unfortunately, the aromatic polyurethane used in the previous studies releases toxic particles during degradation, therefore the material was altered and an
aliphatic polyurethane was used.[108] Both defect healing and total replacement were tested with this new material.[108] It was found to induce fibrous tissue ingrowth by 15 weeks, whereas the aromatic polyurethane’s induction time was 20 weeks.[108] The only problem found with this material was the weak tear strength, which would cause the implant to loosen.[108] Complex suturing techniques were used to help alleviate this problem, though the problem still needs to be addressed.[108] Unfortunately, at the end of the 52 week canine study some degeneration of the articular cartilage was noted.[108] The next study done by this group used a 50/50 copoly(L-lactide/ε-caprolactone) scaffold with varying compressive modulus (40 and 100kPa).[109] This material was sutured into a partial defect in the lateral meniscus of the canine and tissue ingrowth was determined; an aliphatic polyurethane was also used as a comparison.[109] The copolymer with a compression modulus of 40 kPa had no fibrous tissue ingrowth, whereas the 100kPa sample had a fibrous tissue ingrowth of 50-70%, and the aliphatic polyurethane had 80-100% fibrous tissue ingrowth.[109] The one other group doing a large number of studies on meniscal tissue engineering is Ibarra and coworkers.[110, 111] One unique thing that this group is doing is that all of their scaffolds, either PGA or PGLA, are seeded. Due to the poor mechanical properties of these materials Ibarra and coworkers seed the scaffolds for a few days, implant them subcutaneously for a period of time (depending on animal model), and then place them in the defect. The most recent test consisted of a sheep model with a 4 week subcutaneous implantation time and 6 weeks in situ.[111] The resultant tissue was rich with proteoglycans and had a organized collagen fiber matrix.[111] These results are quite promising, though more studies need to be performed. One important study has been done that can be applied to any of the porous
scaffold materials; Klompkamer and coworkers[112] have performed a study on the effects of pore size for tissue ingrowth. They did a 12 month rabbit study where different pore sizes (50-90, 90-150, 150-250, and 250-500 microns) were constructed into an aromatic polyurethanes. The macropore volume on these implants varied between 48-55% and the total pore volume was 84-86%. Optimal pore size was found to be between 150-500 microns; pore sizes smaller than this were not found to promote fibrous tissue ingrowth.[112] One other study, done by Webber and coworkers,[113] has shown that the RGD peptide enhances the attachment of canine fibrochondrocytes to artificial surfaces, showing great potential for tissue engineering efforts.

_Growth Factors_

Numerous growth factors have been used on meniscal fibrochondrocytes to test their effects on the healing of tears or defects, or on protein synthesis under tissue or cell culture conditions. Table IV summarizes the different growth factors that have been used. All of these have the potential to help in tissue engineering of the meniscus.

Most experiments have used cell culture approaches, which consist of putting fibrochondrocytes or small tissue explants onto a petri dish and then checking the proliferative response of the cells or protein synthesis. One growth factor that has potential is transforming growth factor-β (TGF-β). Studies by Tanaka and coworkers[33] and Collier and coworkers[38] showed that TGF-β increases the proteoglycan synthesis of fibrochondrocytes from all different sections of the meniscus in a dose-dependent manner. In these studies Tanaka used human fibrochondrocytes and Collier used ovine
fibrochondrocytes.[33, 38] Studies by Spindler and coworkers[114] and Bhargava and coworkers[115], et al. tested the effect of human platelet-derived growth factor-AB (PDGF-AB) on ovine and bovine cells. The ovine study showed that PDGF-AB only affected the mitogenic response from the peripheral third of the meniscus; there was no effect on the inner 2/3 of the tissue.[114] Bhargava and coworkers[115] bovine test showed that PDGF-AB stimulated the migration of fibrochondrocytes from the inner, middle and outer thirds of the meniscus; PDGF-AB was also shown to increase DNA synthesis by the cells from all three sections. Bhargava and coworkers also found DNA synthesis increases when hepatocyte growth factor (HGF) or bone-morphogenic protein-2 (BMP-2) was used; HGF also increased the cell migration rate similar to PDGF-AB. BMP-2 and insulin-like growth factor-1 stimulated the migration of fibrochondrocytes from the middle zone by 40-50%. This study also tested the effects of two other growth factors: interleukin-1, which stimulated migration of cells taken from the peripheral third of the tissue; and epidermal growth factor, which stimulated migration of cells from the inner and outer zones by 40-50%.[115] An earlier study by Webber and coworkers[25] tested the effect that fibroblastic growth factor (FGF) and human platelet lysate (PL) had on proliferation of fibrochondrocytes; both were found to stimulate growth.

Other studies have been performed to check the effect that growth factors have on healing open defects and tears in the meniscus. Hyaluronic acid, hyaluronan and endothelial cell growth factor (ECGF) have been studied in both tear healing and defect repair.[116-119] Suzuki and coworkers[116] created a cylindrical defect in a rabbit anterior lateral horn and then made weekly injections of hyaluronic acid, which increased the rate of healing.
A study by Sonoda and coworkers[117] tested hyaluronan’s effect on the healing of peripheral and avascular tears in the rabbit meniscus. They found that hyaluronan stimulated collagen remodeling in the peripheral zone and inhibited swelling in the avascular zone.[117] Another tear style study, which tested the effect of ECGF in the healing of an allograft to the joint capsule, found that ECGF increases short term healing, but over the long term no difference was found.[118] A last study, performed by Hashimoto and coworkers[119], tested the effect of ECGF on assisting the healing of a cylindrical defect placed in the meniscus of a dog. The defect was filled with a fibrin sealant (some containing ECGF) and then allowed to heal over a 24 week period. The defects that contained both the fibrin sealant and ECGF showed the best results; roughly 90% of the defect was filled at the end of the study.[119]

**Animal Models**

There have been a wide variety of animal models that have been used in meniscus studies, each with certain advantages and disadvantages, but, not surprisingly, no model has yet been found that is similar to human meniscal tissue in all aspects (summarized in Table III). The type of study usually limits the animal model to a few choices. For mechanical studies the best choices are bovine and ovine models. Bovine models have the advantage of being rather large, making it easy to create sample specimens and do tests from different locations within the meniscus.[29, 51, 52] The ovine model is an excellent model because certain mechanical properties closely match that of human tissue.[57] Canine and rabbit models have also been used, but these models are much more common in wound healing studies, with canine being the most common.[94, 96,
If the rabbit is used for a wound healing study, it is usually only for tests involving the horns or the medial meniscus; the lateral meniscus should be avoided due to the lack of a transverse ligament and the posterior horn attaching to the femur, not the tibia. There are a large number of different models used for biochemical tests of the meniscus, such as rabbit, dog, sheep, bovine, ovine, and pig. In general, the data from these tests are useful for comparing data between different sections of the meniscus, but not for cross-species tests. Another style of test that sees a wide variety of models is the culturing of fibrochondrocytes, with the most common model being the rabbit model, which has been used for checking the response of cells to different media and culturing conditions. When checking the effect of growth factors on fibrochondrocytes the rabbit model, along with the ovine, canine, and bovine models, have been used. Overall, there is no clear choice for an animal model when it comes to tissue engineering the meniscus: size, cost, and specific characteristics of the animal models must all be taken into consideration.

**Culturing Conditions**

There has been a relatively limited amount of work done on culturing fibrochondrocytes. Fibrochondrocytes have been cultured in monolayer, in alginate beads, and in agarose suspension. Collier and coworkers showed that cells seeded in monolayer produce smaller proteoglycans than cells cultured in alginate beads. Studies by Webber and coworkers have shown that fibrochondrocytes cultured in agarose suspension are capable of expressing their differentiated phenotype. Webber and coworkers has also done a series of studies that tested the effects of different media on
fibrochondrocytes. In one study the group tested how a media containing either Dulbecco’s modified Eagle’s medium (DMEM) or Ham’s F-12 media, both supplemented with fetal bovine serum (FBS), affected the cells.[25] The media containing Ham’s F-12 seemed to cause faster growth of the fibrochondrocytes. The cells also took on different appearances depending on the media used; the cells cultured in the DMEM media were polygonal in shape, whereas the cells cultured in the Ham’s F-12 media were elongated or fusiform. It has been theorized that these two shapes could correlate with the two distinct cell types found within the meniscus, but this has not been proven.[25] Webber and coworkers[123, 124] has also developed a serum-free media; it performs better than the serum-free media’s available commercially, but still doesn’t perform as well as media supplemented with FBS. Fibrochondrocytes grown in the serum-free media were also found to be more chondrocytic.[124] Webber and coworkers[123] has also developed an organ culture model, which gives an in vitro method to test meniscal repair. This model consists of removing a meniscus from a rabbit model, creating a defect, filling the defect with a scaffold, and placing the meniscus in media. Over time tissue repair can be monitored.[123] The use of bioreactors in meniscal tissue engineering has been limited. Ibarra and coworkers[111] have expanded on this organ culture model; the explant was implanted subcutaneously or put on a dynamic culture system, which consists of a orbital shaker placed inside of a CO₂ incubator. Their results showed better healing from the subcutaneous procedure.[111]
Non-Invasive Imaging

Magnetic Resonance Imaging (MRI) and computed tomography (CT) are two non-invasive procedures that have been used to check injuries in the meniscus.[63, 125-128] MRI has been used extensively to check for tears in the meniscus, but there are some problems that need to be addressed before this technique could be used to evaluate a tissue engineered meniscus.[125-128] A study by Deutsch and coworkers[126] has shown that a signal is present that could be interpreted as a tear, even after the tear has successfully healed. Another study, done by Stone and coworkers,[128] showed that while MRI could be used to measure volume changes in regenerated meniscal tissue it does not correctly portray the actual size of the meniscal tissue. CT techniques, while not as heavily researched, do show some promise in meniscal imaging.[63] Manco and coworkers[63] have shown that CT can be helpful in discovering tears in the meniscus, though improvements need to be made for discovering horizontal and non-displaced peripheral tears. Both of these two techniques, with a little more development, could be helpful in evaluating the efficacy of a tissue engineered meniscus.

Conclusion

Successfully tissue engineering the meniscus would be a great help for the treatment of meniscal defects, but much needs to be done before this state can be reached. More characterization of the native tissue needs to be done, both biochemically and biomechanically in a variety of animal models. Current scaffolds need to undergo tests that are specific to meniscus structure-function, or new meniscus-specific scaffolds need
to be developed. Properties such as degradation time and mechanical characteristics need to be optimized, as does growth factor use for improving cellular response within these scaffolds. If cells are seeded onto these scaffolds, then cell type, seeding conditions, and culturing conditions need to be determined. A suitable bioreactor needs to be developed, which takes into consideration the biomechanical and biochemical environment, along with nutrient transport requirements. Methods to assess the effectiveness of tissue engineering approaches also need to be developed, to ascertain that indeed meniscus regeneration satisfies functionally over a suitably extended period of time. Hopefully, all necessary components of the approach described above will be combined in an effective way to achieve a viable solution to the difficult problem of meniscal regeneration.
Tables

Table I. Human Tensile Modulus by Location[58]

<table>
<thead>
<tr>
<th>Location</th>
<th>Tensile Modulus (MPa ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Anterior</td>
<td>159.07 ± 47.4</td>
</tr>
<tr>
<td>Lateral Central</td>
<td>228.79 ± 51.4</td>
</tr>
<tr>
<td>Lateral Posterior</td>
<td>294.14 ± 90.4</td>
</tr>
<tr>
<td>Medial Anterior</td>
<td>159.58 ± 26.2</td>
</tr>
<tr>
<td>Medial Central</td>
<td>93.18 ± 52.4</td>
</tr>
<tr>
<td>Medial Posterior</td>
<td>110.23 ± 40.7</td>
</tr>
</tbody>
</table>

Table II. Biomechanical Properties of Human Menisci using a Creep Indentation Apparatus (unpublished results)

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$v_s$</th>
<th>$k \times 10^{15}$ $m^4 / N*s$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>0.15 ± 0.03</td>
<td>0 ± 0</td>
<td>1.84 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>0.10 ± 0.03</td>
<td>0 ± 0</td>
<td>1.63 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>Posterior</td>
<td>0.11 ± 0.02</td>
<td>0.01 ± 0.02</td>
<td>2.74 ± 2.49</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.12 ± 0.03</td>
<td>0.00 ± 0.01</td>
<td>2.07 ± 1.56</td>
<td></td>
</tr>
<tr>
<td>Tibial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>0.16 ± 0.05</td>
<td>0 ± 0</td>
<td>1.71 ± 0.48</td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>0.11 ± 0.04</td>
<td>0 ± 0</td>
<td>1.54 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>Posterior</td>
<td>0.09 ± 0.03</td>
<td>0 ± 0</td>
<td>1.32 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.12 ± 0.05</td>
<td>0 ± 0</td>
<td>1.52 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>Material &amp; Reference</td>
<td>Seeded?</td>
<td>Test Duration</td>
<td>Animal Model</td>
<td>Degree of Success</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>---------</td>
<td>---------------</td>
<td>----------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>Natural Scaffolds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perichondral Tissue (1998)[95]</td>
<td>No</td>
<td>12 Month</td>
<td>Ovine</td>
<td>Low tensile modulus</td>
</tr>
<tr>
<td>Periosteal Tissue (1999)[94]</td>
<td>No</td>
<td>24 Week</td>
<td>Rabbit</td>
<td>Bone present in repair tissue</td>
</tr>
<tr>
<td>S.I.S. (1999)[96]</td>
<td>No</td>
<td>12 Weeks</td>
<td>Canine</td>
<td>Promising results</td>
</tr>
<tr>
<td>Collagen (I) Sponge (1999)[94]</td>
<td>No</td>
<td>24 Weeks</td>
<td>Rabbit</td>
<td>Fibrous tissue, osteoarthritic degeneration</td>
</tr>
<tr>
<td>Collagen (I) Sponge (1999)[94]</td>
<td>Yes</td>
<td>24 Weeks</td>
<td>Rabbit</td>
<td>Fibrocartilaginous tissue, osteoarthritic degeneration</td>
</tr>
<tr>
<td>Collagen (I)-GAG (1999)[97]</td>
<td>Yes</td>
<td>3 Weeks</td>
<td><em>in vitro</em></td>
<td>Cells at periphery, contraction of scaffold</td>
</tr>
<tr>
<td>Collagen (II)-GAG (1999)[97]</td>
<td>Yes</td>
<td>3 Weeks</td>
<td><em>in vitro</em></td>
<td>Cells throughout scaffold, good GAG content</td>
</tr>
<tr>
<td>Collagen (I)-GAG (1992, 1997, 1999)[98-100]</td>
<td>No</td>
<td>3 Years+</td>
<td>Canine, Human</td>
<td>Phase II clinical trial, slight shrinkage</td>
</tr>
<tr>
<td>Synthetic Scaffolds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Teflon-net (1983)[1]</td>
<td>No</td>
<td>12 Months</td>
<td>Canine</td>
<td>Degeneration of AC</td>
</tr>
<tr>
<td>Carbon Fibers (CF) (1983)[101]</td>
<td>No</td>
<td>17 Weeks</td>
<td>Rabbit</td>
<td>Fibrosis</td>
</tr>
<tr>
<td>CF-PU-PLLA (1986)[102]</td>
<td>No</td>
<td>8 Weeks</td>
<td>Canine</td>
<td>Fibrous tissue growth</td>
</tr>
<tr>
<td>CF-PU-PLLA (1986)[103]</td>
<td>No</td>
<td>20 Weeks</td>
<td>Canine</td>
<td>Carbon particles found</td>
</tr>
<tr>
<td>PLLA fibers-PU-PLLA (1986)[103]</td>
<td>No</td>
<td>20 Weeks</td>
<td>Canine</td>
<td>Fibrous tissue growth</td>
</tr>
<tr>
<td>CF-polyester (1990)[104]</td>
<td>No</td>
<td>6 Months</td>
<td>Rabbit</td>
<td>Carbon particles, degeneration</td>
</tr>
<tr>
<td>PU (1996)[107]</td>
<td>No</td>
<td>50 Weeks</td>
<td>Canine</td>
<td>Degeneration of AC</td>
</tr>
<tr>
<td>PU (1996)[108]</td>
<td>No</td>
<td>52 Weeks</td>
<td>Canine</td>
<td>Degeneration of AC</td>
</tr>
<tr>
<td>PLLA-ε-capralactone (1997)[109]</td>
<td>No</td>
<td>26 Weeks</td>
<td>Canine</td>
<td>Ingrowth of fibrocartilage</td>
</tr>
<tr>
<td>PGLA (2000)[111]</td>
<td>Yes</td>
<td>6 Weeks</td>
<td>Ovine</td>
<td>Organized collagen matrix, good proteoglycan amount</td>
</tr>
<tr>
<td>Type &amp; Reference</td>
<td>In Vitro or In Vivo (Animal)</td>
<td>Cells</td>
<td>Result</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------</td>
<td>-------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>FGF (1985)[25]</td>
<td>in vitro</td>
<td>Rabbit</td>
<td>Stimulate proliferation</td>
<td></td>
</tr>
<tr>
<td>Human PL (1985)[25]</td>
<td>in vitro</td>
<td>Rabbit</td>
<td>Stimulate proliferation</td>
<td></td>
</tr>
<tr>
<td>ECGF (1992)[119]</td>
<td>Dog</td>
<td>No</td>
<td>Improve healing in cylindrical defect</td>
<td></td>
</tr>
<tr>
<td>ECGF (1995)[118]</td>
<td>Dog</td>
<td>No</td>
<td>Increase short term healing in tears</td>
<td></td>
</tr>
<tr>
<td>PDGF-AB (1995)[114]</td>
<td>in vitro</td>
<td>Ovine</td>
<td>Affect mitogenic response from outer 1/3 of meniscus</td>
<td></td>
</tr>
<tr>
<td>TGF-β (1995)[38]</td>
<td>in vitro</td>
<td>Ovine</td>
<td>Increased Proteoglycan synthesis</td>
<td></td>
</tr>
<tr>
<td>Hyaluronic Acid (1998)[116]</td>
<td>Rabbit</td>
<td>No</td>
<td>Increase rate of healing in a cylindrical defect</td>
<td></td>
</tr>
<tr>
<td>TGF-β(1999)[33]</td>
<td>in vitro</td>
<td>Human</td>
<td>Increased Proteoglycan synthesis</td>
<td></td>
</tr>
<tr>
<td>PDGF-AB (1999)[115]</td>
<td>in vitro</td>
<td>Bovine</td>
<td>Stimulates cell migration, Increased DNA synthesis</td>
<td></td>
</tr>
<tr>
<td>HGF (1999)[115]</td>
<td>in vitro</td>
<td>Bovine</td>
<td>Stimulates cell migration, Increased DNA synthesis</td>
<td></td>
</tr>
<tr>
<td>BMP-2 (1999)[115]</td>
<td>in vitro</td>
<td>Bovine</td>
<td>Some cell migration, increased DNA synthesis</td>
<td></td>
</tr>
<tr>
<td>IGF-1 (1999)[115]</td>
<td>in vitro</td>
<td>Bovine</td>
<td>Some cell migration</td>
<td></td>
</tr>
<tr>
<td>Interleukin-1 (1999)[115]</td>
<td>in vitro</td>
<td>Bovine</td>
<td>Some cell migration</td>
<td></td>
</tr>
<tr>
<td>Epidermal GF (1999)[115]</td>
<td>in vitro</td>
<td>Bovine</td>
<td>Some cell migration</td>
<td></td>
</tr>
<tr>
<td>Hyaluronan (2000)[117]</td>
<td>Rabbit</td>
<td>No</td>
<td>Stimulate collagen remodeling in peripheral zone</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Strategy for tissue engineering the knee meniscus.
Fig. 2. Human meniscus (shown attached to tibia)
Fig. 3. Meniscus collagen structure
Fig. 4. Free body diagram of the meniscus
Chapter 3: Biodegradable Scaffolds for Meniscus Tissue Engineering*

Abstract

This review details the biodegradable scaffold materials that have been used in the attempt to successfully tissue engineer the knee meniscus. The meniscus is a fibrocartilaginous tissue found within the knee joint that is responsible for shock absorption, load transmission, and stability within the knee joint. If this tissue is damaged, either through tears or degenerative processes, then deterioration of the articular cartilage can occur. Unfortunately, there is a dearth in the amount of work done to tissue engineer the meniscus when compared to other musculoskeletal tissues, such as bone. An overview of different biodegradable materials, both natural and synthetic, is given in depth. In particular, the review concentrates on collagen, small intestine submucosa, periosteal tissue, perichondrial tissue, polyurethanes, copoly(L-lactide/ε-caprolactone), and poly(lactice-co-glycolic) acid.

Introduction

The meniscus, fibrocartilaginous tissue found within the knee joint, is responsible for shock absorption, load transmission, and stability within the knee joint.[1-5] According to the National Center for Health Statistics, over 600,000 surgeries each year are the result of complications with the meniscus.[6] The meniscus has the intrinsic ability to heal itself; unfortunately, this property is limited to the vascular portions of the tissue.[7] For damage outside of these areas and overall degeneration of the tissue, methods need to be developed that will assist the meniscus in healing itself; tissue engineering is a potential solution. Figure 1 depicts our overall philosophy for addressing the problem of successful regeneration of the knee meniscus. Of particular importance is the choice of the scaffold material. This scaffold must be biodegradable, support tissue growth, and protect the articular cartilage surfaces until new tissue is fully developed.

Meniscus Anatomy

The meniscus is a tissue consisting of two wedge-shaped semilunar sections of fibrocartilaginous tissue between the tibial and femoral bearing surfaces of the knee joint (Fig. 2). On gross inspection the meniscus is a white, glossy, and smooth tissue; this smoothness is also present at the microscopic level.[8] The peripheral portion of the meniscus is vascularized, whereas the inner portion is avascular.[9] The meniscus is attached at the medial collateral ligament, the meniscofemoral ligaments, the transverse ligament, and the anterior and posterior horns. The anterior and posterior horns are
where the meniscus joins with the tibial plate; these attachments are usually considered the most important. [10] These horns are also the site of the highest amount of innervation within the meniscus. [11, 12] The rest of the innervation occurs in the outer 1/3 of the meniscus. [11, 12]

*Cells in the Meniscus*

There are two main zones that the meniscus can be divided into, the superficial zone and the deep zone. The cell type present in each of these zones is different. The superficial zone contains cells that are oval or fusiform, have few processes, and a scant amount of cytoplasm, resulting in the nucleus of the cell looking abnormally large. [8, 9] The deep zone cells are rounded or polygonal, are usually alone, but occasionally groups of two or three can be found, and have a large amount of rough endoplasmic reticulum. [8, 9] In 1985 Webber and coworkers [13] coined the term fibrochondrocytes to describe the complex fibroblastic and chondrocytic nature of these cells.

*Extracellular Matrix of the Meniscus*

The extracellular matrix can be separated into four different categories: water, fibrillar components, proteoglycans, and adhesion glycoproteins. Human meniscal tissue has been shown to be 72% water, 22% collagen, 0.8% glycosaminoglycans, and the rest is made up of DNA and adhesion molecules. [14] These numbers can vary depending on age, animal, and location within the tissue. [15, 16]
Fibrillar Components

The main type of fibrillar component found in the meniscus is collagen. Collagen types I, II, III, V, and VI have been found within meniscal tissue, which account for 60-70% of the dry weight.[15] Type I collagen is by far the most predominant, accounting for >90% of the collagen within the tissue.[17] A study done by Cheung[18] has shown that in bovine menisci the outer 2/3 of the menisci is predominantly type I collagen, whereas the inner 1/3 is 60% type II collagen and 40% type I collagen. The meniscus has a unique collagen structure orientation that is related to its function and consists of three different layers. The superficial layer consists of a thin layer of randomly oriented fibers.[19] The lamellar layer, situated just inside the superficial layer, also consists of randomly oriented fibers, with the exception of the peripheral portions at the anterior and posterior sections; here the fibers are oriented radially.[19] The deep zone consists of circumferentially oriented fibers with a small amount of radially oriented fibers, also referred to as tie fibers.[19] The unique fiber orientation found in the knee meniscus leads to the tissue’s exceptional properties, which will be described later.

Proteoglycans

Proteoglycans are responsible for hydration within the meniscus and the compressive properties of the tissue.[20, 21] The concentration of proteoglycans in meniscal tissue is 8 fold less than the concentration found in articular cartilage.[22, 23] Proteoglycans are responsible for tissue hydration and various studies have been performed on this component.[21] The inner 2/3 of the meniscus produces more proteoglycans than the outer third and the lateral side produces more proteoglycans than the medial side, though
the glycosaminoglycan makeup of the proteoglycans stays the same at all of these locations.[24, 25] Normal human meniscal tissue consists of 40% chondroitin-4-sulfate, 10-20% chondroitin-6-sulfate, 20-30% dermatan sulfate, and 15% keratan sulfate.[26]

**Adhesion Glycoproteins**

As the name suggests, adhesion molecules are partly responsible for binding with other matrix molecules and cells. There are three of these molecules that have been identified within the meniscus: Type VI collagen, fibronectin, and thrombospondin.[15] While the exact nature of these three glycoproteins has not yet been discovered, the RGD sequence, which plays a central role in cell adhesion, has been found in Type VI collagen, fibronectin, and thrombospondin.[15]

**Meniscal Biomechanics**

Functionally, the meniscus is a shock absorber, helps with load bearing and transmission in the knee joint, improves stability in the knee, and helps with lubrication.[1-5] Because of all these different functions and the geometry of the tissue, the meniscus is subjected to compressive, tensile, and shear stress. Whenever a load is applied to the knee joint, the meniscus is compressed, but due to its wedge-shaped architecture it is also displaced away from the center of the femoral condyles, resulting in tensile stress because of the anterior and posterior attachment to the tibial plate.[4, 27, 28] If these attachments are not present then the biomechanical function of the meniscus is altered and degeneration of the tissues in the area can result.[29-31] In terms of shear properties of the meniscus, it is known that they depend heavily on the collagen orientation of the meniscus and the
low circumferential shear strength is thought to be partly responsible for the occurrence of longitudinal tears.\[32, 33\]

Movement of the Meniscus and Force Transmission

The menisci partially cover the articular cartilage on the tibial plate and are responsible for absorbing some of the load transmitted through the knee. Application of these loads causes the meniscus to be displaced, and different flexion angles also cause displacement of the tissue.\[3, 4, 27, 28\] In general, the lateral meniscus is displaced more than the medial meniscus during compression, showing that while the menisci both have the same general function they do react differently.\[27\]

Mechanical Properties of Meniscal Tissue

Tensile, compressive, and shear tests have been run on meniscal tissue. Tensile tests run by Proctor and coworkers\[16\] have shown the following:

1. Tensile specimens taken from the surface layer, where collagen orientation is random, are isotropic.
2. Tensile specimens taken from the deep zone, where the majority of the collagen fibers are oriented circumferentially, are anisotropic, and are the stiffest in the circumferential direction.
3. Circumferential specimens from the posterior section are stiffer than specimens from the anterior section.
Tensile tests of the attachments have also been performed, showing that on a rabbit model the anterior attachment is stronger than the posterior attachment and both of these attachments are vital to keep mechanical function.[31, 34] Compression tests of the meniscus, although not performed as frequently as tensile tests, have yielded a wealth of information. It has been shown that the meniscus has a lower compressive stiffness and lower permeability than articular cartilage, suggesting that the meniscus is an excellent shock absorber.[35] Joshi and coworkers[36] have shown a wide difference in the compressive characteristics of the meniscus between different animals, suggesting caution when designing a scaffold for a particular animal model. The shear properties of the meniscus are theorized to contribute to the larger occurrence of longitudinal tears.[35, 37] It has been shown that shear properties are anisotropic, with the stiffness being the lowest in the planes that run parallel to the major collagen orientation.[35] These complex mechanical properties of the meniscus need to be taken into consideration when designing a scaffold that will emulate the native tissue.

Injuries and Repair of the Meniscus

Injuries to the meniscus usually consist of tears in the tissue, though separation from the tibial attachments and degeneration of the tissue also occur.[5, 7, 31, 38, 39] Different styles of tears can occur, such as longitudinal and bucket-handle tears (most common), radial tears, and complex tears.[38, 39] In 1936, King[7] was the first to show that tears in the vascularized portion of the meniscus would naturally heal, whereas tears in the avascular portion would not. It has also been shown that longitudinal tears, if they heal, restore native mechanical function, but radial tears, where the collagen structure is
disrupted, do not have restored mechanical function after healing.[5] While some repair techniques, such as suture, meniscal arrows, fibrin sealant, laser welding, and abrasion therapy, can help heal longitudinal tears in the vascular region, other techniques need to be developed to heal avascular tears and other complications.[40, 41] Tissue engineering offers one way to do this.

Tissue Engineering of the Meniscus

When compared to other musculoskeletal tissues such as bone or articular cartilage, there is a dearth in the number of studies done to-date in the attempt to tissue engineer the meniscus. Many components, such as cells, growth factors, animal models, culturing conditions, and evaluation techniques, must be considered when attempting to tissue engineer the meniscus. Of particular importance is the scaffold, on which more information on past and current efforts is given below.

Biodegradable Scaffolds

Several different biodegradable scaffolds have been used to tissue engineer the meniscus. Results have varied, with some materials warranting no further research to other materials that are still undergoing testing in animal models and in humans. All of the scaffolds that have been attempted can be separated into two major categories, natural and synthetic. Table I gives a summary of different scaffold materials that have been attempted and their effectiveness in tissue engineering the meniscus.
Natural Scaffolds

Four natural scaffolds have been attempted for tissue engineering of the meniscus: collagen, small intestine submucosa (SIS), periosteal tissue, and perichondrial tissue. Of these four, periosteal tissue had the least acceptable results. In a study done by Walsh and coworkers[42] a partial anterior medial meniscectomy was performed in a rabbit model and the defect was filled with a periosteal autograft. After unrestricted activity, the rabbits were sacrificed at 6, 12, and 24 weeks to evaluate the regenerated tissue via gross and histological observations.[42] Results at 6 weeks were promising, but at 12 and 24 weeks bone was noted in the repair tissue and there were severe degenerative changes in the joint.[42] The results from a perichondrial tissue study was not much better.[43] In this study a complete medial meniscectomy was performed on an ovine model and then the defect was filled with autologous perichondrial tissue obtained from the lower rib of the animals.[43] Sacrifice was performed at 3, 6, and 12 months; the regenerated tissue was examined by gross examination, histology, microscopy, and biomechanical testing.[43] The regenerated meniscus showed good results grossly and microscopically, though the histology showed a small area of calcification in the middle of the regenerated tissue.[43] Of greatest concern was the mechanical properties of the regenerated tissue, because the failure stress and tensile modulus of the regenerated tissue were significantly lower than native meniscal tissue.[43] SIS is another material that has undergone brief testing as a scaffold for tissue engineering the meniscus.[44] Cook and coworkers[44] have performed a study where porcine SIS was used to fill the defect created by a partial medial meniscectomy in a canine model. The study, which was performed over a 12 week period, showed similarity to native tissue with respect to type II collagen content,
zonal architecture, and overall size. [44] No mechanical testing of the tissue was performed and, to the authors’ knowledge, no studies over a longer term have been performed. [44] By far the most promising results for a natural scaffold have come from using collagen as a base material for the scaffold. Walsh and coworkers [42] have performed a study where the effect of a bovine type I collagen sponge was tested to evaluate the regenerative effects under the same conditions listed above for the periosteal autograft. [42] They found that the sponge gave better results than the periosteal tissue, though the repair tissue was largely fibrous. [42] They also found that better results could be obtained by seeding the scaffolds with mesenchymal stem cells, though this is the only study that had mesenchymal stem cells seeded on a natural scaffold. [42] One study, done by Mueller and coworkers [45], consisted of seeding meniscal cells on type I collagen-GAG scaffolds and type II collagen-GAG scaffolds and incubating them over a 21 day period. The scaffolds were evaluated via histology, immunohistochemistry, GAG and DNA analysis, and the degree of matrix contraction. [45] Results showed that the type I collagen-GAG scaffold exhibited cells growing at the boundaries of the scaffold only, whereas the type II collagen-GAG scaffold exhibited cells throughout. [45] The type II matrix showed increased GAG and DNA content when compared to the type I matrix at the end of the three week trial. [45] The type II matrix also did not show evidence of significant shrinkage, whereas the type I matrix shrunk to half its initial size. [45] Walsh and coworkers [45] concluded that the type II matrix should be tested in vivo, though to the author’s knowledge this has never been done.
The best results appear to have come from using a collagen-GAG scaffold developed by Stone, Rodkey and coworkers.[6, 46-50] To create this scaffold, collagen is harvested from bovine Achilles tendons, purified, and then molded into a circumferential orientation via manual mold rotation.[46, 47] After a series of in vitro studies, the scaffold was tested in both the porcine and canine animal model.[6, 47, 48] The initial study, using an immature porcine model, showed that the scaffold had no ill effects toward the healing of the meniscus.[48] Following this study, a mature canine model was tested over a 12 month period, where 80% of the meniscus was removed and replaced with the scaffold. Histological and biochemical tests showed that the scaffold induces regeneration of the meniscus.[47] The excellent results of these studies has led to phase II clinical trials of this product.[50] The phase I and II clinical trials consisted of removing the damaged portion of the meniscus, trimming the scaffold to the appropriate size, and then suturing it in place, with the replaced section varying between 35-85% of the total meniscus.[49, 50] Nine patients finished the phase I 36 month trial, and results showed a decrease in the amount of pain noted by the patient and an increase in mobility.[49] The phase II clinical trial, which consisted of eight patients, lasted for at least 24 months and showed similar trends to the phase I trial.[50] Gross examination by arthroscopy showed tissue regeneration in the patients and preservation of the joint articulating surfaces.[50] No adverse effects were noted with the use of this collagen scaffold.[50]
**Synthetic Scaffolds**

There is a wider variety of synthetic scaffolds that have been tried, though none of them have yet reached the state that collagen scaffolds have in the development cycle. A study done by Klompmaker and coworkers[51] showed that the optimal pore size in a scaffold for inducing ingrowth of fibrocartilage is 150-500μm. This group has also done a large amount of research on using an aliphatic polyurethane for a biodegradable synthetic scaffold.[52-54] This material, which degrades 50-75% after one year, was first used in the attempt to improve healing in an avascular longitudinal tear of a canine’s meniscus.[52] The porous polyurethane (86% porosity, 31% being macropores from 250-300 μm and the rest being micropores under 90 μm) was sutured into the defect and healing was evaluated over a 52 week period.[52] Histological and biochemical tests showed both type I and type II collagen present, and some healing of the tear did occur.[52] Degeneration of the articular cartilage surface was also noted in some of the animals.[52] The porous polyurethane was later used to replace the lateral canine meniscus, though there were a larger number of macropores (43%) in this study and the compression modulus was tested and found to be 150 kPa.[54] The scaffold was implanted and the animals were unrestricted for the 28 week period of the study.[54] Histology showed the presence of fibrocartilage in the scaffold after 28 weeks, though degeneration of the articular cartilage bearing surfaces did occur.[54] The investigators theorized that this degeneration could be due to the long period of time that elapses before there is sufficient tissue ingrowth into the scaffold.[54] This same group did another lesion study very similar to the first study where they found that vascularization approaches a lesion, and then when the wound is healed the vascularization retracts.[53]
Unfortunately, the aromatic polyurethane used in the previous studies releases toxic particles during degradation, therefore the material was altered and an aliphatic polyurethane was used.[55] The aliphatic polyurethane scaffold had a compression modulus of 150 kPa and a porosity of 65%, with 35% of the pores being between 250-300 \( \mu \text{m} \) and 35% of the pores ranging in size from 50-90 \( \mu \text{m} \).[55, 56] Results over a 52 week study showed that fibrocartilage was present by 18 weeks after implantation in the canine model, though the tear strength of the implant was weak, leading to loosening of the implant.[55] Complex suturing techniques were used to help alleviate this problem.[55] Unfortunately, at the end of the study some degeneration of the articular cartilage was noted, and they theorized that the scaffold needed a higher compressive modulus to help alleviate this problem.[55] The next study done by this group used a 50/50 copoly(L-lactide/\( \varepsilon \)-capralactone) scaffold with varying compressive modulus (40 and 100kPa).[56] This material was sutured into a partial defect in the lateral meniscus of the canine and tissue ingrowth was determined; an aliphatic polyurethane, like the one used in the above study, was also used as a comparison.[56] The copolymer with a compression modulus of 40 kPa had no fibrous tissue ingrowth, whereas the 100kPa sample had a fibrous tissue ingrowth of 50-70%, and the aliphatic polyurethane had 80-100% fibrous tissue ingrowth.[56] Adhesion of the implant to the native tissue was noted to be higher in the 50/50 copoly(L-lactide/\( \varepsilon \)-capralactone) implants, and was theorized to be due to its faster degradation time when compared to the aliphatic polyurethane.[56]

Ibarra and coworkers[57, 58] have done a series of studies where they use seeded polyglycolic acid (PGA) or poly(lactice-co-glycolic) acid (PLGA) scaffolds in the
attempt to tissue engineer the meniscus. Due to the poor mechanical properties of these materials they seed the scaffolds for a few days, implant them subcutaneously for a period of time (depending on the animal model), and then place them in the defect. In one of their preliminary studies, bovine fibrochondrocytes were harvested and expanded in a supplemented Ham’s F-12 culture media and then seeded onto PGA scaffolds at a concentration of $2.5 \times 10^7$ cells/mL.[57] These scaffolds were then implanted subcutaneously in nude mice for a period of 16 weeks.[57] At the end of 16 weeks the tissue from the implant was found to grossly and histologically resemble meniscal tissue.[57] Biomechanical tests of some of these subcutaneous implants have also been performed; results showed a compressive modulus that was about 40% of the native tissue value.[58] The most recent test performed by the group consisted of implanting a seeded PGLA scaffold in a sheep model for 4 weeks subcutaneously and 6 weeks in situ.[58] The resultant tissue was rich with proteoglycans and had a organized collagen fiber matrix.[58] These results are quite promising, though more studies need to be performed.

In one study, done by Webber and coworkers[59], it was shown that the RGD peptide enhances the attachment of canine fibrochondrocytes to artificial surfaces. This shows great potential for enhancement of current scaffolds that are under development.

**Conclusion**

Successfully tissue engineering the meniscus would be a great help for the treatment of meniscal defects, but much needs to be done before this state can be reached. In
particular, scaffolds need to undergo more testing, and the repair tissue that these scaffolds create needs to be tested grossly, histologically, biochemically, and biomechanically. If none of the current scaffolds being researched give acceptable results then new meniscus-specific scaffolds need to be developed. Peptides could be used in a scaffold for directed cell attachment, and growth factors could be used for aiding in differentiation and synthesis. The scaffold could also be combined with cells and the tissue could be grown in a bioreactor (Fig 1). Central to this approach is the use of a suitable scaffold that can allow the implementation of innovative strategies to tissue engineer the meniscus.
## Tables

### Table I. Meniscal Scaffolds

<table>
<thead>
<tr>
<th>Material &amp; Reference</th>
<th>Seeded?</th>
<th>Test Duration</th>
<th>Animal Model</th>
<th>Degree of Success</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural Scaffolds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perichondral Tissue (1998)[43]</td>
<td>No</td>
<td>12 Month</td>
<td>Ovine</td>
<td>Low tensile modulus</td>
</tr>
<tr>
<td>Periosteal Tissue (1999)[42]</td>
<td>No</td>
<td>24 Week</td>
<td>Rabbit</td>
<td>Bone present in repair tissue</td>
</tr>
<tr>
<td>S.I.S. (1999)[44]</td>
<td>No</td>
<td>12 Weeks</td>
<td>Canine</td>
<td>Promising results</td>
</tr>
<tr>
<td>Collagen (I) Sponge (1999)[42]</td>
<td>No</td>
<td>24 Weeks</td>
<td>Rabbit</td>
<td>Fibrous tissue, osteoarthritic degeneration</td>
</tr>
<tr>
<td>Collagen (I) Sponge (1999)[42]</td>
<td>Yes</td>
<td>24 Weeks</td>
<td>Rabbit</td>
<td>Fibrocartilaginous tissue, osteoarthritic degeneration</td>
</tr>
<tr>
<td>Collagen (I)-GAG (1999)[45]</td>
<td>Yes</td>
<td>3 Weeks</td>
<td>in vitro</td>
<td>Cells at periphery of scaffold, contraction of scaffold</td>
</tr>
<tr>
<td>Collagen (II)-GAG (1999)[45]</td>
<td>Yes</td>
<td>3 Weeks</td>
<td>in vitro</td>
<td>Cells throughout scaffold, good GAG content</td>
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<td>3 Years+</td>
<td>Canine, Human</td>
<td>Phase II clinical trial, slight shrinkage</td>
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<tr>
<td><strong>Synthetic Scaffolds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PU (1996)[54]</td>
<td>No</td>
<td>28 Weeks</td>
<td>Canine</td>
<td>Degeneration of AC</td>
</tr>
<tr>
<td>PU (1996)[53]</td>
<td>No</td>
<td>50 Weeks</td>
<td>Canine</td>
<td>Degeneration of AC</td>
</tr>
<tr>
<td>PU (1996)[55]</td>
<td>No</td>
<td>52 Weeks</td>
<td>Canine</td>
<td>Degeneration of AC</td>
</tr>
<tr>
<td>PLLA-ε-capralactone (1997)[56]</td>
<td>No</td>
<td>26 Weeks</td>
<td>Canine</td>
<td>Ingrowth of fibrocartilage</td>
</tr>
<tr>
<td>PGA (1997)[57]</td>
<td>Yes</td>
<td>16 Weeks Subcutaneous</td>
<td>Rat</td>
<td>Resembled meniscal tissue, Lower compressive modulus</td>
</tr>
<tr>
<td>PLGA (2000)[58]</td>
<td>Yes</td>
<td>6 Weeks</td>
<td>Ovine</td>
<td>Organized collagen matrix, good proteoglycan amount</td>
</tr>
<tr>
<td></td>
<td>(Meniscal)</td>
<td>in situ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. General principle for tissue engineering the meniscus.
Fig. 2. Human meniscus (shown attached to tibia)
Chapter 4: Fibrochondrocytes and their Use in Tissue Engineering of the Meniscus

Abstract

The meniscus is a fibrocartilaginous tissue within the knee joint responsible for shock absorption, load transmission, lubrication, and stability. Damage to the meniscus can result in loss of some or all of the above functions. Unfortunately, current repair techniques do not adequately address the issue of meniscus regeneration. Tissue engineering is one possible solution to fix this difficult problem. Meniscal cells, also known as fibrochondrocytes, have the potential to play a central role in the tissue engineering approach. This article provides a description of various studies performed to-date, such as fibrochondrocyte characterization and their reaction to different culturing environments, peptides, and growth factors. The current tissue engineering attempts performed are also examined.

Introduction

The meniscus is a fibrocartilaginous structure found within the knee joint responsible for shock absorption, load transmission, and stability [1-5]. According to the National Center for Health Statistics in the United States, over 600,000 surgeries each year are the result of complications with the meniscus [6]. Regions of the meniscus, namely those in the vascular zone, have an intrinsic healing capability, whereas the avascular zone does not heal [7]. To repair damage in the avascular region and overcome tissue degeneration, methods that will assist the meniscus in healing itself need to be developed, and tissue engineering is a potential solution. Fibrochondrocytes constitute a major component of tissue engineering attempts. Fibrochondrocytes are responsible for filling a scaffold material with matrix, organizing the matrix in response to the mechanical stimuli present in the joint, and helping the tissue construct integrate with the surrounding tissue. A thorough understanding of the mechanics and anatomy of the tissue is necessary before attempting to tissue engineer the meniscus.

Meniscus Anatomy

The meniscus is a tissue consisting of two wedge-shaped semilunar sections of fibrocartilaginous tissue between the tibial and femoral bearing surfaces of the knee joint (Fig. 1). On gross inspection the meniscus is a white, glossy, and smooth tissue; this smoothness is also present at the microscopic level [8]. The peripheral portion of the meniscus, also known as the red zone, is vascularized, whereas the inner portion, known
as the white zone, is avascular (Fig. 2) [9]. The meniscus is attached to the medial collateral ligament, the meniscofemoral ligaments, the transverse ligament, and the anterior and posterior horns. The anterior and posterior horns are where the meniscus joins with the tibial plate; these attachments are usually considered the most important [10].

**Cells in the Meniscus**

Most meniscal cell characterization has been carried out in humans and the rabbits [8, 9, 11-13]. Meniscal cells are generally considered to be a cross between chondrocytes and fibroblasts. The cells have a rounded morphology and are protected by a territorial matrix, as are chondrocytes, yet the cells produce type I collagen, like fibroblasts. In 1985, Webber and coworkers [13] coined the term ‘fibrochondrocytes’ to describe these unique cells. There are two relatively distinct fibrochondrocyte populations found in different locations within the human meniscus. Oval or fusiform fibrochondrocytes with a few small processes are found in the superficial layers (Fig. 3). Fibrochondrocytes from the deeper zone are rounded or polygonal and contain more processes [9]. In-depth studies have also been carried out on the rabbit meniscus. A study by Moon et al. [12] found that the periphery of the meniscus contains cells that are fibroblast-like, whereas chondrocyte-like cells were found in the inner rim of the meniscus. This correlates with the functional aspects of the tissue. The inner rim of the meniscus is a bearing surface for the knee joint, is subjected to compressive forces, and contains a combination of type I and II collagen. The periphery is responsible for shock absorption, is subjected mainly to tensile forces,
and contains large quantities of type I collagen. A recent study on rabbit fibrochondrocytes [11] provides an in-depth look at the different locations of the meniscus. This study found four morphologically distinct classes of fibrochondrocytes within the meniscus. Cells in the superficial layer were found to be fusiform in shape, as shown in other studies [8, 9]. Cells from the inner rim, also known as the white zone, contained rounded cells that lacked projections. The last two cell types, found in the red zone and red-white zone, had long cellular processes (larger quantities in the red zone) and a large number of gap junctions. Cells within these two regions were also organized into rows, something not seen in the white zone. This row-like organization of cells along the collagen fibers is also noted in other fibrous tissues, such as tendon. It is believed that the large number of processes helps the tissue organize the extracellular matrix so that it can withstand the tensile hoop-stress. It should also be noted that the processes observed in fibrochondrocytes in vivo are not found in these cells if grown in culture, indicating that culture conditions result in morphological changes.

*Extracellular Matrix of the Meniscus*

The extracellular matrix is made of four different components: water, fibrillar proteins, proteoglycans, and adhesion glycoproteins. Biochemical analysis has shown that human meniscal tissue contains 72% water, 22% collagen, 0.8% glycosaminoglycans; the rest is made up of DNA and adhesion molecules [14]. These numbers can vary depending on age, species, and location within the tissue [15, 16].
Fibrillar Components

The main type of fibrillar component found in the meniscus is collagen. Collagen types I, II, III, V, and VI have been found within meniscal tissue, and account for 60-70% of the dry weight [15]. Type I collagen is by far the most predominant, accounting for more than 90% of the collagen within the tissue [17]. For example, in bovine menisci the outer 2/3 of the tissue collagen is predominantly type I collagen, whereas the inner 1/3 is 60% type II collagen and 40% type I collagen [18]. The meniscus has a unique collagen fiber orientation that is related to its function and consists of three different layers. The superficial layer consists of a thin layer of randomly orientated fibers [19]. The lamellar layer, situated just inside the superficial layer, also consists of randomly orientated fibers, with the exception of the peripheral portions at the anterior and posterior sections; here the fibers are orientated radially [19]. The deep zone consists of circumferentially orientated fibers with a small amount of radial fibers, also referred to as tie fibers [19]. The combination of the different mechanical stimuli most likely cause the cells to organize the collagen fibers in such a fashion.

Proteoglycans

Proteoglycans are responsible for hydration within the meniscus and the compressive properties of the tissue [20, 21]. The concentration of proteoglycans in meniscal tissue is 8 fold lower than the concentration found in articular cartilage [22, 23]. Various studies have been performed on meniscal proteoglycans, which are responsible for tissue hydration [21]. The inner 2/3 of the meniscus produces more proteoglycans than the outer 1/3. More proteoglycans are produced laterally than medially, though the
glycosaminoglycan makeup of the proteoglycans stays the same at all of these locations [24, 25]. This higher proteoglycan content in the inner third correlates with the bearing surface nature in the avascular portion of the tissue.

**Adhesion Glycoproteins**

As the name suggests, adhesion molecules are partly responsible for binding with other matrix molecules and cells. There are three of these molecules that have been identified within the meniscus: type VI collagen, fibronectin, and thrombospondin [15]. While the exact nature of these three glycoproteins has not yet been described, the RGD peptide, which plays a central role in cell adhesion, has been found in type VI collagen, fibronectin, and thrombospondin [15].

**Meniscal Biomechanics**

Functionally, the meniscus acts as a shock absorber, helps with load bearing and transmission in the knee joint, improves stability in the knee, and helps with lubrication [1-5]. Because all of these different functions and the geometry of the tissue, the meniscus is subjected to compressive, tensile, and shear stress. Whenever a load is applied to the knee joint, the meniscus is compressed, but due to its wedge-shape it is also displaced away from the center of the femoral condyles, resulting in tensile stress because of the anterior and posterior attachment to the tibial plate [3, 26, 27]. In terms of shear properties of the meniscus, it is known that they depend heavily on the collagen orientation of the meniscus and the low circumferential shear strength is thought to be
partly responsible for the occurrence of longitudinal tears [28, 29]. The menisci partially cover the articular cartilage on the tibial plate and are responsible for absorbing some of the load transmitted through the knee and protecting the articular cartilage within the joint [3, 5, 26, 27, 30, 31]. In general, the lateral meniscus is displaced more than the medial meniscus during compression, showing that while both menisci have the same general function they do react differently [27]. These differences can also be noted in the biomechanical and biochemical properties of the meniscus among various animal models [30, 32].

Tissue Engineering of the Meniscus

The amount of work that has been performed in the attempt to tissue engineer the meniscus has been limited when compared to other musculoskeletal tissues, such as bone or articular cartilage. Many components, such as the fibrochondrocytes, the animal model, scaffold material, and evaluation techniques, are of importance. Several aspects of the cellular components have been studied, such as cell isolation, cell culture, the effect of peptides, and the effect of growth factors. Much of this information has been used in the few attempts to engineer the meniscus carried out to-date.

Fibrochondrocyte Isolation Methods

A variety of different methods have been used to isolate the fibrochondrocytes from the surrounding matrix for tissue culture applications [24, 33-38]. Webber et al. [34] used a
method adopted from Green [33] for articular chondrocytes. This isolation method consisted of sequential treatment of minced tissue with 0.05% hyaluronidase, 0.2% trypsin, and 0.2% clostridial collagenase, in conjunction with mechanical stirring, to release the cells, followed by filtering and washing the fibrochondrocytes several times to remove the debris [33, 34]. Tanaka et al. [24] treated meniscal tissue with 0.8% pronase for 25 minutes, followed by digestion with 0.4% collagenase for 40-60 minutes to isolate fibrochondrocytes from the meniscus. Mueller et al. [36] used a simple collagenase digestion (0.2%), in conjunction with mechanical stirring, for a period of six hours to release the fibrochondrocytes from the meniscal tissue. Nakata et al. [35] looked at each of the three above methods and determined that a simple collagenase digestion provided the most consistent results in terms of cell number and phenotype. In their studies a 0.4% collagenase solution was used for a period of 2-3 hours. Ibarra et al. [37, 38] also used a simple collagenase digestion method.

**In Vitro Cell Culture**

The majority of *in vitro* studies have been performed in monolayers. Fibrochondrocytes from several different species have been used, such as the human, rabbit, dog, and pig, but the majority of studies have been performed on the human and rabbit models [13, 34, 35, 39, 40]. Human fibrochondrocytes grown in monolayer culture have shown three distinguishable types: elongated fibroblast-like cells, polygonal cells, and round chondrocyte like cells [35]. There are many different factors which can have an effect on
the cells, such as the media type, the age and sex of the animal, and the amount of CO$_2$ in the culture environment.

**Media Type**

The type of media used in monolayer culture can have a profound effect on morphology, proliferation, and protein synthesis capabilities of fibrochondrocytes [13, 35, 39, 40]. A study performed by Nakata et al. [35] compared morphology, proliferation ability, and mRNA synthesis of fibrochondrocytes in three different culture mediums: Ham’s F-12 with 10% fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, and a 1:1 mixture of Ham’s F-12 and DMEM supplemented with 10% FBS. Fibrochondrocytes proliferated the fastest in DMEM and the slowest in Ham’s F-12. Morphology was maintained in the Ham’s F-12 media and the Ham’s F-12:DMEM media, but the elongated fibroblast like cells became predominant in the DMEM media within one week. Fibrochondrocytes grown in the Ham’s F-12:DMEM mixture also showed expression of mRNA for types I, II, III, and IX collagen, and aggregan. Additional studies looking into the effect of the different media types on fibrochondrocytes have been carried out by Webber et al. [13, 39, 40] In a study from 1985 [13] fibrochondrocytes were grown in either a DMEM/10% FBS media or a Ham’s F-12/10% FBS media. The cells were then passaged into secondary culture and analyzed for morphology, growth, and proteoglycan synthesis. The morphology of the fibrochondrocytes varied depending on the initial media type. The cells grown in the DMEM had a polygonal shape and resembled chondrocytes, whereas the cells from the F-12 media were fusiform, with a fibroblast-like morphology. Cells grown in the F-12
media had a slightly faster growth rate in primary culture, but in secondary culture the
growth rate of the cells from the DMEM was more rapid. The addition of ascorbate to the
media had a drastic effect on the population doubling time of the fibrochondrocytes. In
secondary culture the addition of ascorbate to the F-12 media increased the population
doubling time (PDT) from 20 hours to greater than 24 hours, whereas the PDT of the
cells grown in the DMEM decreased from 15 hours to 8.8 hours with the addition of
ascorbate. The addition of ascorbate also decreased the uptake of $^{35}$SO$_4$, a marker used for
estimating proteoglycan synthesis, though it should also be noted that the uptake was
higher in the F-12 media than the DMEM media. In another study, performed by Webber
et al. [39], a serum-free culture media was developed that gives similar results to 10%
FBS. The serum-free media developed consists of a 1:1 ratio of DMEM:Ham’s F-12,
transferrin (1 μg/ml), selenium (1 pg/ml), trace metal mix (1:100), dexamethasone (100
ng/ml), insulin-like growth factors I and II (50 ng/ml each), pituitary fibroblastic growth
factor (100ng/ml), and lactalbumin hydrolysate (2 μg/ml). The one major difference
between the serum-free media and the media with 10% FBS was the morphology.
Fibrochondrocytes from the serum-free media had a more polygonal morphology. This
trait continued for the length of the experiment (20 days).

**Age and Gender**

Webber et al. [34, 41] have also performed two studies examining the effect of age and
gender on fibrochondrocyte quantity, proliferation, and proteoglycan synthesis. A 1986
study [34] looked into these characteristics in rabbit fibrochondrocytes. Results showed
that female menisci contain more fibrochondrocytes than male menisci, regardless of age.
Gender and age did not have a significant effect on the PDT, but an effect was noted on proteoglycan synthesis. Fibrochondrocytes from six-month-old male rabbits incorporated four times as much $^{35}$SO$_4$ as the female fibrochondrocytes, though this trend was reversed in older rabbits (24 months), with the fibrochondrocytes from the females exhibiting twice as much uptake as the fibrochondrocytes from the males. A later study [41], performed using human explants, showed that ingrowth of fibrochondrocytes into a fibrin clot was significantly quicker in skeletally immature individuals (14 and 16 years old) when compared to skeletally mature individuals (>22 years old).

**CO$_2$ Levels**

Different CO$_2$ levels have been used in fibrochondrocyte culture both for fibrochondrocyte characterization studies and tissue engineering attempts. Ten percent CO$_2$ was used by Webber et al. [13, 34, 39, 40] for all of their cell characterization work, whereas Nakata et al. [35] and Bhargava et al. [42], Ibarra et al. [43, 44], and Mueller et al. [36] used 5% CO$_2$ for their tissue engineering efforts. No direct comparison has been performed to determine the effect of these two culture environments on fibrochondrocytes.

**Fibrochondrocytes and Peptides**

It has been shown that the RGD peptide enhances the attachment of fibrochondrocytes [40]. Canine fibrochondrocytes were harvested and seeded on surfaces either coated with chondroitin sulfate alone, or with chondroitin sulfate conjugated to a peptide containing
the RGD sequence. A large quantity of cells attached to the surface containing the RGD peptide, but not on the surface with chondroitin sulfate alone [40]. Overall, the RGD sequence shows great potential for supporting fibrochondrocytes attachment to a scaffold for tissue engineering.

**Fibrochondrocytes and Growth Factors**

Numerous growth factors have been used on meniscal fibrochondrocytes to test their effects on the healing of tears or defects, and on protein synthesis in tissue and cell culture. Table I summarizes the different growth factors that have been used. All of these have the potential to help in tissue engineering of the meniscus.

Most experiments have used cultures of fibrochondrocytes or small tissue explants to examine the proliferative response of the cells or protein synthesis. One growth factor that has a potential application is transforming growth factor-β (TGF-β). Studies by Tanaka et al. [24] and Collier et al. [25] showed that TGF-β increases proteoglycan synthesis in fibrochondrocytes from all different regions of the meniscus in a dose-dependent manner. In these studies Collier and Ghosh used ovine fibrochondrocytes and Tanaka et al. used human fibrochondrocytes [24, 25]. Studies by Spindler et al. [45] and Bhargava et al. [42] tested the effect of human platelet-derived growth factor-AB (PDGF-AB) on ovine and bovine cells. The ovine study showed that PDGF-AB only affected the mitogenic response from the peripheral third of the meniscus; there was no effect on the inner 2/3 of the tissue [45]. Bhargava et al. [42] bovine test showed that PDGF-AB
stimulated the migration of fibrochondrocytes from the inner, middle and outer 1/3 of the meniscus; PDGF-AB was also shown to increase DNA synthesis by the cells from all three sections. Bhargava et al. [42] also found increased DNA synthesis when hepatocyte growth factor (HGF) or bone-morphogenic protein-2 (BMP-2) was used. HGF also increased the cell migration rate similar to PDGF-AB. BMP-2 and IGF-I (insulin-like growth factor-1) stimulated the migration of fibrochondrocytes from the middle zone by 40-50%. This study also tested the effects of two other growth factors: interleukin-1, which stimulated migration of cells taken from the peripheral 1/3 of the tissue, and epidermal growth factor (EGF), which stimulated migration of cells from the inner and outer zones by 40-50% [42]. A study by Imler et al. [46] found that in bovine explants the addition of IGF-1 increased proteoglycan synthesis in a dose dependent manner. An earlier study by Webber et al. [13] tested the effect of fibroblast growth factor (FGF) and human platelet lysate (PL) on proliferation of fibrochondrocytes and both were found to stimulate their growth.

Other studies have been performed to check the effects of growth factors on healing of open defects and tears in the meniscus. Hyaluronic acid, hyaluronan and endothelial cell growth factor (ECGF) have been studied in both tear healing and defect repair [47-50]. Suzuki et al. [47] created a cylindrical defect in a rabbit anterior lateral horn and then made weekly injections of hyaluronic acid, demonstrating increased rate of healing. A study by Sonoda et al. [48] tested hyaluronan’s effect on the healing of tears in the peripheral and avascular region of the rabbit meniscus. They found that hyaluronan stimulated collagen remodeling in the peripheral zone and inhibited swelling in the
avascular zone [48]. Another study tested the effect of ECGF, a member of the acidic FGF family[51], on the healing of an allograft to the joint capsule, found that ECGF increases short-term healing, but over the long term no difference was found [49]. A study by Hashimoto [50] tested the effect of ECGF on assisting the healing of a cylindrical full-thickness defect placed in the meniscus of a dog. The defect was filled with a fibrin sealant (some containing ECGF) and then allowed to heal over a 24-weeks period. The defects that contained both the fibrin sealant and ECGF showed the best results. Roughly, 90% of the defect was filled at the end of the study [50].

*Tissue Engineering Attempts*

Only a limited amount of work has been carried out to attempt to tissue engineer the meniscus with fibrochondrocyte seeded scaffolds [35-37, 52]. Ibarra et al. [37] used polyglycolic acid (PGA) scaffolds seeded with bovine fibrochondrocytes in an attempt to engineer the meniscus. The cells were seeded onto the PGA scaffold, implanted subcutaneously in nude mice, and evaluated after 12 weeks. Grossly, the construct resembled meniscal tissue, and histology indicated meniscal repair tissue. Unconfined compression on the constructs demonstrated compressive properties that were 40% of the native tissue levels. These studies were also performed using ovine, canine, and human cells. In a later study, ovine cells were seeded onto a copolymer of PGA and polylactic acid (PLA), implanted subcutaneously in a sheep, and later implanted within the knee joint. Histological evaluation showed the presence of fibroblastic and chondrocytic cells within the repair tissue, along with collagen and proteoglycans. In a study by Ertl et al.
[52] a PGA mesh was seeded with rabbit fibrochondrocytes, grown in culture for one month, and implanted into a full-thickness defect for a period of one year. Histological results showed healing at one year, but no biochemical or biomechanical testing was performed. Collagen scaffolds have also been examined for tissue engineering of the meniscus. A study by Nakata et al. [35] showed that human fibrochondrocytes can attach to a collagen sponge and infiltrate some of the scaffold. Mueller et al. [36] examined the effect of collagen-glycosaminoglycan (GAG) scaffolds made from either type I collagen or type II collagen. The scaffolds were seeded with canine fibrochondrocytes and evaluated over a period of 21 days. Results showed that in the type I collagen matrix the cells remained near to the periphery of the scaffold, whereas they were evenly distributed throughout the type II scaffold. The type II scaffold contained 50% more GAGs than the type I scaffold, and did not contract, whereas the type I scaffold shrunk to half of its original size. To the authors' knowledge, no seeded collagen scaffold has yet been implanted in an animal model.

**Conclusion**

Successful tissue engineering of the meniscus would provide great help for the treatment of meniscal defects, but much work needs to be carried out before this can be achieved. More characterization of the cells is needed, particularly with respect to their culture environment and growth factors. The use of bioreactors for small and large scale cell production needs to be examined, and more animal studies need to be performed.
Hopefully, fibrochondrocytes can be used to tissue engineer constructs that will successfully address the difficult problem of meniscal regeneration.
### Tables

**Table I. Growth factors that were studied for meniscal tissue engineering**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Cells</th>
<th>In Vitro or In Vivo (Animal)</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF</td>
<td>Lapine</td>
<td><em>In vitro</em></td>
<td>Proliferation was stimulated</td>
<td>[13]</td>
</tr>
<tr>
<td>Human PL</td>
<td>Lapine</td>
<td><em>In vitro</em></td>
<td>Proliferation was stimulated</td>
<td>[13]</td>
</tr>
<tr>
<td>ECGF</td>
<td>No cells</td>
<td>Dog</td>
<td>Improved healing in cylindrical defect</td>
<td>[50]</td>
</tr>
<tr>
<td>ECGF</td>
<td>No cells</td>
<td>Dog</td>
<td>Increased short term healing in menisc tears</td>
<td>[49]</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>Ovine</td>
<td><em>In vitro</em></td>
<td>Affected mitogenic response from the outer 1/3 of meniscus</td>
<td>[45]</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Stimulated cell migration and the increased DNA synthesis</td>
<td>[42]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Ovine</td>
<td><em>In vitro</em></td>
<td>Increased proteoglycan synthesis</td>
<td>[25]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Human</td>
<td><em>In vitro</em></td>
<td>Increased proteoglycan synthesis</td>
<td>[24]</td>
</tr>
<tr>
<td>Hyaluronic Acid</td>
<td>No cells</td>
<td>Rabbit</td>
<td>Increased rate of healing in a cylindrical meniscal defect</td>
<td>[47]</td>
</tr>
<tr>
<td>HGF</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Stimulated cell migration, increased DNA synthesis</td>
<td>[42]</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Some cell migration and increased DNA synthesis</td>
<td>[42]</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Some cell migration</td>
<td>[42]</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Increased proteoglycan synthesis</td>
<td>[46]</td>
</tr>
<tr>
<td>Epidermal GF</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Some cell migration</td>
<td>[42]</td>
</tr>
<tr>
<td>Interleukin-1</td>
<td>Bovine</td>
<td><em>In vitro</em></td>
<td>Some cell migration</td>
<td>[42]</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>No cells</td>
<td>Rabbit</td>
<td>Stimulated collagen remodeling in peripheral zone</td>
<td>[48]</td>
</tr>
</tbody>
</table>
Fig. 1. Meniscus attached to the tibia.
Fig. 2. Schematic drawing of the meniscus showing the vascular profile and tibial attachment locations. The white zone is avascular, the red-white zone is a transitional area, and the red zone is vascularized.
Chapter 5: Tensile and Compressive Properties of the Medial Rabbit Meniscus

Abstract

Quantification of the material properties of the meniscus is of paramount importance, creating a “gold-standard” reference for future tissue engineering research. The purpose of this study was to determine compressive and circumferential tensile properties in the rabbit meniscus. Creep and recovery indentation experiments were performed on the meniscus using a creep indentation apparatus and analyzed via a finite element optimization method to determine the compressive material properties at six topographical locations. Tensile properties of samples taken circumferentially from the rabbit meniscus were also examined. Results show that the femoral side of the anterior portion exhibits the highest aggregate modulus (510 ± 100 kPa) and shear modulus (240 ± 40 kPa), while the lowest aggregate modulus (120 ± 30 kPa) and shear modulus (60 ± 20 kPa) were found on the femoral side of the posterior location. A Young’s modulus of 156.6 ± 48.9 MPa and an ultimate tensile strength of 21.6 ± 7.0 MPa were found from the tensile samples, which is similar to the values found in other animal models. These baseline values of material properties will be of help in future tissue engineering efforts.

Introduction

The meniscus (Fig. 1) has been recognized as a crucial structural element in the knee joint that is responsible for load distribution, shock absorption, knee stabilization, and lubrication.[1-7] Damage to this tissue can lead to osteoarthritic changes, suggesting that the meniscus protects the knee joint from degenerative joint disease.[7-9] Owing to its biomechanical significance, tissue engineering efforts have been used in the attempt to repair or replace the damaged meniscus. Unfortunately, successful replacement has yet to occur.

In an effort to better understand pathologic and traumatic processes in the knee meniscus, many studies have investigated the biomechanical behavior of this tissue. Tensile and compressive tests have been performed, but there has been variability in testing methods, including differences in specimen size, specimen harvest location, test conditions, and animal models.[6, 10-16] Most of the studies performed have been tensile studies,[10, 14, 17-21] with some compressive testing having also been performed.[10, 12, 13, 22]

Due to the size of the tissue, most biomechanical characterization of the meniscus has been performed on large animal models, which allows for topographical examination of the material properties. Tensile studies performed by Fithian and coworkers,[15] Tissakht and Ahmed,[20] and Proctor and coworkers[10] have found that the Young’s modulus varies on circumferential samples taken from the anterior, central, and posterior portions of the meniscus, though which location exhibits the highest value seems to depend on the animal model, with the anterior portion the highest in the human and the
posterior portion the highest in the bovine. Another significant variation, which was studied in human menisci by Lechner and coworkers,[21] is that the thickness of the tensile specimen has a significant effect on the tensile material properties, with thinner specimens exhibiting higher values than thicker specimens. One testing parameter that does not have significant effect on the tensile properties is the strain rate, as discovered by Uezaki and coworkers[23] and Newton and coworkers.[24] This is probably due to the low amount of proteoglycans within the tissue.[25] Topographical compressive characterization has also been performed on the meniscus. One of the first compressive tests, performed by Proctor and coworkers,[10] used confined compression to determine the aggregate modulus and permeability of the bovine meniscus at different locations and depths. Results showed that the aggregate modulus from the superficial zone did not vary significantly depending on location, though specimens from the deep zone were found to be stiffer in the posterior portion than the anterior portion. Hacker and coworkers[13] tested disks from the anterior, central, and posterior portions of the human meniscus under confined compression. Their results showed that the posterior portion of the meniscus had the highest aggregate modulus and the anterior portion had the lowest aggregate modulus.

Unfortunately, all of the above studies have been performed in larger animal models. Little characterization has occurred in small animal models, such as the rabbit, which are an ideal animal model for preliminary tissue engineering efforts. The purpose of this investigation was to examine the compressive and tensile properties of the medial rabbit meniscus. Specifically, via a creep indentation experiment, the compressive
biomechanical properties (aggregate modulus - $H_A$, permeability - $k$, Poisson’s ratio - $\nu_S$, and shear modulus - $\mu_S$) were quantified at six topographical locations. Also, after determination of the native tissue’s collagen fiber alignment, tensile properties of circumferential specimens were examined to determine the Young’s modulus (E) and ultimate tensile strength (UTS) of the medial rabbit meniscus.

Materials and Methods

Specimen Preparation

All medial menisci used for this study were harvested from skeletally mature 3.5-4.0 kg male New Zealand white rabbits. 14 medial menisci from eight different rabbits were removed from the knee joint, wrapped in gauze soaked with a 0.15 M NaCl solution with protease inhibitors (N-ethylmaleimide, 10 mM; benzamidine HCl, 5 mM; EDTA, 2 mM; and PMSF, 1 mM) and frozen at -20°C until time of testing. Six medial menisci from six different rabbits were removed from the knee joint and processed immediately for tensile testing. All samples underwent one freeze-thaw cycle before testing (the freeze-thaw cycle for the tensile specimens occurred during the tissue preparation stage, described below).

SEM Preparation

Two menisci were fixed in 10% formalin and subdivided into various sections so that different areas could be evaluated. Ohtani’s procedure[26] was used to uncover the collagen fibrils. Briefly, the deep zone section of the meniscus was exposed via mechanical separation. The specimens were then placed in a 2N NaOH solution for 6
days, rinsed with water, and saturated in a 1-2% tannic acid solution for 5 hours. After this step, they were rinsed again, counter-fixed in OsO4, dehydrated via an ascending alcohol series, and dried using the critical point method. The specimens were then sputtered with gold and viewed on a Philips XL-30 ESEM. The size of the collagen fibers and bundles were then quantified using these SEM images.[27, 28]

*Creep Indentation*

The anterior, central, and posterior portion of each meniscus, on either the femoral or tibial side, was tested biomechanically to determine each meniscal specimen’s aggregate modulus, Poisson's ratio, permeability, and shear modulus. A creep indentation apparatus[29, 30] was used to quantify the creep and recovery deformation behavior of each specimen. The testing apparatus is able to load and unload the meniscus specimen automatically through a closed-loop control system. To test, each specimen (Fig. 2a) was thawed for an hour in normal saline containing protease inhibitors, separated into anterior, central, and posterior portions (Fig. 2b), and then each section (Fig. 2c) was attached with cyanoacrylate cement to a sample holder (Fig. 2d). The sample holder was positioned with a spherical joint and lead screw assembly (Fig. 2e), allowing the meniscal surface to be oriented normal to the loading shaft. A tare load of 0.005 N was then applied with a 0.8 mm diameter, flat-ended, cylindrical rigid, porous indenter tip (50% porous, ~50 μm pore diameter), and the tissue was allowed to reach tare creep equilibrium. The tare load, test load, and indenter tip size were selected to ensure that the response of the tissue was within its linear range. Equilibrium was automatically determined when the slope of the creep curve became smaller than 1x10^{-6} mm s^{-1}. Once
creep equilibrium from the tare load was reached, the tissue was loaded with a step force of 0.02 N. The tissue's deformation was monitored with a linear variable differential transformer (LVDT) using a computer-based data acquisition system at a 0.25 μm deformation-resolution. The frictional resistance of the system was reduced with air bearings to less than 9.81 x 10^-4 N. The bearings were driven with pressurized air (552 kPa) and the air was cleaned with a 5 μm particle filter and two 0.1 μm coalescing filters. The creep response of the specimen under the step force was monitored until equilibrium (defined as slope<1x10^-6 mm s^-1, or two hours) was reached. At this point, the force was automatically removed and the recovery phase began. When recovery equilibrium was achieved (defined as slope<1x10^-6 mm s^-1, or one hour), data acquisition was stopped automatically. Overall, the automated creep indenter yielded the creep and recovery deformational behaviors of each meniscal specimen in response to a 0.02 N step load.

*Thickness of Compressive Samples*

The thickness was measured using two methods: First we simply used a micrometer to measure the overall approximate thickness at the test site. Following specimen mounting and creep indentation testing, thickness was also measured using a needle probe attached to a force transducer and an LVDT. The entire probe assembly was moved downward with a linear motor until the needle touched the meniscal surface. At this point, the force transducer noted a significant change in force on the needle probe and the needle continued to move through the sample. When the needle contacted the sample holder underneath the meniscal sample another significant change in the force on the needle
probe was noted. The difference between the measured needle positions at the two force readings corresponded to specimen thickness.

**Finite Element Modeling of Compressive Specimens**

The experimental data for each test site were analyzed using a finite element/non-linear optimization modeling (FEO) method. This approach, which is based on biphasic finite element routines and nonlinear optimization techniques, uses the entire creep curve to calculate the intrinsic material properties of articular cartilage[30] and the meniscus[31]. The output of this procedure depends significantly on an initial estimate of the tissue’s properties. Thus, we used a semi-analytical/semi-numerical biphasic procedure to obtain an estimate of the tissue’s properties, before applying the finite element/non-linear optimization routine.[30] More details can be found in a series of papers by Mow and coworkers.[32-34]

**Tensile Sample Preparation**

Fresh (unfrozen) menisci were removed from the rabbit (Fig. 3a) and the inner 1/3 of the tissue was mechanically removed with a scalpel (Fig. 3b). The peripheral edge of the sample was then flattened against the freezing stage of a Microm HM 500 M cryotome (Microm International; Germany) by gently applying pressure to the anterior and posterior horns (Fig. 3c). Multiple 100 μm thick slices of the tissue were then removed from each meniscus and placed in saline with protease inhibitors to keep hydrated (Fig. 3d). The thickness of the samples and the width at the narrowest portion of the sample were then measured via a stereomicroscope and digital calipers to obtain the cross-
sectional area (Fig. 3e). The samples were not cut into dog-bone shapes due to their small size. The test specimens were then kept in saline with protease inhibitors until testing to keep the tissue hydrated. Due to the small size of the medial rabbit meniscus, topographical samples could not be retrieved from the tissue.

**Tensile Test**

Tensile testing was performed with a electromechanical materials testing system (Model 5565, Instron; Canton, Massachusetts) with a 50 N load cell. For gripping the specimen, two surgical wire twisters (Miltex; York, Pennsylvania) were attached to the materials testing system (Fig. 4) and the samples were held in the textured ends of the wire twisters with a 3 mm grip-to-grip separation. After a 0.05 N tare load was applied, the tissue was preconditioned to 2% strain at a displacement rate of 0.06 mm s\(^{-1}\) for 10 cycles. The sample was then pulled at a rate of 0.06 mm s\(^{-1}\) until failure. Multiple samples from each meniscus were tested, and only samples that fractured between the grips (Fig. 5) were used in the analysis; no interface fracture samples were used in the analysis. The tissue was misted with saline during all tests to keep the tissue hydrated.

**Tensile Sample Data Analysis**

Stress-strain curves were created from the load-displacement curves and the cross-sectional areas of each sample. From each stress-strain curve, the Young’s modulus (E) and ultimate tensile strength (UTS) were calculated. All successful samples were averaged for each individual meniscus, and the six menisci were used for the statistical analysis.
Statistical Analysis

For the compressive data one-way analysis of variance (ANOVA) was used to determine statistical significance, with an n=6 for each sample set. The aggregate modulus, Poisson's ratio, permeability, and shear modulus were set as the dependent variable and the topographical location as the independent variable. If the F-test showed a significant difference (p < 0.05), a post hoc test was performed (Fisher’s Protected Least Significant Difference) to compare sample sets. A significance level (α = 0.05) was used to determine statistical significance between the test locations. For the tensile data, the mean value ± standard deviation was calculated from a sample size of n=6, and this data was compared to previous tensile results in other animal models. Due to the small size of the medial rabbit meniscus, topographical variations of the tensile properties could not be tested.

Results

From the SEM pictures the collagen fiber structure, orientation, fiber size, and collagen bundle size were determined. The predominate fiber orientation is in the circumferential direction (Fig. 6a), with some radial tie fibers being noted. The collagen fibers are arranged in bundles in the deeper sections of the tissue (Fig. 6b, 6c), with the surface of the meniscus exhibiting randomly oriented collagen fibers (Fig. 6d). The diameter of the collagen fibers was found to be between 100 and 150 nm, and the size of the collagen bundles varied between 10 and 70 μm. No variation was found for either the orientation
or size of the collagen fibers and bundles among the anterior, central, and posterior portions of the tissue.

From the creep-relaxation curve (Fig. 7) the four material properties (aggregate modulus, Poisson's ratio, permeability, and shear modulus) were determined. The intrinsic material properties exhibited wide topographical variation (Table I), with a general trend that the anterior portion of the tissue had higher material properties than the central and posterior portions (Fig. 8). The aggregate modulus and shear modulus were statistically higher in the anterior segment on the femoral side of the tissue compared to the central and posterior locations (p<0.0001). The statistically highest permeability and Poisson’s ratio were found on the anterior portion on the tibial side of the tissue when compared to the central and posterior locations of the tissue (p<0.0008). If only the anterior, central, and posterior aspects of the tissue are examined (the values were averaged from the femoral and tibial side), the anterior portion is statistically higher than the central and posterior portions (p<0.0007). If only the femoral and tibial sides are examined (anterior, central, and posterior portions averaged) then no statistical significance is noted (p>0.23).

The results of the tensile testing can be found in Table II. The Young’s modulus, which was calculated from the linear portion of the stress-strain curve (Fig. 9), was found to be 156.6 ± 48.9 MPa. The ultimate tensile strength, which was the stress at the point of fracture, was found to be 21.6 ± 7.0 MPa.
Discussion

While some studies have examined the tensile and compressive properties of the meniscus in large animal models, no studies have examined smaller animal models, such as the rabbit. This study was conducted to determine the tensile properties of the medial rabbit meniscus and the variation in the compressive properties at different topographical locations. For tensile characterization, the collagen fiber structure was examined via SEM, and circumferential samples were pulled to failure to determine the Young’s modulus and ultimate tensile strength. For the compressive characterization, a creep indentation technique was used to determine the aggregate modulus, Poisson’s ratio, permeability, and shear modulus (calculated from $H_A$ and $v_S$) at six different locations: the anterior, central, and posterior portions of the meniscus on both the femoral and tibial surfaces of the tissue.

Scanning electron microscopy studies performed by Petersen and Tillmann[27] showed that the human meniscus has a unique collagen structure orientation consisting of three different layers. They found the superficial surface of the tissue to consist of randomly oriented collagen fibers. A lamellar layer, situated just inside the superficial layer, also consists of randomly oriented fibers, with the exception of the peripheral portions at the anterior and posterior sections, where the fibers are oriented radially. The deep zone consists of circumferentially oriented fibers with a small amount of radially oriented fibers. The scanning electron microscopy performed in the current study also showed a superficial layer consisting of randomly oriented fibers and a deep zone consisting of predominantly circumferentially oriented collagen fibers with some radial fibers present.
A lamellar layer was not noted in the rabbit meniscus, but it is unclear if it was not present or just not successfully exposed during the tissue processing.

Tensile tests have been applied recently in large animal models such as the human, bovine, canine, and ovine models (Table II).[10, 15, 20, 21, 35, 36] Several of these studies also performed topographical comparisons of the tensile properties. Unfortunately, due to the small size of the rabbit meniscus, this could not be performed in the current study. Most tensile characterization has been performed on the human meniscus. The most recent study, performed by Lechner and coworkers,[21] examined human meniscal samples at three different thicknesses to determine if specimen thickness had an effect on the properties of the tissue. Results show that the thickness of the specimen does effect the Young’s modulus observed from the tissue, with thicker specimens (3 mm: 52.5 ± 44.5 MPa) exhibiting lower values than thinner specimens (0.5 mm: 116.9 ± 58.2 MPa). The current study on the rabbit exhibited a Young’s modulus of 156.6 ± 27.9 MPa in 0.1 mm thick specimens. This variation from the Lechner and coworkers study could be due to the difference in specimen thickness and animal model, and it should be noted that the high value of aggregate modulus in the thin rabbit specimens matches the trend noted by Lechner and coworkers.[21] Tissakht and Ahmed[20] also examined the human meniscus in a variety of different topographical locations. Using 1.5-2 mm thick specimens, they found a Young’s modulus of 57.97 ± 19.82 MPa and an ultimate tensile strength of 11.83 ± 1.63 MPa in the central portion of the medial meniscus, which is the location closest to what was tested in the current study. The Young’s modulus is once again higher in the current study (156.6 ± 27.9 MPa), as
was the ultimate tensile strength \((21.6 \pm 7.0 \text{ MPa})\). The variation between these values and could again be due to the difference in specimen size and animal model. It should be noted that the values between the Lechner and coworkers study[21] and the Tissakht and Ahmed study[20] are similar. A study by Fithian and coworkers[15] also found similar values for the human meniscus when compared to the Lechner and coworkers study.[21] This study examined 0.4 mm thick samples and found a Young’s modulus of \(93.18 \pm 52.4 \text{ MPa}\). Samples of the same thickness from the bovine model were examined by Proctor and coworkers.[10] A Young’s modulus of \(139.0 \pm 79.2 \text{ MPa}\) was found, which is higher than the value found in the human meniscus, indicating variation between animal models. The canine model has also been tested with 0.4 mm thick samples, with results exhibiting a Young’s modulus of \(99.46 \pm 38.13 \text{ MPa}\) and a ultimate tensile stress of \(11.92 \pm 9.27 \text{ MPa}\).[35] The Young’s modulus between the human and canine are very similar, indicating that animal size is not a determining factor in the variation between tensile properties. The sheep model has also been examined by in a study performed by Andersen and coworkers.[36] In this study, 0.35 mm thick tensile samples were found to have a Young’s modulus of \(217 \pm 37 \text{ MPa}\) and a ultimate tensile strength of \(22.9 \pm 1.0 \text{ MPa}\) in the anterior portion of the tissue. This Young’s modulus is significantly higher than the other animal models tested, though the ultimate tensile strength is very similar to the rabbit model tested in the current study. Overall, tensile properties of meniscal tissue vary significantly between animal model and specimen thickness, with the rabbit tensile properties being similar to the values found in the human and canine models, after taking specimen thickness into consideration.
Compressive characteristics of the medial meniscus have been examined in the human, bovine, canine, and porcine models. Results show variations both topographically and among animal models. One study that found topographical variations, performed by Hacker and coworkers,[13] used a confined compression technique on the human medial meniscus to determine the aggregate modulus and permeability at the anterior, central, and posterior portions of the tissue (tibial side). They found the aggregate modulus to be 200 kPa, 220 kPa, and 280 kPa at the anterior, central, and posterior portions of the tissue, respectively.[13] Our results showed aggregate moduli of 410 ± 110 kPa, 190 ± 80 kPa, and 150 ± 60 kPa for the respective locations. It should be noted that the posterior portion of the Hacker and coworkers[13] study found the highest aggregate modulus, whereas the current study found the highest value in the anterior portion. Differences between the two studies were also noted for the permeability values. The Hacker and coworkers[13] study found permeability values of 0.9 x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}, 0.8 x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}, and 0.9 x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1} for the anterior, central, and posterior locations. The current study found the permeability at each of the locations to be 4.44 ± 2.05 x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}, 0.94 ± 0.40 x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}, and 0.99 ± 0.29 x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}. These variations are probably due to the animal model, testing method, and different curve-fitting methods (our study used a finite element optimization procedure for curve fitting, the Hacker and coworkers[13] study used a biphasic theory which does not take into consideration the full creep curve). Topographical variations were also examined in the bovine model by Proctor and coworkers,[10] who tested the femoral side of the medial meniscus at four different locations (anterior, anterior-central, central-posterior, and posterior). Testing in the superficial zone found an aggregate modulus of 393 ± 109 kPa
in the posterior portion and 440 ± 108 kPa in the anterior portion. Our results for the femoral side of the rabbit meniscus show aggregate modulus values of 120 ± 30 kPa for the posterior portion and 510 ± 100 kPa for the anterior portion. If the permeability between the Proctor and coworkers[10] study and this study is compared it is found that the Proctor and coworkers[10] study found lower values (0.7 - 1.0 x10^{-15} m^4 N^{-1} s^{-1}) than our study (0.9 – 4.0 x10^{-15} m^4 N^{-1} s^{-1}). These variations are probably due to variations in the testing method, test location, and curve-fitting procedure (this study used the same method as the Hacker and coworkers study[13]). The current study of the rabbit meniscus shows a significantly higher aggregate modulus in the anterior portion when compared to the other topographical locations, something not seen in the other animal models. This topographical variation could be due to the bent-knee resting stance of the rabbit and the frequency with which the animal jumps. Joshi and coworkers[12] performed a confined compression study where the aggregate modulus and permeability of the meniscus was examined at the tibial-posterior location among a variety of animal models (human, canine, bovine, porcine, monkey, and ovine). Their human results found an aggregate modulus of about 220 kPa and a permeability of 1.99 ± 0.79 x10^{-15} m^4 N^{-1} s^{-1}, whereas our results in the rabbit show an aggregate modulus of 150 ± 60 kPa and a permeability of 0.99 ± 0.29x10^{-15} m^4 N^{-1} s^{-1}. The bovine model exhibited an aggregate modulus of about 120 kPa and a permeability of about 3.3 x10^{-15} m^4 N^{-1} s^{-1}. For the canine and porcine models, an aggregate modulus of about 150 kPa and 270 ± 40 kPa were found and the permeability was 3.5 x10^{-15} m^4 N^{-1} s^{-1} and 1.74 ± 0.19 x10^{-15} m^4 N^{-1} s^{-1}, respectively. The monkey and ovine models also exhibited a wide variation between the two models, with an aggregate modulus of 110 ± 20 kPa and a permeability of 6.78 ±
1.12 \times 10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1} found in the monkey and an aggregate modulus of about 230 kPa and a permeability of $1.91 \pm 0.46 \times 10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}$ in the ovine model. This study showed that large variations in the compressive properties of the meniscus existed among different animal models. When compared to the current study, the canine model exhibited results most similar to the lapine aggregate modulus, and the porcine model exhibited results close to the lapine permeability values. It should also be noted that all of the above studies used confined compression to determine the material properties, whereas this study used a indentation technique, which we believe is a more physiologically relevant testing method due to the presence of native tissue surrounding the test site and the lack of edge effects when testing the tissue sample.

The most common location for injury in the meniscus is the posterior region.[37, 38] Our results show a trend of the posterior region having the lowest shear modulus and, to a lesser extent, aggregate modulus. This trend in material properties could help explain the frequency of tears in this location. The other interesting trend noted was that the aggregate modulus, shear modulus, and permeability of the anterior portion of the lapine model were much greater than the central and posterior portions. We believe that this characteristic is due to the bent-knee resting stance of the rabbit and the frequency that the animal jumps, something not seen in other animal models.

Conclusion

Overall, this study examined the medial rabbit meniscus using scanning electron microscopy to determine collagen structure and orientation, tensile testing to determine
the Young’s modulus and ultimate tensile strength, and indentation testing to determine the aggregate modulus, Poisson’s ratio, permeability, and shear modulus. Results indicate that the structure is similar to the human meniscus, tensile properties similar to other animal models, and topographical variations are present in the compressive properties, most likely due to the bent-knee resting stance of the rabbit and the frequency that the animal jumps. These results can be used as a “gold-standard” baseline for future tissue engineering efforts.

Acknowledgments

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### Tables

**Table I. Lapine meniscal biomechanical properties (±SD)**

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (kPa)</th>
<th>$\nu_S$</th>
<th>$k$ ($10^{15}$ m$^4$ N$^{-1}$ s$^{-1}$)</th>
<th>$\mu_S$ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral</td>
<td>Anterior</td>
<td>510 ± 100</td>
<td>0.04 ± 0.04</td>
<td>4.03 ± 1.49</td>
<td>240 ± 40</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>130 ± 20</td>
<td>0.00 ± 0.00</td>
<td>0.92 ± 0.20</td>
<td>60 ± 10</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>120 ± 30</td>
<td>0.00 ± 0.00</td>
<td>1.13 ± 0.47</td>
<td>60 ± 20</td>
</tr>
<tr>
<td>Tibial</td>
<td>Anterior</td>
<td>410 ± 110</td>
<td>0.08 ± 0.07</td>
<td>4.44 ± 2.05</td>
<td>190 ± 60</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>190 ± 80</td>
<td>0.01 ± 0.01</td>
<td>0.94 ± 0.40</td>
<td>90 ± 40</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>150 ± 60</td>
<td>0.00 ± 0.00</td>
<td>0.99 ± 0.29</td>
<td>80 ± 30</td>
</tr>
</tbody>
</table>

a. $H_A$ = aggregate modulus, $\nu_S$ = Poisson's ratio, $k$ = permeability, $\mu_S$ = shear modulus
### Table II. Meniscal tensile properties from the central region (±SD)

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Young’s Modulus (MPa)</th>
<th>Ultimate Tensile Strength (MPa)</th>
<th>Sample Thickness (mm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapine</td>
<td>156.6 ± 27.9</td>
<td>21.6 ± 7.0</td>
<td>0.1</td>
<td>current study</td>
</tr>
<tr>
<td>Human</td>
<td>116.9 ± 58.2</td>
<td></td>
<td>0.5</td>
<td>[21]</td>
</tr>
<tr>
<td>Human</td>
<td>73.5 ± 49.9</td>
<td></td>
<td>1.5</td>
<td>[21]</td>
</tr>
<tr>
<td>Human</td>
<td>52.5 ± 44.5</td>
<td></td>
<td>3.0</td>
<td>[21]</td>
</tr>
<tr>
<td>Human</td>
<td>57.97 ± 19.82</td>
<td>11.83 ± 1.63</td>
<td>1.5-2.0</td>
<td>[20]</td>
</tr>
<tr>
<td>Human</td>
<td>93.18 ± 52.4</td>
<td></td>
<td>0.4</td>
<td>[15]</td>
</tr>
<tr>
<td>Bovine</td>
<td>139.0 ± 79.2</td>
<td></td>
<td>0.4</td>
<td>[10]</td>
</tr>
<tr>
<td>Canine</td>
<td>99.46 ± 38.13</td>
<td>11.92 ± 9.27</td>
<td>0.4</td>
<td>[35]</td>
</tr>
<tr>
<td>Ovine</td>
<td>217 ± 37</td>
<td>22.9 ± 1.0</td>
<td>0.35</td>
<td>[36]</td>
</tr>
</tbody>
</table>
Figures

Fig. 1. Schematic diagram of the knee. The joint space is elongated to show the location of the medial and lateral menisci.
Fig. 2. A schematic diagram of the compressive testing procedure. The medial meniscus (a) is separated into anterior, central, and posterior regions (b). Each portion of the meniscus (c) is then attached to the sample mount (d) and tested in the creep indentation apparatus (e).
**Fig. 3.** A schematic diagram of the tensile testing procedure. The medial meniscus is removed from the rabbit knee (a). The inner portion of the meniscus is then removed with a scalpel (b) and the outer portion is sliced into thin sections via a cryotome (c). The samples are then stored in saline with protease inhibitors (d) until measurement and testing (e).
Fig. 4. Picture of the specimen grips in the electromechanical materials testing system.

Fig. 5. Example of a tensile fracture in the center of the specimen.
Fig. 6. Scanning electron microscopy of the meniscal collagen fibers. A) Circumferential fiber orientation (50x). B) Close-up of collagen bundle, showing individual fibers (8000x). C) Cross-section of collagen bundle, showing the ends of the individual fibers (10000x). D) Random fiber orientation on the outer surface of the meniscus (20000x).
Fig. 7. A typical creep-recovery curve from the anterior portion (femoral side) of the medial rabbit meniscus.
Fig. 8. Aggregate modulus at the six tested locations of the medial rabbit meniscus. Values are mean ± standard deviation. (#) indicates statistical significance between the femoral-anterior location and all other locations (p<0.03). (@) indicates statistical significance between the tibial-anterior location of the medial meniscus and the central and posterior portions of the tissue (p<0.0001).
Fig. 9. A representative stress-strain curve of the tensile specimens.
Chapter 6: Biochemical Properties of the Medial Rabbit

Meniscus*

Abstract

The purpose of this investigation was to examine the biochemical properties of the medial rabbit meniscus at a variety of topographical locations to create a “gold-standard” baseline for future meniscal repair research. Specifically, the inner 1/3 and outer 2/3 of the anterior, central, and posterior portions of the medial meniscus were examined for degree of hydration, quantity of sulfated glycosaminoglycans, level of hydroxyproline, and collagen type distribution. The hypothesis was that the inner 1/3 of the tissue would have a statistically significant variation compared to the outer 2/3 of the tissue and that the anterior portion of the tissue would vary significantly from the central and posterior portions of the meniscus. Results indicate that, indeed, the inner 1/3 of the tissue exhibits a greater level of hydration, sulfated glycosaminoglycans, collagen II content, and lower amount of hydroxyproline. The anterior portion of the tissue exhibits a higher sulfated glycosaminoglycan content and lower hydroxyproline content. These baseline values of biochemical properties will be of help in future meniscal repair efforts, using the rabbit as the animal model.

* To be submitted to Matrix Biology.
Introduction

The meniscus, a semilunar fibrocartilaginous tissue attached to the tibial plateau (Fig. 1), is responsible for weight-bearing, load distribution, shock absorption, knee stabilization, and lubrication within the knee joint.[1-5] In the human meniscus the inner 1/3 of the meniscus is avascular, consists of randomly oriented collagen type I and II fibers, and is considered to be more cartilaginous in nature. The outer 2/3 of the meniscus is partially vascularized, consists of circumferentially oriented collagen type I fibers, and is considered more fibrous in nature.[6-8] Loss of normal function of the meniscus due to trauma or degenerative wear often results in significant morbidity for the patient.[9-11] Owing to this tissues significance in proper knee function, salvage of the damaged meniscus has drawn clinical attention. Unfortunately, due to the fact that this tissue was originally believed to be functionless,[11, 12] and that excision of the tissue was assumed to ameliorate any complications, limited examination of the baseline biochemical properties has been performed, particularly in smaller animal models such as rabbit.

Most biochemical characterization of the meniscus has been performed in the human model, but the canine, porcine, bovine, lapine, and equine models have also been examined. Water content, sulfated glycosaminoglycan (GAG) content, collagen content, and collagen type distribution have all been examined in meniscal tissue, but there has been variability in testing methods, including differences in topographical test locations and animal models. Results from these animal studies have demonstrated that meniscal biochemical properties are inhomogeneous, with variations occurring depending on the animal model and test location. Most studies have been performed on the larger animal
models, with very little characterization performed on small animal models, such as the lapine model. Larger animal model characterization has shown that the inner, cartilaginous portion of the meniscus has higher water content and lower collagen content than the outer, fibrous portion of the meniscus, which consists of a lower water content and greater concentration of collagen.[7, 13, 14] No variation in these properties between the anterior, central, and posterior portions has been noted in the limited characterization on the canine and human models. It should also be noted that the anterior portion of the rabbit meniscus is significantly stiffer than the central or posterior portions of the tissue, something not seen in other animal models.[15]

Unfortunately, the lack of characterization on smaller animal models, such as the lapine model, has limited the ability to perform preliminary studies for various meniscal repair techniques, such as tissue engineering. The purpose of this investigation was to examine the biochemical properties of the medial rabbit meniscus at a variety of topographical locations to create a standard baseline for future meniscal repair research. Specifically, the inner 1/3 and outer 2/3 of the anterior, central, and posterior portions of the medial meniscus were examined for degree of hydration, quantity of sulfated glycosaminoglycans, levels of hydroxyproline, and distribution of collagen type. The hypothesis was that the inner 1/3 of the tissue would have a statistically significant variation compared to the outer 2/3 of the tissue and that the anterior portion of the tissue would vary significantly from the central and posterior portions of the meniscus.
Materials and Methods

Tissue Harvest

A total of 13 male New Zealand white rabbits, weighing between 3.5 and 4.0 kg, were used for this study. The rabbits were anesthetized with ketamine (25 mg/kg) and xylazine (10 mg/kg), and sacrificed with an injection of Beuthanasia (0.22 mL/kg). The medial menisci were harvested from the knee joints, wrapped in saline soaked gauze, and stored at −20°C until testing. For water content and biochemical analysis a sample size of 6 was used. For each tested parameter, three menisci were obtained from the right leg of three rabbits, and the remaining three menisci were obtained from the left leg of three different rabbits.

Histological Testing and Immunohistochemistry

Routine histological processing was performed using 5-20 μm thick sections of the meniscal tissue and staining with Hematoxylin & Eosin, Masson’s trichrome, and Safranin O/Fast Green. The stained tissue was observed for histological appearance, proteoglycan staining, and collagen staining. Immunohistochemistry was also performed on slices of the tissue to determine the distribution of collagen types I and II. The sections of tissue were fixed in chilled acetone for 20 min and then stored at −20°C until staining. Immunostaining was then performed using a Biogenex i6000 (San Ramon, CA) autostainer. Slides were placed in the autostainer and rehydrated for 5 min in PBS. Endogenous peroxidase activity was quenched with 1% hydrogen peroxide in methanol for 30 min, blocked with serum from the secondary antibody host for 20 min (3% horse
serum) incubated with the primary antibody (mouse monoclonal IgG anti-collagen I antibody (Accurate Chemical and Scientific: Westbury, NY) and mouse monoclonal IgG anti-collagen II antibody (Chondrex, LLC: Redmond, WA)) for 60 min. The specimens were then incubated with a secondary antibody in 3% horse serum (and 2% porcine serum to prevent non-specific binding) for 30 min, followed by the avidin-biotinylated enzyme complex for 30 min and then 3,3'-diaminobenzidine for 4 min. Slides were removed from the autostainer, counterstained with hematoxylin, dehydrated in graded ethanol and mounted for viewing.

Water content

The water content of the meniscus was measured at six locations: the inner 1/3 (white zone) and outer 2/3 (red zone and red-to-white zone) of the anterior, central, and posterior regions of the tissue (Fig. 2). Water content was determined in six medial menisci using the following procedure. Each meniscal specimen was weighed on a balance after gentle blotting to remove excess water. The specimen was then placed in a lyophilization unit for twenty-four hours to remove all water and dry weight was measured. The water content was then expressed as the percent difference between the wet weight and dry weight of the tissue:

\[
\text{Water content (\%)} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}}
\]
Biochemical Characterization

Two biochemical assays were performed to determine the composition of the medial rabbit meniscus. A dimethylmethylene blue assay was used to determine the sulfated glycosaminoglycan content of the tissue, and a hydroxyproline assay was used to approximate the total collagen content. Both tests were performed at the six testing locations (anterior, central, posterior; inner 1/3 and outer 2/3) used for the water content study. Six medial menisci were separated into these sections, minced with a scalpel, weighed, and digested in a papain solution (125 µg/ml papain in a 0.1M sodium phosphate, 5 mM EDTA, and 5 mM N-acetyl-L-cysteine solution[16]) at 60ºC for the hydroxyproline assay and an additional six menisci were processed using the same method for the dimethylmethylene blue assay.

Dimethylmethylene Blue Assay

A dimethylmethylene blue assay was performed on a portion of the papain digest described above. Samples were tested via the Blyscan™ glycosaminoglycan assay kit (Accurate Chemical, Westbury, NY) and viewed at a wavelength of 656 nm on a Bio-Tek Instruments Powerwave 340 plate reader.

Hydroxyproline Assay

Hydroxyproline quantification has been used widely to estimate the amount of collagen.[17-21] A procedure reported by Woessner[22] that improves the sensitivity and stability of the assay and eliminates contribution from other amino acids was used to determine hydroxyproline content. Samples of the papain digest described above were
hydrolyzed using 4N sodium hydroxide at 121 °C for 1h. After neutralization with 4N hydrochloric acid, the samples were placed in a buffer and then hydroxyproline content was determined by reacting the samples with chloramine T and dimethylaminobenzaldehyde, which allows for a colorimetric comparison.[22] Absorbency was determined spectrophotometrically at a wavelength of 550nm on a Bio-Tek Instruments Powerwave 340 plate reader.

*Statistical Analysis*

For the water content and biochemical tests sample size was set as n=6 and a one-way analysis of variance (ANOVA) was used to determine statistical significance. The dependent variables were set as the tested parameters and the topographical locations were the independent variables. If the F-test showed a significant difference (p < 0.05), a post hoc test was performed (Fisher’s Protected Least Significant Difference) to compare sample sets. A significance level (α = 0.05) was used in all the statistical tests performed.

*Results*

*Histology and Immunohistochemistry*

Hematoxylin & Eosin staining performed on sections of meniscal tissue (Fig. 3) showed short rows of fibrochondrocytes situated between the collagen fibers, as is typical of rabbit meniscal tissue.[23] Safranin O/Fast Green staining exhibited a greater degree of staining in the inner 1/3 of the tissue and the anterior portion of the tissue (Fig. 4). Masson’s trichrome staining indicated a large amount of collagen at all tissue locations.
Immunohistochemistry revealed collagen I staining throughout the tissue, whereas the collagen II staining was nonhomogeneous, with staining noted in the inner 1/3 of the tissue and at the edges of the anterior and posterior locations (Fig. 5).

**Water Content**

Variation of the water content was found among the six test locations (Table I), with the inner 1/3 of the anterior position exhibiting more hydration than the other locations (p<0.03) (Fig. 6). If the anterior, central, and posterior portions of the tissue are compared there is no statistically significant difference between these locations (p>0.13). If the inner 1/3 and outer 2/3 of the tissue is compared, it is found that the inner 1/3 is more hydrated (72.9 ± 2.5%) compared to the outer 2/3 (67.7 ± 1.9%) (p<0.002).

**Dimethylmethylene Blue Assay**

Significant variation was found among the six test locations (Table II, Fig. 7) with the inner 1/3 of the anterior portion exhibiting the greatest amount of sulfated glycosaminoglycans (114.5 ± 33.9 μg/mg dry weight, p<0.0002). If the anterior, central, and posterior portions of the tissue are compared, it is found that the anterior portion exhibits a higher sulfated glycosaminoglycan level (31.4 ± 5.1 μg/mg dry weight) than the central and posterior portions (16.8 ± 4.5 μg/mg dry weight, 17.8 ± 4.1 μg/mg dry weight, p<0.0003). If the inner 1/3 and outer 2/3 of the tissue is compared, it is found that the inner 1/3 exhibits a higher amount of sulfated glycosaminoglycans (83.1 ± 22.2 μg/mg dry weight) compared to the outer 2/3 (19.2 ± 2.1 μg/mg dry weight) (p<0.0001).
Hydroxyproline Assay

Significant variation was found among the six test locations (Table III, Fig. 8), with the outer 2/3 of the posterior location exhibiting the highest hydroxyproline content (67.7 ± 6.9 μg/mg dry weight) and the inner 1/3 of the anterior location exhibiting the lowest hydroxyproline content (30.0 ± 6.7 μg/mg dry weight). If the anterior, central, and posterior locations of the tissue are compared, it is found that the posterior portion (58.6 ± 14.6 μg/mg dry weight) contains a statistically significant variation (p<0.01) compared to the anterior location (40.9 ± 12.4 μg/mg dry weight). If the inner 1/3 and outer 2/3 of the tissue are compared, the inner 1/3 (37.9 ± 14.5 μg/mg dry weight) is significantly lower than the outer 2/3 (61.6 ± 9.5 μg/mg dry weight) (p<0.0001).

Discussion

While some studies have examined various biochemical properties of the meniscus in a variety of larger animal models, no study has examined the topographical variation of these properties in smaller animal models, such as the rabbit. This study was conducted to determine the degree of hydration, quantity of sulfated glycosaminoglycans, levels of hydroxyproline, and distribution of collagen types I and II in the medial rabbit meniscus. Specifically, the inner 1/3 and outer 2/3 of the anterior, central, and posterior portions of the medial meniscus were examined via a wet-to-dry weight comparison, a dimethylmethylene blue assay, a hydroxyproline assay, and immunohistochemistry. Due to the more cartilaginous nature of the inner 1/3 of the tissue, and the variation in compressive properties between the anterior, central, and posterior locations,[15] it was
hypothesized that the inner 1/3 of the tissue would have a statistically significant variation compared to the outer 2/3 of the tissue and that the anterior portion of the tissue would vary significantly from the central and posterior portions of the meniscus.

The water content of the meniscus has been previously examined in the canine, porcine, and human models.[19, 24-26] Adams and coworkers[25] found the canine medial meniscus to be 66.65 ± 1.64% water, and in an earlier study found the central portion of the meniscus to be 62.8 ± 1.2% water, whereas a pooled portion of the anterior and posterior locations consisted of 63.5 ± 1.4% water.[24] The current study found a water content of 68.7 ± 1.8%, 67.8 ± 2.8%, and 70.0 ± 2.5%, in the anterior, central, and posterior locations, respectively, of the medial rabbit meniscus. Overall, the water content is higher in the rabbit meniscus. An even greater water content was found in the human meniscus, with the anterior portion consisting of 72.1 ± 9.7% water, the central consisting of 76.8 ± 2.7% water, and the posterior portion consisting of 74.9 ± 7.3% water.[19] Nakano and Aherne[26] examined the inner 1/3, middle 1/3, and outer 1/3 of the medial porcine meniscus and found that the inner 1/3 had the higher degree of hydration (74.0 ± 1.4%) compared to the middle (69.2 ± 0.3%) and outer (66.2 ± 0.8%) portions. This trend was also noted in the medial rabbit meniscus, with the inner 1/3 hydrated at 72.9 ± 2.5% and outer 2/3 at 67.7 ± 1.9%. One other interesting note is that the inner 1/3 of the anterior portion of the rabbit meniscus is more hydrated than any other location within the meniscus. This might be due to the high loads placed on this portion of the tissue (due to the flexed knee resting stance of the rabbit, unlike humans, dogs, and pigs).
Proteoglycan / glycosaminoglycan analysis has been performed via histology and dimethylmethylen blue assays. To determine the distribution of proteoglycans, Nakano and coworkers[18] have performed safranin-O staining on the porcine meniscus. Using a scoring system to determine the intensity at different topographical locations, they showed a significantly (p<0.05) higher degree of staining in the inner 1/3 of the tissue, and a higher degree of staining in the anterior locations, compared to the central and posterior locations (p<0.05). These results match the histological findings of this study in the rabbit meniscus (Fig. 4). Fithian and coworkers,[19] in addition to the water content mentioned above, also performed a dimethylmethylen blue assay on the human meniscus to determine the sulfated glycosaminoglycan distribution between the anterior, central, and posterior regions. They found a sulfated GAG content of 22.0 ± 10.1 μg/mg dry weight in the anterior region, 20.6 ± 6.8 μg/mg dry weight in the central region, and 19.4 ± 8.3 μg/mg dry weight in the posterior region. The results in the rabbit showed a sulfated GAG level of 31.4 ± 5.1 μg/mg dry weight in the anterior region, 16.8 ± 4.5 μg/mg dry weight in the central region, and 17.8 ± 4.1 μg/mg dry weight in the posterior region. These values are roughly in the same range, though topographical variation is seen in the rabbit data, with the anterior portion exhibiting a higher concentration of sulfated GAGs. This might be due to the high loads placed on this portion of the tissue (due to the flexed knee resting stance of the rabbit and its propensity to jump, something not seen in the human).
Analysis of the hydroxyproline content of meniscal tissue has been previously performed on the human and porcine models.[17-21] One of the first hydroxyproline assays run on the human meniscus, performed by Peters and Smille,[17] found a hydroxyproline content of 105 ± 16 μg/mg dry weight. A later human study, performed by Ingman and coworkers,[20] examined the variation between the inner 1/3 and outer 2/3 of the meniscus, and found hydroxyproline contents of 105 ± 1 μg/mg dry weight and 102 ± 1 μg/mg dry weight, respectively. The variation between the anterior, central, and posterior locations has also been examined in the human model. Fithian and coworkers[19] found a hydroxyproline content of 132 ± 36 μg/mg dry weight, 139 ± 34 μg/mg dry weight, and 139 ± 36 μg/mg dry weight for the anterior, central, and posterior locations, respectively. Both the Ingman and coworkers study[20] and the Fithian and coworkers[19] study found no statistical significance between any of the test locations. However, the current study in the rabbit model did find statistical significance between locations, with the outer 2/3 of the tissue exhibiting a higher hydroxyproline level (61.6 ± 9.5 μg/mg dry weight) than the inner 1/3 of the tissue (37.9 ± 14.5 μg/mg dry weight). Variation was also noted between the anterior location (40.9 ± 12.4 μg/mg dry weight) and the posterior location (58.6 ± 14.6 μg/mg dry weight). Variations in the sulfated glycosaminoglycan level, water content, and compressive properties show that topographical variations in the rabbit meniscus are common.[15] The large variation between the hydroxyproline levels in the human (100-140 μg/mg dry weight) and the rabbit (30-70 μg/mg dry weight) was unexpected; the animal model is the most likely the source of this discrepancy. The hydroxyproline content has also been examined in the porcine model in two studies by Nakano and coworkers.[18, 21] In the first study,[18] the hydroxyproline content was
examined at the same six locations used in the current rabbit study. All hydroxyproline values were between 104-111 μg/mg dry weight, with the anterior 1/3 and posterior 1/3 of the tissue containing a statistically significant lower value than the other four locations. It should also be noted that the inner 1/3 of the tissue had lower hydroxyproline values than the outer 2/3, but this difference was not statistically significant. In the more recent study performed by Nakano and coworkers,[21] the hydroxyproline content of the porcine meniscus was examined at the inner 1/3, middle 1/3, and outer 1/3 of the tissue. Results show that the inner 1/3 of the meniscus had a hydroxyproline level of 103.3 ± 9.1 μg/mg dry weight, the middle 1/3 was 119.6 ± 5.1 μg/mg dry weight, and the outer 1/3 was 125.8 ± 5.5 μg/mg dry weight. The inner 1/3 was statistically lower than the middle 1/3 and outer 1/3, following the same trend seen in the rabbit meniscus. Attention should be noted to the difference in values between the two Nakano and coworkers papers,[18, 21] with there being a 15 μg/mg dry weight higher level of hydroxyproline in the more recent study in the outer locations of the meniscus. This would seem to indicate significant variation within even the same animal models.

The distribution of collagen types I and II has been examined in the canine, rabbit, and bovine models.[7, 27-29] Immunohistochemical results from Naumann and coworkers[29] indicate the presence of both collagen types I and II in the rabbit meniscus, which correspond to the results of the current study. However, no topographical variations are mentioned in the Naumann and coworkers study.[29] Topographical variations were noted in a study on the canine model performed by Kambic and McDevitt.[28] Results showed higher amount of collagen type II in the inner portion of
the meniscus, and heavy collagen I staining throughout the tissue. The higher degree of collagen II staining in the inner 1/3 of the tissue was also noted in the current study. A quantitative study, performed by Cheung[7], found that the inner 1/3 of the bovine meniscus consists of about 60% collagen type II and 40% collagen type I. However, none of the three previous studies mentioned collagen II staining at the anterior and posterior horns. A study performed by Gao[27] in the rabbit meniscus examined the degree of collagen I and II staining in the anterior and posterior horns of the rabbit meniscus. Results indicate the presence of both collagen types I and II, which would explain the noted collagen II staining at the outer edges of the anterior and posterior locations in the current study. Immunohistochemical staining of the rabbit meniscus matches the results seen in other studies and in other animal models.

Overall, this study examined the medial rabbit meniscus using a variety of biochemical techniques to determine the native properties of the tissue. Specifically, the ultrastructure was examined via histology and immunohistochemistry, the degree of hydration was examined via a wet-to-dry weight comparison, the level of sulfated glycosaminoglycans was examined via a dimethylmethylen blue assay, and the hydroxyproline content was examined via a hydroxyproline assay. Results indicate topographical variations within the medial rabbit meniscus, with significant variations in the anterior portion of the tissue and in the inner 1/3 of the tissue. These variations are most likely due to the bent-knee resting stance of the rabbit, the frequency that the animal jumps, and the more chondrocytic nature of the inner 1/3 of the tissue. These results can be used as a “gold-standard” baseline for future tissue engineering efforts.
Acknowledgments

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Tables

Table I. Water content at the anterior, central, and posterior portions of the inner 1/3 and outer 2/3 of the medial rabbit meniscus.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Region</th>
<th>% Water ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>Anterior</td>
<td>75.9 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>70.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>71.4 ± 4.7</td>
</tr>
<tr>
<td>2/3</td>
<td>Anterior</td>
<td>67.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>67.4 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>69.5 ± 3.1</td>
</tr>
</tbody>
</table>

Table II. Sulfated glycosaminoglycan content at the anterior, central, and posterior portions of the inner 1/3 and outer 2/3 of the medial rabbit meniscus.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Region</th>
<th>Sulfated GAGs (µg/mg dry weight) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>Anterior</td>
<td>115.2 ± 37.5</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>65.9 ± 24.8</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>55.1 ± 16.7</td>
</tr>
<tr>
<td>2/3</td>
<td>Anterior</td>
<td>25.2 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>14.4 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>14.7 ± 3.1</td>
</tr>
</tbody>
</table>
Table III. Hydroxyproline content at the anterior, central, and posterior portions of the inner 1/3 and outer 2/3 of the medial rabbit meniscus.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Region</th>
<th>Hydroxyproline (µg/mg dry weight) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3</td>
<td>Anterior</td>
<td>30.0 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>34.0 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>49.6 ± 14.9</td>
</tr>
<tr>
<td>2/3</td>
<td>Anterior</td>
<td>51.7 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>65.4 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>67.7 ± 6.9</td>
</tr>
</tbody>
</table>
Fig. 1. Schematic diagram of the knee. The medial and lateral menisci are shown attached to the tibia.

Fig. 2. Topographical testing locations.
Fig. 3. H&E of the inner 1/3 of the central region. Note the rows of cells among the collagen fibers (100x).
Fig. 4. Safranin O / Fast Green staining of the medial rabbit meniscus. Note the high amount of staining at the inner 1/3 of the anterior region. All figures at 200x magnification.
Fig. 5. Immunohistochemical staining of the medial rabbit meniscus. All figures at 200x magnification.
**Fig. 6.** Water content at six different locations in the medial meniscus. (#) indicates statistical significance between the inner 1/3 of the anterior location and all other locations (p<0.03). (@) indicates statistical significance between the inner 1/3 of the posterior location and either the outer 2/3 of the anterior portion or the outer 2/3 of the central portion (p<0.05).
**Fig. 7.** Sulfated glycosaminoglycan content at six different locations of the medial meniscus. (#) indicates statistical significance between the inner 1/3 of the anterior location and all other locations (p<0.0001). (@) indicates statistical significance between the inner 1/3 of the central location and any of the outer 2/3 locations (p<0.0009). (&) indicates statistical significance between the inner 1/3 of the posterior location and any of the outer 2/3 locations (p<0.02).
Chapter 7: The Feasibility of a Novel Hydrogel as a Scaffold Material for Meniscal Tissue Engineering

Abstract

The aim of this study was to determine the feasibility of the hydrogel poly(propylene fumarate-co-ethylene glycol), modified with the GRGDS peptide, to serve as a scaffold material for meniscal tissue engineering. Fibrochondrocytes were seeded on five different substrata: 1) hydrogel, 2) hydrogel with low peptide level, 3) hydrogel with medium peptide level, 4) hydrogel with high peptide level, and 5) tissue culture plastic. The fibrochondrocytes were allowed to attach and proliferate on the five substrata, after which qualitative (visual) and quantitative (change in cell numbers) data were analyzed. Results show that while the GRGDS peptide assisted in the attachment and spreading of the fibrochondrocytes on the hydrogel, cell attachment was not sustained on the material. This indicates that the hydrogel is not a suitable scaffold material in its present configuration and will need to be modified for future meniscal tissue engineering efforts.
Introduction

The meniscus (Fig. 1) is a tissue consisting of two wedge-shaped semilunar sections of fibrocartilage between the tibial and femoral bearing surfaces of the knee joint, and is responsible for load transmission, shock absorption, stability, and lubrication.[1-5] The meniscus can be split into two major sections, the vascular and avascular zone. The vascular zone, which is found in the outer third of the tissue, exhibits a spontaneous healing response to injury.[6] The avascular zone, found in the inner two-thirds of the tissue, does not have this intrinsic healing capability.[6] Damage to the meniscus, such as tears or degeneration of the tissue, particularly in the avascular zone, can lead to joint degeneration and osteoarthritis.[7-9] Current repair techniques, such as meniscectomy, sealing of tears, and transplantation, have had limited success at halting the development of osteoarthritis.[10-13] One repair technique that shows promise in repairing meniscal damage is tissue engineering, an experimental process where cell-seeded scaffolds are implanted to assist the body in regenerating healthy repair tissue. Several scaffold materials for meniscal tissue engineering efforts, such as polyglycolic acid (PGA), poly(lactic-co-glycolic) acid (PLGA), polyurethane, perichondrial tissue, periosteal tissue, small intestine submucosa (SIS), and collagen, have been utilized.[14-24]

The use of PGA and PLGA has been investigated as a possible approach to assist meniscal regeneration.[20, 21] Due to the poor mechanical properties of these materials the scaffolds were seeded for a few days, implanted subcutaneously for a period of time, and then placed in a meniscal defect. In one of the preliminary studies, bovine fibrochondrocytes were seeded onto PGA scaffolds and implanted subcutaneously in
nude mice for a period of 16 weeks.[21] At the end of 16 weeks the tissue from the implant was found to grossly and histologically resemble meniscal tissue.[21] Biomechanical tests of similar subcutaneous implants showed a compressive modulus that was about 40% of the native tissue value.[20] The most recent test performed by the group consisted of implanting a seeded PLGA scaffold in a sheep model for 4 weeks subcutaneously and 6 weeks in situ.[20] The resultant tissue was rich with proteoglycans and had a organized collagen fiber matrix.[20] These results are quite promising, though more studies need to be performed.

Various formulations of porous polyurethanes have also been investigated for meniscal tissue engineering.[18, 19, 25, 26] These studies aimed at repairing a tear in the meniscus with an unseeded porous polyurethane scaffold. The scaffold material has gone through many revisions, with the most recent being a 50/50 copoly(L-lactide/ε-caprolactone) scaffold.[26] Two formulations of this material, with a compressive modulus of 40 or 100kPa, were sutured into a partial defect in the lateral meniscus of a canine and tissue ingrowth was determined.[26] The copolymer with a compression modulus of 40 kPa had no fibrous tissue ingrowth, whereas the 100kPa sample had a fibrous tissue ingrowth of 50-70%.[26] More studies need to be performed, including biomechanical and biochemical analysis of the repair tissue, to determine if this material is a viable scaffold material.

Another approach has been to use tissue from other locations, such as periosteal tissue, perichondrial tissue, and small intestine submucosa, as a scaffold material for tissue
ingrowth.[15, 22-24] Periosteal tissue was found to encourage both bone and cartilage tissue growth in the scaffold, leading to degeneration of the joint surfaces.[15] Perichondrial tissue, while showing microscopic similarities to native tissue, had much lower tensile properties than the native meniscus.[24] Small intestine submucosa, which has undergone more evaluation than the previous two materials, has shown some promising results.[22, 23] After 12 weeks implantation, histological results exhibited repair tissue that was similar to the native tissue, but damage to articular cartilage was noted and no mechanical characterization of the repair tissue was performed.[22, 23] In-depth studies, including biomechanical and biochemical analysis, need to be performed on the SIS scaffold to determine if it is a suitable material for meniscal tissue engineering.

A wide variety of collagen scaffolds have been examined for meniscal tissue engineering. Walsh and coworkers[15] examined a type I collagen sponge, both unseeded and seeded with mesenchymal stem cells. Results showed fibrous repair tissue in the unseeded scaffold and tissue histologically similar to the native tissue in the seeded scaffolds. However, damage to the articular cartilage surfaces was also noted, and no mechanical testing was performed.[15] Mueller and coworkers[16] examined both type I collagen and type II collagen-GAG scaffolds, seeded with cells and examined after 21 days of *in vitro* culture. Results showed that the type II collagen-GAG scaffold exhibited better cell proliferation, GAGs synthesis, and collagen I synthesis than the type I collagen-GAG scaffold.[16] The collagen-GAG scaffold developed by Stone, Rodkey and coworkers[17, 27, 28] has undergone the most rigorous testing. This scaffold, already in phase II clinical
trials, is made from collagen retrieved from bovine tendons and then molded into a circumferential orientation.[17] The phase I and II clinical trials consisted of removing the damaged portion of the meniscus, trimming the scaffold to the appropriate size, and then suturing the unseeded scaffold into the defect, with the replaced section varying between 35-85% of the total meniscus.[17, 28] After three years, the results were promising, with no visual degeneration of the articular cartilage noted.[17, 28] Animal studies carried out in the dog showed that after six months the regenerated tissue’s biomechanical and biochemical properties were not identical to native tissue.[27] The water content of the repair tissue was 77.1±5.32% versus 66.6±1.35% for native tissue, and the permeability of the repair tissue was lower than the native tissue (roughly 2.7x10^{-15} m^{4}/Ns versus 3.7x10^{-15} m^{4}/Ns).[27] The compressive stiffness and glycosaminoglycan content were similar, with a aggregate modulus of roughly 0.18MPa and a glycosaminoglycan content of approximately 2.9%.[27]

Despite the exciting, biomaterial-based approaches described above, no ideal scaffold material has yet been determined. The ideal scaffold material should allow for unrestricted cell proliferation into and on the material, diffusion of nutrients, and be able to assume the biomechanical responsibilities of the native meniscus, until the tissue regenerates to sustain the mechanical forces within the knee joint. One versatile hydrogel, poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), exhibits mechanical properties that can be adjusted over a wide range during material formulation.[29, 30] This material is biocompatible,[31, 32] biodegradable,[32, 33] can be formed into a macroporous structure,[33, 34] and modified with the RGD peptide to aid in cell
attachment. Marrow-derived osteoblasts, endothelial cells, and smooth muscle cells have been shown to attach to the P(PF-co-EG) material, indicating potential as a scaffold material for tissue engineering efforts. The presence of the RGD peptide aids in cell attachment and has also been shown to enhance fibrochondrocyte attachment to artificial surfaces.

The objective of this study was to investigate if P(PF-co-EG) is a viable scaffold material for meniscal tissue engineering and to determine if incorporating the GRGDS peptide into the scaffold material aids in cell attachment and proliferation. To this end, fibrochondrocytes were seeded onto five different substrata (hydrogel, hydrogel with low peptide level, hydrogel with medium peptide level, hydrogel with high peptide level, and tissue culture plastic), and the attachment and proliferation of the cells on the substrata were monitored. For the attachment assay, fibrochondrocytes were seeded near confluence on the five substrata and allowed to attach for a period of 12 hours, slightly under the population doubling time of fibrochondrocytes. For the proliferation assay, fibrochondrocytes were seeded at a lower density on the same series of substrata and allowed to proliferate until 80-90% confluence was reached on one of the test groups.

Materials and Methods

Reagents and Cell Culture Materials

Methoxy poly(ethylene glycol) (mPEG, M_n~2000), poly(ethylene glycol)-diacrylate (PEG-DA; M_n~700), diethyl fumarate, zinc chloride, hydroquinone, and L-ascorbic acid were purchased from Aldrich Chemical Company (Milwaukee, WI). Propylene glycol,
methylene chloride, ethyl ether, sodium bicarbonate, and Corning 12-well tissue culture plates were obtained from Fisher Scientific (Pittsburgh, PA). Ammonium persulfate was acquired from Acros (Pittsburgh, PA). Acryloyl-PEG-N-hydroxysuccinimide (Ac-PEG-NHS; MW 3.4K) was obtained from Nektar Therapeutics (San Carlos, CA). The Gly-Arg-Gly-Asp-Ser (GRGDS) peptide was purchased from Bachem California (Torrance, CA). The Spectra/Por-7® membrane (MWCO:2000) was acquired from Spectrum Laboratories Inc. (Rancho Dominguez, CA). Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 with GlutaMAX (DMEM/F-12; 1:1), MEM Non-essential Amino Acids (NEAA), and trypsin-EDTA were purchased from Gibco (Grand Island, NY). Penicillin/streptomycin/fungizone (PSF) was obtained from Cambrex (Walkersville, MD). Fetal bovine serum (FBS) was acquired from Mediatech (Herndon, VA). Collagenase type II was obtained from Worthington Biochemical Corporation (Lakewood, NJ). The PicoGreen® dsDNA Quantitation Kit was purchased from Molecular Probes (Eugene, OR). Phosphate buffered saline (PBS), Trizma®-base, and L-ascorbic acid (AA) were acquired from Sigma (St. Louis, MO). TPP T-285 tissue culture flasks were obtained from Phenix Research Products (Hayward, CA).

**Synthesis of Poly(Propylene Fumarate)**

Poly(propylene fumarate) (PPF) was prepared by first making bis-(2-hydroxypropyl) fumarate through a reaction of diethyl fumarate and propylene glycol at 160°C with zinc chloride and hydroquinone, as described previously.[39] The product was then transesterified under vacuum at 150°C to form PPF. The molecular weight was monitored
via gel permeation chromatography until a number average molecular weight of 2000 was achieved.

*Synthesis of Poly(Propylene Fumarate-co-Ethylene Glycol)*

Synthesis of the polymer was accomplished as described previously.[39] Methoxy poly(ethylene glycol) (mPEG) was added to the PPF in a 1:2 PPF to mPEG molar ratio. A transesterification reaction was then carried out under vacuum at a temperature of 160°C until the starting prepolymer was depleted and the copolymer had formed. To purify the copolymer, the product was dissolved in methylene chloride, filtered, and precipitated in ethyl ether. The precipitate was then filtered off and dried under vacuum at room temperature to remove any residual organic solvent. The P(PF-co-EG) copolymer was stored at −20°C until use.

*Synthesis of Acryloyl-Poly(Ethylene Glycol)-GRGDS*

The synthesis of acryloyl-poly(ethylene glycol)-GRGDS (Ac-PEG-GRGDS) is described in other work.[35, 40] Briefly, Ac-PEG-NHS and GRGDS peptide were dissolved in a sodium bicarbonate buffer (pH 8.2) and reacted under stirring for 2.5 hours at room temperature. The reaction mixture was then dialyzed in deionized distilled water (DDW) for a period of 2 days using a dialysis membrane with a molecular weight cutoff of 2000. The solution was then flash frozen in liquid nitrogen, lyophilized to remove water, and stored at −20°C until use.
Hydrogel Fabrication

P(PF-co-EG) was mixed with PEG-DA at a 1:1 ratio (wt %) and dissolved with vigorous mixing in DDW (final polymer concentration of 66%). The Ac-PEG-GRGDS was then dissolved in the polymer solution, at a concentration of 0, 100, 1000, or 2000 nmoles/mL swelled volume and centrifuged to remove small bubbles. Ammonium persulfate and L-ascorbic acid were added consecutively so that final concentrations of 0.01 M were achieved. The solution was then placed between two glass plates with a 0.5 mm spacer, resulting in the creation of thin films of the hydrogel. The solution was allowed to crosslink overnight at 37°C, then placed in PBS to swell for several hours. Hydrogel disks 21 mm in diameter were cut out (Fig. 2A) and placed in a 70% ethanol solution for 24 hours to sterilize (Fig. 2B). The disks were then rinsed with sterile PBS containing PSF several times over four days to remove all ethanol from the hydrogels (Fig. 2C).

Fibrochondrocyte Isolation and Culture

Knee meniscal fibrochondrocytes were obtained from a 3.5 kg New Zealand white rabbit following a harvest protocol approved by the institution’s animal care and use committee. First, the knee joint was exposed under aseptic conditions, the medial menisci removed, and placed in a beaker of sterile PBS. The meniscal horns, synovium, and joint capsule were separated from the menisci and discarded, leaving only meniscal tissue. The tissue was then minced into 1 mm³ pieces and digested in a collagenase solution. After 24 hours, the cells were seeded on T-285 culture flasks and supplemented with a DMEM/F12 cocktail containing 10% FBS, PSF, AA (50 μg/mL), and NEAA. The cells
were incubated under standard culture conditions (37°C, 5% CO₂, 95% relative humidity) and passaged once to obtain 2° passage cells.

Fibrochondrocyte Attachment and Proliferation

The hydrogel films were placed in 12 well tissue culture plates and held in place with stainless steel annuli (7.2 mm inner diameter, 15.8 mm outer diameter, 16 mm height) (Fig. 2D). Five different substrata were tested (hydrogel with four levels of Ac-PEG-GRGDS, TC plastic), as can be seen in Table I. There were six samples for each experimental group, and three for the background comparison (preliminary tests indicated that the peptide level did not change the background noise for the P(PF-co-EG) hydrogel). Fibrochondrocytes were then seeded onto the substrata in one of two ways (Fig. 2E). For the attachment assay, 75000 fibrochondrocytes were placed in each well (~80% confluent) and allowed to attach for a period of 12 hours. For the proliferation assay, 25000 cells were placed in each well and allowed to proliferate until 80-90% confluence was reached on one of the substrata. At this point, the medium was removed and a lysis buffer (25 mM Trizma® base, pH 7.4) was added. A freeze-thaw cycle followed by sonication was then used three times to suspend the double stranded DNA in the lysis solution. To determine the level of double stranded DNA in each well, PicoGreen analysis was performed in 96-well plates using an automated plate reader (FLx800, Bio-Tek Instruments, Winooski, VT). The degree of attachment and proliferation were then calculated as the percentage of cell numbers on the experimental surface compared to cells on the tissue culture plastic controls for each of the experimental groups.
Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine statistical significance, with a sample size of n=6 and an alpha level of 0.05 defined as significant. If the F-test showed a significant difference (p<0.05), a post hoc test was performed (Fisher’s Protected Least Significant Difference) to compare sample sets.

Results

Fibrochondrocytes cultured on the five different substrata were visualized (Fig. 3) at 12 hours (attachment) and 72 hours (proliferation). At 12 hours, the fibrochondrocytes on the hydrogel with 0 GRGDS peptide level had a rounded morphology, whereas both fibrochondrocytes with a fusiform morphology and a rounded morphology were found on the hydrogels with the GRGDS peptide. Only the fusiform morphology was noted for the fibrochondrocytes on the tissue culture plastic. The cells on the 2000 GRGDS hydrogel also appeared to be at a higher density when compared to the 100 and 1000 GRGDS hydrogels. At 72 hours, the fibrochondrocytes on the tissue culture plastic were well spread, had a large number of processes, and had proliferated until 80-90% confluent, whereas there were scant numbers of cells on the hydrogels and any cells that remained were lightly attached and had a rounded morphology (for all GRGDS levels).

PicoGreen analysis verified these qualitative results, as can be seen in Fig. 4. For all comparisons, the cell number was calculated as a percentage of cells compared to the tissue culture plastic substrata, as seen in Table II. For the 12-hour time period, the
addition of the GRGDS peptide caused a statistically higher (p<0.005) level in cell number when compared to the hydrogel with no GRGDS peptide. The 2000 GRGDS hydrogel was also statistically (p<0.04) higher than the 100 and 1000 GRGDS hydrogels. For the 72-hour time period, there were no statistically significant differences (p>0.05) among the experimental groups. There was also a statistically lower (p<0.0001) number of cells found at the 72-hour time period compared to the 12-hour time period for each of the substrata tested, with the exception of the 0 GRGDS hydrogel.

Discussion

The objective of this study was to determine the feasibility of P(PF-co-EG) as a candidate scaffold material for tissue engineering of the knee meniscus. To determine this, fibrochondrocytes were seeded onto the hydrogel material, both with and without the GRGDS peptide, and allowed to attach and proliferate. Attachment and proliferation of cells on a tissue engineering scaffold is of paramount importance, allowing for growth of new tissue in the defect and integration with the surrounding tissue.

Previous meniscal tissue engineering studies show the necessity of fibrochondrocyte attachment and proliferation on the material surface. A study by de Groot and coworkers,[26] using 50/50 copoly(L-lactide/e-capralactone) as a scaffold material, found that scaffold compressive stiffness plays a significant role in fibrocartilage ingrowth. Another study by the same group showed that a porous scaffold material needed to have a pore size of at least 150 μm to allow for optimal tissue ingrowth.[41] If the biomaterial does allow for tissue growth on the scaffold and ingrowth from the surrounding tissue,
such as a PGA or PLGA scaffold[20, 21] or a collagen-GAG scaffold[17, 28], then favorable results can be shown. Implanted PGA and PLGA scaffolds have histologically elicited meniscal tissue growth and biomechanical properties that are 40% of the native tissue values.[20, 21] The collagen-GAG scaffolds[17, 27, 28] have shown enough promise to result in phase II clinical trials, though the constructs biomechanical and biochemical properties are not identical to native tissue.[17, 27] However, the attachment and proliferation results for fibrochondrocytes in this study indicate that the P(PF-co-EG) hydrogel, if used as a scaffold material, would likely not promote development and ingrowth of meniscal tissue. The results of this study show that while fibrochondrocytes do attach onto the hydrogel with the GRGDS peptide, they do not establish themselves on the material to achieve proliferation, which would hinder tissue development. This result was unexpected, considering the previous studies that have shown cell attachment to this material[30, 35-37] and the ability of the RGD peptide to enhance fibrochondrocyte attachment to artificial surfaces.[38] The present study was originally designed to also examine biosynthesis (collagen and sulfated glycosaminoglycan content) via a hydroxyproline assay and a dimethylmethylene blue assay. This would allow for a comparison to some of the above studies. Unfortunately, due to the lack of cell proliferation, these assays could not be performed.

Cell attachment to the P(PF-co-EG) hydrogel has been demonstrated with endothelial cells,[30] smooth muscle cells,[37] and marrow-derived osteoblasts.[35, 36] In the endothelial cell study, attachment of these cells was monitored on the P(PF-co-EG) hydrogel. Depending on the formulation of the hydrogel, attachment ranged from 3.9 to
31.1% of the cells seeded on the material.[30] In the smooth muscle cell study, the composition of the hydrogel was varied to monitor its effects on cell attachment. It was found that attachment levels varied between 20 to 78% of the initial seeding density, although the cells did not spread on the hydrogel material.[37] If you compare these two studies, it can be seen that varying the formulation of the polymer can have a great effect on the cell attachment, as can the cell type. When compared to the fibrochondrocyte results, it can be seen that there was very little attachment on the hydrogel with no GRGDS peptide (21.5±13.5% of the tissue culture plastic control), and that the rounded morphology was similar to the smooth muscle cell results. These results indicated that the GRGDS peptide would most likely aid in the attachment of cells to the P(PF-co-EG) material.

The two studies investigating the response of marrow-derived osteoblasts to the P(PF-co-EG) material used the GRGDS peptide to aid in cell attachment and proliferation.[35, 36] It was found that the presence of the GRGDS peptide in sufficient quantities (100 nmol/mL) resulted in 84% of the initial seeding of marrow-derived osteoblasts attached to the material, compared to 9% for the hydrogel with no peptide.[35] The fibrochondrocyte results also show a significant difference between the hydrogel with and without the GRGDS peptide; 62.0±27.8% (100 nmol/mL GRGDS concentration) vs. 21.5±13.5% (no peptide on material) of cells compared to tissue culture plastic control. However, the results differ between the proliferation results for the two cell types. After three days of culture, marrow-derived osteoblasts exhibited a decrease in cell number for low peptide levels (10, 50 nmol/mL) and an increase in cell numbers for high peptide
levels (100, 1000 nmol/mL).[35] Fibrochondrocyte results show a decrease in cell number, with no statistical difference (p>0.05) among the four peptide levels (0, 100, 1000, 2000 nmol/mL). The cause for this discrepancy between the proliferation results is not known, though some possibilities include steric hindrances or the inability of fibronectin secreted from the fibrochondrocytes to adhere to the hydrogel.[42, 43]

Considering the previous studies exhibiting cell attachment to the P(PF-co-EG) hydrogel,[30, 35-37] and the ability of the RGD peptide to enhance fibrochondrocyte attachment to artificial surfaces,[38] the results of this study were unexpected. While the steric hindrances of the hydrogel material was expected to hinder fibrochondrocyte attachment to the unmodified P(PF-co-EG) material,[30, 35, 42] the addition of the GRGDS peptide was expected to help overcome this shortcoming. While the peptide did aid in attachment and spreading, it failed to maintain attachment of the fibrochondrocytes to the scaffold material and allow them to proliferate. Possible reasons for this reaction, especially considering the previous success with other cell types, would be that the GRGDS peptide was not sufficiently exposed when the polymer was cross-linked or that there was not sufficient protein absorption on the material surface to aid in cell attachment. A lack of fibrochondrocyte proliferation on the hydrogel indicates an inability to allow for tissue growth and integration with surrounding tissue if used as a scaffold material for meniscal tissue engineering. More characterization of this hydrogel needs to be performed to determine peptide exposure and protein absorption. If both of these aspects are not the cause of the lack of cell proliferation, then modification of the
P(PF-co-EG) hydrogel will need to be performed for this material to be used in meniscal tissue engineering efforts.

Acknowledgments

This study was partially supported by a grant from the National Institutes of Health (Grant# R01 AR47839-2).
### Tables

#### Table I. Substrata testing groups.

<table>
<thead>
<tr>
<th></th>
<th>Attachment (12 hours)</th>
<th>Proliferation (72 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell-seeded substrata</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=6</td>
<td>0 nmoles/mL Ac-PEG-GRGDS</td>
<td>0 nmoles/mL Ac-PEG-GRGDS</td>
</tr>
<tr>
<td></td>
<td>100 nmoles/mL Ac-PEG-GRGDS</td>
<td>100 nmoles/mL Ac-PEG-GRGDS</td>
</tr>
<tr>
<td></td>
<td>1000 nmoles/mL Ac-PEG-GRGDS</td>
<td>1000 nmoles/mL Ac-PEG-GRGDS</td>
</tr>
<tr>
<td></td>
<td>2000 nmoles/mL Ac-PEG-GRGDS</td>
<td>2000 nmoles/mL Ac-PEG-GRGDS</td>
</tr>
<tr>
<td><strong>Background substrata</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(no cells), n=3</td>
<td>0 nmoles/mL Ac-PEG-GRGDS</td>
<td>0 nmoles/mL Ac-PEG-GRGDS</td>
</tr>
<tr>
<td></td>
<td>TC plastic</td>
<td>TC plastic</td>
</tr>
</tbody>
</table>

#### Table II. Percentage of fibrochondrocyte attachment and proliferation on the P(PF-co-EG) hydrogel, normalized to tissue cultured plastic. Percentage shown as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Attachment (12 hours)</th>
<th>Proliferation (72 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nmoles/mL Ac-PEG-GRGDS</td>
<td>21.5±13.5</td>
<td>2.3±3.3</td>
</tr>
<tr>
<td>100 nmoles/mL Ac-PEG-GRGDS</td>
<td>62.0±27.8</td>
<td>3.3±2.5</td>
</tr>
<tr>
<td>1000 nmoles/mL Ac-PEG-GRGDS</td>
<td>53.9±19.1</td>
<td>3.4±2.8</td>
</tr>
<tr>
<td>2000 nmoles/mL Ac-PEG-GRGDS</td>
<td>85.3±36.9</td>
<td>4.6±8.2</td>
</tr>
</tbody>
</table>
Fig. 1. Schematic diagram of the knee. The menisci are shown attached to the tibial plateau.
Fig. 2. A schematic diagram of the testing procedure. First, disks are removed from the swollen hydrogel (a) and placed in a 70% ethanol solution (b). After 24 hours, the disks are rinsed several times in sterile PBS (c), after which the disks are placed in a 12-well plate and held down with a stainless steel annulus (d). The hydrogels are then seeded with the desired concentration of fibrochondrocytes (e) and allowed to attach and proliferate.
Fig. 3. (Preceding page) Representative images of fibrochondrocyte attachment and proliferation on the hydrogel with no GRGDS peptide, hydrogel with the GRGDS peptide at a concentration of 100 nmoles/mL, and tissue culture plastic. Images are at 100x magnification.

Fig. 4. Fibrochondrocyte attachment (12 hours) and proliferation (72 hours) to the four different hydrogels. Data are shown as the percentage of cells compared to the cells on the tissue culture plastic. Data shown as mean ± standard deviation for n=6. (#) indicates a significant difference (p<0.005) between the 0 GRGDS hydrogel and the other three substrata at 12 hours. (&) indicates a statistical significance (p<0.04) between the 2000 GRGDS hydrogel and the 100 and 1000 GRGDS hydrogels at 12 hours. (*) indicates a significant difference (p<0.0001) between the 12 hour and 72 hour cell percentage for the 100, 1000, and 2000 GRGDS hydrogel.
Chapter 8: Biomechanical Characteristics of the Normal Medial and Lateral Porcine Knee Menisci

Abstract

The purpose of this investigation was to examine the compressive properties of the porcine meniscus at a variety of topographical locations using a creep indentation experiment. Three different solution techniques were used to analyze the creep response of the tissue. Specifically, the indentation stiffness, aggregate modulus, permeability, Poisson’s ratio, and shear modulus were determined at six different testing locations (anterior, central, posterior regions; femoral and tibial side) of both the medial and lateral porcine menisci. Results indicate topographical variations among the testing locations, with the femoral-anterior portion of the medial meniscus having the highest indentation stiffness (350±110 kPa), aggregate modulus (270±90 kPa), and shear modulus (140±40 kPa). The posterior-tibial region of the medial meniscus exhibited the lowest indentation stiffness (170±40 kPa), aggregate modulus (130±30 kPa), and shear modulus (60±20 kPa). No statistical differences were found at the six tested locations of the lateral meniscus.

* Accepted for publication in the Proceedings of the Institution of Mechanical Engineers Part H: Journal of Engineering in Medicine.
Introduction

Over the past century the knee meniscus has been recognized as an important anatomic structure, with significant functions such as weight-bearing, load distribution, shock absorption, knee stabilization, facilitation of joint rotation, and lubrication.[1-6] There are two menisci in each knee joint: the medial meniscus and the lateral meniscus (Fig. 1). The menisci are attached to the tibia by strong insertions into the anterior and posterior intercondylar regions of the tibia spine. They are also attached to the joint capsule peripherally and have other relatively minor restraints. Each meniscus has distinct features, which are probably due to the variations in the mechanical environment between the two structures. The lateral meniscus is smaller in diameter, thicker about the periphery, and usually wider than the medial. The body of the medial meniscus is both wider and thicker in the posterior region than the anterior region. The lateral meniscus is more symmetrical and wider on average. The radius of curvature of the medial meniscus in the transverse plane is larger than that of the lateral, resulting in the tibial insertion points of the medial meniscus to be farther apart than the lateral meniscus. The relative proximity of the lateral meniscus tibial insertions allows for a greater degree of mobility. The menisci cover substantial areas of the tibial plateau, with the medial meniscus occupying 51-74% of the medial plateau and the lateral meniscus occupying 75-93% of the lateral plateau.[7, 8] The menisci also move on the plateau with flexion and extension, with the menisci sliding posteriorly during flexion and anteriorly during extension. The range of this anterior-posterior movement is greater for the lateral meniscus than for the medial meniscus.[8, 9] This capacity to move allows the meniscus to absorb some of the load in the knee joint. The menisci transmit between 40% to 90%
(in human) of the load during walking,[2, 10] and this fraction has been estimated to be 40-50% on the medial side and 60-70% on the lateral.[2, 11] The shock absorbing properties of the menisci protect the articular cartilage surfaces from damage and degeneration.

Loss of normal function of the meniscus due to trauma or degenerative wear often results in significant morbidity for the patient. The medial meniscus is damaged or diseased much more frequently than the lateral meniscus,[12] and perhaps not surprisingly as the human medial meniscus is weaker than the lateral meniscus under tensile load.[13] In athletic injuries the ratio of medial meniscus lesions to those of the lateral meniscus is 3 to 1; in miners we find a ratio which can go as high as 20 to 1. In a mixed series the average ratio is approximately 8 to 1.[14]

In an effort to better understand pathologic and traumatic processes in the knee meniscus, many studies have investigated the tissue’s biomechanical behavior. Tensile, compressive, and shear tests have been performed on meniscal tissue, but there has been variability in testing methods, including differences in specimen size, specimen harvest location, test conditions, and animal models.[6, 13, 15-20] Results from these studies have demonstrated that meniscal material properties are anisotropic and inhomogeneous, with variations occurring depending on the animal model, test location, loading direction, and testing method. While tension has been the most widely used testing modality, some compression studies have yielded interesting results. A study by Joshi and coworkers[16] examined the difference in material properties at the posterior region of the medial
meniscus in six animal models, and discovered significant variations. Proctor and coworkers[15] examined the aggregate modulus at four different locations of the medial bovine meniscus, and found that the posterior portion of the tissue was the stiffest. Unfortunately, no compressive studies have been performed at different testing locations of both the medial and lateral meniscus, which would be of interest considering the differences in anatomy and movement between the menisci and the greater occurrence of tears in the medial meniscus.

The purpose of this investigation was to examine the compressive properties of the porcine meniscus at a variety of topographical locations using a creep indentation experiment. The creep indentation data will be analyzed using three solution techniques to determine the compressive material properties at six different testing locations (anterior, central, posterior regions; femoral and tibial side) of both the medial and lateral porcine meniscus (Fig. 2). Specifically, the data will be analyzed via a elasticity solution to determine the indentation stiffness (E), and by a semi-analytical/semi-numerical biphasic solution to determine the aggregate modulus (H_A), permeability (k), Poisson’s ratio (v_S), and shear modulus (\mu_S). The solutions to the semi-analytical/semi-numerical biphasic solution will then be used as initial estimates for a finite element/non-linear optimization modeling technique, which yields a more accurate aggregate modulus, permeability, Poisson’s ratio, and shear modulus.
Materials and Methods

Specimen Preparation

Six right porcine knee joints were obtained from skeletally mature animals (about one year old). The medial and lateral menisci were dissected out, bisected in the axial plane into tibial and femoral aspects, which were then further divided into anterior, central and posterior segments. Each specimen was then wrapped in gauze soaked in 0.15 M NaCl solution with protease inhibitors (N-ethyl maleimide, 10 mM; benzamidine HCl, 5 mM; EDTA, 2 mM; and PMSF, 1 mM) and stored at -20°C until time of mechanical testing. The preparation and testing of specimens required two freeze-thaw cycles, which are not believed to affect the tissue’s intrinsic materials properties. A total of 72 mechanical tests were performed.

Creep Indentation

Menisci were tested mechanically to determine each meniscal specimen’s indentation stiffness, aggregate modulus, Poisson's ratio, permeability, thickness, and shear modulus. An automated creep indentation apparatus (Fig. 3) [21, 22] was used to quantify the in situ creep and recovery deformation behavior of each specimen. The testing apparatus was able to load and unload the meniscus specimen automatically through a closed-loop control system. Before testing, each specimen was thawed for an hour in normal saline containing protease inhibitors and then attached with cyanoacrylate cement to a sample holder. A fiber optic light positioning system in line with the loading shaft emitted light on the section of the meniscus surface to be tested. The sample holder was positioned with a spherical joint and lead screw assembly, until the reflected light indicated that the
loading shaft was normal to the meniscal surface. A tare load of 0.01 N was then applied with a 1.0 mm diameter, flat-ended, cylindrical, rigid, porous indenter tip, and the tissue was allowed to reach tare creep equilibrium. Equilibrium was automatically determined when the slope of the creep curve became smaller than $1 \times 10^{-6}$ mm s$^{-1}$. Once creep equilibrium was reached, the tissue was loaded with a step force of 0.038 N. The tissue's deformation was monitored with a linear variable differential transformer (LVDT) using a computer-based data acquisition system at a 0.25 μm deformation-resolution. Data points were collected and plotted on the screen every 2.5 μm or every 50 s, whichever occurred first. The frictional resistance of the system was reduced with air bearings to less than $9.81 \times 10^{-4}$ N. The bearings were driven with pressurized air (552 kPa) that was cleaned with a 5 μm particle filter and two 0.1 μm coalescing filters. The creep response of the specimen under the step force was monitored until equilibrium (defined as slope $< 1 \times 10^{-6}$ mm s$^{-1}$). At this point, the force was automatically removed and the recovery phase began. When recovery equilibrium was achieved, data acquisition was stopped automatically. Overall, the automated creep indenter yielded the creep and recovery deformational behaviors of each meniscal specimen in response to a 0.038 N step load.

**Thickness**

The thickness was measured using two methods: First we simply used a micrometer to measure the overall approximate thickness at the test site. Following specimen mounting and creep indentation testing, thickness was also measured using a needle probe attached to a force transducer and an LVDT. The entire probe assembly was moved downward
with a linear motor until the needle touched the meniscal surface. At this point, the force transducer noted a significant change in force on the needle probe and the needle continued to move through the sample. When the needle contacted the sample holder underneath the meniscal sample another significant change in the force on the needle probe was noted. The difference between the measured needle positions at the two force readings corresponded to specimen thickness.

Solution Techniques

The experimental data for each test site were analyzed using three methodologies, as described below:

a) Elasticity Solution (B-P)

This solution is based on a mixed boundary value problem of the theory of elasticity, considering axisymmetric testing of a linearly elastic, semi-infinite solid with a plane-ended cylindrical indenter.[23] Using Boussinesq-Papkovich potential functions, the solution reduces to, \( E = \frac{p(1-\nu_S^2)}{(d\omega)} \), where, \( E \): Young’s modulus or indentation stiffness (MPa), \( p \): test load (N), \( \nu_S \): Poisson’s ratio, \( d \): diameter of tip (mm), and \( \omega \): displacement (mm). For this study, \( \nu_S \) is assumed to be 0.3 and the term indentation stiffness is used to denote \( E \).

b) Semi-Analytical/Semi-Numerical Biphasic Solution (SA/SN)

The creep indentation problem of the meniscus assumes that a frictionless, porous, rigid tip is used to apply a step load, and then solved using the linear biphasic theory[24] by Mak and associates.[25] A numerical algorithm is used to curve-fit the indentation data using a bi-cubic spline function, which represents the “master solution” of the
probem.[26] The described solution scheme allows simultaneous computation of the three independent intrinsic properties of soft hydrated tissues: the aggregate modulus - \( H_A \) (measure of compressive stiffness; MPa), Poisson's ratio - \( \nu_S \), (apparent compressibility; dimensionless quantity), and permeability - \( k \) (ease or difficulty with which the tissue's interstitial fluid moves past the solid matrix; \( \text{m}^4 \text{N}^{-1} \text{s}^{-1} \)). This methodology has been used with the creep indentation experiment to successfully obtain the intrinsic material properties of articular cartilage.[21, 27-31] The shear modulus, \( \mu_S \) can be calculated from the aggregate modulus and Poisson's ratio: \( \mu_S = 0.5H_A(1-2\nu_S)/(1-\nu_S) \).

c) Finite Element/Non-Linear Optimization Modeling (FEO)

Following the solution of each creep indentation problem using the SA/SN procedure described in the previous section, a finite element approach is employed.[22] This approach, which is based on biphasic finite element routines and nonlinear optimization techniques, uses the entire creep curve to calculate the intrinsic material properties of the meniscus. Although this procedure is more accurate, its output depends significantly on an initial estimate of the tissue’s properties. Thus, the SA/SN procedure is used to obtain an estimate of the tissue’s properties, before applying the finite element/non-linear optimization routine.

The tissue is modeled as an axisymmetric cylinder of uniform depth of a material described by the linear biphasic theory,[24] with unknown properties \( K_i \), where, \( K_i = [H_A^i, \nu_S^i, k^i]^T \). Although the meniscus is not uniform in thickness, for our analysis where infinitesimal strain criteria will be satisfied, the fact that the bottom surface may not be
parallel with the top surface is not crucial, since the stress field is concentrated near the indenting tip (St. Venant's principle) and the indenting tip is perpendicular to the top surface.

The meniscus is subjected to a step load $P(t) = P_0H(t)$, where $P_0$ is a constant force and $H(t)$ is the Heaviside step function, through a frictional, rigid, porous, cylindrical indenter with material properties $K_2 = [3 \times 10^{36} \text{ MPa}, 0.25, 2.30 \times 10^{-10} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}]^T$. The sample holder is modeled as rigid and impervious to fluid flow. Each FE mesh is generated for each specimen by specifying the tip radius, specimen thickness, and test load. The creep experiment yields the solid axial displacement ($U_c$) as a function of time. This creep problem was solved using a finite element formulation.[22] Numerical curvefit is provided by an optimization technique. The objective of the optimization technique is to obtain the unknown vector $K_1$. Our code consists of a main program, the finite element subroutine, and the optimization subprograms. In the main program, we read $U_c$, decide on an initial guess ($K_1^0$), apply the finite element solution, post process the solid axial displacement ($U_i$), set up and calculate the objective function $F(K_1) = \sum (U_c - U_i)^2$, and apply the optimization procedure to minimize $F(K_1)$. The objective function is the sum of the square of the differences of the experimental meniscus compressive deformation to that obtained through application of the finite element solution. This sum represents the error between the actual and predicted displacements. Thus, the goal is to find the optimal combination of the aggregate modulus, permeability, and Poisson's ratio that minimizes this error. Function $F(K_1)$ is minimized, subject to side constraints $X_j^1 \leq X_j \leq X_j^l$ ($j = 1, 2, 3$), which define the feasible space of search for $K_1$. The aggregate modulus
is allowed to vary between 0.001 and 3 MPa, the permeability between 10^{-17} and 10^{-13} m^4/Ns, and the Poisson's ratio between 0 and 0.5. Once \( F(K_t) \) is calculated, a search direction \( (S^p) \) is established using the 1st order Broydon-Fletcher-Shanno-Goldfarb variable metric method for unconstrained minimization. The design vector is then updated via \( X^p = X^{p-1} + a^p S^p \), where \( a^p \) indicates the amount of move in search direction \( S^p \). The scalar parameter \( a^p \) is obtained through an interpolation scheme using polynomial approximations. Thus, the problem is reduced to minimization of a function of one variable \( (a^p) \).

**Statistical Evaluation**

The meniscus material properties for the three solution methodologies (aggregate modulus, Poisson's ratio, permeability, shear modulus, and indentation stiffness) were examined using analysis of variance (ANOVA). Fisher's Protected Least Significant Difference multiple comparisons test of the means was applied when the F-test in ANOVA was significant. The statistical significance level was set at \( p < 0.05 \) for all tests.

**Results**

Following application of the three methods described above, the material properties of the medial and lateral porcine menisci were obtained. Results are shown in Tables I-VI. The paragraphs that follow discuss the statistical comparisons of the data from the Elasticity solution (BP) and Finite element/non-linear optimization modeling (FEO).
Medial Meniscus

The femoral anterior section of the medial meniscus was found to have a larger indentation stiffness, aggregate modulus, and shear modulus than the other five tested locations of the medial meniscus (p<0.006). The aggregate modulus and indentation stiffness of the tibial-anterior section was also found to be statistically larger than the tibial-posterior section (p<0.05). No statistical significance was found between the six test locations for the Poisson’s ratio and permeability. When the medial data were analyzed to compare the anterior, central, and posterior sections (with the femoral and tibial orientation not taken into account), the anterior section was found to have statistically the highest indentation stiffness, aggregate modulus, and shear modulus (p<0.003). When the medial data were analyzed to compare the femoral and tibial surfaces (with the anterior, central, and posterior location not taken into account), the femoral side was found to have a statistically higher indentation stiffness, aggregate modulus, shear modulus, and a lower permeability than the tibial side (p<0.03).

Lateral Meniscus

Statistically no significant differences were observed in the indentation stiffness, aggregate modulus, permeability, and shear modulus for the lateral meniscus at the six tested locations (p>0.08). The femoral-central location had a statistically higher Poisson’s ratio than the femoral-anterior location and all locations on the tibial side (p<0.01). There were also no statistical differences when comparing the anterior, central, and posterior sections (with the femoral and tibial orientation not taken into account).
(p>0.16). If split into the femoral and tibial surfaces (with the anterior, central, and posterior location not taken into account), again no statistical differences were noted for any of the tested parameters (p>0.22), with the exception of the Poisson’s ratio, which was found to be higher on the femoral side (p<0.03).

*Medial and Lateral Comparison*

Tables VII-IX show the statistical significance for the indentation stiffness, aggregate modulus, and shear modulus among the 12 tested locations on the medial and lateral meniscus (p<0.05). The femoral-anterior location of the medial meniscus had the highest indentation stiffness, aggregate modulus, and shear modulus of the 12 test locations (Fig. 4). The femoral-central location of the lateral meniscus has a statistically higher Poisson’s ratio than the other tested locations (p<0.03). No statistical significance was noted for the permeability among the 12 test locations (p>0.09). If the average medial result is compared to the average lateral result (with no topographical variation within each meniscus taken into account), statistical significance is only found for the permeability (p<0.04). If the data is analyzed to compare the anterior, central, and posterior aspects of the medial and lateral meniscus (6 test groups), then statistical significance is found for the indentation stiffness, aggregate modulus, and shear modulus (p<0.04). Statistically significant differences were also found for the Poisson’s ratio, permeability, and shear modulus for the femoral and tibial aspects of the medial and lateral meniscus (p<0.05).
Discussion

In recent years, the meniscus has been recognized clinically as a crucial structural element in the knee joint due to its exceptional biomechanical functions. For example, animal studies have shown that after total meniscectomy, knee articular cartilage develops osteoarthritic changes, suggesting that the meniscus protects the knee joint from degenerative joint disease.[32] Owing to its biomechanical significance, salvage of the damaged meniscus has drawn clinical attention.[33] To elucidate and improve healing of the meniscus, various animal models for studying the repair process of this soft tissue have been developed.[34-38] However, these and other animal models used to investigate the repair of the knee meniscus require detailed knowledge and baseline data of the biomechanical properties of normal and repaired menisci. Only limited data on meniscal biomechanical properties of various animals are currently available. To the authors’ knowledge, no study has been performed that compares the compressive properties of both the medial and lateral meniscus in a single animal model.

A series of confined compression testing of the meniscus has been previously performed on the porcine, bovine, and human models.[15-17] A study performed by Joshi and coworkers[16] examined the compressive properties in several animal models, including the pig. Their results show a higher aggregate modulus (270±40 kPa vs. 130±30 kPa) and lower permeability (1.74±0.19×10^{-15} m^4 N^{-1} s^{-1} vs. 6.32±4.20×10^{-15} m^4 N^{-1} s^{-1}) than the current study. One possible reason for this discrepancy is the difference in testing methodology, with Joshi and coworkers[16] using confined compression and this study using creep indentation. Proctor and coworkers[15] examined the medial bovine
meniscus using confined compression samples from the anterior and posterior portions of the femoral surface. Their results exhibited a higher aggregate modulus (anterior: 440±110 kPa vs. 270±90 kPa; posterior: 390±110 kPa vs. 140±30 kPa) and lower permeability (anterior: 0.63±0.47x10^{-15} m^4 N^{-1} s^{-1} vs. 3.62±1.41x10^{-15} m^4 N^{-1} s^{-1}; posterior: 0.76±0.47x10^{-15} m^4 N^{-1} s^{-1} vs. 3.66±1.20x10^{-15} m^4 N^{-1} s^{-1}) than the current study. Differences in value are likely due to the different testing method and animal model, but it is interesting to note that no statistical significance was found for the Proctor study (p>0.05), whereas the current study found a significant difference for the aggregate modulus between the anterior and posterior locations (p<0.0001). This would seem to indicate that there are different topographical variations in different animal models. A study by Hacker and coworkers[17] also shows an animal dependent variation in compressive properties. Hacker and coworkers[17] examined the anterior, central, and posterior portions (tibial side) of the medial human meniscus using confined compression testing. Their results show an increasing aggregate modulus from the anterior to posterior portions of the tissue (roughly 200 kPa for the anterior portion, 230 kPa for the central portion, and 270 kPa for the posterior portion), whereas the current study found a higher aggregate modulus in the anterior portion of the tissue (anterior: 180±20 kPa, central: 130±20 kPa, posterior: 130±30 kPa).

Topographical variations in meniscal mechanical properties have also been noted in tensile testing, and while direct comparison to compression results is not feasible, trends can be compared. One of the original tensile studies on the meniscus was performed by Mathur and coworkers.[13] Load to fracture was examined for both the medial and
lateral meniscus in the human and canine model. Their results show that, in the human model, the lateral meniscus is significantly stronger than the medial meniscus \( (p<0.0001) \), whereas in the canine model no statistical significance was observed \( (p>0.27) \). The results of the current study also exhibit no statistical significance between the medial and lateral meniscus in the porcine model \( (H_A: p>0.78) \). A more recent study by Fithian and coworkers\[18, 19\] examined the circumferential tensile properties at the anterior, central, and posterior portions of the medial and lateral human meniscus. For the medial meniscus, both their study and the current study found the highest mechanical properties in the anterior portion of the tissue. However, Fithian and coworkers\[18, 19\] found the highest mechanical properties in the posterior portion of the lateral meniscus, whereas the current study found no significant difference between the anterior, central, and posterior portion of the lateral meniscus. Differences between the Proctor and coworkers study\[15\] and the current study are also noted. The tensile testing performed on the medial bovine meniscus found the highest mechanical properties in the posterior section of the tissue, whereas the current study found the highest mechanical properties in the anterior section of the tissue.

The most common location for injury in the human meniscus is the posterior region of the medial meniscus.\[12, 39\] The results from the current study show that this region also has the lowest indentation stiffness, aggregate modulus, and shear modulus in the porcine meniscus. While the lower compressive properties of this region could explain the frequency of tears, it may be difficult to make comparisons among animal models, as described in the above discussion.
Conclusion

Overall, this study has used a biphasic creep indentation experiment on the medial knee meniscus and lateral knee meniscus to determine topographical variations in the compressive properties in the porcine model. Results indicate topographical variations among the testing locations, with the femoral-anterior portion of the medial meniscus having the highest indentation stiffness, aggregate modulus, and shear modulus. The posterior-tibial region of the medial meniscus exhibited the lowest indentation stiffness, aggregate modulus, and shear modulus. The femoral-central location of the lateral meniscus exhibited a statistically higher Poisson’s ratio than the other tested locations. These results provide detailed baseline data that can be used in future repair studies of the knee meniscus.
Tables

Table I. Biomechanical properties of the medial porcine meniscus (Elasticity solution)

<table>
<thead>
<tr>
<th>Segment</th>
<th>Femoral aspect</th>
<th>Tibial aspect</th>
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<tr>
<td></td>
<td>E (kPa)</td>
<td>E (kPa)</td>
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<td>Central</td>
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Table II. Biomechanical properties of the medial porcine meniscus (SA/SN solution)

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<th>k (10^{15} m^4 N^{-1} s^{-1})</th>
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### Table III. Biomechanical properties of the medial porcine meniscus (FEO solution)

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### Table IV. Biomechanical properties of the lateral porcine meniscus (Elasticity solution)

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### Table V. Biomechanical properties of the lateral porcine meniscus (SA/SN solution)

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### Table VI. Biomechanical properties of the lateral porcine meniscus (FEO solution)

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### Table VII. Statistical comparison of the indentation stiffness

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a. First letter denotes side (L=lateral, M=medial), second denotes surface (F=femoral, T=tibial), and third letter denotes location (A=anterior, C=central, P=posterior).

b. "X" denotes statistical significance between test groups (p<0.05)

### Table VIII. Statistical comparison of the aggregate modulus

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b. “X” denotes statistical significance between test groups (p<0.05)
Fig. 1. Schematic diagram of the knee. The menisci are shown attached to the tibial plateau.
Fig. 2. The six testing locations on the medial and lateral menisci (A=anterior, C=central, P=posterior).

Fig. 3. Picture of the creep indentation apparatus.
Fig. 4. Aggregate modulus at the 12 test locations. First letter denotes side (L=lateral, M=medial), second letter denotes surface (F=femoral, T=tibial), and third letter denotes location (A=anterior, C=central, P=posterior). Values are mean ± standard deviation. (@) indicates statistical significance between the femoral-anterior location of the medial meniscus and all other locations (p<0.05). (*) indicates statistical significance between the femoral-anterior location of the lateral meniscus and the other indicated locations (p<0.05). (#) indicates statistical significance between the tibial-posterior location of the medial meniscus and the other indicated locations (p<0.05). (&) indicates statistical significance between the tibial-anterior and tibial-central locations of the medial meniscus (p<0.05).
Chapter 9: Intraspecies and Interspecies Comparison of the Compressive Properties of the Medial Meniscus

Abstract

Quantification of the compressive material properties of the meniscus is of paramount importance, creating a “gold-standard” reference for future research. The purpose of this study was to determine compressive properties in six animal models (baboon, bovine, canine, human, lapine, and porcine) at six topographical locations. It was hypothesized that topographical variation of the compressive properties would be found in each animal model and that interspecies variations would also be exhibited. To test these hypotheses, creep and recovery indentation experiments were performed on the meniscus using a creep indentation apparatus and analyzed via a finite element optimization method to determine the material properties. Results show significant intraspecies and interspecies variation in the compressive properties among the six topographical locations, with the moduli exhibiting the highest values in the anterior portion. For example, the anterior location of the human meniscus has an aggregate modulus of 160±40 kPa, whereas the central and posterior portions exhibit aggregate moduli of 100±30 kPa. Interspecies comparison of the aggregate moduli identifies the lapine anterior location having the highest value (450±120 kPa) and the human posterior location having the lowest (100±30 kPa). These baseline values of compressive properties will be of help in future meniscal repair efforts.

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Introduction

The meniscus (Fig. 1) has been recognized clinically as a crucial structural element in the knee joint due to its exceptional biomechanical functions. For example, animal studies have shown that after total meniscectomy, knee articular cartilage develops osteoarthritic changes, suggesting that the meniscus protects the knee joint from degenerative joint disease.[1, 2] Owing to its biomechanical significance, salvage of the damaged meniscus has drawn clinical attention.[3, 4] To elucidate and improve healing of the meniscus, various animal models for studying the repair process of this soft tissue have been developed.[5-8] However, these and other animal models used to investigate the repair of the meniscus require detailed knowledge and baseline data of the biomechanical properties of normal and repaired menisci.

In an effort to better understand pathologic and traumatic processes in the knee meniscus, many studies have investigated the biomechanical behavior of this tissue. The meniscus is a viscoelastic material that undergoes compressive, tensile, and shear stresses during normal function. A multitude of studies have observed the effect of these stresses on meniscal tissue.[9-20] The results of these studies demonstrate that meniscal biomechanical properties are anisotropic and inhomogeneous, and vary with location (anterior, central and posterior), testing direction (radial, axial and circumferential), and surface (femoral and tibial). Most of the studies performed have been tensile studies,[9, 12-15, 17, 20] with some compressive[9, 16, 18, 19] and shear studies.[10, 11, 17]
One of the first compressive tests performed on the meniscus was performed by Proctor and coworkers.[9] This study used confined compression to determine the aggregate modulus and permeability of the bovine meniscus at different locations and depths. Results showed that the aggregate modulus from the superficial zone did not vary significantly depending on location, though specimens from the deep zone were found to be stiffer in the posterior portion than the anterior portion. Hacker and coworkers[18] tested disks from the anterior, central, and posterior portions of the human meniscus under confined compression. Their results showed that the posterior portion of the meniscus had the highest aggregate modulus and the anterior portion had the lowest aggregate modulus. Unfortunately in the two aforementioned studies, the topographical compressive properties were only examined in bovine and human menisci. However, in a later study performed by Joshi and coworkers,[16] biomechanical comparisons were performed among six species: human, bovine, monkey, canine, ovine, and porcine. Properties were determined through confined compression testing of plugs taken only from the posterior portion of the meniscus. This study found statistically significant differences among the animal models and showed that the ovine aggregate modulus most closely resembled the human aggregate modulus.[16] While this study did compare the interspecies variation among the animal models, only one location was used for the testing site.

While some biomechanical studies of the meniscus have been performed, no study has compared either the topographical variation in a multitude of different animal models or the variation at different testing locations among these animal models. However, two
general trends have been noted in the previously performed studies as mentioned above: 1) the posterior portion of the human and bovine medial menisci had the highest aggregate modulus and 2) interspecies variation in biomechanical properties occurred in the posterior portion of the tissue. The purpose of this investigation was to test the following two hypotheses: (a) for intraspecies variation, the aggregate and shear moduli will be the greatest in the posterior portion of the tissue, and (b) for interspecies variation, the animal model will have a significant effect on the compressive material properties of the tissue. To test these hypotheses we quantified the compressive biomechanical properties (aggregate modulus - $H_A$, permeability - $k$, Poisson's ratio - $\nu_S$, and shear modulus - $\mu_S$) of the medial meniscus and their topographical distribution in six animal models (human, bovine, baboon, canine, lapine, and porcine) using a creep indentation technique, which we believe may be a more physiologically relevant loading configuration. This approach, which uses both experimental and theoretical means, was then applied to quantify the biomechanical behavior at six topographical locations (anterior, central, and posterior portions; femoral and tibial side) of the medial meniscus.

Materials and Methods

Specimen Preparation

Ten bovine medial menisci (1-2 year old animals), eight canine medial menisci (4 year old animals), nine human medial menisci (average 33.3 years old), fifteen baboon medial menisci (average 12.8 years old), six porcine medial meniscus (about 1 year old), and ten lapine medial meniscus (about 9 months old) were harvested from the knee joint. For storage between harvest and testing, the specimens were wrapped in gauze soaked with
0.15 M NaCl solution with protease inhibitors (N-ethylmaleimide, 10 mM; benzamidine HCl, 5 mM; EDTA, 2 mM; and PMSF, 1 mM) and frozen at -20°C until time of testing. For testing, the menisci were bisected in the axial plane into the tibial and femoral aspects (with the exception of the rabbit, which was too small to perform this technique on reliably), and were then further divided into anterior, central and posterior segments. A total of 318 mechanical tests were performed. Figure 2 shows a schematic layout of the testing procedure.

**Creep Indentation**

Menisci were tested mechanically to determine each meniscal specimen’s aggregate modulus, Poisson's ratio, permeability, and shear modulus. A creep indentation apparatus[21, 22] was used to quantify the creep and recovery deformation behavior of each specimen. The testing apparatus is able to load and unload the meniscus specimen automatically through a closed-loop control system. To test, each specimen was thawed for one hour in normal saline containing protease inhibitors and then attached with cyanoacrylate cement to a sample holder. The sample holder was positioned with a spherical joint and lead screw assembly, allowing the meniscal surface to be oriented normal to the loading shaft. A tare load of 0.005 - 0.01 N was then applied with a 0.8 - 1.0 mm diameter, flat-ended, cylindrical rigid, porous indenter tip (50% porous, ~50μm pore diameter), and the tissue was allowed to reach tare creep equilibrium. Different combinations of the tare load, test load, and indenter tip were used to ensure that the response of the tissue was within its linear range, with the strain applied to the tissue being under 10%. Samples were tested within a narrow range of strain to minimize
variation in testing among animal models. Equilibrium was automatically determined when the slope of the creep curve became smaller than $1 \times 10^{-6}$ mm s$^{-1}$. Once creep equilibrium from the tare load was reached, the tissue was loaded with a step force of 0.02 - 0.04 N. The tissue's deformation was monitored with a linear variable differential transformer (LVDT) using a computer-based data acquisition system at a 0.25 µm deformation-resolution. The frictional resistance of the system was reduced with air bearings to less than $9.81 \times 10^{-4}$ N. The bearings were driven with pressurized air (552 kPa) and the air was cleaned with a 5 µm particle filter and two 0.1 µm coalescing filters. The creep response of the specimen under the step force was monitored until equilibrium (defined as slope$<1 \times 10^{-6}$ mm s$^{-1}$, or 2 hours) was reached. At this point, the test force was automatically removed and the recovery phase began. When recovery equilibrium was achieved (defined as slope$<1 \times 10^{-6}$ mm s$^{-1}$, or 1 hour), data acquisition was stopped automatically. Overall, the automated creep indenter yielded the creep and recovery deformational behaviors of each meniscal specimen in response to a 0.02 - 0.04 N step load.

**Thickness**

The thickness was measured using two methods: First we simply used a micrometer to measure the overall approximate thickness at the test site. Following specimen mounting and creep indentation testing, thickness was also measured using a needle probe attached to a force transducer and an LVDT. The entire probe assembly was moved downward with a linear motor until the needle touched the meniscal surface. At this point, the force transducer noted a significant change in force on the needle probe and the needle...
continued to move through the sample. When the needle contacted the sample holder underneath the meniscal sample another significant change in the force on the needle probe was noted. The difference between the measured needle positions at the two force readings corresponded to specimen thickness.

**Finite Element Modeling**

The experimental data for each test site were analyzed using a finite element/non-linear optimization modeling (FEO) method. This approach, which is based on biphasic finite element routines and nonlinear optimization techniques, uses the entire creep curve to calculate the intrinsic material properties of articular cartilage[22] and the meniscus. The output of this procedure depends significantly on an initial estimate of the tissue’s properties. Thus, we used a semi-analytical/semi-numerical biphasic procedure to obtain an estimate of the tissue’s properties, before applying the finite element/non-linear optimization routine.[22] More details can be found in a series of papers by Mow and coworkers.[23-25] It should be noted that the tissue was modeled as an axisymmetric cylinder, which is only partially correct for meniscal architecture. The surface of the meniscus consists of isotropically oriented collagen fibers, whereas the deeper tissue has an anisotropic fiber alignment. Due to the small degree of strain placed on the tissue, we believe that the compressive testing only occurs in the isotropic portion, but this might not be the case. However, without knowing the thickness of this surface layer in all of the animal models, more complex modeling cannot be performed.
Statistical Analysis

For the intraspecies and interspecies data one-way analysis of variance (ANOVA) was used to determine statistical significance. In the intraspecies analysis, the aggregate modulus, Poisson's ratio, permeability, and shear modulus were set as the dependent variable and the topographical location as the independent variable. For the interspecies analysis the independent variables were split into the anterior, central, and posterior portions (femoral and tibial sides averaged) of each animal model, and the dependent variables were the same as for the intraspecies study. For both the intraspecies and interspecies analysis, if the F-test showed a significant difference ($p<0.05$), a post hoc test was performed (Fisher's Protected Least Significant Difference) to compare sample sets. A significance level ($\alpha=0.05$) was used in all the statistical tests performed.

Results

From the creep-relaxation curve (Fig. 3) the four material properties (aggregate modulus, Poisson's ratio, permeability, and shear modulus) were determined. It should be noted that the time constant found for the tested animal models was much shorter than found by Mow and coworkers[25] for articular cartilage. This is probably due to articular cartilage having an eight-fold higher concentration of proteoglycans than the meniscus.[26] The intrinsic material properties of the six tested animal models exhibited a wide variation in both intraspecies and interspecies values (Tables I-VI). The size of the testing groups were as follows: baboon, $n=15$; bovine, $n=10$; canine, $n=8$; human, $n=9$; lapine, $n=5$; porcine, $n=6$. 
Intraspecies Topographical Variations

Aggregate Modulus

No statistical difference (p>0.14) was found among the aggregate moduli at the six different locations on the meniscus (femoral side: anterior, central, posterior; tibial side: anterior, central, posterior) in the canine and baboon models. In the bovine model the femoral-anterior location was stiffer than any other location on the meniscus (p<0.04). The tibial-anterior location was also stiffer than the femoral-posterior and tibial-central locations (p<0.03). In the porcine model the femoral-anterior location was stiffer than any other location on the meniscus (p<0.002). The tibial-anterior portion was also statistically different than the tibial-posterior location (p<0.05). In the human and lapine models the anterior portion (both femoral and tibial side) was stiffer than the central or posterior portions of the meniscus (p<0.007; Fig. 4).

Poisson's Ratio

No statistical difference (p>0.16) was found for the Poisson's ratio at the six different locations on the meniscus (femoral side: anterior, central, posterior; tibial side: anterior, central, posterior) in all of the models with the exception of the lapine and baboon. In the lapine model, the tibial-anterior portion had a higher Poisson’s ratio than the central and posterior portions of the tissue. In the baboon model, the central portion of the tissue had a higher Poisson's ratio than the anterior and tibial-posterior portions of the tissue (p<0.03).
Permeability

No statistical difference (p>0.15) was found for the permeability at the six different locations on the meniscus (femoral side: anterior, central, posterior; tibial side: anterior, central, posterior) in all of the models with the exception of the lapine model. In the rabbit, the anterior portion of the tissue had a higher permeability than the central and posterior portions of the tissue (both femoral and tibial side) (p<0.0005).

Shear Modulus

No statistical difference (p>0.10) was found among the shear moduli at the six different locations on the meniscus (femoral side: anterior, central, posterior; tibial side: anterior, central, posterior) in the canine and baboon models. In the bovine model the femoral-anterior location had a higher modulus than any other location on the meniscus (p<0.04); the tibial-anterior location was also higher than the femoral-posterior and tibial-central locations (p<0.03). In the porcine model the femoral-anterior portion had a higher shear modulus than all other locations (p<0.002). In the human model the anterior locations had a higher modulus than the central and posterior locations (both femoral and tibial) (p<0.007). This pattern could also be found in the lapine model (p<0.0007), though the femoral-anterior portion was also statistically higher than the tibial-anterior portion (p<0.03).

Interspecies Topographical Variations

Statistical variations in the compressive properties of the meniscus were observed among the different animal species. General trends noted were that the lapine model exhibits the
highest aggregate modulus (Fig. 5), Poisson’s ratio, and shear modulus, whereas the bovine model exhibited the highest permeability (Fig. 6). The central and posterior portions of the lapine model exhibited the lowest aggregate and shear modulus, whereas the central and posterior portions of the lapine model exhibited the lowest permeability. Tables VII-X show where statistical significance (p<0.05) was noted among the testing locations and models.

Discussion

While some intraspecies and interspecies compressive property comparisons have been performed on the meniscus previously, no studies have looked at intraspecies variations in a diversity of animal models and interspecies variation at a variety of different locations. This study was conducted to determine the variation in compressive properties of the medial meniscus at different topographical locations in a variety of animal models. To fulfill this goal, medial meniscal samples were tested using an indentation technique at six different locations: the anterior, central, and posterior portions of the meniscus on both the femoral and tibial surfaces of the tissue. These tests were performed on tissue from six different animal models (baboon, bovine, canine, human, lapine, and porcine). The creep indentation testing used for this study is based on biphasic finite element optimization of the creep response of the medial meniscus and allows for four material properties to be determined: the aggregate modulus, Poisson's ratio, permeability, and shear modulus (calculated from $H_A$ and $v_S$). We believe this is a more physiologically relevant testing method than many other testing methods, such as disk compression, due to the presence of native tissue surrounding the test site and the lack of edge effects when
testing the tissue sample. A limitation of this testing method is that the meniscus requires excision from the joint before testing, possibly altering the native resting state of the meniscus. It should be noted that this limitation would also be exhibited by disk compression techniques.

Our original hypotheses were that (a) for intraspecies variation, the aggregate and shear moduli will be the greatest in the posterior portion of the tissue, and (b) for interspecies variation, the animal model will have a significant effect on the compressive material properties of the tissue. Results from the study found the intraspecies hypothesis to be incorrect. Our results showed either no variation in the aggregate and shear modulus depending on location or that the anterior portion of the tissue had the highest values, not the posterior portion. However, our data show that the second hypothesis, that the animal model would have a significant effect on the compressive properties of the tissue, was correct.

The compressive characteristics of the bovine meniscus have now been observed in three different studies, including this one. The other two studies were performed by Proctor and coworkers[9] and Joshi and coworkers[16]. Proctor and coworkers,[9] who tested the femoral side of the meniscus at four different locations (anterior, anterior-central, central-posterior, and posterior) found in the superficial zones an aggregate modulus of 0.393 ± 0.109MPa in the posterior portion and 0.440 ± 0.108MPa in the anterior portion. Our results for the femoral side of the bovine meniscus show lower aggregate modulus values: 0.11 ± 0.04MPa for the posterior portion and 0.21 ± 0.06MPa for the anterior
portion. If the permeability between the Proctor and coworkers[9] study and this study is compared it is found that the Proctor and coworkers[9] study found lower values (0.7 - 1.0x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}) than our study (5.4 - 6.2x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}). These variations are probably due to variations in the testing method, test location, and curve-fitting procedure (our study used a finite element optimization procedure for curve fitting, the Proctor and coworkers[9] study used a biphasic theory which does not take into consideration the full creep curve). The intraspecies hypothesis was based on the deep-tissue results from the Proctor and coworkers[9] study, which exhibited a higher aggregate modulus in the posterior portion of the tissue, which turned out to be opposite of the results from the current study. This variation is probably due to the difference in testing location. Due to the minor degree of force placed on the meniscal tissue in the current study, only the compressive response at the surface was examined. It is possible that, while the surface of the meniscus consists of an isotropic collagen alignment, the deeper zone, with its anisotropic collagen alignment in the circumferential direction, might exhibit differences in compressive properties. In the Joshi and coworkers[16] study, the aggregate modulus and permeability of the tibial-posterior location were obtained in several different animal models, including the bovine model. Their results show an aggregate modulus of about 0.12MPa and a permeability of about 3.3x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}, whereas our results from that section of tissue show an aggregate modulus of 0.13 \pm 0.06MPa and a permeability of 5.40 \pm 5.36x10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}. The results of the aggregate modulus are almost identical, and the permeability results are quite similar.[16]
The compressive characteristics of the human meniscus have been observed in three different studies, including this one. A study performed by Hacker and coworkers[18] found the aggregate modulus and permeability of the meniscus at the anterior, central, and posterior portions of the meniscus (tibial side). They found the aggregate modulus to be 0.2MPa, 0.22MPa, and 0.28MPa at the anterior, central, and posterior portions of the tissue, respectively.[18] Our results showed an aggregate modulus of 0.16 ± 0.05MPa, 0.11 ± 0.04MPa, and 0.09 ± 0.03MPa for the respective locations. Our results were lower, and this is probably due to the testing method and different curve-fitting methods. The Hacker and coworkers[18] study used the same curve-fitting method as the Proctor and coworkers[9] study. There were differences noted in the permeability values also. The Hacker and coworkers[18] study found permeability values of 0.9x10^-15 m^4 N^-1 s^-1, 0.8x10^-15 m^4 N^-1 s^-1, and 0.9x10^-15 m^4 N^-1 s^-1 for the anterior, central, and posterior locations. The current study found the permeability at each of the locations to be 1.71 ± 0.48x10^-15 m^4 N^-1 s^-1, 1.54 ± 0.49x10^-15 m^4 N^-1 s^-1, and 1.32 ± 0.61x10^-15 m^4 N^-1 s^-1. The other study was performed by Joshi and coworkers[16] and is part of the study described above. Their results show an aggregate modulus of about 0.22MPa and a permeability of about 1.99 ± 0.79 x10^-15 m^4 N^-1 s^-1 for the tibial-posterior location, whereas our results from that section of tissue show an aggregate modulus of 0.09 ± 0.03MPa and a permeability of 1.32 ± 0.61x10^-15 m^4 N^-1 s^-1. The permeability results are quite similar, though there is a significance difference between the aggregate modulus found in the two studies.[16]
The Joshi and coworkers[16] study also found the aggregate modulus and permeability in the canine and porcine model. For the canine model, Joshi and coworkers[16] found an aggregate modulus of about 0.15MPa and a permeability of about $3.5 \times 10^{-15}$ m$^4$ N$^{-1}$ s$^{-1}$, whereas our results were $0.19 \pm 0.08$MPa and $2.76 \pm 1.10 \times 10^{-15}$ m$^4$ N$^{-1}$ s$^{-1}$, respectively. For the porcine model, Joshi and coworkers[16] found an aggregate modulus of about $0.27 \pm 0.04$MPa and a permeability of about $1.74 \pm 0.19 \times 10^{-15}$ m$^4$ N$^{-1}$ s$^{-1}$, whereas our results were $0.13 \pm 0.03$MPa and $6.32 \pm 4.21 \times 10^{-15}$ m$^4$ N$^{-1}$ s$^{-1}$, respectively. These values are not very similar, though the difference is slight compared to the variations found in the Proctor and coworkers[9] study. It should be mentioned that in the Joshi and coworkers[16] study a trend of increasing aggregate modulus with decreasing permeability was noted among the different animal models. This trend was not noted in the tibial-posterior location or any other location in our study. Overall, this study found significant variations in the compressive properties of the different animal models, as was hypothesized.

The most common location for injury in the meniscus is the posterior region.[27, 28] Our results show a common trend in the tested animals of the posterior region having the lowest shear modulus and, to a lesser extent, aggregate modulus. This trend in material properties could help explain the frequency of tears in that location. The other interesting trend noted was that the aggregate modulus, shear modulus, and permeability of the anterior portion of the lapine model were much greater than the central and posterior portions. We believe that this characteristic is due to the bent-knee resting stance of the
rabbit and the frequency that the animal jumps, something not seen in any of the other animal models tested.

The significant variations in the material properties among these different animal models suggest caution when using an animal model to study the human knee joint. By comparison of the four material properties between the human and the other animal models, it can be noted that no model is ideal in all cases. The aggregate modulus and shear modulus in the human are the most similar to the bovine model, but when looking at permeability the canine and baboon values are the closest to human values. The Poisson's ratio indicates that any of the tested animal models have similar values, with the exception of the lapine and baboon model.

Acknowledgments

This study was partially supported by a grant from the National Institutes of Health (Grant# R01 AR47839-2).
## Tables

### Table I. Baboon meniscal biomechanical properties (±SD)

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$\nu_S$</th>
<th>$k$ $(10^{15} m^4 N^{-1} s^{-1})$</th>
<th>$\mu_S$ (MPa)</th>
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<tbody>
<tr>
<td>Femoral</td>
<td>Anterior</td>
<td>0.17±0.05</td>
<td>0±0</td>
<td>1.16±0.56</td>
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<td>Central</td>
<td>0.18±0.06</td>
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<td>1.08±0.71</td>
<td>0.09±0.03</td>
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<tr>
<td></td>
<td>Posterior</td>
<td>0.18±0.05</td>
<td>0.01±0.03</td>
<td>1.36±0.40</td>
<td>0.09±0.03</td>
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<tr>
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<td>Anterior</td>
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<td>0±0</td>
<td>1.05±0.23</td>
<td>0.08±0.03</td>
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<td>0.18±0.07</td>
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<td>0.09±0.03</td>
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<tr>
<td></td>
<td>Posterior</td>
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<td>0±0</td>
<td>1.36±0.60</td>
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</tr>
</tbody>
</table>

a. $H_A$ = aggregate modulus, $\nu_S$ = Poisson's ratio, $k$ = permeability, $\mu_S$ = shear modulus

### Table II. Bovine meniscal biomechanical properties (±SD)

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<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$\nu_S$</th>
<th>$k$ $(10^{15} m^4 N^{-1} s^{-1})$</th>
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<td>5.73±6.19</td>
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<td>Posterior</td>
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<td>0.06±0.02</td>
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<td>5.79±4.31</td>
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<td>5.65±4.13</td>
<td>0.06±0.02</td>
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<td></td>
<td>Posterior</td>
<td>0.13±0.06</td>
<td>0±0</td>
<td>5.40±5.36</td>
<td>0.07±0.03</td>
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</tbody>
</table>

a. $H_A$ = aggregate modulus, $\nu_S$ = Poisson's ratio, $k$ = permeability, $\mu_S$ = shear modulus
Table III. Canine meniscal biomechanical properties (±SD)

<table>
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<tr>
<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$\nu_S$</th>
<th>$k$ ($10^{15}$ m$^4$ N$^{-1}$ s$^{-1}$)</th>
<th>$\mu_S$ (MPa)</th>
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<td>Posterior</td>
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<td>Posterior</td>
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<td>2.76±1.10</td>
<td>0.10±0.04</td>
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</tbody>
</table>

a. $H_A$ = aggregate modulus, $\nu_S$ = Poisson's ratio, $k$ = permeability, $\mu_S$ = shear modulus

Table IV. Human meniscal biomechanical properties (±SD)

<table>
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<tr>
<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$\nu_S$</th>
<th>$k$ ($10^{15}$ m$^4$ N$^{-1}$ s$^{-1}$)</th>
<th>$\mu_S$ (MPa)</th>
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<td>Femoral</td>
<td>Anterior</td>
<td>0.15±0.03</td>
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<td>Central</td>
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<td>1.54±0.71</td>
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<td>Posterior</td>
<td>0.11±0.02</td>
<td>0.01±0.02</td>
<td>2.74±2.49</td>
<td>0.05±0.01</td>
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<tr>
<td>Tibial</td>
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<td>0.16±0.05</td>
<td>0±0</td>
<td>1.71±0.48</td>
<td>0.08±0.02</td>
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<tr>
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<td>Central</td>
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<td>0±0</td>
<td>1.54±0.49</td>
<td>0.06±0.02</td>
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<tr>
<td></td>
<td>Posterior</td>
<td>0.09±0.03</td>
<td>0±0</td>
<td>1.32±0.61</td>
<td>0.05±0.01</td>
</tr>
</tbody>
</table>

a. $H_A$ = aggregate modulus, $\nu_S$ = Poisson's ratio, $k$ = permeability, $\mu_S$ = shear modulus
### Table V. Lapine meniscal biomechanical properties (±SD)

<table>
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<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$\nu_s$</th>
<th>$k$ ($10^{15}$ m$^4$ N$^{-1}$ s$^{-1}$)</th>
<th>$\mu_s$ (MPa)</th>
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<td>Anterior</td>
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<td>Central</td>
<td>0.13±0.02</td>
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<td>Posterior</td>
<td>0.12±0.03</td>
<td>0.00±0.00</td>
<td>1.20±0.49</td>
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<td>Posterior</td>
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<td>0.97±0.32</td>
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</table>

a. $H_A =$ aggregate modulus, $\nu_s =$ Poisson's ratio, $k =$ permeability, $\mu_s =$ shear modulus

### Table VI. Porcine meniscal biomechanical properties (±SD)

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Segment</th>
<th>$H_A$ (MPa)</th>
<th>$\nu_s$</th>
<th>$k$ ($10^{15}$ m$^4$ N$^{-1}$ s$^{-1}$)</th>
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a. $H_A =$ aggregate modulus, $\nu_s =$ Poisson's ratio, $k =$ permeability, $\mu_s =$ shear modulus
Table VII. Interspecies Comparison of Aggregate Modulus

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b. “X” denotes statistical significance between test groups (p<0.05)
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a. First letter denotes animal (M= baboon, B= bovine, C= canine, H= human, L= lapine, P= porcine) and second letter denotes location (A= anterior, C= central, P= posterior).
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a. First letter denotes animal (M=baboon, B=bovine, C=canine, H=human, L=lapine, P=porcine) and second letter denotes location (A=anterior, C=central, P=posterior).
b. "X" denotes statistical significance between test groups (p<0.05)
Fig. 1. Schematic diagram of the knee. The joint space is elongated to show the location of the medial and lateral menisci.
Fig. 2. A schematic diagram of the testing procedure. The medial meniscus (a) is separated into anterior, central, and posterior regions (b). The meniscus is split in the transverse plane (c) and separated so that the femoral and tibial surfaces can be tested (d). The meniscal sample is then attached to the sample mount (e) and tested in the creep indentation apparatus (f).
**Fig. 3.** A typical creep-recovery curve from the anterior portion (femoral side) of the medial rabbit meniscus.
Fig. 4. Comparison of the aggregate modulus (femoral and tibial sides combined) at the anterior, central, and posterior portions of the human and lapine meniscus. Data shown as mean ± standard deviation. (*) indicates a significant difference between either the human central or human posterior regions and the human anterior region (p<0.05). (#) indicates a significant difference between either the lapine central or lapine posterior regions and the lapine anterior region (p<0.05).
Fig. 5. Comparison of the aggregate modulus (femoral and tibial sides combined) at the anterior location in the six tested animal models. Data shown as mean ± standard deviation. (@) indicates statistical significance between the lapine aggregate modulus and the aggregate modulus of the other five animal models (p<0.0001). (*) indicates an additional significant difference between either the bovine, baboon, or human aggregate modulus and the canine aggregate modulus (p<0.0002). (#) indicates an additional significant difference between either the baboon or human aggregate modulus and the porcine aggregate modulus (p<0.006).
Fig. 6. Comparison of the permeability (femoral and tibial sides combined) at the central location in the six tested animal models. Data shown as mean ± standard deviation. (#) indicates a significant difference between either the bovine or porcine permeability and the canine, human, baboon, or lapine permeability (p<0.02).
Chapter 10: Conclusion

The work described in this thesis has employed ultrastructural, biomechanical, biochemical, and cellular techniques to provide a fully characterized lapine model that can serve as a reference and guide for meniscal tissue engineering studies. It also generated a biomechanical baseline for future studies of the knee meniscus using large animal models. This study consisted of five stages: 1) evaluation of ultrastructural properties of the medial rabbit meniscus, 2) quantification of biomechanical properties of the medial rabbit meniscus, 3) measurement of biochemical properties of the medial rabbit meniscus, 4) characterization of properties of rabbit fibrochondrocytes on a P(PF-co-EG)-co-GRGDS hydrogel, and 5) quantification of the compressive creep properties of the knee meniscus in several animal models.

Stage 1, *evaluation of ultrastructural properties of the medial rabbit meniscus*, was completed through the use of two steps. In the first step, histological processing of meniscal tissue via Hematoxylin & Eosin, Masson’s trichrome, and Safranin O/Fast Green staining, determined that the medial rabbit meniscus contains abundant levels of collagen, fibrochondrocytes are aligned between collagen fibers, and proteoglycan levels vary topographically within the tissue. Proteoglycan staining was greater in the inner 1/3 of the tissue (as expected due to the cartilaginous nature of this region), particularly in the anterior portion. These results can be found in Chapter 6. In the second step, evaluation of collagen fiber structure within the knee meniscus, was established by chemically and mechanically exposing the collagen fibers and viewing their structure via scanning
electron microscopy. Results showed that the surface consists of randomly oriented collagen fibers, and the deep zone consists of collagen fibers arranged in 10-70 μm diameter bundles positioned in a circumferential direction throughout the meniscus (Chapter 5).

Stage 2, *quantification of biomechanical properties of the medial rabbit meniscus*, was completed via tensile testing and creep compressive testing. Tensile testing, which consisted of dissecting circumferential specimens (oriented along the direction of collagen fiber alignment) and pulling them to failure, found that the medial rabbit meniscus has a Young’s modulus of $156.6 \pm 48.9$ MPa. Compressive creep testing, which consisted of performing creep indentation tests at the anterior, central, and posterior portions of both the femoral and tibial sides of the tissue, found topographical variations of the material properties. Specifically, it was determined that the femoral side of the anterior portion exhibited the highest aggregate modulus ($510 \pm 100$ kPa) and shear modulus ($240 \pm 40$ kPa), while the lowest aggregate modulus ($120 \pm 30$ kPa) and shear modulus ($60 \pm 20$ kPa) were found on the femoral side at the posterior location. More details on these studies can be found in Chapter 5.

Stage 3, *measurement of biochemical properties of the medial rabbit meniscus*, consisted of three parts, as described in Chapter 6. In the first part, determination of water content was accomplished through comparison of the wet and dry weight of the tissue. Results show that the inner 1/3 of the tissue is more hydrated ($72.9 \pm 2.5\%$) than the outer 2/3 ($67.7 \pm 1.9\%$), and the inner 1/3 of the anterior location has the greatest hydration overall
(75.9 ± 3.9%). In the second part, determination of the sulfated glycosaminoglycan content was performed through the use of a dimethylmethylen blue assay. Results showed that the inner 1/3 of the tissue had the greatest amount of sulfated GAGs, particularly in the anterior portion (114.5 ± 33.9 µg/mg dry weight). The distribution found in this assay matched the distribution noted in the Safranin O/Fast Green staining performed in Stage 1 and this comparison can be found in Chapter 6. In the third and final part, determination of collagen content was performed through a hydroxyproline assay. Results show that the outer 2/3 of the posterior location exhibited the highest hydroxyproline content (67.7 ± 6.9 µg/mg dry weight) and the inner 1/3 of the anterior location exhibited the lowest hydroxyproline content (30.0 ± 6.7 µg/mg dry weight). The inner 1/3 (37.9 ± 14.5 µg/mg dry weight) had a significantly lower hydroxyproline content than the outer 2/3 (61.6 ± 9.5 µg/mg dry weight). The significant variation of the biochemical properties in the inner 1/3 of the anterior location might be due to the bent-knee resting stance of the rabbit and the propensity of the animal to jump.

Stage 4, characterization of properties of rabbit fibrochondrocytes on a P(PF-co-EG)-co-GRGDS hydrogel, was performed to determine if this material could be a suitable scaffold for meniscal tissue engineering (Chapter 7). To determine this, the attachment and proliferation of rabbit meniscal fibrochondrocytes was monitored on monolayers of the P(PF-co-EG) hydrogel and on P(PF-co-EG) hydrogel with varying levels of the GRGDS peptide attached. Results showed that while the fibrochondrocytes initially attached to the hydrogel, particularly if it contained the GRGDS peptide, they did not continue to stay attached over a 72 hour time period. This would indicate that the
References

References: Chapter 1

There are no references for this chapter.

References: Chapter 2


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References: Chapter 3


References: Chapter 4


References: Chapter 5


References: Chapter 6


References: Chapter 7


References: Chapter 8


References: Chapter 9


References: Chapter 10

There are no references for this chapter.