

Recent Advancements in Ligament Tissue Engineering: The Use of Various Techniques and Materials for ACL Repair

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Abstract: The anterior cruciate ligament (ACL) is important for knee stability and kinematics. Unfortunately, it is also the most commonly injured ligament of the knee and due to its poor healing potential, severe damage warrants surgical intervention including complete replacement. Current ACL replacements have a host of limitations that prevent their extensive use. Thus, investigators have begun to pursue tissue engineering techniques to create new options for the repair, regeneration, and replacement of the ACL. These options involve devices that are mechanically functional tissue engineered scaffolds and as such are designed to withstand normal mechanical loads while promoting ligament development. This article presents background on the ACL and its replacement, novel replacement approaches utilizing natural polymers, synthetic polymers, and natural tissues, and recent patent coverage.

Keywords: Anterior cruciate ligament (ACL), silk, poly (L-lactic acid) (PLLA), xenograft, scaffold, polymer, tissue engineering.

INTRODUCTION

BACKGROUND ON THE ACL

The anterior cruciate ligament (ACL) is the major intra-articular ligament of the knee and the most commonly injured ligament of the knee. There are between 100,000 and over 250,000 (or 1 in 3,000 in the general population) patients per year diagnosed with ACL disruptions with approximately 50,000 ligament reconstructions performed annually [1-6]. Its main functions are to support and strengthen the knee and prevent extreme translation of the tibia relative to the femur. ACL injury is a growing problem; in a recent study that included 17,397 patients with 19,530 sport injuries over a 10-year period, 37% of the patients had knee injuries. The ACL was damaged in 45.4% of these cases with 33.9% of them requiring surgery [3]. A number of repair techniques are currently available, and the success rates for long term clinical outcome are 85-90% [7-10].

The ACL is a dense, highly organized, cable-like tissue composed of types I, III, and V collagen, elastin, proteoglycans, water, and cells. Ligaments have a hierarchical structure with increasing levels of organization; these levels include collagen molecules, fibrils, fibril bundles, and fascicles all arranged parallel to the long axis of the ligament [11]. The collagen fibrils also have a periodic change in direction called a crimp pattern. In ACL, this crimp pattern repeats every 45-60 nm [12, 13]. The fascicles contain collagen fibrils, proteoglycans, and elastin. The ligament is surrounded by an epiligament sheath [14]. The ACL is also twisted approximately 180° from the femoral attachment site to the tibial attachment site and has antromedial and posterolateral bands [13].

Ligaments display unique mechanical behavior due in part to the crimp pattern of the collagen fibers in the ligament. The presence of the crimp pattern allows ligaments to increase in length under low strains without straining the collagen molecules and plastically deforming the collagen fibers, (Fig. 1). This enables the tissue to respond to the presence of maintained stress and still recover (up to a certain amount of strain).

The stress-strain curve of ligament can be divided into 3 sections (as seen in Fig. 1). The first section is the toe region. This region is characterized by a low slope and, as mentioned earlier, is caused by the straightening of the crimp pattern in type I collagen when the ligament is placed under strain. The next section is the linear region; this section has an increased slope and linear stress-strain behavior. In the linear region the type I collagen fibers are straightened in the direction of the tensile stress and are being stretched. The last section is the yield region; this section is identified by a decrease in slope. It represents defibrillation of the ligament and ligament failure [13, 15-17].

INTRODUCTION TO ACL REPLACEMENT

There are a number of options available to surgeons for ACL replacement including autografts, allografts, and synthetic devices. Autografts (tissue removed from the patient) are typically considered to be the gold standard in treating ACL injuries. Autografts possess the necessary amount of initial mechanical strength and promote cell proliferation and new tissue growth. There is no risk of rejection or disease transmission associated with autografts since the tissue comes from the patient. However, autografts have disadvantages as well. Autografts are limited in availability and require additional surgery for tissue harvest, which may cause donor site morbidity [5, 18]. Allografts (tissues from cadavers) have many of the same advantages as autografts such as initial mechanical strength and promotion of cell and tissue growth [5, 18, 19]. They also do not require a second

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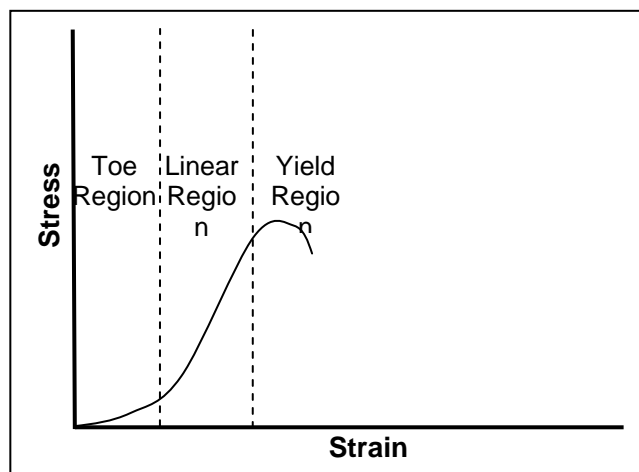


Fig. (1). Example of a stress-strain curve for ligament. Note the toe, linear, and yield regions.

surgery for the patient and there is no limit to their supply. However, autografts suffer from problems such as the potential for disease transfer, bacterial infection, and complications from host immunogenic response. Sterilizing these grafts can also change their mechanical properties [5, 18, 20, 21].

Past attempts have also been made to use synthetic materials in ligament replacements. Non-degradable synthetic materials that have been used for ACL repair include polyethylene terephthalate (Leeds-Keio ligament), carbon fibers, polytetrafluoroethylene (Gore-Tex), and polypropylene (Kennedy Ligament Augmentation Device) [22-33]. These synthetic ligament replacements have been conditionally approved by the FDA for testing and augmentation but are not recommended for primary ACL repair [11]. Two of these devices, the Leeds-Keio ligament and the Kennedy Ligament Augmentation Device, are braided polymer structures [13, 29-31]. Non-degradable synthetic grafts can be divided into three categories: permanent replacements, augmentation devices, and scaffolds. Permanent replacements must supply the function of the ligaments that they replace and are not amendable to tissue ingrowth. As a result, they are susceptible to long-term mechanical failure due to creep and fatigue. Augmentation devices are used to protect autografts and allografts from high loads during the early postoperative period when they are at their weakest [18, 33]. These devices may shield the biological graft from stress, which leads to poor long-term neoligament formation. Devices in the last category, scaffolds, are designed with a porous structure to allow tissue ingrowth; they also are designed to transfer loads to the new tissue.

Although these synthetic devices initially supply the function of the ligaments that they replace or protect the ligament that they augment, these devices fail over time because they cannot duplicate the mechanical behavior of the native ligament [18, 33]. Repeated elongation of these devices leads to permanent deformation at the points of stress. Contact with the sharp edges of the bone tunnel causes abrasions, which weaken the implant and create debris that can cause synovitis in the joint. Woven prostheses

face the additional problems of axial splitting, low tissue infiltration, low extensibility, and abrasive wear [18, 33].

Synthetic augmentation devices may bear most of the stress placed on the repaired ligament, which leads to stress shielding and a reduction in strength of the remaining tissue and any new tissue. Devices that are designed for tissue ingrowth, such as the Leeds-Keio ligament remain implanted while the new tissue grows. This leads to the production of new tissue with reduced strength because the implant bears most of the applied load. In both cases, if the implant fails the remaining tissue does not have the strength to withstand the load. The presence of these scaffolds leads to poor long-term neoligament formation.

In light of these limitations, a number of researchers have created additional tissue engineered options for ACL replacement and augmentation. A successful ACL device must be designed to successfully support the mechanical loads experienced in the knee. The tissue engineered replacements should also support cell and tissue growth leading to the regeneration of a new ligament. In addition, they must slowly degrade over time allowing the new tissue to slowly bear the load without risk of sudden rupture.

Each of these design criteria are extremely important in the development of a successful tissue engineered construct. A variety of new devices have been developed that fulfill each of these criteria; they vary in their structure and base materials. These devices can be classified based on the materials used to produce them such as natural polymers, synthetic polymers, and natural tissue (autografts, allografts, xenografts). This paper will review some of the more recent tissue engineered replacement devices in each of these areas.

NEW OPTIONS FOR ACL REPLACEMENT

Natural Polymers

Among the materials used in biodegradable tissue-engineered grafts are type I collagen and silk [2, 22, 34-36]. Some of the advantages of collagen are the capability of altering resorption rate and mechanical properties of scaffolds through crosslinking and its low antigenicity. These scaffolds experience an early decrease in mechanical strength followed by tissue remodeling between 10 and 20 weeks with a strength gain similar to autografts. Fibrous proteins such as silk or collagen are composed mainly of specific amino acid sequences repeated throughout the primary structure, this creates homogeneity in the protein's secondary structure (collagens exhibit triple helical structures and most silks display β -sheet conformations) [2, 34, 35, 37]. The rigid, extended structure of these proteins also gives them the mechanical properties necessary for the replacement of load bearing materials making them excellent materials for ACL replacements [2, 34, 37].

One recent tissue-engineered device for ACL replacement developed by Altman *et al.* is a twisted fibrous matrix composed of silk fibers [2, 34, 37, 38]. This silk fiber matrix is a hierarchical structure consisting of bundles of silk fibers wound into strands; these strands are wound together into cords and wound together to form the matrix. Each layer of the hierarchy is wound in a different direction [2, 34, 35].

Studies have shown that this matrix is not cytotoxic and is conducive to the proliferation of cells as seen in tests with bone marrow stromal cells (BMSCs) [2, 34, 35]. These scaffolds have mechanical properties similar to ACL. The matrices have maximum loads of over 2 kN, strains at failure of approximately 39%, and elastic moduli of over 350 N/mm [34]. All of these values are similar to those seen in tests with native ACL. The scaffolds also demonstrate the three phase mechanical behavior seen in ligament and tendon. The scaffolds demonstrate a toe region (low stress per unit strain) followed by a linear region (high stress per unit strain) [34]. As mentioned earlier, natural ligament is characterized by this behavior [13, 39].

This characteristic curve is important for the prevention of scaffold damage due to fatigue and creep. In other studies, Altman *et al.* have increased matrix biocompatibility and regenerative ability by coating the surface with RGD sequences [35]; this greatly increase cellular attachment, cellular proliferation, and extracellular matrix production of by BMSCs [35].

Synthetic Polymers

Many artificial biodegradable polymers have been investigated for ACL repair including poly glycolic acid (PGA), poly lactic acid (PLA), their copolymers, poly desamino-tyrosyl-tyrosine ethyl carbonate (poly (DTE carbonate)), and poly caprolactone (PCL) [11, 41]. The use of synthetic biodegradable polymers has several benefits. There is no limit to the supply of grafts (as opposed to autografts) and there is no risk of disease. These polymers are designed to degrade over time and therefore do not cause a long term foreign body response. The mechanical properties of the device may also be controlled by altering the degree of polymer crystallinity, changing the polymer molecular weight, or changing the ratio of each polymer in the copolymer.

Laurencin and his colleagues have developed a cell seeded, degradable, three-dimensional (3-D) braided poly L-lactic acid (PLLA) scaffold [5, 11, 26, 41, 42]. PLLA, has been approved by the FDA for different clinical applications including sutures; it does not elicit a permanent foreign body reaction and degrades gradually (allowing it to be replaced by natural tissue) [11]. Unlike autografts, use of these devices carries no risk of disease transmission and there is no limit to the number of these devices due to their synthetic source. These scaffolds can also be sterilized easier than grafts made from natural materials without sacrificing the mechanical properties of the device [11].

Unlike the synthetic polymers used in earlier ACL replacements (such as the Leeds-Keio ligament and the Kennedy Ligament Augmentation Device), the fatigue properties of PLLA are not a concern; the polymer degrades over time *in vivo* and the scaffold is eventually replaced by natural tissue. In a degradation study, PLLA fibers showed only a slight change in mechanical properties over an 8-week period in media [5].

The scaffolds produced using this 3-D braiding technique have mechanical properties comparable to the natural ACL. The braiding method also provides wear and rupture resistance to the scaffolds [5, 11]. The 3-D braiding technique causes the fibers to be woven throughout the entire thickness

of the braid giving it strength and reinforcing the structure, thus preventing total scaffold failure if some of the fibers are damaged.

Past ligament prostheses made of flexible composites consisting of fibers that have been woven or braided into structures have had long-term outcomes that were disappointing [28, 32]. Many of the scaffolds were limited by poor tissue integration, poor abrasion resistance, and structural fatigue [28, 32]. The weaving process also creates a network of integrated pores of regulated size. This network of pores aid in the flow of nutrients and removal of waste [40]. Both of these properties enhance cell proliferation and tissue ingrowth. The presence of pore interconnectivity throughout the entire implant also increases the overall surface area for cell attachment and allows for tissue ingrowth throughout the entire scaffold [11, 41].

In addition, this braided scaffold also has a fibrous, hierarchical structure. The scaffold is composed of micro-fibers that are similar in diameter to collagen fibers in natural ligament. These microfibers are grouped together to form fibers. The fibers are arranged into bundles and braided throughout the entire thickness of the scaffold [5, 11, 26, 41]. The braids are split into three sections: femoral tunnel attachment site (bony attachment end), ligament region (intra-articular zone), and tibial tunnel attachment site (bony attachment end). The angle of the fibers at the attachment sites is higher than the angle of the intra-articular zone. These differences in fiber orientation create differences in pore sizes of the regions [5, 11, 41]. This is important for the proliferation of cells and growth of tissues in each region; studies have shown that a minimum pore diameter of 150 μm is necessary for bone ingrowth and 200-250 μm for soft tissue ingrowth [43, 44]. The pore sizes of the different sections reflect these tissues preferences order to encourage ingrowth of the appropriate tissue in the appropriate area as well as capillary supply.

Upon implantation in New Zealand white rabbit studies, the device, when seeded with cells displayed the ability to regenerate new ligament tissue with oriented, mature collagen fibers [41]. Scaffolds seeded with primary ACL cells prior to implantation displayed a decreased fibrous capsule, blood vessels, and the presence of mature, oriented collagen fibers 12 weeks after the surgery. The collagen fibers were also able to infiltrate the full thickness of the graft [41].

Biological Tissues

Concerns with devices produced from biological tissues are based on the tissue source. The main concerns include potential transmission of disease to the patient, possible unfavorable immunogenic response, and bacterial infection [11, 45]. As mentioned earlier, it is also difficult to sterilize these devices without altering their mechanical properties. On the other hand, their use does not require a second surgery for tissue harvest. There is no limit to the supply of the graft tissue for these devices and they have the appropriate initial mechanical strength (depending on the source of tissue) [11]. Devices based on biological tissues also promote cell proliferation and new tissue growth.

Recently, xenografts (tissues from animals) have been presented as an option for ACL repair. Xenografts have the same advantages and disadvantages as allografts. They may also carry the additional risk of transferring a disease that is normally seen in an animal to their human hosts as well as the risk of rejection. Despite these risks, the work of Stone *et al.* have shown that treated xenografts may be a viable option for ACL repair [11, 46-48]. In their studies, Stone and colleagues have used chemically modified grafts from cloned pigs as ACL replacements. These studies note the successful use of immunochemically modified, chemically crosslinked porcine grafts for ACL reconstruction [11, 57, 48, 49].

In order to prevent rejection of the graft, the α -gal epitopes were enzymatically removed from the grafts. The interaction between the natural anti-Gal antibody and α -gal epitopes has been an obstacle in the use of xenotransplantation with porcine tissues [50,51]. Recently, the cloning of pigs lacking α -gal epitopes has eliminated this obstacle [51-53]. Along with eliminating the α -gal epitopes, the ACL grafts used in these studies have been pulse lavaged to remove cellular components and crosslinked with 0.10% glutaraldehyde for 12 hours; this treatment was followed by glycine endcapping to block unreacted glutaraldehyde molecules and sterilization by electron beam irradiation at 17.8 kGy [51,53].

In an *in vivo* study treated porcine grafts were implanted into a rhesus monkeys [51]. Twenty animals were reconstructed with treated grafts for 2, 6 and 12 months; 3 monkeys were used at the 2 month time point, 5 monkeys were used at the 6 month time point and another 5 monkeys were used at the 12 month time point. The controls consisted of 1 untreated porcine allograft and 1 rhesus allograft at 2 months along with 5 rhesus allografts at 12 months. The grafts and corresponding intact ligaments from the other leg were biomechanically tested at 6 months (3 grafts) and 12 months (10 grafts); a histological examination followed the mechanical tests. Treated porcine bone-patellar tendon-bone grafts and fresh frozen rhesus bone-patellar tendon-bone devices were cut into bone-tendon constructs. The devices for implantation were 30-mm long by 4-mm wide (tendon material) grafts with bone plug ends of 5 mm diameter by 7 mm in length.

The implants were shown to promote the regeneration of new ligament tissue [51]. Mingozi has recently patented anchors for tendons used in the reconstruction of ligament [54]. There were signs of graft remodeling extending from the graft periphery to the graft center. In addition, after 12 months the porcine grafts displayed comparable ultimate load, yield load, stiffness and ultimate displacement. The strength of the implanted treated grafts increased from 43% to 58% between 6 and 12 months.

Unfortunately, the grafts also demonstrated lower values for ultimate strength, yield strength, ultimate strain, and modulus. Though these final values need improvement, the values based on load and displacement show that this graft system is well on its way to becoming a viable option for ACL replacement. Feanny has also recently patented bone and ligament graft modification system [55].

Along with mechanical tests the animals were tested for the rejection of the porcine grafts [48]. Blood samples were taken prior to surgery and at days 10, 14, 21, 28, 42, 56 as well as at 3, 6, 9, and 12 months for analysis of anti-Gal and anti-non-Gal antibodies such as antibodies to proteins present in the porcine grafts. Serum immunoglobulin (Ig) anti-Gal IgG and IgM activity was determined by ELISA. There was a greater increase in anti-Gal titers (>200%) in the monkey engrafted with untreated porcine graft when compared to anti-Gal titers from the monkey implanted with the treated graft (95% lower than the untreated) within 2 weeks following implantation. The strong response with the untreated grafts is an indicator of acute rejection and can lead to graft destruction and resorption. It is thought that the small increase in anti-Gal titers in monkeys with the treated grafts may be due to an immune response to α -gal epitopes on the porcine bone marrow cells in cancellous bone interstices of the bone-ligament-bone graft. The anti-Gal titers reached resolved preimplantation values by 8 to 12 weeks after implantation. Tuke has patented a replacement ligament prosthesis [56]. Wenstrom has also patented a ligament fixation device [57].

Whittaker has recently patented an implant with integral fixation devices [58]. In a separate study, the porcine grafts were implanted into human subjects for ACL replacement [51]. Analysis using Western blotting and ELISA showed that the subjects produced anti-non-gal antibodies against multiple pig xenoproteins. Their level of production peaked from two to 6 months; all antibodies were no longer produced after two years. No antibodies were produced against human ligament proteins. After two years five of the six patients in the study showed had no problem with the function of the porcine graft.

CURRENT & FUTURE DEVELOPMENTS

The ACL is a complex, highly ordered tissue with mechanical properties that are important for normal knee kinematics. In order to maintain knee function after injury, repair devices must be able to bear the appropriate amount of load and display similar mechanical properties in the short term while promoting the growth of new mature ligament to bear the load in the long term. The devices listed above represent some of the advancements that have been made in ACL tissue engineering. They display the range of options available in tissue engineered scaffolds; structures can be made from natural polymers, synthetic polymers, and treated tissues. They are all designed to fulfill the needs of a tissue engineered device, these include structural stability and appropriate mechanical strength, promotion of cell and tissue growth, and the ability to slowly degrade and allow the new tissue to bear the load. This blend of biological activity and mechanical stability make these devices excellent options for ACL replacement.

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