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An Investigative Study Toward the Development of a Crosslinked Porcine Xenograft Meniscus Total Replacement

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An investigative study toward the development of a crosslinked porcine xenograft meniscus total replacement

By

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An investigative study toward the development of a crosslinked porcine xenograft meniscus total replacement

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Meniscus damage is very common and eventually leads to the deterioration of the entire knee joint. The goal of this study was to provide evidence that supports a proof of concept for a decellularized porcine meniscal xenograft to be used as a treatment method for meniscal injury as a partial or full meniscus transplant. This research adapted an antigen removal protocol for articular cartilage to produce decellularized xenografts in 48% of the time and with no significant difference in DNA content as other current methods. DNA and GAG content, and the compression moduli were significantly lower in the xenograft than the control, but collagen content remained the same. Tensile modulus and ultimate tensile stress were significantly higher for the xenograft than the control. Crosslinking analysis was performed and 0.2% genipin was found to have a significantly higher degree of crosslinking than the rest.

DEDICATION

This body of work is dedicated to my wonderful wife Britton and our son Ehren. She has given so much of her time and effort to support me throughout graduate school. Without her, I wouldn't be half the man that I am today. I am so happy to have you in my life. J'taime beaucoup, tu es l'amour de ma vie!

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CHAPTER I

LITERATURE REVIEW

The overall goal of this study was to investigate and determine the ideal meniscus replacement implant to be used in conjunction with a total meniscectomy. When a meniscus suffers significant damage due to disease or injury, it eventually leads to the breakdown of the articular cartilage of the knee. This deterioration of the articular cartilage leads to the overall deterioration of the whole joint. If the damaged meniscus could be identified in the early stages of deterioration, it can be resected and replaced by an implant that would prevent further deterioration. This study first analyses the meniscus as a whole, its tissue components, role in the functionality of the joint, and the biomechanical properties of the meniscus. Next, the ways that a meniscus can become damaged, either through injury or disease, and how these damages are treated is described and assessed. Finally, this study evaluates the current implant types in development and recommends the most ideal choice of implant.

The Meniscus

The meniscus is a crescent, or "C" shaped cartilaginous tissue in the knee joint. It is comprised of a lateral and a medial component and is situated between the femoral condyles and the tibial plateau. The main functions of the meniscus are to act as a load-bearer, shock absorber, to evenly distributing load transmission through the knee joint, and to provide lubrication and nutrition to the articular cartilage of the femur and tibia[1].

Anatomical Review

The "C" shape of the meniscus provides the effective coverage and protection for the articular cartilage of the femur and tibia while leaving the innermost portion of the knee joint, between the femoral condyles, exposed. It is in that exposed region where the insertion and origin points for cruciate ligaments, the main stabilizing ligaments of the knee, are located.

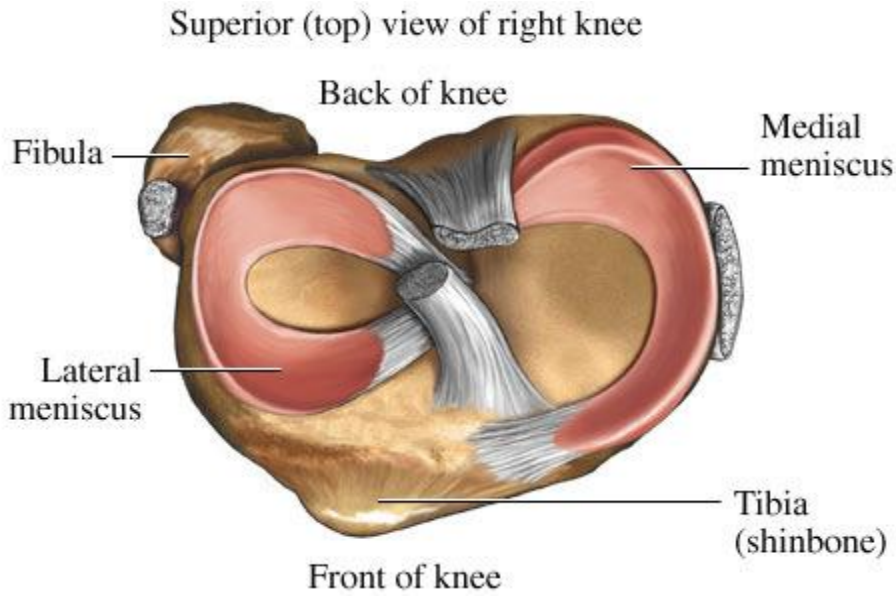


Figure 1.1 Superior view of the knee and menisci.[6]

The menisci are wedge shaped with concave proximal surfaces that are aligned directly inferior to the lateral and medial condyles of the femur. The articular cartilage of the femur rests directly on the proximal surface of the meniscus. The distal surface of the meniscus is flat and lies superior to the articular surfaces of the lateral and medial condyles of the head of the tibia. The menisci are connected to the tibial plateau by the horn attachments on the anterior and posterior horns of the crescent. The anterior horn is

attached anteriorly to the anterior cruciate ligament (ACL) and the posterior horn is attached anteriorly to the posterior cruciate ligament (PCL).

Comparing the two menisci, the lateral meniscus is larger, has more variation in size, and covers more of the tibial plateau than the medial meniscus[2,3,4,5]. The lateral meniscus also has more ligament connections than the medial meniscus. The anterior and posterior horns of the lateral meniscus are connected by the transverse (intermeniscal) ligament. The posterior horn has three additional connections, the first two being connections to the PCL and medial condyle of the femur by the meniscofemoral ligaments of Wrisberg, posteriorly to the PCL, and Humprey, anteriorly to the PCL. The third connection is to the popliteal tendon which can act on the posterior horn and move the lateral meniscus during flexion[6]. The medial meniscus lacks any attachment to the corresponding lateral cruciate ligament but does have an additional attachment to the tibia via the coronary ligament[7].

Biochemical Content

Meniscus tissue is very hydrated, with 72% of the weight being water. The remaining 28% of the wet weight is fibroblast cells in an extracellular matrix (ECM) composed of collagen, proteoglycans, DNA, adhesion glycoproteins, and elastin[8,9]. The main types of collagen found in meniscus tissue are type I and type II collagen. There are also trace amounts of types III, IV, VI, and XVIII collagen and elastin found in different regions of the meniscus tissue[10]. Aggrecan is the main large proteoglycan molecule in meniscus tissue and biglycan and decorin are the main smaller proteoglycan molecules[11]. Proteoglycan molecules are extremely important to the functionality of the meniscus. Structurally, proteoglycans have heavily branched and negatively charged

glycosaminoglycan (GAG) molecules bound to a core protein. The negative charges on the heavily branched regions attract water into the meniscus, maintaining the tissue's hydration, the visco-elastic behavior, compressive stiffness, and surface friction reduction of the tissue[12,13].

Zones of the Meniscus

The meniscus has two main zones, the outer zone and the inner zone, with regards to collagen content. The collagen fibers change in orientation and type moving from the most superficial layer inwards. There is also an increase in the concentration of proteoglycans toward the center of the meniscus[14].

The outer zone of the meniscus is characterized by an ECM comprised of mostly type I collagen and small concentrations of GAGs. The chondrocytes in the outer zone are oval and fusiform[15]. These chondrocytes produce the ECM and lay the type I collagen circumferentially along the "C" shape of the meniscus. There are two distinct layers of collagen, the superficial and surface layers, found in the outer zone.

The inner zone of the meniscus is categorized by an ECM composed from mostly type II collagen, with a smaller but significant percent of type I collagen, and a higher concentration of GAGs. The chondrocytes in the inner zone are more round than the chondrocytes of the outer zone and produce an ECM that includes the circumferential pattern of the type I collagen with the addition of radially arranged collagen to resist longitudinal splitting[16,6].

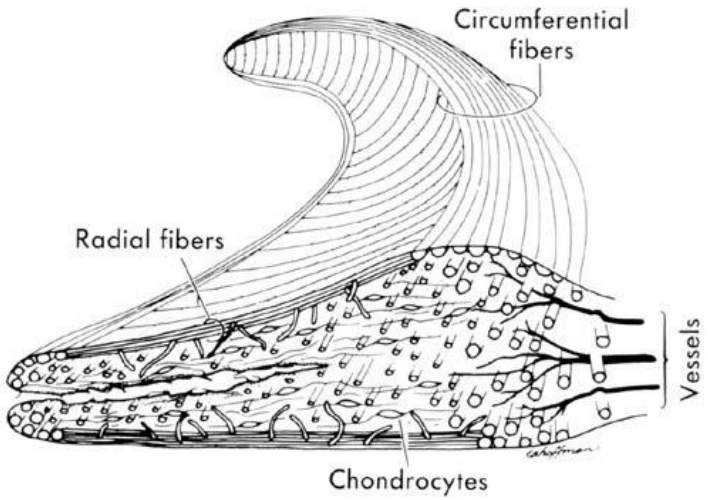
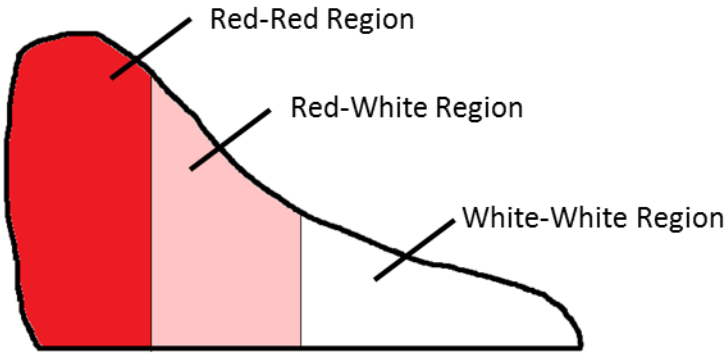


Figure 1.2 Collagen orientation creates and inner and outer zone of the meniscus.[91]

The meniscus can also described by zones of vascularity. The peripheral meniscus is vascularized by the geniculate arteries through the anterior and posterior horn attachments[17]. The peripheral rim of the meniscus, termed the "red-red zone", is the most vascularized zone with up to 30% vascularity. This percentage continuously decreases with age until the inner two thirds of the menisci are completely avascular[7]. The middle zone is called the "red-white zone" because some vascular tissue is able to penetrate deeper into the meniscus tissue. The most central zone of the meniscus is called the "white-white zone" and is totally avascular, receiving nutrition through synovial fluid diffusion[18].

Figure 1.3 The vascular zones of the meniscus.



The differing vascularities throughout the meniscus have a significant effect on the healing rates of injured meniscus. Areas with higher vascularity have better repair and healing potential than lower or avascular areas[14].

Biomechanical Properties

The knee joint is a highly active, load bearing joint that exerts a variety of dynamic forces on the meniscus. The meniscus withstands shear, tension, and compressive forces during flexion, extension, and load bearing[1]. The wedge-shape of the meniscus allows for these forces to be stabilized between the curved femoral condyles and the flat surface of the tibial head. The wedge-shape effectively converts the vertical compressive forces exerted on the meniscus from the femur into hoop stress that is contained as tension within the meniscus. Shear forces develop between the collagen fibers as the meniscus is deformed radially by compression[19,20,21].

Values for these forces have been calculated and documented in many studies. The shear forces on the meniscus is approximately 120 kPa[22]. The tensile properties of the meniscus are significantly different depending on the direction of the tensile force. The circumferential tensile strength is approximately 100-300 MPa, while the radial tensile strength is approximately 10-30 MPa. The meniscus resists axial compression

with an approximate stress of 100-150 kPa[23]. The meniscus occupies 60% of the contact area between the femoral and tibial articular cartilage and transmits over 50% of the compression forces on the joint at full extension and 85% of the load at 90° of flexion[24,25].

Meniscus Injury

Knee injuries, with meniscal lesions in particular, are the most common injuries requiring surgical intervention in the United States[26,27,28]. These injuries usually occur during sports or sports-related activities that involve cutting and twisting movements, hyperextensions, or high impact[4]. The most common motion that causes a tear in the meniscus is an internal rotation of the femur as the knee moves from a flexed to an extended position, splitting the meniscus longitudinally[6]. Trauma induced meniscal injury usually occurs in conjunction with anterior cruciate ligament tearing, occurring in >80% of cases, and is more common in the medial meniscus[1].

Tear Classification

Classification of meniscal tears is determined by location and tear pattern. Tears can be denoted by the vascular region in which they occur. Tears in the peripheral attachment sites, either meniscofemoral or meniscotibial, are called red-red tears. Tears located in the middle zone are called red-white tears. These tears typically occur at the junction of the red-red zone and the red-white zone, approximately 4mm from the meniscal attachment. The inner most zonal tears in the avascular region are called white-white tears[1]. Classifying tears due to their vascular zone is important because the healing potential is significantly different in the different regions. A red-red tear has a much

greater chance of healing naturally than a white-white tear, which almost always requires surgical repair.

Tear Patterns

There are five types of tear patterns for meniscal lesions caused by traumatic injury and a specific tear pattern for gradual degeneration. The five tears caused by traumatic injury can either be partial or full tears of the tissue, with partially torn tissue having a greater healing potential than fully torn tissue.

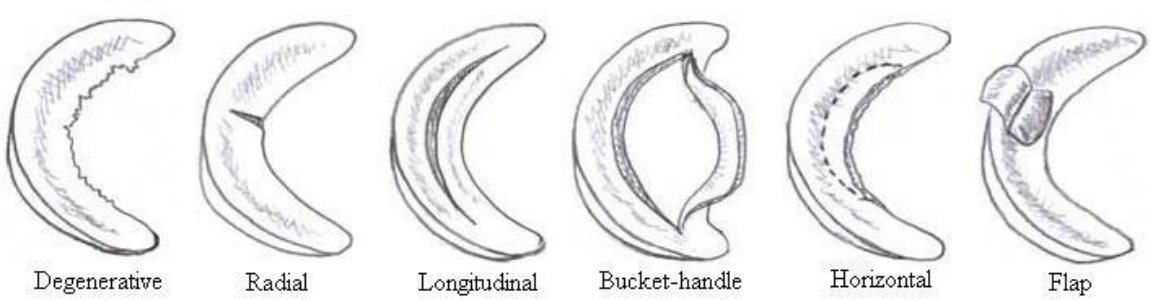


Figure 1.4 Visual representations of the different types of meniscal tears.[6]

Radial tears occur perpendicular to the circumferentially oriented collagen and often occur along with ACL injury. Longitudinal tears run along circumferentially oriented collagen fibers along the length of the meniscus. A bucket-handle tear is a full depth longitudinal tear that causes additional problems because it creates a long segment of the meniscus that has separated from the main body of the meniscus. This segment, which resembles a "bucket handle", can get caught on the medial side of the femoral condyle and cause joint locking. Horizontal tears form in the body of the meniscus and eventually bisect the tissue into a superior portion and an inferior portion. These tears are difficult to identify and are even more difficult to repair. Flap tears are horizontal tears that occur in a close proximity to the surface and cause a flap of the surface tissue to open

and close with movement. Complex tears occur when there are multiple tears in multiple planes. These types of tears are difficult to repair and are commonly found in degenerative cases. Complex tears are normally too damaged to repair and require a meniscectomy[6].

Degenerative injury causes a specific tear pattern, referred to as a degenerative tear, and is characterized by a frayed edge appearance of the medial portion of the meniscus. Meniscal lesions that develop as a result of gradual degeneration usually appear in tandem with other degenerative problems in the knee. These types of tears are very common, especially in subjects with knee pain or osteoarthritis. One study of patients over the age of 65 found that 91% of patients with symptomatic osteoarthritis had degenerative tears[29]. The study also found that 67% of patients who were asymptomatic for knee pain also had degenerative tears. Because these tears occur in the medial zone of the meniscus, they are entirely in the white-white zone and have almost no healing capability. The only treatment option for degenerative tears is a partial or full meniscectomy. The high prevalence of degenerative tears in the population combined with the only treatment option being a meniscectomy, means that there is a large demand for an effective meniscus implant.

Diagnosis

A comprehensive examination is required in order to diagnose a meniscal tear. Tibiofemoral joint-line tenderness is the primary indicator of a meniscal tear[24]. Symptoms of joint tenderness include joint stiffness, swelling, pain, lack of full extension, and deformity. The assessment of the tibiofemoral joint line is performed with the patient lying on a flat surface with the knee positioned at a 90° flexion.

Osteoarthritis

The primary long term consequence of meniscal injury is the development of osteoarthritis in the knee joint. Osteoarthritis (OA) is the most common degenerative joint disease that affects more than 25% of the adult population[30]. Symptoms of OA include chronic pain, joint instability, and stiffness. This is due to a progressive loss and destruction of articular cartilage, thickening of the subchondral bone in response to greater shear forces on the bone, formation of bone spurs in the joint, significant increase in inflammation in the synovial cavity, and a degeneration of the ligaments and meniscus leading to a narrowing of the joint space[31]. There is significant data that supports that past meniscus injury has a strong correlation to the prevalence of OA[32]. A study of former NFL athletes found that athletes with a history of meniscal repair were almost 3 times more likely to develop OA, and that players who underwent a partial meniscectomy were 6 times more likely to develop OA[33]. Other factors that contribute to the development of OA include obesity, aging, and heredity[34].

The molecular mechanisms that facilitate the start and progression of OA are not well understood and there are currently no known treatments to restore degraded cartilage or stop the progression of the disease[30]. Nonsurgical treatment of OA is limited to anti-inflammatory drugs including NSAIDs and hyaluronic acid or corticosteroid injections[35,36]. Once the disease progresses to include severe articular cartilage damage and bone fragmentation, surgical intervention is needed. Arthroscopic surgery can be performed to remove rough edges and bone fragments in the joint, but more severe cases require a more invasive option. An osteotomy can be performed to remove a severely damaged portion of either the tibia or the femur to relieve pressure on the joint. An

osteotomy will only delay the need for a total knee arthroplasty, the eventual end treatment for OA. The degradation of the joint will eventually become so severe that a partial or total knee replacement will need to be performed. The surgically reconstructed joint is made of metal, plastic, or combination of the two and is anchored into the shafts of tibia and femur after the diseased tissue has been removed[37,38].

Treatment Options

There are three main treatment options for a meniscus tear: surgical repair, meniscectomy, and meniscus replacement. In order to determine the proper course of treatment, an arthroscopic survey and MRI scan of the tissue is performed. The course of treatment is dependent on the location and the severity of the tear, as well as the age and overall health of the patient. The end goal of the treatment option chosen is to delay the onset of OA for as long as possible if not indefinitely. Some tears do not require surgical intervention. These tears are typically red-red tears and tears smaller than 8mm that have minimal damage to the main body of the meniscus[6]. Because these tears occur in the vascularized region, their potential for healing is far greater.

Surgical Repair

Meniscus tears that are good candidates for meniscal repair are unstable red-white tears that are longer than 10-12mm in active patients under the age of 50[24,25]. Typical tear patterns that have higher success rates of repair are longitudinal, radial, or bucket-handle tears[39]. Longitudinal tears are generally the most successful with nearly an 85% healing rate[40]. Longitudinal tears are caused by excessive compression and tear the meniscus along the circumferential collagen fibers, which remain intact. These fibers are

vital in the strength and force dissipation of the meniscus. Suturing these back together, most closely resembles an uninjured tissue, which leads to the high rates of success[41]. There are two main types of meniscal surgical repair, arthroscopic and open repair.

Arthroscopic Repair

There are three techniques that can be used during an arthroscopic repair of a meniscus tear, inside-out, outside-in, and all-inside[39]. The inside-out and the outside-in techniques require a 2.5-4cm accessory posteromedial or posterolateral incision for suture retrieval. The all-inside technique is completely arthroscopic and does not require an incision[25]. In all surgeries, the meniscus is prepared by performing a granulation tissue debridement with a meniscal rasp on the edges of the meniscal tear and perimeniscal tissue surrounding the tear[42]. This increases the vascular infiltration to the tear and in the adjacent tissue that significantly aids in the healing process.

The inside-out technique is the considered the gold standard classical technique for meniscus repair[42]. The technique is very versatile, with the ability to repair most tear patterns, and allows for the use of smaller diameter needles. The disadvantages of the inside-out technique include an increased risk of neurovascular injury to the popliteal vessels, an increased surgical time, post-operative pain, necessity for a surgical incision, and needlestick injury to the surgeon. Using a cannula to guide a long needle, the sutures are started from within the meniscus and pass through the tissue toward posterior. On each pass, the needle exits either through the posteromedial or posterolateral incision so another throw can be initiated[43]. Once complete the sutures are tied over the capsules on the periphery of the meniscus.

The outside-in technique is typically only used for longitudinal tears of the anterior horn of the meniscus because there is limited arthroscopic access in the region[40]. Tears are sutured with spinal needles that are pushed through the outer rim of the meniscus and then through the torn fragment. One main advantage of this technique is the ability to avoid neurovascular injury of the surrounding tissue without creating a large posterior incision[18]. The two main disadvantages of this technique is the use of larger diameter needles, which cause more damage when passed through the body of the meniscus, and difficulty of tying perpendicular sutures in tears adjacent to the posterior horn attachment.

The all-inside technique is a completely arthroscopic procedure characterized by the use of bioabsorbable repair devices including arrows, screws, darts, and staples instead of sutures[18]. Currently the most frequently used devices are suture-like anchors that are fixed to the meniscus fragments and then connected using a polyester nonabsorbable suture. These anchors can be oriented many different ways and thus can repair many types of tears. All-inside repairs are becoming more popular because the procedure does not require the need of an accessory incision and has a shorter surgical time[42]. The all-inside technique has significantly developed since its invention in 1991, but historically some of the devices have been found to be mechanically inferior to sutures[18,44]. The newer suture-like anchors have shown to be comparable to the sutures used in the inside-out technique in strength, flexibility, and load-to-failure[42,18].

Open Repair

Open repair was the original method of meniscal repair. This technique requires a 20-30cm incision, through tendon and muscle, to visualize the entire joint. Vertical

sutures are usually used to repair any tears[39]. Due to the size of the incision, there is a 3 month recovery along with increased risk of infection, blood clotting within the joint, and further cartilage damage. Open repairs have become less and less common as arthroscopic techniques have developed, but they are performed when the medial compartment is too tight or if the knee is already opened for a separate procedure[40].

Meniscectomy

In cases with more significant meniscal damage or a white-white tear, surgical repair is not recommended and a partial meniscectomy is performed. In a meniscectomy, the overly damaged portion of the meniscus is resected, leaving the healthy portions to maintain the function of the knee joint and compensate for the missing portion. A partial meniscectomy is also the recommended treatment option for patients over 50, sedentary patients, or patients unwilling to comply with the rehabilitation procedures required after a meniscal surgical repair. Complex tears and degenerative tears must be treated via partial meniscectomy, making it the most commonly performed surgical treatment for a meniscal tear[45]. Horizontal tears, caused by excessive shear forces, are usually degenerative and must also be removed with a partial meniscectomy[46]. Partial meniscectomies speed up the progression of OA within the knee joint and are generally only performed on older patients. However, due to the shorter rehabilitation period of a partial meniscectomy, it is often chosen over a meniscal repair by competitive athletes who wish to resume training as soon as possible[12].

Meniscus Replacement

Due to the significant correlation of early OA development with meniscal repair and partial meniscectomy, alternative methods to replace rather than repair the meniscus have been investigated. Replacing the damaged meniscus with healthy tissue or tissue substitute would prevent further damage to the articular cartilage and potentially halt the onset of OA. The advancement of tissue engineering has allowed for the development of tissue or tissue-like scaffolds that mimic the collagenous framework of the native meniscus tissue. These scaffolds can then either be populated by chondrocytes, creating a living tissue implant, or promote healing and regeneration of the host tissue within the scaffold. The scaffold is then implanted into the patient as a functional, biologically active transplant. The development of tissue engineering has been so significant that there are now several different methods to custom design a fully functioning scaffold to fit to a patient's knee[1,47].

There are two general types of tissue engineered scaffolds, categorized by the amount of the damaged meniscus initially removed. The first type of tissue engineered scaffolds is designed for partial meniscectomies. These scaffolds are designed to integrate fully into the host tissue and facilitate the reconstruction of the meniscal tissue. Due to the widespread use of partial meniscectomy, meniscus reconstruction scaffolds have increasingly become in demand. After removing the damaged meniscus tissue, a custom-sized scaffold is fitted and sutured to the body of the original meniscus[48]. As the injury heals, the highly porous scaffold facilitates the regeneration of meniscus-like tissue to infiltrate and eventually replace the scaffold with native tissue[49]. Short and long term studies have both shown that the reconstruction implants have improve knee function,

pain reduction, and restoration of activity levels[49,50,51]. Generally, meniscus reconstructions are more effective for trauma induced meniscal tears, rather than degenerative or complex tears.

The second type of tissue engineered scaffolds is a total meniscus replacement scaffold. When the damage to the meniscus is too great or the meniscus is riddled with degenerative tears, a total meniscectomy of the body of the meniscus should be performed. Because there is none of the body of the host meniscus remaining, the full replacement must be able to perform all of the mechanical functionalities of a meniscus upon implantation, while promoting cellular migration to eventually allow the graft to perform all of the biological functionalities as well. The total replacement also needs to promote host tissue integration from the remaining vascular peripheral tissue to fully be assimilated as a transplant. Clinical evidence shows that total meniscus allograft transplantations (MAT) have proven to be very successful treatment methods for meniscus repair.[52]

Cell Sources for Tissue Engineering

One trait that all tissue engineered scaffolds have in common is that the final product of implant production and processing is acellular. An effective acellular implant must allow for the host to facilitate healing and integration via cellular migration and vascularization. This can either be achieved by the host after the implantation of a decellularized scaffold or the scaffold can be pre-seeded with cells before transplantation. Cell compatibility is a major concern because any cell, protein, or enzyme that is seen as foreign will trigger an immune response and cause the scaffold to be rejected. Cell

sources are assessed by their immune response limitation and the availability of healthy cells.

Autologous Chondrocytes

Autologous chondrocytes are the obvious first cell source to investigate because they are directly collected from the patient and there is no need for additional processing of the cells. The main drawback for autologous chondrocytes is cell availability. In order to collect cells for the autologous chondrocyte implantation (ACI), a pre-transplant surgery must be performed to gather thin slices of cartilage from minor weight bearing areas of the joint[1,53]. This allows for the maximum amount of chondrocytes to be harvested while minimizing the trauma to the joint. The number of cells harvested is limited because the ratio of chondrocytes to collagenous ECM in a meniscus is extremely small and the cells producing the desired GAG matrix located only within the inner collagenous zone of the meniscus[54,55,56]. This small number of cells can be expanded via a monolayer culture. However, this monolayer causes the cells to differentiate into the chondrocyte morphology found in the outer collagenous zone which have a significant downregulation of ECM gene expression, causing them to produce less ECM matrix with the desired GAGs[57]. Another concern with using autologous cells is the possibility of cells already being in a diseased state due to OA[58]. Once in the diseased state, the chondrocytes will continue to break down the articular cartilage in the joint as well as an implanted scaffold.

Recent studies of chondrocytes in articular cartilage, which also dedifferentiate when proliferated in a monolayer culture, show that the cells can redifferentiate using a 3D pellet model[59]. The study was performed with OA chondrocytes as well and showed

that after redifferentiation, the cells began exhibiting normal chondrocyte expression. Although this research shows that more viable differentiated cells can be produced, the use of autologous cells is still restricted in clinical practice due to their high cost to collect and prepare.

Allogenic Chondrocytes

In an attempt to avoid the complications that have risen with using autologous cells, researchers have looked to allogenic cells as a cell source for tissue engineering meniscus scaffolds. Research has shown that allogenic cells are equally effective as autologous cells in functionality and promotion of healing of meniscal lesions[60]. Benefits of allogenic cells are that they can be harvested from cadavers in greater number than autologous sources because the entire tissue can be resected and used. Also, the host immune response to allogenic cells is very limited due to the low vascularity in the region and the dense ECM that surrounds the allogenic cells[56]. Even though meniscus tissue is deemed "immune-privileged", there have been studies that have shown that over time the summation of the limited immune responses from allogenic cells can lead to the overall destruction of the tissue by macrophages, natural killer cells, and T cells[61].

Drawbacks to allogenic cells include possible disease transmission and availability. Even though allogenic cells can be collected in greater number than autologous cells, the availability of cadaver sources with healthy fresh cells is still limited. There are also limitations with the methods of cryogenically preserving or storing cells in a refrigerated state for a prolonged time. Both storage methods has been shown to reduce the viability, effectiveness, and metabolism of the chondrocytes[62].

Xenogeneic Chondrocytes

The main drawback of autologous and allogeneic cell sources is cell availability. Xenogeneic cell sources have the potential to provide for the high demand for chondrocytes to reseed scaffolds and repair lesions. However, using a xenogeneic source of cells has a major drawback in that it generates an immune response from the host. A recent study suggests that that the immune response can be prevented by removing the cellular and molecular mechanisms that trigger an immune response through genetic engineering[53]. This study identified the molecular targets of the human immune system on pig chondrocytes and the pathways of rejection for each target. With the molecular targets and immune system pathways identified, the pig chondrocytes can be genetically engineered to not express these targets and thus avoid the immune response. Researchers have already shown that this can be plausible by using homologous recombination and vector cloning to eliminate the expression of the carbohydrate antigen Gal, a common target against pig tissue by a humoral immune response[63]. Pig tissue is considered to be the best source for xenogeneic cells and tissues because it is domesticated, easily reproducible in large qualities, and has similar physiology and size of cells and tissues to humans[53].

Mesenchymal Stem Cells

Another more recent source of cells that has been researched is the adult mesenchymal stem cells (MSC). MSCs are desirable because they naturally have the ability to differentiate into chondrocytes[64]. These cells can be found in bone marrow, adipose tissue, synovium, periosteum, skeletal muscle, skin, amniotic fluid, or umbilical cord blood[65]. Both autologous and allogenic MSCs can be used in research due to their

immunomodulary characteristics[66]. Allogenic MSCs are a promising avenue because patients receiving allogenic MSCs only undergo one surgical treatment instead of the two needed to use autologous MSCs. Research shows that both types of MSCs can be either seeded in a scaffold or injected into the lesion to promote healing and/or tissue integration by the host[65,67].

A major concern when using MSCs is triggering proper chondrogenesis and differentiation. In order to trigger MSC chondrogenesis there must be a high cellular density with a biological environment stimulated by growth factors[68]. Proper MSC differentiation is driven by a combination of biological and mechanical factors which interact to ensure that the correct cellular differentiation is happening in the correct areas of the meniscus[1]. Growth factors play a vital role in differentiation, but the mechanical stresses to which the cells are exposed also has an impact on cellular differentiation. Properly differentiated chondrocytes are important because they need to be producing the correct ECM matrix that mirrors and integrates with the native tissue surrounding it. The wrong ECM matrix in the wrong area will not be able to maintain the biomechanical functionality of the tissue.

Types of Meniscus Scaffolds

There are four main classes of meniscus scaffold implants: synthetic polymers, hydrogels, ECM components, and tissue grafts[1]. These classes are based on the method of construction, how the tissue is processed, and the material used to create it. The classes are not mutually exclusive and some researchers have experimented with hybrid combinations of different materials in order design the best model scaffold. A successful scaffold should restore the functionality of the knee (mechanically and biologically),

allow for host tissue integration, degrade once the host tissue has regenerated, and have the ability to be replicated successfully and cost effectively on a large scale.

Synthetic Polymer Implants

Synthetic polymer scaffolds are created from durable polymers not normally found in the body but that also are nontoxic and biodegradable. Synthetic scaffolds generally excel at replicating the mechanical functions of the meniscus[1]. The most common types of synthetic scaffold are polyurethane (PU), polycaprolactone (PCL), polylactic acid (PLA), polyglycolic acid (PGA), and polylactic co-glycolic acid (PLGA). These synthetic scaffolds benefit from being able to be fabricated by a variety of different methods, have an almost unlimited supply, and they can be specifically designed to create specific pore sizes and fiber thicknesses to customize the mechanical properties of the scaffold.

The main weakness of synthetic scaffolds is that they have little to no biological activity. This has lead synthetic scaffolds to be designed to maximize the host's regenerative potential to occur within the scaffold's framework. With this design, synthetic scaffolds have been effectively used as partial meniscus reconstructions. Actifit® (Orteq Biologics, London UK) is one of the most successful synthetic meniscus implants on the market. The Actifit® is a honeycomb polyurethane scaffold that provides the maximum space for host tissue reintegration, while still maintaining the minimum mechanical function needed until the host tissue can begin to reassume the function[69]. This minimum level, however, does not provide the load support equivalent to a native meniscus as it only serves to act as scaffolding for new tissue regeneration. Studies have shown that Actifit® excels as a partial meniscus reconstruction, with tissue

reintegration and significant improvement in knee functionality in almost all of the patients tested[70]. However, due to the biological inactivity of the polyurethane and the inability to provide load support equivalent to the native tissue, the Actifit® implant would not be a sufficient total meniscus replacement. The Actifit® implant does prove that an acellular scaffold can be used to regenerate a large portion of the meniscus without having to pre-seed cells directly into the transplant.

Synthetic scaffolds have a place as treatments for partial meniscus reconstructions after a partial meniscectomy. But the biological inactivity and structural weakness of the scaffold prevents its usage as a total meniscus replacement. In order to create a viable synthetic scaffold for total meniscus replacement, a synthetic material or a composite of synthetic and more biologically active material that could provide better structural support would need to be investigated.

Hydrogel Implants

Hydrogel scaffolds were a result of researchers attempting to create a biologically active meniscus scaffold that most closely resembles the molecular environment of native tissue. Hydrogels have a very high water content, much like native meniscus tissue, and have the ability to have chondrocytes and growth factors seeded directly into the scaffold during creation[71,72]. Hydrogels can be created from synthetic materials or natural components, but the most successful have been made from collagen. Collagen is preferred because it is the main structural component of meniscus tissue leading to a high biocompatibility, is readily available, and scaffold formation can be controlled with pH and temperature[71,73,74]. Collagen based hydrogels are made from dissolving collagen in acid which then are allowed to reassemble into a triple helix structure. These types of

collagen hydrogens have been developed for partial meniscus reconstruction as an implantable scaffold or an injectable ECM hydrogel[75,76].

The main drawback with hydrogels is their weak mechanical properties. Without modification, hydrogels are unable to withstand the physical and mechanical demands of the tissue and are very difficult to be handled in a clinical setting[71]. There is also an overall shrinking of the scaffold once cells are seeded due to cell-collagen interactions. This leads to a reduced overall size and shape of the scaffold, limiting the scaffolds ability to mimic the native tissue precisely.

In order to overcome the mechanical disadvantages, hydrogels have been supplemented with collagen crosslinkers that significantly increase the stability and mechanical properties of the scaffold. Traditional crosslinkers include glutaraldehyde and formaldehyde, but these chemicals are extremely cytotoxic. Researchers have investigated and found success with less cytotoxic crosslinkers like genipin, epigallocatechin-3-gallate (EGCG), and photo-induced crosslinking with riboflavin[77,78,75]. There are several ongoing studies aimed at finding the best balance between mechanical strength and functionality while minimizing cytotoxicity to optimize the potential for hydrogel scaffolds.

Another significant drawback with hydrogels is the difficulty of promoting proper cell differentiation in the scaffold and inducing ECM synthesis[1]. Cells tend to be uniform throughout the matrix, and not vary with location as found in natural tissue. This causes ECM production and tissue regeneration to be biologically different than native tissue. Researchers have attempted to use cellular adhesion to help promote correct cellular morphology within hydrogels to address this issue[79].

Hydrogels tend to have many more drawbacks than advantages. The high biocompatibility and close-to-natural cellular environment is countered by the difficulty of promoting proper cellular morphology and ECM production. The structural weakness of the tissue can be supplemented with collage crosslinking making hydrogels potentially effective for partial meniscus reconstruction. A significant structural weakness of injectable hydrogels is that there is currently no way to mold the scaffold into any particular shape. Injectable hydrogels could be very successful in filling lesions or resected areas with structural boundaries to hold the hydrogel in place, but they cannot be used for a total replacement. The geometry of the meniscus is too important to the function in the knee joint to not have proper means of shaping or designing it. The combination of inefficient cell differentiation, improper ECM deposition, and mechanical weaknesses, even with crosslinking supplementation, it is not recommend for hydrogels to be used as total meniscus replacements.

ECM Component Implants

ECM component implants are characterized by the fact that they are constructed mainly with macromolecules commonly found in the native meniscus matrix. The most common ECM component implants are made from collagen or hyaluronic acid. Collagen scaffolds have been the most successful. Collagen scaffolds can be created via several manufacturing methods and have strength comparable to synthetic scaffold[1]. In terms of bioactivity, collagen scaffolds can create an effective natural environment for seeded cells much more effectively than synthetic scaffolds and hydrogels. The cellular microenvironments are not exactly identical to the natural tissue, but seeded MSCs in

acellular CMIs have been shown to still be able to produce significant amounts of fibrocartilaginous ECM that integrated with the host tissue[80].

The first ECM component implant cleared for usage in human patients was a collagen meniscus implant (CMI) called Menaflex®. This CMI is made of bovine Achilles tendon type I collagen and has proven effective at alleviating pain and restoring normal knee function and physical activity along with host tissue integration[14,81,82,83,84]. Disadvantages with the Menaflex® is that the use of bovine collagen carries the risk of disease transmission and possible immunogenic reaction[85]. Another disadvantage is that once the CMI is moistened, it becomes very fragile, making the surgical procedure much more difficult[69]. ECM scaffolds in general tend to have poor cellular infiltration which can make seeding the scaffold difficult[76].

ECM scaffolds are successful because they strive to be as identical to native meniscus tissue as possible. Structurally they provide mechanical stability and functionality comparable to native tissue. The cellular environments of ECM scaffolds are biologically active and closely resemble native tissue, allowing for proper cellular differentiation and ECM production. ECM scaffolds have been used as partial meniscus reconstructions and also have the potential to be used as a total meniscus replacement.

Tissue Graft Implants

The final category of meniscus scaffolds are tissue grafts. The ideology behind the first three categories, synthetic, hydrogel, and ECM scaffolds, is an attempt to completely recreate meniscus tissue, in form and function, from various materials through tissue engineering. Mimicking the natural tissue has proven to be difficult due to the subtle differences in collagen content/ orientation and properly dispersed and

differentiated chondrocytes. The ideology of using tissue graft implants is different in that it seeks to use tissue engineering to convert natural meniscus tissue, which is already properly structured, into an acellular implant that can be reseeded with chondrocytes compatible with the host[86]. This alleviates the structural and biocompatibility issues faced by other grafts, but it also creates some unique challenges in creating the implant.

There are two types of tissue grafts that can be used to create an acellular scaffold, categorized by their original donor. Allografts are collected from human cadavers and xenografts are harvested from anatomically similar species of animal. Both types of grafts are processed similarly and must be decellularized. Decellularization is a process in which all cellular components of the past host are removed to prevent an immunogenic response to the new tissue once implanted. There are several methods of decellularization, but generally the cells within the graft are forced to burst by freezing or submersion in either a detergent or a hypotonic solution[87,88]. Then the cellular components are either washed away or digested using DNase and RNase enzymatic activity. The resulting acellular tissue is mechanically weakened through the decellularization process, but this can be countered with the addition of collagen crosslinking to add stability to the tissue[88,77]. The end product is a structurally sound acellular implant.

Allografts at face value seem like the most logical candidate to create a tissue derived scaffold. Ideally it would be a like-for-like change between the donor and host. Originally, allograft menisci were used as a direct transplant and cellular survival was extremely important and various methods of cryopreservation or fresh-frozen protocols were investigated[89]. These transplants had high failure rates due to the preservation

methods killing cells so decellularized allograft scaffolds have become the preferred method[88].

An implication that should be noted for allografts is the possibility for disease transmission. This was more of a disadvantage when allografts were being used as a direct transplantation. The decellularization and sterilization techniques that would normally be used to create a safe allograft scaffold would prevent disease transmission.

Allograft scaffolds are definitely capable of providing a successful treatment for a partial meniscus reconstruction and a total meniscus replacement. The main disadvantage with allograft scaffolds is tissue availability[80]. With the high incidence of OA in older patients, finding a cadaver with an intact and healthy meniscus can be difficult. This problem is further compounded by the need to size match the donor to the recipient. This causes allograft scaffold creation to very expensive and not an ideal candidate for widespread use of meniscus replacement.

Immunogenic rejection has traditionally prevented the use of xenografts as a transplantable tissue source. Advancement in decellularization techniques has led to the serious possibility of using a xenograft meniscus as a meniscus scaffold. Because the decellularization process removes all traces of cellular material, there is nothing for the host's immune system to generate an immune response to. One key area that needs to be addressed with xenografts is the anatomical differences between the donor species and humans. In a partial meniscus reconstruction, the anatomical difference would not make a significant impact because the desired portion can be cut from the xenograft. In a full meniscal replacement, the anatomical differences could be very significant. Studies on the anatomical similarities between humans and animals have shown that pigs, goats, and

cows have menisci that would be anatomically suitable for usage by humans[90]. The attachment sites are the main anatomical differences in the animal menisci. However, this issue can be solved by designing and applying appropriate attachment sites during creation of the scaffold so they can be easily attached during implantation.

A xenograft scaffold implant is a versatile treatment method for a partial meniscus reconstruction and the most promising treatment option for a total meniscus replacement. The framework of the collagen fibers from the tissue graft is already properly oriented to encourage the development of proper cell morphology within the scaffold. This allows the cells to produce the appropriate microenvironment and ECM matrix to begin the regeneration and host integration process. The availability of xenograft meniscus tissue is almost unlimited, especially with porcine tissue, and decellularized scaffolds can be frozen and stored[91]. This allows for large scale production of scaffolds to accommodate the large need for meniscus reconstruction and replacement. In terms of cost, availability, and functionality, a xenograft derived scaffold would be the best direction for researchers to develop as the main treatment for a total meniscus replacement.

Conclusion

There is a great need for the development of a reliable and effective total meniscus replacement. This is due to the great number of knee related surgeries performed each year and the high prevalence of OA in the population. While meniscus repair surgeries have proven effected in repairing the tissue, only minor tears can be repaired and further complications develop that eventually lead to a heightened development of OA and joint instability. Total meniscus replacement can be a viable option that replaces not only severely damaged tissue but also previously repaired

meniscus tissue before the onset of OA. A xenograft tissue based meniscus scaffold is the most promising avenue for total replacement because the collagenous framework is already in the natural orientation allowing for proper cellular differentiation and morphology. Proper differentiation and morphology allow the tissue to readily resume the biochemical functions of the natural tissue. The natural orientation is also beneficial in maintaining the natural mechanical properties of the tissue within the joint. Supplemented with collagen crosslinking, the xenograft tissue based scaffold would be more than be stable enough to allow for tissue regeneration of the host while maintaining joint functionality. Further development of the decellularization process and antigen removal would relieve the immunogenic stress normally placed on a xenograft and would cement its place as the best treatment option for a total meniscus replacement.

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CHAPTER II

RESEARCH

Introduction

The previous chapter established that a xenograft tissue based meniscus scaffold, coupled with a partial or full meniscectomy, is the most promising solution for damaged meniscus repair. The purpose of this research was to provide a proof of concept that a viable decellularized meniscus xenograft scaffold implant can be produced to fill this demand. The first challenge of developing a xenograft meniscus scaffold implant was first and foremost, antigen removal. Removing antigens, which would cause a host immune response and transplant rejection, is paramount to the success of the concept. Secondly, because a meniscus provides such a crucial role in locomotion, acting as the shock absorber and friction reducer in the knee, the biomechanical properties of the tissue are also critical to the overall functionality of the transplant. The design of this proof of concept was comprised of three experiments; the first experiment is to develop an antigen removal protocol, and the last two experiments explore the understanding and manipulation of the biomechanical properties of the tissue.

The goal of the first, and most important experiment, was the development of an optimal antigen removal protocol. Current research has shown that antigen removal within thick connective tissue, like meniscus, to acceptable levels of immunotolerance is possible.[1] However, these published protocols are time consuming, expensive, and/or

damaging to the structural properties of the tissue, making these current protocols not conducive to expanding the research on xenograft meniscus transplants. This study proposed modifying a less invasive antigen removal protocol that uses SDS for antigen removal, originally designed and proven effective for articular cartilage,[2] to create a faster and more cost-efficient method to create a decellularized scaffold. These less harsh conditions will, in theory, maintain the structural integrity of the graft, while also reducing production time and cost.

The second experiment investigated the biochemical and biomechanical differences between the native meniscus tissue and the decellularized meniscus scaffold. This experiment was vital because any antigen removal protocol has some effect on the biochemical and biomechanical properties of the tissue. First, a more in depth study on the effectiveness of the antigen removal protocol was conducted to include DNA and GAG removal. GAG performs a critical role in shock absorbance through water retention in the native tissue, and although it would be beneficial to maintain, it is mildly immunogenic and is almost impossible to retain during efficient antigen removal. However, one benefit of GAG removal is that it increases porosity in the scaffold that will ease cellular migration into the scaffold. Collagen content was also assessed to determine if the antigen removal protocol was also removing collagen from the tissue. If collagen was being removed, it would indicate that the structural integrity of the tissue was being compromised by the antigen removal. Compression and tensile testing was performed on both the fresh native tissue and the decellularized scaffold to determine if the decellularized tissue would be able to withstand normal loading associated with weight bearing and gait. Comparing the mechanical properties of the two tissues will

help determine if the tissue would be too rigid and inflexible such that it would damage the femoral articular cartilage or overall knee functionality. Comparing the biochemical and biomechanical analyses of the two tissues will give a greater understanding of the antigen removal process and serve as a spring board for further research.

The third experiment was designed to investigate the feasibility of further treating the tissue via collagen crosslinking. Crosslinking the tissue could be an important aspect of the development of the xenograft as a whole because it inhibits rapid enzymatic degradation of the scaffold. In terms of biomechanics, crosslinking would also be expected to restore some of the compressive resistance lost as a result of the GAG removal. Crosslinking may also increase tensile stiffness, which might be an undesirable side effect if antigen removal alone increases the tensile modulus. By being able to consciously adapt the biomechanical properties of the xenograft, the transplant can be engineered to match the specifications of the native host tissue. The goal of this experiment was to determine the effect of various chemical crosslinkers, genipin and EGCG, and their concentrations on the xenograft meniscus. Genipin and EGCG were chosen for this experiment due to their low cytotoxicity and their proven collagen crosslinking ability in literature.[2,3] Additionally, due to the fact that menisci are large and dense pieces of connective tissue, and previous research on crosslinking similar tissues, such as tendons and ligaments, has shown thorough crosslinking of these tissues to be difficult,[4] two pre-crosslinking treatment protocols were designed to increase the permeability of the crosslinkers into the meniscus tissue. The two pre-crosslinking treatment protocols developed for this experiment are freeze drying the tissue before crosslinking and soaking the tissue in a super saturated saline solution, then freeze drying

the tissue before crosslinking. Both pre-crosslinking treatment protocols utilize osmotic gradients to attempt to increase permeability of the aqueous crosslinkers to the inner most portions of the meniscus.

The goal of this study is that the data collected from the three experiments will build a strong foundation of fundamental knowledge about the development and biomechanical behaviors of a decellularized xenograft meniscus scaffold. It is hypothesized that an effective and more efficient antigen removal protocol can be developed to create a xenograft meniscus replacement that is comparable to current protocols, and that the decellularized scaffold created from that protocol can bioengineered to mimic native tissue in its mechanical properties.

Experimental Methods

Experiment 1: Antigen Removal Protocol

The original antigen removal protocol, Protocol 1 (P1), was adapted from the antigen removal procedure used for osteochondral bone plugs fused with articular cartilage taken from the articular surfaces of the tibia and femur inside the knee joint.[2] Articular cartilage is much thinner than meniscus tissue, so the original protocol was modified to penetrate the thicker tissue. The sequential protocols, Protocol 2 (P2) and Protocol 3 (P3), made adjustments to soaking times and additional antigen removal steps were added or removed to optimize the procedure. To assess antigen removal, DNA content was measured in 39 total samples from 13 different menisci for P1, P2, and the FTC each, and 24 total samples from 8 menisci for P3 and SP each.

Stabile Protocol

The Stabile Protocol (SP) is a well-established protocol that was used to compare the protocol developed in this research. Eight menisci were placed in distilled DNase/RNase-free water in a shaker at 200rpm at 37˚C for 48 hours to lyse the cells. Then the menisci were treated with a 0.05% trypsin-ethylenediaminetetraacetic acid on the shaker for 24 hours. The menisci were then treated with Dulbucco's modified Eagle's medium with high glucose, 10% fetal bovine serum, and 1% antibiotic-antimycotic solution for 24 hours to neutralize the trypsin. The menisci were then treated with a 2% aqueous Triton X-100 and 1.5% peracetic acid solution for 48 hours. The meniscus were then washed in dH_2O once for an hour and then for 72 hours, changing the water every 24 hours. Finally, the menisci were washed in PBS for 24 hours.

Protocol 1

Thirteen menisci were cleaned after extraction in PBS twice for one hour, then in 10% hydrogen peroxide for one hour, and finally in dH_20 for an hour. The menisci were then treated with a 2.0% SDS, 10mM Tris-HCl (pH 7.6), 1mM PMSF, 5mM $MgCl_2$, 0.5mM $CaCl_2$, 0.5 mg/ml DNase I, 0.05mg/ml RNase, 1% antibiotic-antimycotic solution for 48 hours, changing the solution every 24 hours. The menisci were then washed in 10mM Tris-HCl (pH 7.6) for 90 minutes and then twice in dH_2O for 30 minutes. All treatments were performed in a shaker at 37˚C.

Protocol 2

The second protocol adaptation was designed to maximize the decellularization potential by including several different antigen removal steps. The protocol further

increased treatment times in certain solutions, added EtOH and hyaluronidase treatments, and the menisci were sonicated for 10 minutes after each step of the protocol. Thirteen menisci were cleaned after extraction twice in PBS for one hour, a 10% hydrogen peroxide solution for 8 hours, and then degreased in EtOH for three hours. The menisci were treated with a 0.2% hyaluronidase, 10 mM Tris-HCl (pH 7.6) solution for 48 hours, changing the solution every 24 hours. The menisci were then treated with a 2.0% SDS, 10mM Tris-HCl (pH 7.6), 1% antibiotic-antimycotic solution for 72 hours, changing the solution every 24 hours. The menisci were then treated in a 10mM Tris-HCl (pH 7.6), 1mM PMSF, 5mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5mg/ml DNase I, 0.05mg/ml RNase, 1% antibiotic-antimycotic solution for 48 hours, changing the solution every 24 hours. Finally the menisci were washed in dH_2O twice for 30 minutes. All treatments were performed in a shaker at 37˚C.

Protocol 3

The third and final protocol was designed to use a hypotonic osmotic gradient to induce cell lysis in addition to the SDS detergent. Eight menisci were cleaned after extraction twice in PBS for one hour, then in 10% hydrogen peroxide for 8 hours, and finally twice in dH_20 for one hour. The cells were lysed in dH_20 for 48 hours, changing solution every 24 hours. Then the menisci were treated in a 2.0% SDS, 10mM Tris-HCl (pH 7.6), 1mM PMSF, 5mM $MgCl_2$, 0.5mM $CaCl_2$, 0.5mg/ml DNase I, 0.05mg/ml RNase, 1% antibiotic-antimycotic solution for 48 hours, changing the solution every 24 hours. The menisci were then washed in dH2O twice for one hour and then overnight in a large volume of dH2O for 8 hours. All treatments were performed in a shaker at 37˚C.

This protocol was found to be superior to all of the previous protocols and was used for all subsequent tests for the decellularized xenografts.

Experiment 2: Biochemical and Biomechanical Analysis of Protocol 3

A vertical cross section was taken from a random meniscus from both the FTC and P3 test groups and was plated onto a slide. The slide was stained with Safranin-O and was analyzed using light microscopy. Biochemical analysis was performed to quantify antigen removal with DNA content and GAG content, and to ensure that collagen was not being removed during the antigen removal protocol. Removal of collagen would indicate a loss of structural integrity and function. Biochemical analysis was performed on 12 total samples from the body of 6 FTC menisci and 12 total samples from 6 menisci treated with P3.

Papain Digestion

Meniscus samples had to be digested in papain in order to perform the biochemical analysis of DNA content, GAG content, and collagen content. The same samples of papain digested menisci were used to complete all three biochemical analysis tests. The papain digestion buffer is a 10mM cysteine solution in PBE buffer with 50µL of papain. Samples are digested in papain solution overnight at 60˚C in a water bath and then spun at 10,000rpm for 3 minutes. The supernatant was then be used for various biochemical analysis.

Biochemical Analysis

DNA content, GAG content, and collagen content analyses were performed on the papain digested samples as previously described, with minor modifications to accommodate the larger sample sizes.[2]

Compression Testing

A stress-relaxation compression test was performed to determine the change in biomechanical properties of the decellularized samples compared to the control meniscus samples, with regards to compressive forces. The compression testing was performed using a Mach 1 machine. A total of 24 samples collected from both 4 FTC menisci and 4 P3 menisci using a deli slicer to take even horizontal cross sections of approximately 3mm thickness, and then a 4mm biopsy punch was used to punch out round disks from the cross sections.

The test was performed with the sample being submerged in a bath of PBS between smooth impermeable plates. The thickness of each disk was determined by applying an initial load of 10 gf and measuring the distance between compression plate and the base of the bath. A 5% ramp strain was calculated from the thickness of each sample. Five compressions were applied to the sample, each increasing by the 5% strain, from 5%-25%, allowing the tissue to relax in between ramps until the slope of the values, measured at 15 second intervals, was less than 0.2500 gf/min.

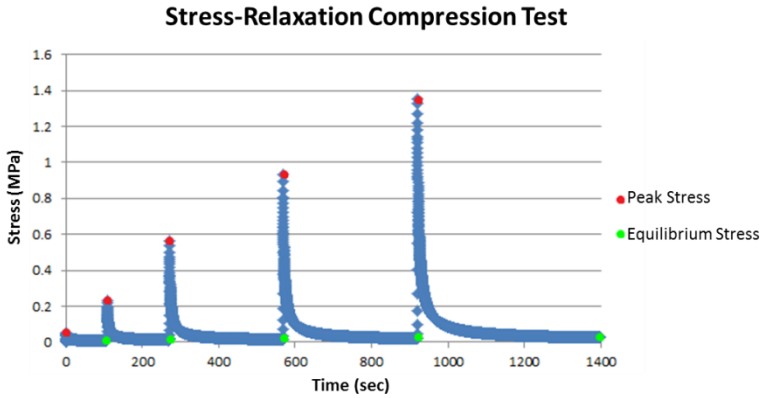


Figure 2.1 Stress-Relaxation Compression Test: Five compressions, increasing in 5% increments, allowing for the tissue to relax in between intervals.

The peak stress modulus, also referred to as the instantaneous modulus, and equilibrium stress moduli were calculated by plotting the 5 peak stress points, depicted in Figure 2.1 in red, and 5 equilibrium stress points, depicted in Figure 2.1 in green, against strain and determining the slopes of the respective stress vs strain plots.

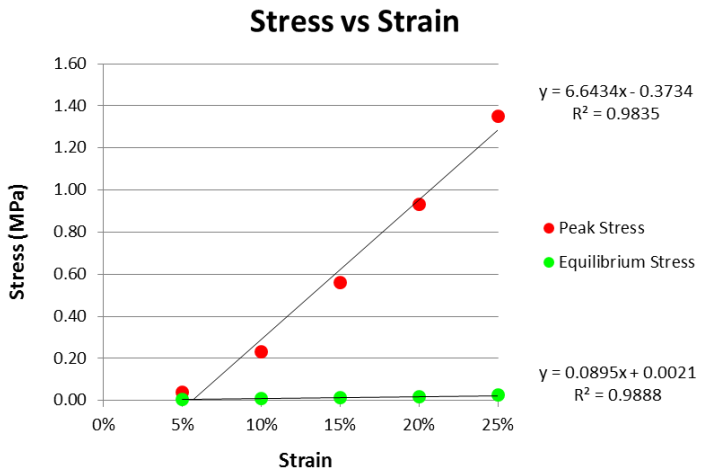


Figure 2.2 Stress vs strain graph to calculate the instantaneous and equilibrium moduli from compression data.

Tensile Testing

A tensile test was performed to determine the change in tensile properties of the decellularized samples compared to the control meniscus sample. Tensile testing was performed using an MTI-2K machine. Samples were collected from both control and decellularized meniscus using a deli slicer to take even horizontal cross sections, then a custom designed and built stamp was used to punch out dumbbell shaped sections of the tissue. The dimensions of the stamp are 9mm wide at the ends, 3mm wide in the middle section, with a gauge length of 18mm. The overall length of the sample was determined by the size of the meniscus, and it varied for each sample. Three thickness measurements were taken were taken at various points of the middle gauge length with digital calipers to calculate an average thickness for the sample.

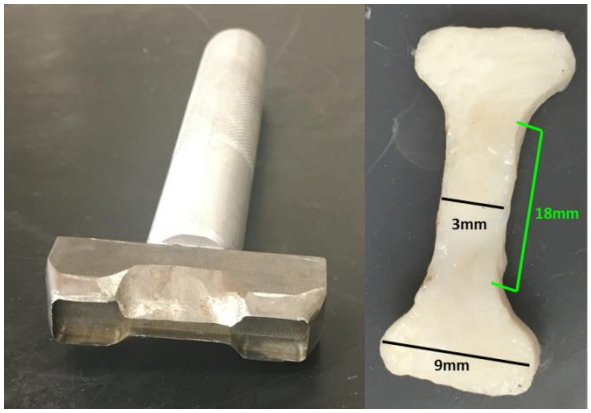


Figure 2.3 The custom made tissue punch and the resulting dumbbell shaped tissue removed from the original meniscus tissue.

The dumbbell shaped tissue's was secured at both ends into a set of clamps attached to a 200N load cell and the MTI-2K machine. A 5N preload was applied to the tissue to remove any slack in the tissue before testing. The sample was pulled at 5

mm/min until failure. Only samples where the failure occurred in the middle portion of the tissue were kept, any failure resulting from the grips was discarded.

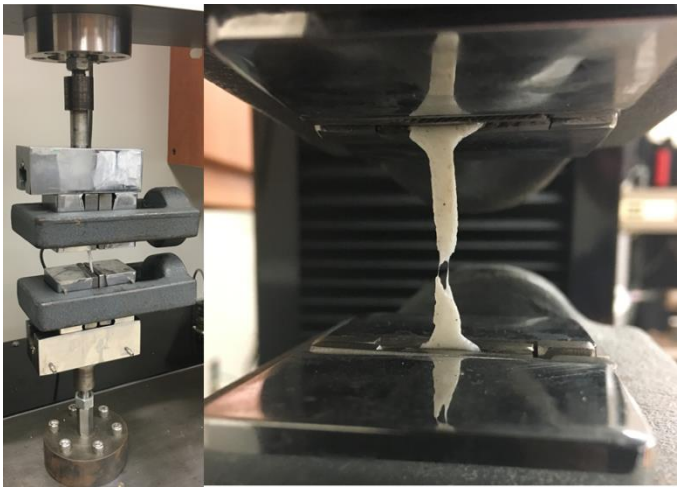


Figure 2.4 Tensile Testing Setup: The sample was clamped in place at both ends on the MTI-2K machine (left). An excepted test with structural failure occurring in the middle portion of the tissue sample (right).

Strain was calculated by dividing displacement by the gauge length of the tissue sample. Stress was calculated by dividing load by the average thickness of the tissue sample. Stress vs strain was plotted and the modulus of elasticity, which was defined for this experiment to be the maximum slope in the elastic region of the graph, was determined.

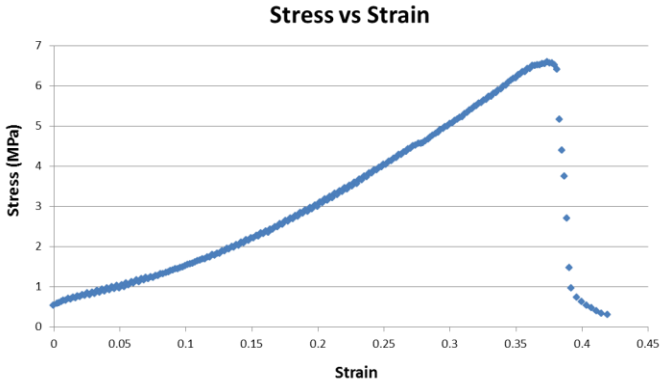


Figure 2.5 Stress vs Strain Graph: The stiffness modulus was defined as the maximum slope of the elastic region.

The ultimate tensile stress, strain at failure, and energy absorbed until failure were also determined and/or calculated from their corresponding points on the graph.

Experiment 3: Crosslinking Assessment and Permeability

Decellularized menisci were treated with two different nontoxic crosslinkers, genipin and epigallocatechin gallate (EGCG). Three concentrations of genipin were tested to produce a wide range in the degree of crosslinking, based on previous research on articular cartilage,[2] 0.2%, 0.04%, and 0.008%, and one concentration, 1%, of EGCG was tested. Eleven menisci were randomly assigned to the 5 different treatment methods, with two menisci for each of the four crosslinking treatments and 3 mensici assigned as the fresh tissue controls. The menisci were treated with the appropriate solutions for 72 hours, changing the solution every 24 hours. Samples were then taken from each menisci to determine the permeability of the various crosslinkers and concentrations. Vertical cross sections were taken from each sample and a 4.0mm biopsy punch was used to punch out the centermost portion of the cross section.

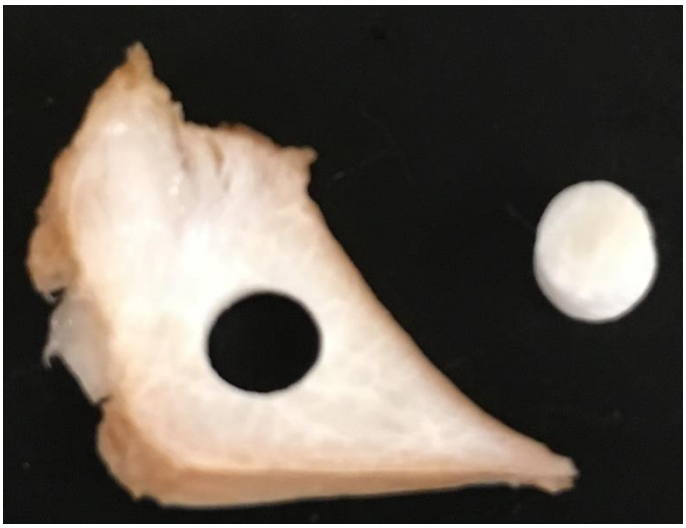


Figure 2.6 Vertical cross section of a meniscus with the inner portion removed. Both portions are assed for percent crosslinking to determine permeability.

The difference in crosslinking percentage between the outer portion and inner portion was then calculated to determine the permeability of the crosslinker used.

Crosslinking Assessment

The percentage of crosslinking in a sample was determined using a Ninhydrin Assay, which indicates the amount of free amino groups. Two solutions were prepared, the first solution was created by adding 1.05g citric acid, 10mL (1.0M) aqueous NaOH, and 0.04g $SnCl_2 \bullet 2H_20$ and then adding dH_2O until 25mL. The second solution was created by adding 1g ninhydrin to 25mL of 2-methoxyethanol. The two solutions were blended and stirred for 45 minutes to complete the ninhyrdin solution.

Test samples were freeze-dryed for 24 hours and weighed to determine their dry weigh. The sample was then placed in a tube with 3mL of ninhydrin solution in a 100˚C water bath for activation four minutes, a time that was determined using a positive control sample as a reference. The tubes were cooled in a cool water bath and then were

read with a microplate spectrophometer at 570nm. The degree of crosslinking was calculated as previous described in previous research.[3]

Crosslinking Permeability

Two adaptations to the administration of the crosslinking treatment were tested to attempt to increase permeability. Three groups of 10 decellularized menisci were subject to different pretreatment conditions before all of the groups were individually treated with a 0.04% genipin solution for 72 hours, changing the solution every 24 hours. The first group of decellularized menisci was freeze-dried for 24 hours in a vacuum prior to the crosslinking treatment. The second group of decellularized menisci was treated in a super saturated 28.1% NaCl solution for 72 hours, changing the solution every 24 hours, and then freeze-dried for 24 hours in a vacuum prior to crosslinking. The control group of decellularized menisci was only treated with the crosslinking treatment. Samples were then taken from each meniscus in each group to determine if there was a difference in permeability due to the different pre-crosslinking treatments. Vertical cross sections were taken from each sample and a 4mm biopsy punch was used to punch out the centermost portion of the cross section. Differences in crosslinking percentages between the outer and inner portions were calculated and assessed.

Results

Experiment 1: Antigen Removal Protocol

The average DNA content for the FTC, P1, P2, P3, and SP, in ng DNA/mg, were calculated to be 26.62 ± 12.91, 30.57 ± 9.70, 28.83 ± 7.42, 18.05 ± 4.61, and 17.53 ± 9.31, respectfully, as can be seen in Table 2.2. Statistical analysis was performed using

SAS calculated the critical value of t to be 1.97509 with a least significant difference of 4.7773. P1 and P2 were not significantly different from FTC, and are therefore not an effective antigen removal protocol. P3 was found to have significantly lower average DNA content than FTC and was also not significantly different than SP.

Table 2.1 DNA Content in ng DNA/mg of three antigen removal protocols (P1, P2, P3), a fresh tissue control (FTC), and the Stabile antigen removal protocol (SP).

	FTC	P1	P2	P3	SP
Average DNA Content (ng DNA/mg)	26.62	30.57	28.83	18.05	17.53
Standard Deviation	12.91	9.70	7.42	4.61	9.31

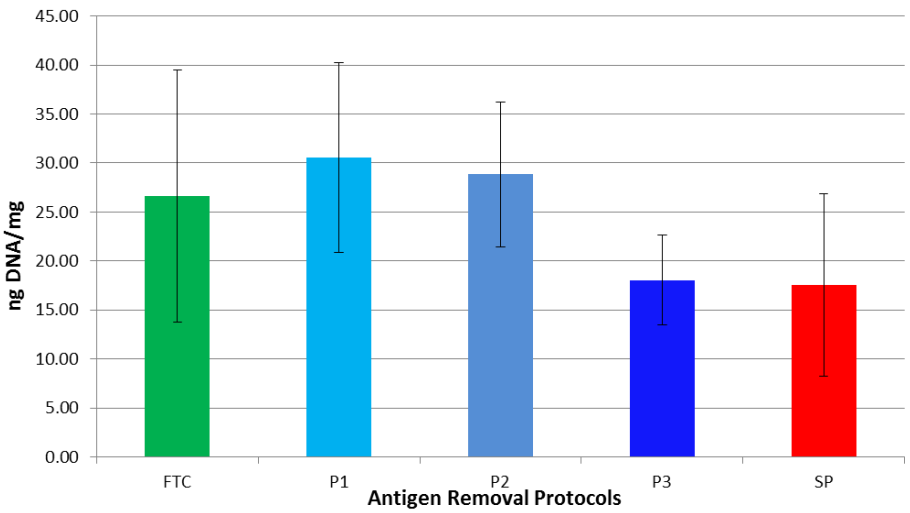


Figure 2.7 DNA content in ng DNA/mg for the three antigen removal protocols (P1, P2, P3) a fresh tissue control (FTC) and the Stabile antigen removal protocol (SP).

Table 2.2 T-Tests (LSD) for DNA Content in ng DNA/mg from SAS Output.

T Tests (LSD) for DNA Content			
t Grouping	**Mean**	**N**	**PROTOCOL**
A	30.574	37	P1
A			
A	28.834	39	P2
A			
A	26.625	39	FTC
B	18.045	24	P3
B			
B	17.525	24	SP

Experiment 2: Biochemical and Biomechanical Analysis of Protocol 3

Microscopy

Under 20x magnification, the Safranin-O stained slide, clearly shows evidence of successful decellularization. As can be seen in Figure 2.8, at 20x magnification there is strong evidence that P3 is an effective decellularization protocol.

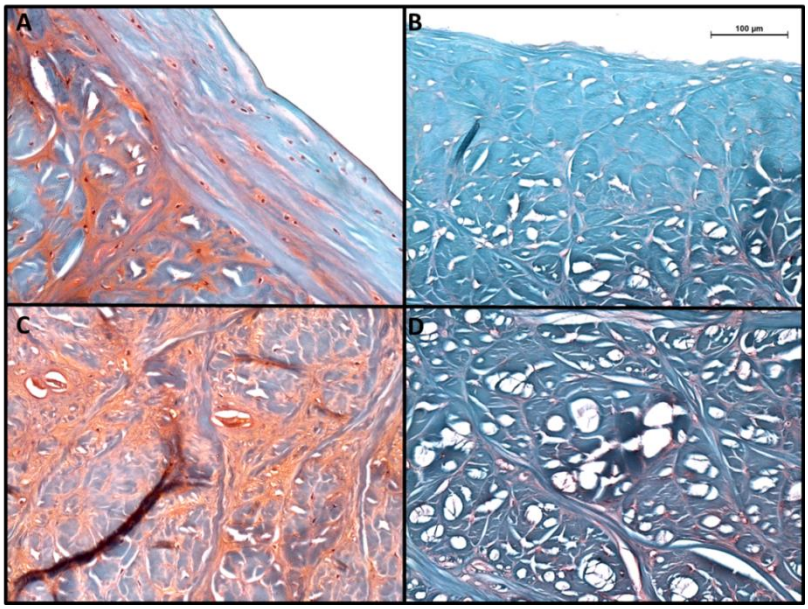


Figure 2.8 20x image of Safranin-O stained vertical cross section of FTC (left) and P3(right) meniscus. A) FTC femoral surface B) P3 femoral surface C) FTC midsection D) P3 midsection

The darkest staining regions of the FTC are the cell nuclei of fibrochondrocytes habiting their lacunae in the tissue. The P3 meniscus clearly shows an overall absence of these dark staining areas and empty lacunae. The P3 meniscus also shows a significant reduction in the amount of GAG and ECM, the red staining portion of the FTC. This effect can be better seen in Figure 2.8, a 5x magnification mosaic image of the entire cross section created using ImageJ.

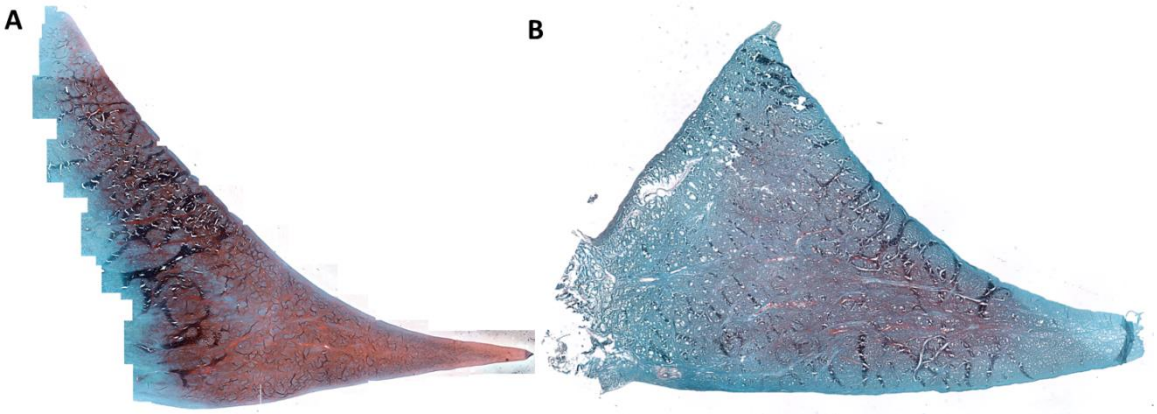
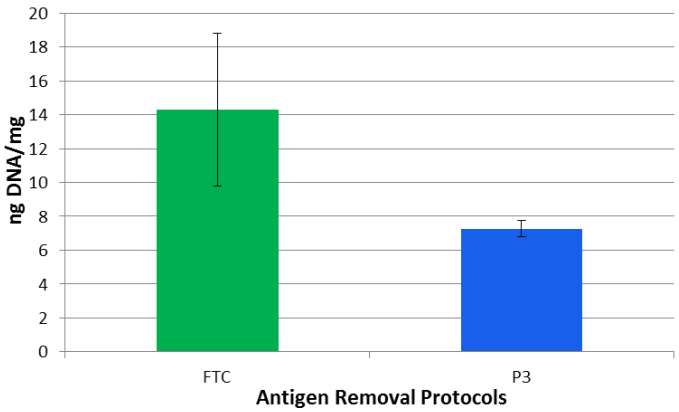


Figure 2.9 5x magnification compiled mosaic of the FTC (left) and P3 (right) menisci. A) FTC meniscus B) P3 meniscus

The outside portions of the menisci appear to be completely devoid of GAG and only a small residual amount is leftover in the inner portion. There is a stark difference in the overall amount of GAG in the P3 meniscus compared to the FTC meniscus, which provides evidence to the antigen removal effectiveness of P3.

DNA Analysis

The average DNA content of the FTC was calculated to be 14.2926 ng DNA/mg and the P3 average DNA content was calculated to 7.27355 ± 0.47699 ng DNA/mg. A two sample t-Test assuming unequal variances with a hypothesized mean difference of 0 at the significance level of $\alpha = 0.05$ was performed on the data collected from the two groups. Menisci treated with P3 were found to have a significantly lower average DNA content than the FTC menisci with a p-value of 0.000225.

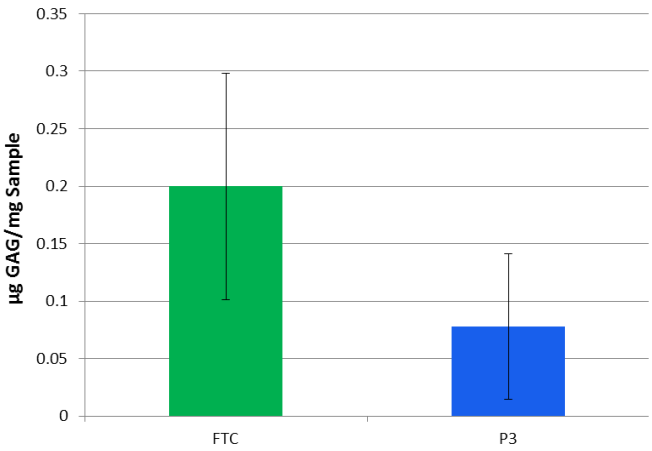


	FTC	P3
Mean DNA Content (ng DNA/mg)	14.2926	7.27355
Std. dev.	4.49934	0.47699
P(T<=t) two-tail	0.000225	

Figure 2.10 DNA Content Comparison between FTC and P3. Menisci treated using P3 have a significantly lower DNA Content (ng DNA/mg) than FTC.

GAG Analysis

Menisci treated with P3 had 0.7787 μg GAG/ mg of sample compared to the 0.199667 μg GAG/mg of sample of the FTC menisci. A two sample t-Test assuming unequal variances with a hypothesized mean difference of 0, at the significance level of $\alpha=0.05$ was performed on the data collected from the two groups. The mean GAG content of menisci treated with P3 was significantly lower than the FTC, with a p-value of 0.001846. The data supports the significant difference seen in the Safranin-O slides of the FTC and P3.



t-Test: Two-Sample Assuming Unequal Variances		
	FTC	*P3*
Mean	0.199667	0.07787
Std. dev.	0.0982	0.0631
P(T<=t) two-tail	0.001846	

Figure 2.11 GAG Content Analysis between FTC and P3.

Collagen Content

The Safranin-O stained slide was viewed under cross polarization in order to see the orientation of the collagen bundles of the P3 meniscus. The collagen bundles in the P3 menisci, seen in both 5x and 20x magnification, are not disturbed by the treatment and maintain a tight bundle and natural orientation.

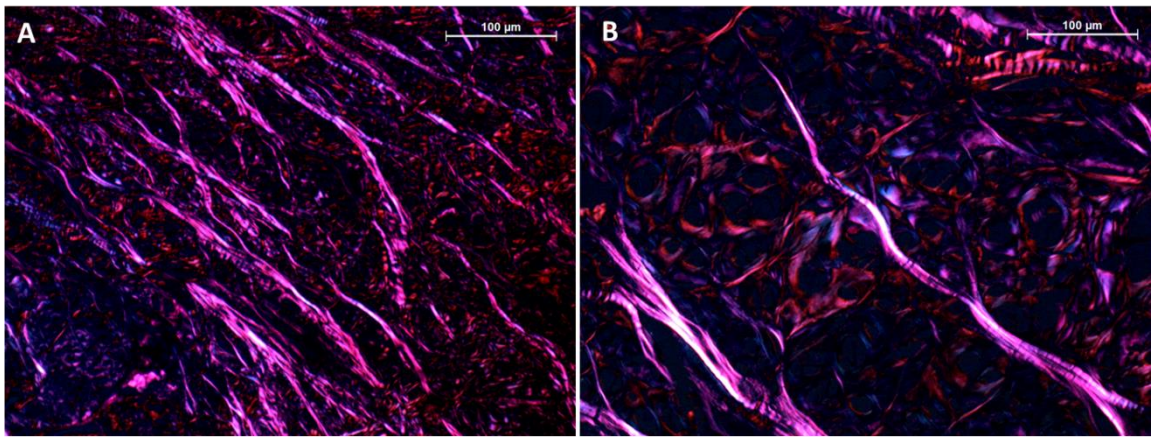
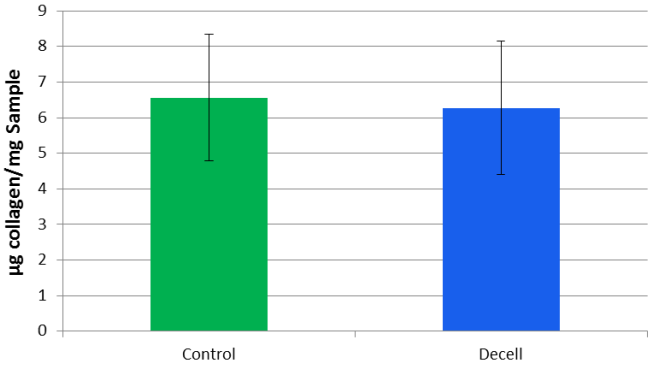


Figure 2.12 P3 meniscus cross section under cross polarization to show collagen bundle structure and orientation.

The average collagen content of the FTC menisci was calculated to be 6.563657 ± 1.779202 µg collagen/mg and the P3 menisci was 6.268484 ± 1.8728 µg collagen/mg. A two sample t-Test assuming unequal variances with a hypothesized mean difference of 0, at the significance level of α=0.05 was performed on the data collected from the two groups.



t-Test: Two-Sample Assuming Unequal Variances		
	FTC	***P3***
Mean	6.563657	6.268484
Std. dev.	1.779202	1.8728
P(T<=t) two-tail	0.696043	

Figure 2.13 Collagen Content Analysis between FTC and P3.

Compression Testing

The instantaneous modulus for the FTC was calculated to be 2.5523 MPa and the equilibrium modulus was calculated to be 0.0529 MPa. The instantaneous modulus for

P3 menisci was calculated to be 0.8019 MPa and the equilibrium modulus was 0.1013 MPa. A two sample t-Test assuming unequal variances with a hypothesized mean difference of 0, at the significance level of $\alpha = 0.05$ was performed for the instantaneous modulus and equilibrium modulus independently. The p-value for the instantaneous modulus, as seen in Table 2.4, was calculated to be 0.0013 and the p-value for the equilibrium modulus was 0.0036.

Table 2.3 Average Compression Moduli Comparison between FTC and P3.

Mean Compression Moduli (MPa)		
	FTC	**P3**
Instantaneous Modulus	2.5523	0.8019
P(T<=t) two-tail	0.0013	
Equilibrium Modulus	0.0529	0.1013
P(T<=t) two-tail	0.0036	

Both the instantaneous modulus and the equilibrium modulus for the FTC menisci were significantly higher than the P3 menisci.

Tensile Testing

The FTC menisci were found to have an average tensile modulus of 29.6519 MPa, average ultimate tensile stress of 7.3234 MPa, average strain at failure was 0.3534, and the energy absorbed was calculated to be 1.1856 $J \cdot m^{-3} \cdot 10^4$. The P3 menisci were found to have an average tensile modulus of 72.3724 MPa, average ultimate tensile stress of 12.1215 MPa, average strain at failure was 0.2256, and the energy absorbed was calculated to be 1.3380 $J \cdot m^{-3} \cdot 10^4$.

Table 2.4 Comparison of tensile properties of FTC and P3 calculated from testing data.

	Tensile Modulus (MPa)	Ultimate tensile stress (MPa)	Strain at Failure	Energy Absorbed ($J \cdot m^{-3} \cdot 10^4$)
FTC	29.6519	7.3234	0.3534	1.1856
P3	72.3724	12.1215	0.2256	1.3380
P(T<=t) two-tail	0.00003	0.00071	0.00003	0.39495

The P3 menisci had a significantly higher tensile modulus and ultimate tensile stress than the FTC menisci. The P3 had a significantly lower strain at failure than the FTC menisci. There was no significant difference in the energy absorbed by the P3 and FTC menisci.

Experiment 3: Crosslinking Assessment and Permeability

Crosslinking Assessment

A vertical cross section was taken at random from each of the four treatment groups. Pigmentation is a side effect of both genipin and EGCG and it can be used as an estimate for degree of crosslinking, with more intensely pigmented regions having higher degrees of crosslinking. There was evidence that the permeability of genipin is determined by concentration, as the inner portions of the menisci proceed to get lighter as the dilution was increased.

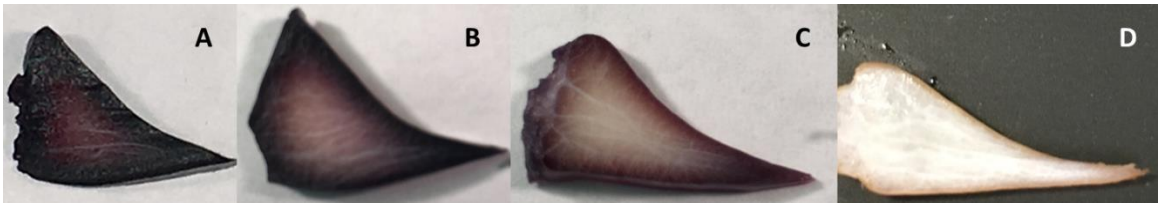


Figure 2.14 Cross sections of four different crosslinking treatments. A) 0.2% Genipin B) 0.04% Genipin C) 0.008% Genipin D) 1% EGCG.

The degree of crosslinking, shown in Figure 2.15, was compared for each treatment. Statistical analysis on the values for the degree of crosslinking was computed using SAS. The critical value of t was found to be 2.08596 with a least significant difference of 23.152 at the significance level of α=0.05. The values from Table 2.6 show that the outer portion of the 0.2% genipin treatment had the highest degree of crosslinking with 78.02%. There was no significant difference between 0.2% genipin and the 1% EGCG treatment with regards to degree of crosslinking in the inner portion of the menisci, and both had significantly higher degree of crosslinking in the inner portion than 0.04% and 0.008% genipin.

Percent Crosslinked				
Crosslinker	Genepin			EGCG
Concentration	0.2%	0.04%	0.008%	1%
Inner Portion	47.74%	3.34%	0.00%	60.82%
Outer Portion	78.02%	24.47%	19.51%	53.98%
Difference	30.28%	21.13%	19.51%	6.84%

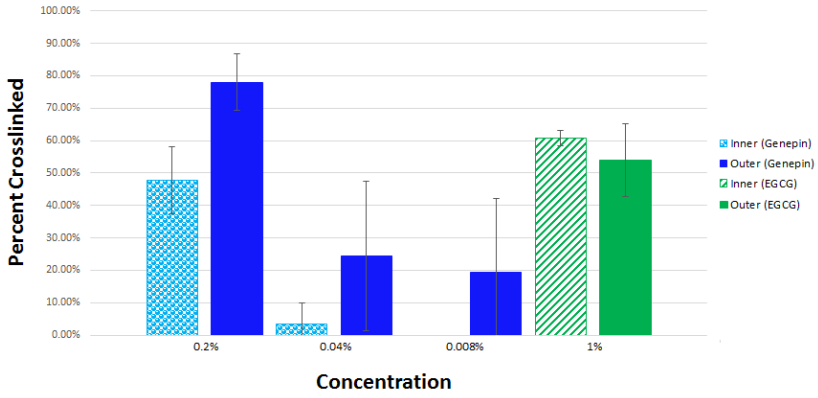


Figure 2.15 The permeability of genipin and EGCG as a crosslinking agent with regards to degree of crosslinking for menisci.

Table 2.5 T-Test (LSD) for degree of crosslinking for four differently crosslinking treatments.

t-Test (LSD) for Degree of Crosslinking			
t Grouping		**Mean**	**Treatment and Portion**
	A	78.02	0.2% Genipin Outer
	A		
B	A	60.82	1% EGCG Inner
B			
B		53.98	1% EGCG Outer
B			
B		47.74	0.2% Genipin Inner
	C	24.47	0.04% Genipin Outer
	C		
D	C	19.51	0.008% Genipin Outer
D	C		
D	C	3.34	0.04% Genipin Inner
D			
D		0.00	0.008% Genipin Inner

The 1% EGCG had the most permeability as a crosslinker, despite not being indicated by a color change due to crosslinking. This can be inferred as it had only a 6.84% difference in the degree of crosslinking between the inner and outer portions, which was the smallest of all four treatments. The permeability of genipin decreased rapidly with dilution, with only 3.34% crosslinking at 0.04% genipin and 0.00% crosslinking at 0.008% genipin. However, after the initial significant decrease in the degree of crosslinking of the outer portion caused by genipin due to dilution from 0.2% to 0.04%, there was not a significant change in the degree of crosslinking as genipin was further diluted to 0.008%.

Crosslinking Permeability

The average degree of crosslinking for control meniscus was 70.11% for the outer portion and 36.78% for the inner portion. The freeze-dry treatment had a degree of crosslinking of 68.64% for the outer portion and 37.61% for the inner portion. The salt solution bath and freezes-dry treatment had a 72.35% degree of crosslinking on the outer portion and a 31.07% degree of crosslinking on the inner portion.

Mean Degree of Crosslinking			
	Control	*Freeze-dry*	*Salted+Freeze-Dry*
Inner	36.78%	37.61%	31.07%
Outer	70.11%	68.64%	72.35%
Diff.	33.33%	31.04%	41.28%
Std. dev. Inner	0.097035	0.131274	0.111308
Std. dev. Outer	0.074347	0.105490	0.122126

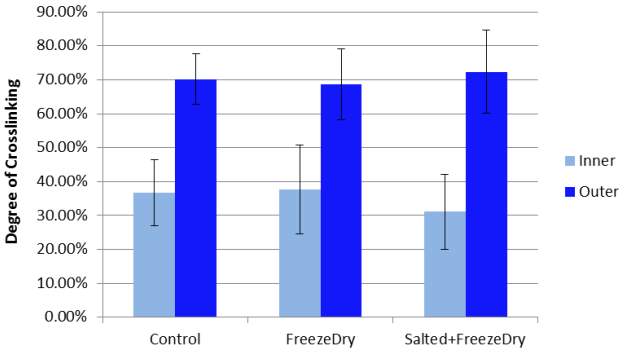


Figure 2.16 Degree of Crosslinking results for the freezes-dry, the salt solution bath and freeze-dry, and control pretreatments for genipin crosslinking.

A statistical analysis of the data was conducted using SAS at the significance level of α=0.05, the critical value of t was calculated to 2.00488 with a least significant difference of 0.0973. The outer portions had a significantly higher degree of crosslinking compared to the inner portions. However, there was no significant difference between pretreatment protocols with either the out or the inner segments.

Table 2.6 T-Tests (LSD) for the Degree of Crosslinking of Pretreatment Protocols.

T Tests (LSD) for Degree of Crosslinking of Pretreatment Protocols		
t Grouping	**Mean**	**TRT**
A	0.72347	Salted+Freeze Dry (outer)
A		
A	0.70110	Control (outer)
A		
A	0.68643	Freeze Dry (outer)
B	0.37607	Freeze Dry (inner)
B		
B	0.36775	Control (inner)
B		
B	0.31069	Salted+Freeze Dry (inner)

Discussion

The goal of this study was to provide strong evidence to the proof of concept for the ability to produce a decellularized xenograft meniscus implant that could be used for a partial or full meniscectomy. It was hypothesized that an effective and more efficient antigen removal protocol can be developed that is comparable to current protocols, and that the decellularized scaffold created from that protocol can bioengineered to mimic native tissue in its mechanical properties. This study provides evidence that there is no significant difference between P3 and SP, a currently published and established protocol, with regards to DNA removal. In terms of production time, SP requires 240 hours (10 days) to complete, while P3 requires less than half, 49.17%, of that time at 118 hours (4 days, 22 hours) to fully process the tissue. The development of P3 supports the initial portion of the hypothesis that an antigen protocol can be developed that is not

significantly different from a well-established antigen removal protocol, but be more efficient in terms of production time and material consumed in production.

The antigen removal analysis showed that P3 significantly lowered the DNA and GAG content in the meniscus receiving the treatment, however, as can be seen in the test data and microscopy, there was residual porcine DNA and GAG content in the treated meniscus. There is currently no published data on the threshold of immunotolerance of a host to these residual antigens. Menisci in general are not very vascular tissues, so the likelihood of an immune response would be very low. The fact that P3 was not significantly different from SP in terms of antigen removal is promising, but further testing would need to be developed to ensure that the residual porcine material falls below the immune threshold so a transplant would not be rejected.

The relationships observed in the compression and tensile moduli of the FTC and P3 menisci support previous literature on decellularized collagen scaffolds.[5] Understanding the degree in which these values changes helps direct further research into the bioengineering of the tissue to the desired specifications. Further research into the coefficient of friction differences in the native tissue and the decellularized tissue would also be beneficial.

The third experiment provided insight into the crosslinking effects, should it be needed to treat a would-be implant. A dose of 0.2% genipin, which is relatively low, proved to induce the greatest degree of crosslinking of the meniscus tissue; however, its low permeability prevented an even degree of crosslinking throughout the tissue. The permeability of EGCG was far superior, which could be due to a different mechanism of crosslinkage than genipin.[3] Further research into the physical requires of the implant

would need to be established, particularly if the tissue must have an even level of crosslinking throughout or if a highly crosslinked outer portion with a softer inner portion would act as a better shock absorber. Research that could test the various crosslinkers *in situ* could be designed using this research's findings.

In conclusion, this study has provided strong evidence to a proof of concept that a decellularized xenograft meniscus scaffold can be effectively and efficiently produced and that it can be modified via collagen crosslinking to fit the specifications needed for it to be used as an implant for a partial or full meniscectomy.

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