Effect of Temporal Onset of Mechanical Loading on Tendon to Bone Healing, Determined with a Novel In-Vivo Device

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ON TENDON TO BONE HEALING,
DETERMINED WITH A NOVEL IN-VIVO DEVICE

by
Mark E. Stasiak

A dissertation submitted to the Graduate Faculty in Biomedical Engineering in partial fulfillment
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This manuscript has been read and accepted for the
Graduate Faculty in Biomedical Engineering to satisfy the dissertation
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ABSTRACT

EFFECT OF TEMPORAL ONSET OF MECHANICAL LOADING ON TENDON TO BONE HEALING, DETERMINED WITH A NOVEL IN-VIVO DEVICE

by Mark E. Stasiak

Advisor: Professor Luis Cardoso

Background: Exposure to tensile loading has been shown to maintain tendon/ligament homeostasis, with the tensile modulus of the tissue increasing in response to greater loading frequency, and even decreasing in the absence of load. However after injury or surgical repair the effect of tensile load is controversial, with many studies implicating mechanical loading with impaired healing. It is currently unknown when the tensile loading imparted during rehabilitation exercise, can be safely reintroduced after surgical tendon repair or ligament reconstruction, and at what time point it can most benefit the remodeling process. To investigate the effect of tensile loading protocols on tendon graft to bone tunnel healing, we developed and validated a novel device to cyclically distract the knee joint in a rat model of anterior cruciate ligament reconstruction (ACL_R), thereby imposing a controlled amount of tensile strain on the tendon graft. Using this device we tested the following hypotheses; H₀ which predicts that the immediate resumption of tensile load after ACL_R surgery will impair tendon-to-bone healing compared to continuous knee immobilization. And H₁ which predicts that after ACL_R surgery, insertion of a 4 to 10 day load shielding period, prior to the resumption of tensile load, will improve tendon-to-bone healing compared with both immediate load resumption or continuous immobilization.

Methods: Male Sprague Dawley rats who had undergone ACL_R surgery via a flexor digitorum longus tendon auto-graft were randomly assigned to either; 1) a continuous-immobilization group to strain
shield the graft or a cyclic knee distraction group using the device to achieve two-percent strain of the tendon graft. The animals receiving cyclic knee distraction were further subdivided into: 2) an immediate-loading group beginning on postoperative day one, 3) a four-day delayed-loading group beginning on postoperative day four, and 4) a ten-day delayed-loading group beginning on postoperative day ten. The above experiment was repeated with the device applying ten-percent strain to additional four-and ten-day delayed-loading groups. All animals were sacrificed on either postoperative day fourteen or twenty-eight for immuno-histochemical, biomechanical, and micro-computed tomography analysis of the femur-graft-tibia complex.

**Results:** Immediate-loading, beginning on postoperative day one did not result in a significantly reduced load-to-failure mechanical response or bone tunnel volume, compared with continuous-immobilization. However a significantly greater number of inflammatory macrophage cells in the immediate-loading group provided partial support for hypothesis H₀. The data supported hypothesis H₁, with the four- and ten-day delayed-loading groups having significantly less inflammatory macrophages and more new bone volume at both two and four weeks. The failure load of the femur-graft-tibia complex was significantly greater in the ten-day delayed-loading group at two weeks, and in the four-day delayed-loading group at four weeks. Repeating the experiment with cyclic knee joint distraction increased to ten-percent of graft length did not recapitulate the significantly greater failure loads in the delayed loading groups.

**Conclusion:** On the basis of greatest failure load, most bone tunnel ingrowth, and lowest inflammatory cell population, the four-day delayed-loading protocol was judged most effective for tendon-to-bone healing in the rat model of ACL_R. These parameters could be extrapolated to a human clinical trial of an improved ACL rehabilitation protocol, provided a careful choice of knee exercises applying a maximum of two-percent tendon graft strain were made.
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Chapter 1:

General Introduction

Surprisingly, a literature search of the phrase “tendon to bone healing” returns little on intrinsic tendon healing. Rather, the results reflect current research interest in the mechanisms of incorporation after the surgical apposition of tendon onto bone. Lacerations and ruptures that fail to heal after conservative treatment often require surgical reattachment of tendon to bone. Additionally, non-healing ligament ruptures of the knee are typically reconstructed using tendon grafts due to the structural and mechanical similarity between tendons and ligaments. In either case, repair or reconstruction, the outcome depends on successful tendon to bone healing [1,2].

The simplicity of the phrase also belies the difficulty of the task from an engineering standpoint. It requires the union of two dissimilar materials; flexible fibrous tendon with a Young’s modulus of 200 MPa solely in tension, onto rigid, brittle bone, with a 20 GPa modulus in both tension and compression. This results in a second order of magnitude disparity in the stress-strain response across the interface, creating stress concentrations and a propensity to delaminate as one material deforms significantly more than the other under identical loading [3].

Operations depending upon effective tendon to bone healing can have varying success rates. Of the more than 250,000 operations each year in the US to repair rotator-cuff tendon tears of the shoulder, an estimated 50 % experience re-tears, with a disproportionate effect on the elderly and their quality of life [4]. The gold standard of care for a ruptured anterior cruciate ligament of the knee, is surgical reconstruction with a tendon graft, and generally achieves good results. However rates of revision surgery in young and active patients are as high as 20 %. Poor tendon to bone healing after ACL reconstruction (ACL_R) can increase knee laxity and predispose the patient to early osteoarthritis [5–7].
While the ACL is a knee ligament and the rotator cuff tendons control humeral head position in the shoulder, they both share a similar structure in their attachment sites to bone and a common synovial joint environment [8]. Unfortunately they also share a poor propensity to heal, reasons for which will be given in chapter II, and both typically require surgical repair/reconstruction upon injury and depend on effective tendon to bone healing for rehabilitation.

The weakest link in a healing ligament reconstruction is not the tendon graft itself, but at its point of attachment to the bone [9]. Failure here is more likely to occur than at a mid-substance. After the initial surgical placement, the interface between tendon graft and bone is rapidly filled with an amorphous granulation tissue during the initial the healing response [10]. Although remodeling of the interface tissue does occur, it does not replicate the structure and superior mechanical properties of the native tendon/ligament insertion site, known as an enthesis [2]. Enthesis type, composition and development, along with the native tendon/ligament wound healing response are described in detail in chapter II.

Because development of the native enthesis is believed to be driven both by genetic cues and as a cellular response to mechanical loading during joint movement, both pre and postnatal, it is thought that after ligament reconstruction or tendon repair, continued tensile loading of the tendon/graft will drive remodeling of the interface tissue into an improved approximation of the native enthesis, with a greater resistant to failure under load [3]. Many studies have been conducted on the augmentation of tendon and ligament repair/or reconstruction using applied growth factors and genetic vectors, yet the few studies on the effect of mechanical loading have used activities such as treadmill running where the applied load on the tendon is difficult to quantify [11–13].

In addition to the basic science questions regarding postsurgical enthesis restoration, there is an urgent clinical need to ascertain when one can safely resume tensile loading of a vulnerable ligament.
reconstruction or tendon repair [14]. To answer these questions we used a previously developed rat model of ACL_R [10] and developed a unique joint fixation and loading system to apply a controlled and quantified amount of tensile load on the healing tendon graft. A rat model of rotator cuff repair was considered but excluded due to the inability of the thin rat scapula to support a joint fixation device. This dissertation will detail the design of that system and elaborate on the results obtained using it to study the role of mechanical load on tendon to bone healing.

**Thesis Organization**

Chapter two presents tendon and ligament mechanical properties and basic science. Explanation of the time dependent aspects of these properties is included to aid novice readers interpreting data from repeated loading. The basic science section covers not only tissue composition and structure but also embryonic development, to introduce those cell signaling molecules guiding tissue genesis that may also have a role in repair. The impaired healing response of intra-articular tendon/ligaments is explained along with the requirement for the surgical reconstruction and rehabilitation addressed by this research. Chapter two concludes by framing the hypotheses and specific aims governing this thesis.

Chapter three details design and validation of a device to provide in-vivo tensile loading of a tendon graft ACL reconstruction, modeling the effect of a rehabilitation exercise protocol on the tendon to bone tunnel healing.

Chapter four will use this device to evaluate the effect of immediate knee motion post ACL_R, and compares it with the effect of continuous knee immobilization.

Chapter five explores the effects of short periods of immobilization prior to the daily knee motion protocol and compares the effects with the data obtained in chapter four.
Chapter six will investigate the effect of increasing the strains applied by the knee distraction device to the tendon graft.

Chapter seven will summarize and evaluate the results, and suggest future research directions.
Chapter 2: Tendon & Ligament

Introduction

While bone provides the rigid support for a body and muscle the power necessary for its locomotion, tendons and ligaments tie the musculoskeletal components into a unified ambulant system. With their morphology determined by the loads they transmit and resist; formed into flexible, fibrous cables and straps, or even wide flat bands, their appearance pale from a limited blood supply, and of a similar internal composition and structure to resist tensile stress, tendons and ligaments are the essential motion enabling skeletal connective tissues [1,15,16].

Ligaments restrain one bone to another, forming flexible skeletal joints. Tendons serve to connect muscles to bone. Typically within the appendicular skeleton, a shorter tendon suspends a muscle from its skeletal origin, while at the distal muscle end a longer tendon emerges to insert at the point of action on a long bone [15]. Both work together as compliments, while a tendon transmits the force of muscle contraction through its substance, resulting tension in the joint spanning ligaments constrains and guides the path of skeletal motion. Injured tendons can limit or disable motion, while injured ligaments result in excessive joint motion that wears the articular surface provoking early onset arthritis [16,17].

Tendons and ligaments reduce the metabolic cost of locomotion by functioning as elastic extension springs; when un-loaded they recoil to their original length, helping return the limb to its starting position. The Achilles tendon and the plantar ligaments of the feet have been calculated to return as much as 50% of the energy expended during running [18–20]. High water content allows for their functioning as speed reducing dashpots, braking motion during rapid stops and changes in direction; the viscous drag force on water exuding from tendon/ligament increases with the speed of their elongation, absorbing energy to protect the skeleton [17]. Enervation not only senses pain but also
enables proprioception. Stretch and pressure receptors in tendons and ligaments provide information on joint position and muscle contraction and even enable reflexive motion [21].

Ligaments guide joint motion by restricting it more in one plane than another.[17] While the anterior cruciate ligament (ACL) allows large degrees of knee flexion and extension in the sagittal plan, its posterior-lateral bundle becomes taught as the tibia rotates internally in the transverse plan, limiting that motion and preventing one from tripping over their own foot. However, the ACL becomes slack as the tibia rotates externally and places less restraint on that motion (Figure 2.1 a, b) [22]. Athletic activities or accidents causing a rapid internal rotation or anterior-posterior translation of the tibia that stretch the ACL past its ultimate strain (breaking length) are a known mechanism for its rupture [23].

Knee flexion-extension is not just a rotation of the tibia about the femur like a simple pin hinge. The tibial plateau also slides on the femoral condyles, changing the instant center of rotation of the tibia throughout the path of knee flexion-extension (Figure 2.1 right). Therefore without a fixed center of tibial rotation in the femur, the ACL must stretch and twist elastically to accommodate all degrees of freedom in the knee joint [24].

**Figure 2.1, (a)** “The ACL is stretched when the tibia is internally rotated relative to the femur, due to its oblique orientation inside the knee. **(b)** The ACL is unwound and becomes slack and unloaded with the tibia externally rotated relative to the femur. Similarly, the ACL may be loaded and unloaded by tibial
adduction and abduction, respectively” [22]. **Right:** Instantaneous center of knee rotation determined via the perpendicular bisectors of the paths A to A’ and B to B’ and their intersection at the instantaneous center C. Note that the femur is sliding in the direction of the vector from point D. As the flexion angle increases, the locations of C within the femur and tibial-femoral contact point D change [24].

Tendons and ligaments must also resist fatigue failure, an accumulation of tears and damage from many cycles of loading/unloading that over time reduce the strength and cross-sectional area of a structure so that it fails unexpectedly at a level far below its expected failure load. Indeed, the accumulation of unrepaired fatigue damage from endurance exercises and repetitive work tasks, coupled with an inability of cellular repair processes to keep up, is a proposed etiology for tendon/ligament degeneration and disorder [25].

**Tendon and Ligament Composition**

Tendons and ligaments are biological fiber reinforced composite materials adapted to withstand tensile stresses while retaining flexibility. Their dry weight consists of from 65 to 75% collagen fibers with a predominantly longitudinal orientation, adjusted to resist the unique tensile stresses at each tendon/ligament’s specific anatomical location. The collagen fibers are surrounded in an amorphous matrix or ground substance composed chiefly of water and proteoglycans that add time dependent viscous properties and provide lubrication for the fibers to glide over each other [1,17]. Sesamoid bones can arise within a tendon where it slides over a bone prominence and the resulting change in direction of tensile force creates a component of compressive force acting on the tissue. Sesamoids serve as friction reducing glides and can provide a fulcrum for mechanical advantage. The patella (knee cap) is a sesamoid bone within the quadriceps tendon/patella ligament that provides a fulcrum for the quadriceps muscle during knee flexion/extension while gliding over the articular cartilage surface of the trochlea, located in between the condyles of the femur [26].
Tendon and Ligament Mechanical Properties

- Force Displacement Relation

If a representative specimen of tendon or ligament is stretched to failure at a constant rate, a plot of the internal force – elongation relation will at first increase slowly, and then gradually begin to curve upward as the internal force grows faster with each equal increment in specimen length. This initial upward curve of the plot is called the toe region and represents the gradual recruitment and alignment of the collagen fibers within as they begin to resist the stretching; it requires an exponential function to represent it mathematically (Figure 2.2). The toe region ends as the slack is taken up from all collagen fibers, now each equal increment in specimen length adds the same proportional increase to the internal force, represented mathematically as: \( F = k^*x \). The plotted force-elongation relation is now a straight inclined line, the constant slope of which is known as the stiffness. The stiffness is due to the material and structural properties of the collagen fibers and other components of the extracellular matrix (ECM) and its magnitude correlates with the amount, thickness and quality (the extent of chemical crosslinking) of the fibers in the specimen [16,17].

![Figure 2.2: Typical force versus elongation relation for a tendon or ligament. The slope (or stiffness) of the curve can increase with the rate of elongation of the tissue.](image-url)
A specimen’s stiffness can be divided by its cross sectional area and then multiplied by its original length, giving its *modulus of elasticity* (stress/strain) in units of Pascals (N/m²). This normalization allows one to compare tendon or ligament specimens from animals differing in size or weight and even make comparisons across species. Unfortunately the non-contact devices required to accurately measure the irregularly shaped and compliant cross sectional areas of tendons and ligaments are costly and not available in many laboratories [27–29].

As stretching of the specimen continues, eventually some of the collagen fibers begin to break or slide past each other and the internal resisting force stops increasing with added length. The curve has reached a plateau and the specimen will not recoil to its original length if released, its elastic behavior has been permanently lost. More fibers begin to break or slide and the specimen will soon completely fail. The highest force reached is its load-to-failure force (*ultimate strength*, if normalized) [16,17].

Tensile testing of human cadaver ACL specimens revealed decreasing structural properties with age, as shown in the table 2.1 below of failure load and stiffness by age group [30]. Specimens consisted of the native femur-ACL-tibia complex with all surrounding soft tissue removed:

<table>
<thead>
<tr>
<th>Age Group (years)</th>
<th>Failure Load (N)</th>
<th>Stiffness (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young Adult (22 - 35)</td>
<td>2,160 ±157</td>
<td>242 ± 28</td>
</tr>
<tr>
<td>Middle Aged (40 - 50)</td>
<td>1,503 ± 83</td>
<td>220 ± 24</td>
</tr>
<tr>
<td>Older (60 – 97)</td>
<td>658 ± 129</td>
<td>180 ±25</td>
</tr>
</tbody>
</table>

For comparison with the subjects used in our studies, a control group of eighteen week old female Sprague-Dawley rats had a mean failure load of 26.7 ± 8.6 N and a stiffness of 41.3 ± 19.3 N/mm [31].
• **Force-Time & Strain-Time Relations:**

Stress relaxation (force relaxation without normalization) occurs when a tendon or ligament specimen is suddenly stretched and held to a constant length within the linear elastic region for an extended period. (Instantaneous elongation and infinite duration are required parameters for mathematical modeling.) As time elapses the internal force will gradually decrease, without any corresponding reduction in length, falling in ever smaller increments to approach but never quite reach a steady state level. A plot of the internal force over time would show a curvilinear drop to approach a horizontal line (Figure 2.3 a) [16]. This is due to the gradual loss of specimen fluid content over time, while the steady state level of internal force \( f_0 \) reflects the static elongation of the collagen fibers \( f_0 = k^*x \) [16].

**Figure 2.3:**

- **A.** Force relaxation curve in tendon or ligament. The force required to maintain a constant length decreases over time until a steady state level is reached.
- **B.** Creep curve - If held under a constant tension, a tendon or ligament will slowly lengthen (creep) until reaching a steady state length.

Conversely, if we held external force on the specimen constant - while allowing its length to vary; say we suspended a weight from its free end. After an initial rapid stretch we would observe the specimen continue to lengthen under constant weight, but in ever diminishing increments. A plot of elongation over time (Figure 2.3 b), would show an initial rapid curvilinear increase, slowing to approach a horizontal limit on specimen length, reflecting the time independent response of the collagen fibers to
the constant weight \((x = f/k)\). This continual but gradually diminishing elongation of tendon/ligament under constant tension is known as \textit{creep}; due to the gradual decay of the viscous component of the internal force as less and less water bleeds out of the tissue until all that is left is the elastic component of the collagen fibers [16].

- Hysteresis and Energy Loss

Stretching a tendon/ligament specimen to a length not exceeding the linear region of its force-elongation curve, then allowing it to recoil to its original length yields a contraction force-elongation curve lower in force at every length point than the initial stretching curve. This decrease in force is known as \textit{hysteresis} and represents the energy lost as heat through the viscous component of the specimen. The area in the loop between the upper “loading” curve and lower “unloading” curve represents the total energy not recovered during recoil to original length. Most reports of tendon and ligament hysteresis have been in the range of 5 to 25 \% energy lost out of the total work performed. The total area below the unloading curve is called rebound resilience and represents the amount of the initial strain energy recovered by elastic recoil. Rebound resilience helps the Achilles tendon and the plantar ligaments of the foot reduce the cost of locomotion [16,17,19,20].

**Figure 2.4:** Hysteresis, the energy lost after stretching an elastic specimen and then allowing it to return to its original length. The total energy lost is the area between the upper elongation curve and the lower contraction curve (Note: the x-axis must be deformation to calculate the energy loss).
Extracellular Matrix: Collagen Fiber Synthesis and Composition

The formation of a collagen molecule requires the cellular synthesis of three individual polypeptide α-chains, of type α1 or α2. This process begins within the fibrocyte nucleus, with the transcription of three sequences of messenger ribonucleic acid (mRNA) nucleotides, from the genome deoxyribonucleic acid (DNA) molecule; one mRNA strand for each α-chain. During transcription, the double strands of the DNA molecule separate at the gene section encoding the required α-chain type, and essentially an RNA polymerase reads each subsequent nucleotide from the DNA template strand and matches it with its complementary nucleotide on the growing mRNA strand. Upon completion each of the three mRNA strands, essentially blueprints for each α-chain, are transported out of the nucleus and attached to a ribosome on the rough endoplasmic reticulum (RER), for peptide assembly [32–34].

In a process known as translation, the ribosome reads each subsequent sequence of three nucleotides - a codon, from the attached mRNA strand. Each codon refers to a specific peptide type, delivered to the ribosome by transfer RNA (tRNA). The order of the codons determines the order in which the ribosome links together the different peptides, ensuring that the specific α chain; α1 or α, encoded by the mRNA strand is produced [33].

In each α chain, the first peptide added is known as the N-terminus because it contains a free amine group (NH2) that marks the start of the chain. The last peptide added is known as the C-terminus and contains a carboxyl group (COOH) marking the chain’s end. The body of each α-chain consists of a repeating sequence of three peptides that conforms into a left handed helix, with the small amino acid glycine in every third position and the other two positions X and Y frequently occupied by amino acids proline and lysine. The N- and C- termini at each end are non-helical (Figure 2.5) [33,35].
**α - chain sequence:**

\[
\text{Start } \text{NH}_2 \rightarrow \text{Glycine} \rightarrow X \rightarrow Y \rightarrow \text{Glycine} \rightarrow X \rightarrow Y \rightarrow \cdots \rightarrow X \rightarrow Y \rightarrow \text{Glycine} \rightarrow X \rightarrow Y \rightarrow \text{COOH} \text{ End}
\]

Non Helical \[\leftarrow \cdots \cdots \cdots \cdots \cdots \cdots \cdots \cdots \rightarrow \] Non helical

Approximately 330 peptides

**Figure 2.5:** Molecular arrangement of a typical α-chain. X & Y refers to either proline or lysine [33].

After creation the three new alpha chains thread into the lumen of the RER for post translational modifications. Many of the proline and lysine residues on the chains are hydroxylated by ascorbic acid dependent enzymes to form hydroxyproline and hydroxylysine. Some of the hydroxylysines are also glycosylated with the monosaccharides glucose or galactose (Figure 2.6). These modifications of proline and lysine are essential for the formation of molecular crosslinks needed to assemble the staggered hierarchical structure of the future collagen fiber. Variations in post translational modifications can result in differences in composition and tensile strength between collagen fibers of the same gene. This explains how vitamin C deficiency can affect diseases of poor collagen quality such as scurvy [33,35].

After post translational modifications are complete, the non-helical C-terminal of one α-chain folds, allowing it to align with recognition sequences within the C-terminals of the other two α-chains [34]. Starting from the C-terminal ends, the helical portions of each of the three chains wrap around each other into a right handed super-helix, forming the procollagen molecule [34,35]. Formation of this triple helix is possible only if glycine occupies every third position in each alpha chain, because it is the only amino acid small enough to fit within the central axis of the triple helix [33]. The right handed winding of three left hand helixes about each other is analogous to modern techniques used increase the tightness of wound steel cables, and reduces the ability of the three α chains to slip past each other. The extent of proline hydroxylation determines the stability of a collagen molecule because it enables
cross-links between adjacent alpha chains. When two α₁ chains wrap with one α₂ chain the resulting procollagen molecule is classified as collagen type I, the predominant collagen type in tendon and ligament [33–35].

The path of the new pro-collagen molecule from the RER to the Golgi complex is unclear since its 300 nm length goes well beyond the 60-80 nm diameter of the typical transport vesicle [35]. (See Figure 2.6). Within the Golgi complex oligosaccharides are added, yet it is not a completed collagen molecule because it still contains the non-helical C-terminus ends and possibly the N-terminus ends. From the Golgi it is transported, (the method again is unclear) along microtubules to the plasma membrane where it is extruded from the cell [33,35] (Figure 2.6).
Figure 2.6: Synthesis of Collagen alpha chain, (from Junqueira L. Basic Histology, 1992).
Extracellular Matrix: Collagen Fiber Self Assembly

Within the cell, persistent attachment of the C terminus has kept the procollagen molecules soluble, preventing them from aggregating into too large of a structure. Once outside the cell, or within an elongated vacuole on the outer plasma membrane, metalloproteinases cleave the non-helical C and remaining N termini from the pro-collagen triple helix. The C-terminus is removed by bone morphogenic protein 1 (BMP1) and the N-terminus by ADAMTs – a disintegrin and metalloproteinase with thrombospondin motifs. With removal of the N and C termini, it is now a tropocollagen molecule, completely helical along its approximately 300 nm length and 1.5 nm diameter [35,36].

As more tropocollagen molecules accumulate outside of the cell they align in parallel, with the cleaved helical ends of the tropocollagen in each row each offset from the helical ends in the adjacent row by approximately 44 nm, creating a lengthwise-staggered arrangement (See figure 2.7). This is due to the action of lysyl-odixase, which adds aldehyde groups between tropocollagen rows, crosslinking the alternating lysines and hydroxylisines of the adjacent molecules together with strong covalent bonds. It is the arrangement of the crosslinks that forces the lengthwise offsets between adjacent rows [35,37].

Simultaneously with the crosslinking, electrostatic forces wind the parallel tropocollagen molecules about a longitudinal axis into a left hand super-super helix, forming the beginning of a microfibril 3.5 to 4 nm in diameter. The stability of the microfibril increases with the number of hydroxylsyine crosslinks formed between the chains. As the microfibril grows in length, the 44 nm staggering between the end of one tropocollagen molecule and the next, creates a regular series of gaps. Stains used in histology readily accumulate within these gaps making them easily visible on electron micrographs, where they appear as railroad track like cross striations with a regular periodicity of 67 nm [34], (See Figure 2.7).
The self-assembly of cleaved tropocollagen into collagen fibrils can be verified easily in vitro. Collagen fibers are easily dissolved into individual molecules within a mild acid solution. When the solution is neutralized and warmed, the molecules readily assemble into fibrils. However, these fibrils coalesce into a random mesh with no preferred orientation. This isotropic structure is unsuitable for tendon and ligament, which depend on a predominantly parallel orientation of fibers to resist tension along their lengthwise axis. Embryonic development of tendon must somehow rely on cellular involvement, however cultures of collagen I synthesizing fibrocytes in vitro do not produce longitudinally ordered ECM [34,36].
Extracellular Matrix: Embryonic Alignment of Collagen Fibers

Canty et al. have produced evidence that fibrocytes can cleave the C-termini from tropocollagen molecules within the cell. The activation cascade for metalloproteinases BMP1 and ADAMTs can occur not only on the cell surface, but also in the secretory pathway, within the “trans-Golgi network’ (TGN) an apparatus large enough to transport procollagen molecules. The N-terminus is likely cleaved first, within or just after leaving the Golgi apparatus. Cleavage of the C-terminus occurs afterwards, possibly within “Golgi to plasma membrane carriers” (GPC) [38].

Normally this would result in the spontaneous assembly of tropocollagen molecules into an uncomfortably long microfibril within the cell membrane. However an elongated vesicle termed a “fibropositor” fuses with the cell membrane providing both space for microfibrils to grow and a tube to extrude them parallel with the fibrocyte’s length (See Figure 2.8). These fibropositors are aligned with the long axis of the spindle shaped fibroblasts found in tendon and ligament and always parallel with the length of the tendon/or ligament. The distal external end of the fibropositor has a larger diameter to accommodate a microfibril growing in into a fibril, which is eventually extruded distally [39].

Fibropositors occur only during a narrow window in embryonic development, when the fibroblasts are stacked in rows to establish the tissue architecture. Unfortunately, according to current knowledge, fibropositors cease to occur postnatal and are unavailable to participate in the repair of injured tendons and ligaments [39].
Figure 2.8 (A) Transverse sections through a branched fibripositor showing an enclosed GPC (left-hand side arm) and another that is open to the ECM (right-hand side arm). Fibrils are depicted as black lines within the lumen of the fibripositors. Blue arrows point to the same fibril in the electron micrographs and in the schematic. A bar shows the height of the fibripositors. (B) A gallery of electron micrographs in which mouse tail tendons had been treated with HRP and DAB before preparation for EM. Darkly contrasted compartments indicate the presence of HRP (closed arrowhead). Compartments lacking HRP-reactive DAB (simple arrowheads) [39].

- Extracellular Matrix: Ground Substance

The ground substance surrounding and filling the spaces between collagen fibers and cells in tendon and ligament is a viscous, amorphous, transparent gel, 60 – 80 % of which is composed of an electrolytic water solution. Its viscosity provides lubrication allowing the collagen fascicles to slide past one another and also serves as a barrier to bacterial invaders. The ground substance helps compensate for the tissue’s lack of vasculartiy by providing a medium for carbon-dioxide-oxygen exchange and for the diffusion of nutrients required by cellular metabolism [40].
Within the ground substance is a complex mixture of three classes of macro-molecules: 1. glycosaminoglycans, 2. proteoglycans and 3. glycoproteins. Although they account for less than 1% of the total dry weight these macro-molecules add to the physical properties of the ground substance, assist cellular activities, enable the formation of crosslinks between tropocollagen molecules and facilitate the hierarchical assembly of fibrils [16,40,41].

1) Glycosaminoglycans (GAGs) also known as mucopolysaccharides are very long repeating chains of disaccharide molecules, typically an amino sugar paired with auronic acid (sugar acid). The amino sugar can be N-acytel glucosamine or N-acytelgalactosamine, and the uronic acid can be glucuronic acid or iduronic acid. GAGs are highly negatively charged making them hydrophilic and polyanionic, binding large amounts of water and cations within the ground substance, and increasing its viscous properties due to their repeating polymer structure [42].

GAGs are classified into four groups based on their predominant disaccharide component; Chondroitin Sulfates are composed of glucuronate and galactosamine. Dermatan sulfates are composed of iduronate and galactosamine. Heparin sulfates are composed of iduronate and glucosamine, and keratan sulfates have no uronic residues and are composed of galactose with glucosamine. The above GAGs are synthesized in the Golgi apparatus where they are covalently attached to protein cores undergoing post translational modifications, together they become part of the second macromolecule, proteoglycans [42].

Hyaluronic acid is the largest GAG with a molecular mass ranging from 6,500 to 10,900 kDa [43] and is composed of glucoronate and glucosamine. Because of its extreme length and high molecular weight of from hundreds to thousands of kilo-Daltons, it must be synthesized by a transmembrane enzyme complex and dynamically extruded from the cell. Unlike the above GAGs it is uniquely non-sulfated and because of its transmembrane synthesis is not attached to a proteoglycan. Instead, it
accumulates in the ground substance where with its immense water binding capacity and considerable viscosity aids in lubrication and also regulates molecular transport by slowing diffusion [41,44–46].

2) Proteoglycans consist of a long core or trunk of proteins translated (linked together) on the RER. During post translational modification in the Golgi apparatus, side chains of sulfated GAGs and oligosaccharides are added which branch out from the protein trunk giving it the look of a bottle brush. Proteoglycans are diverse with differences in the number, length and composition of the added GAG chains due to enzymatic differences between Golgi complexes. The perpendicular orientation of the attached GAG chains increases the available surface area for their abundant negative charges to bind large quantities of water molecules [41,44–46].

Aggrecan and fibromodulin are common proteoglycans in tendon and ligament. The large proteoglycan aggrecan, can weigh as much as 2,500 kDa, and has about 100 chondroitin sulfate and 60 keratin sulfate GAG chains attached in separate domains along its protein core. These highly negatively-charged chondroitin sulfate chains are arranged along the proteoglycans in a bottle brush format, increasing their surface area to attract and hold cations and water molecules. This provides the swelling pressure necessary to support the ground substance and occupy large spaces between collagen fibers. Many aggrecan proteoglycan chains bind with the even longer GAG, hyaluronic acid to form stable complexes that contribute to the mechanical properties of the ground substance [45,47,48].

A subcategory, small leucine-rich proteoglycans (SLRPs) includes decorin and biglycan. Decorin (up to 140 kDa) has a core protein chain of leucine repeats with a single attached GAG chain of derman sulfate. Its leucine core has a high binding affinity for collagen and is almost always found bound to fibers in vivo. Decorin is believed to regulate fibril diameter, length and organization during fibrollogenesis [48]. Biglycan has two GAG chains, hence the prefix “bi”. It has a role similar to decorin in fibrollogenesis and has recently been reported to affect the viscoelastic properties of tendons as well [49].
Proteoglycans also sequester powerful signaling proteins such as fibroblast growth factor (FGF). Degradation of the ground substance during injury and tissue turnover releases these growth factors to stimulate ECM synthesis and cell growth.

Figure 2.9: Glycoproteins and proteoglycans. (a) Glycoproteins are usually globular proteins with branched oligosaccharide side-chains. (b) Shown here is a TEM darkfield image of many proteoglycans linked to an axis of hyaluronan [50]. The core protein of this extremely large proteoglycan has hydrated chondroitin sulfate and keratan sulfate side chains, and is attached to hyaluronan by link proteins.

3) Glyco-proteins are polypeptides with branched oligosaccharide (3-9 sugar) side chains. In contrast with proteoglycans, their molecular weight is lower (50-100 kDa) [15] and the peptides generally outnumber the sugars in proteoglycans. They are distinguished by how the oligosaccharide
side chains are attached: O-glycosylation refers to bonding of the chain to the hydroxyl group (OH) of the peptides hydroxylsine, hydroxyproline, serine or threonine. N-glycosylation refers to bonding of the saccharide chain to the N-terminus (NH2) of the peptide asparagine.

Multi-adhesive glycoproteins are large globular protein molecules including; fibronectin, thrombospondin, tenasin-C and undulin. They are equipped with many binding sites for cells, platelets, collagen fibers and other macro molecules, and can help cells anchor themselves within the ECM ground substance [37,51].

Fibronectin is a multi-adhesive glycoprotein. It is secreted as a soluble inactive dimer of two 230–270 kDa monomers, each composed of a mixed chain of three repeating peptide sequences labeled FNI, FNII and FNIII. Different combinations of these FN modules along the chain serve as binding sites for cells, collagen, fibrin, and heparin and other macromolecules. Binding of fibronectin to the α5β1 integrin on a cell’s surface changes the conformation of the fibronectin molecule to expose additional binding sites, resulting in the further aggregation of additional cells and macromolecules together into an insoluble matrix. Fibronectin is highly expressed in tissues undergoing remodeling repair, and fibrosis, and has been called the “master organizer” by some investigators [37,51].

Figure 2.10: A) Fibronectin structure from N-terminus on left, with binding sites for cells, collagen, fibrin, FN and heparin shown. B) The ribbon structures of FN modules types I, II and III drawn using PyMOL, this illustrates the need for a conformational change to expose binding sites [51].
Tenascin C is a 300 kDa glycoprotein that has a structural relationship to fibronectin but lacks its adhesiveness to cells. Its level of expression is down-regulated in adults, but up-regulated in tissues experiencing high tensile stress and during injury and repair. It is found of the blood of humans with knee injuries and heart disease. It inhibits binding of fibronectin to its co-receptor syndecan-4 and diminishes focal adhesions. It is hypothesized that this de-adhesive effect contributes to tissue reorganization during repair.

Thrombospondins form a group of five modular glycoproteins TSP-1 to 5. TSP-1 (150 kDa) is normally stored in platelets for release after injury, and has been shown to upregulate collagen I expression during tissue repair. TSP-2 is found bound to proteoglycans and cell membrane receptors. It is also involved in collagen fibrillogenesis.

The relatively larger proteoglycan content in ligaments may be explained by their need for greater extensibility in allowing certain joint motions. As an example the intra-articular cruciate ligaments of sheep and rabbit knees have higher proteoglycan content than their respective extra-articular collateral ligaments. While tendons adapted more for skeletal positioning than energy return are in the lower range of proteoglycan content.

**Enthesis composition, morphology and function**

The attachment site of a tendon, ligament or joint capsule into bone is known as an enthesis. More than a simple insertion site, an enthesis must reduce the order of magnitude mismatch in moduli of elasticity at the junction of two dissimilar materials, hard bone and tendon or ligament. Under identical load a compliant soft tissue deforms much more than bone and this unequal strain along their interface causes stress concentrations and even tearing [52].
Two types of entheses have evolved to reduce stress concentrations. The first is the fibrous enthesis originally termed an “indirect” enthesis by Woo [52]. This is because it typically occurs in the appendicular skeleton where a tendon inserts tangentially along the diaphysis of a long bone. A subtype; fibrous periosteal entheses, attach the tendon to the periosteal tissue that wraps around the entire bone surface like a stocking. This spreads the tensile force that the tendon exerts over a wide area, reducing the stress on the interface. Collagen fibers known as Sharpey’s fibers also penetrate from the tendon through the periosteum into the lamellar bone providing further anchorage. A second subtype of fibrous enthesis known as a fibrous boney enthesis occurs where the periosteum is lacking, here the collagen fibers insert right into the bone without any intermediary periosteum. In time a fibrous periosteal insertion can transform into a fibrous boney enthesis if the underlying periosteum is lost through bone maturation [53].

The second type is the fibrocartilaginous enthesis originally referred to as the “direct” enthesis because it typically occurs on the ends of long bones with the ligament or tendon making an almost perpendicular insertion through the articular cartilage surface. Transmission electron microscopy of the fibrocartilaginous enthesis by Cooper and Misol [54] has shown it can be divided into four zones; Zone one just above the interface is of the tendon itself and consists of longitudinal collagen fiber bundles and decorin. Zone two consists of fibrocartilage and of collagen types II and III along with proteoglycans aggrecan and decorin. Zone three consists of mineralized fibrocartilage with type II collagen. And finally zone four is bone with an interdigitated interface between itself and the mineralized fibrocartilage of zone III [52,53,53,54].

This four zone morphology of the fibrocartilaginous enthesis reduces the mismatch in elastic modulus by transferring the force over three separate interfaces of progressively increasing stiffness: tendon/ligament - fibrocartilage; fibrocartilage - mineralized fibrocartilage; mineralized fibrocartilage -
interdigitated with bone. This reduces the stress concentrations at each interface. The anterior and posterior cruciate ligaments have fibrocartilaginous entheses at both ends [52–55].

Figure 2.11: Tendon enthesis. “The four zones of tissue at the tendon insertion to bone: Dense fibrous connective tissue (CT), uncalcified fibrocartilage (UF), calcified fibrocartilage (CF), and bone (B). The calcified and uncalcified fibrocartilage are separated by a tidemark (TM) that is straight and continuous with a similar tidemark in the adjacent articular cartilage (AC)” [16].

Embryonic development of the fibrocartilaginous enthesis begins during endochondral ossification when tendon/ligament attachments are made to the cartilage analog of the developing limb bone. Cartilage under the early enthesis is eroded by endochondral ossification. The deeper area of fibrocartilage is mineralized, and lamellar bone is deposited below. (See the following embryonic development section).

In tendon, the progenitor cells for a fibrocartilaginous enthesis were originally believed to arise from the growing mass of chondrocyte cells filling the bone template. However recent evidence from cell lineage analysis indicates that these future enthesis cells attach to the bone template as a separate module of cells expressing not only the Sox 9 transcription factor required for chondrocyte differentiation, but also the Scleraxis transcription factor (Scx) associated with tendon cell differentiation. Transforming growth factor Beta (TGFβ) signaling regulates the attachment of this
module, and mineralization of its inner layer by endochondral ossification is initiated by Indian hedgehog (Ihh) autocrine signaling along with parathyroid hormone-related protein (PTHrP) paracrine signaling. The exact embryonic origin for this add-on module of cells is not yet known [3,56].

Tendon to bone healing after surgery has so far failed to recreate the native four zone fibrocartilaginous enthesis typical in the rotator cuff, distal patella tendon and also of the ACL. Rather, a tendon graft heals to bone via formation of a fibrovascular scar tissue interface with material properties inferior to the native enthesis. Remodeling brings Collagen fibers that grow transversely across the bone tendon interface. This morphology is more typical of a “fibrous” enthesis such as at the distal medial collateral ligament. In the fibrous enthesis the tendon/ligament enters bone outside of the joint capsule, attaching tangentially along the shaft, with the interface reinforced with transverse collagen fibers known as Sharpey’s fibers growing across the periosteum. [2]

Vascularity

Mature tendon and ligament are generally described as avascular in the literature, as can be inferred from their pale appearance. Vascular supply comes from three locations: through the enthesis (including both ends in ligaments), through the musculotendinous junction in tendons, and from vessels running longitudinally along the peripheral layer known as the epitenon (or epiligament). The epitenon/epiligament is the outermost layer that bundles the collagen fascicles together and is also continuous with the endotenon membrane covering each individual fascicle. The peripheral blood vessels take advantage of this continuity by passing anastomoses, transverse cross connections through the endotenon to reach the interior [57].

Tendons making sharp bends such as the finger flexors are enclosed within a fibrous sheath to guide them. Synovial fluid within this sheath provides lubrication for sliding and may also provide nutrients to the tendon through diffusion. Diffusion through the synovial fluid may also supply nutrition to tendons and ligaments within the intra-articular space [57].
Embryonic development

During embryogenesis, all future tendon and ligament tissue originate from the lateral plate mesoderm (LPM) [58]. Within the early embryo the mesoderm is one of the three primary germ layers differentiating all cell and tissue lineage. Analogous to the cream filling of an Oreo cookie, the mesoderm is sandwiched between the ectoderm and endoderm layers [59–61].

As a newly fertilized oocyte’s cells continue to divide and multiply forming a spherical blastocyst, the germ layers begin as two flat plates of cells - the upper plate the epiblast, the lower plate the hypoblast. Together they form the “bi-laminar disk” - an Oreo without the cream, dividing the blastocyst into upper and lower hemispheres, each containing a fluid filled cavity; the amnion and yolk sack respectively, for cellular nourishment [59–61].

By the end of the second week in humans, dividing cells in the epiblast - the dorsal layer, have begun migrating to its centerline. This crowding of cells creates a ridge known as the primitive streak. A greater proliferation of cells at one end of the streak forms the primitive node, marking its cephalic end. Thus the embryo’s caudal-cranial axis and left-right sides are now defined, and the relative placement of the future musculoskeletal features, including all tendons and ligaments can now proceed [59–61].

During the third week an invagination opens along the crest of the primitive streak as the increasing proliferation of cells pushes them out the bottom of the streak to populate a third middle layer, the cream in our Oreo cookie analogy - the mesoderm layer. Here these undifferentiated, mobile cells loosely organize within a sparse collagen fiber matrix known as mesenchyme, the outer edges of which are known as the lateral plate mesoderm (LPM) [59–61].

The blastocyst has just undergone gastrulation - the creation of all three germ layers. The epiblast layer becomes the ectoderm; giving rise to outer body coverings, the nervous system, neural crest and brain. Mesoderm cells go on to form the musculoskeletal, cardiovascular and urogenital
systems. Additional mesoderm cells displace the hypoblast layer below to become the endoderm layer. Endoderm cells become tightly packed, polar and columnar - to form the epithelial lining of the entire gastro-intestinal tract, associated glands, and parts of the respiratory system [59–61].

Figure 2.12: Gastrulation of the blastocyst into the three primary germ layers

(https://staff.um.edu.mt/acus1/Gastrulation_files/image017.jpg)

Continued proliferation spreads the mesoderm (the Oreo cream) laterally between ectoderm and endoderm layers, and by day 21 (in humans) its mesenchyme has formed into three regions of decreasing thickness; paraxial, intermediate and LPM. Cells in the thick paraxial region group into a columnar epithelial arrangements, forming balls of cells known as somites, arranged symmetrically into pairs about the left and right sides of the notochord. This segmentation into somites proceeds rostral to caudal (head to tail) creating up to 44 paired somites by day 30. Each somite will later segment into groupings of undifferentiated cells known as dermatomes, myotomes, sclerotomes and syndetomes [62] - the precursors of the dermal, muscular, axial skeleton and tendon respectively, at each vertebral level along the spine [59–62].
Secretion of sonic hedgehog (Shh) by the notochord signals cells in the nearby ventral-medial portion of the somites to de-epithelialize and detach. These cells then surround the neural tube and notochord, organizing into a mesenchymal matrix known as sclerotome, where they express the gene *Pax1*. Out of the sclerotome, the cartilage, bone, tendon and ligament of the vertebrae and ribs will arise, forming the axial skeleton. Leaving the neural tube to form the spinal cord and the notochord the nucleus pulposus of the intervertebral discs [62,63].

Remaining cells on the dorsal side of the somites (known as the dermo-myotome) will split into the dermatome and myotome, from which the dermis and skeletal muscle arise. Expression of myogenic basic helix-loop-helix (bHLH) transcription factors Myf5 and Myo1 (formerly MyoD) drive cells from each end of the dermatome to roll underneath it creating the myotome region. Back and axial muscle precursors will emerge from the dorso-medial “epaxial” myotome. And appendicular (limb) muscle precursors emerge from the ventral-lateral “hypaxial” myotome [62–64].

Signaling from within the myotome, possibly via FGF8, induces cells in the adjacent sclerotome to express scleraxis. This Scx⁺ border strip segregates into a fourth somitic compartment of progenitor cells, the syndetome, from which all axial tendons will arise. Its name comes from the Greek syndesis, to bind and is ideally located between the two progenitor tissues it will eventually link, the myotome and sclerotome [62].

![Figure 2.13: Segmentation of the somites.](http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_05/ch05f29.jpg)
Adjacent to the somites, the intermediate mesoderm will form the urogenital structures. Further laterally, the thin LPM continues to spread out under proliferative pressure, splitting into two layers: the visceral splanchnic layer that envelopes the gut tube, and the parietal (body wall) layer, from which the cartilage, bone and, and the ligaments of the appendicular skeleton, will arise [59–61].

At two levels of the developing vertebral column, (the lower-cervical/upper-thoracic, and the lumbar-sacral) increasing expression of several Hox genes drives proliferation of the parietal LPM to the point where it bulges out bi-laterally underneath the ectoderm. These bulges are the developing limb buds of the axial skeleton, consisting of a growing homogenous core of proliferating mesenchymal LPM covered by a stretching outer layer of cuboidal ectoderm cells [59–61,65].

The distal edge of each limb bud flattens into a paddle shape (or flipper for lower limb) as fibroblast growth factor (FGF) 10 expressed by the mesoderm cells induces the ectoderm cells at the distal circumference of the paddle to form the apical ectodermal ridge (AER). Now expression of FGF-4 and FGF-8 by the AER cells feeds back, inducing continued proliferation of the mesoderm as undifferentiated mesenchymal cells, growing the limb from proximal to distal [66].

A second signaling center, the zone of polarizing activity (ZPA) arises in the lower posterior of the limb bud and engages in reciprocal signaling with the distal AER to polarize the anterior-posterior axis (thumb to pinky) of the developing limb. Briefly, FGF from the AER induces the ZPA to produce retinoic acid. Diffusion creates a posterior-anterior concentration gradient of retinoic acid through the mesoderm cells, which affects the relative amount of sonic hedgehog (SHH) these cells express. The resulting posterior-anterior gradient in SHH diminishes the amount of FGF produced along the AER, affecting morphological changes from anterior to posterior in the developing limb [66]. (Figure 2.12) [67]
Figure 2.12: Limb bud showing the apical ectodermal ridge responsible for initial signaling and the zone of polarizing activity responsible for anterior-posterior digital patterning [67].

As the “progress zone” of proliferating mesoderm grows the limb bud in length, the more proximal mesoderm cells, furthest from the influence of AER secreted FGF, soon condense into groups. Local expression of the transcription factor Sox-9 causes these condensing cells to differentiate into chondrocytes to follow the cartilage model of long bone development [59–61].

The new chondrocytes are wrapped together in a tube of perichondrium, a double layer fibrocartilage sleeve that constrains their interstitial growth to follow the limb bud axis. Changes in proximal-to-distal HOX gene expression pattern these cartilage cell condensations into templates for the individual limb bones. For example, increased expression of HOX11A at the end of the humeral template signals the beginning of the radius and ulna, while later expression of HOX12A, D signals the beginning of the carpal bones of the hand [65]. (Figure 2.13)[67].

Figure 2.13: Changes in HOX gene expression signal changes in the cartilage analog [67].
• Ligament development

This change in HOX gene expression coincides with a local change in cell differentiation from chondrocyte to fibroblast, creating a fibrocyte border between chondrocyte bone templates, and the local expression of growth and differentiation factor 5 (Gdf-5) believed to mediate formation of articular joint structure [68]. The center of the border will develop into a synovial cavity, with the adjacent chondrocytes on either side forming articular cartilage. Condensations of fibroblasts within will bridge this cavity and develop into both the ligaments and the synovial tissue lining of the new articular joint. Evidence for the role of Gdf-5 in joint development can be found in mice Cruciate ligaments that tested positive for Gdf-5 antibodies [69].

Concurrent with limb bud development, muscle precursor cells under the influence of transcription factors Pax-3 and Pax-7 begin to delaminate from the hypaxialmyotomes of those somites adjacent to the limb buds. Upon the induction of FGF and myostatin these cells are determined as myogenic progenitors and migrate into the limb bud as dorsal (future extensor) and ventral (flexor) muscle masses. With Myogenic regulatory factors Myf-5 and Myod1 expression (bHLH transcription factors) they coalesce into muscle fibers and under myogenin they specify into extensor or flexor muscle [62,64].

Tendon development is initiated by signaling from the ectoderm within a broad sub-ectodermal layer above and below the developing cartilage model. This signaling induces cellular expression of tendon specific markers such as follistatin and scleraxis - a basic helix-loop-helix (bHLH) transcription factor essential for tendon differentiation. However the depth of tendon cell specification is limited by BMP signaling from the interior mesenchyme which blocks scleraxis. Patterning of the tendon is aided by a scaffold of mesenchymal lamina on both dorsal and ventral sides of the limb bud [64,69,70].
Complex spatial and temporal changes in gene expression direct the morphogenesis of the distal tendons, which develop apart from (and even without) their respective muscles, although the maturation and segregation into individual tendons requires muscle cell migration into the limb bud. In contrast the development of the proximal tendons is tightly coupled to that of the muscle precursors. However the complex reciprocal signaling that directs the formation of the myotendinous junction and the muscle/proximal tendon to its appropriate skeletal origin is still unknown [63,65,71].

Ridges and prominences on bones appear as the beginning of entheses, tendon insertion sites. Originally the projections on the cartilage model, the developing entheses, were thought to consist of cells separating out of the cartilage template, however recent cell lineage tracing studies show evidence that the entheses cells derive from an external pool of progenitors that are added on shortly after the existing bone template is formed. These cells express both Sox9 and Scx and appear to arrive at the attachment site as separate module from the chondro-progenitors within the bone template [3,56,72].

Like tendon cells, the enthesis progenitors co-express Sox-9 and scleraxis, setting them apart from the chondrocyte fate which requires only Sox-9. If the enthesis does form as a separate module which attaches to the bone analog, this could offer new opportunities for tendon and ligament reconstruction. Could a stem cell patch, seeded with Sox9 and Scleraxis be anatomically placed to create a new enthesis for a torn ligament or tendon?

**Mechanism of Injury**

Tendon and ligament injuries can be classified by their location and etiology. One of three locations are reported: 1) through the mid-substance of the tendon/ligament, 2) at the enthesis, or 3) an avulsion – a fracture through the bone below the enthesis, which can result in a detached tendon/or ligament with a fragment of periosteum or enthesis still attached [73].
The etiology can be immediate from trauma and acute overload or it occurs over time due to the accumulation of micro and macro tears, with cellular repair efforts lagging. Unfortunately, the most common cause of flexor tendon injuries is accidental laceration, often during work and food preparation activities. Most acute ruptures of the strongest human tendon, the Achilles occur in fit men during athletic activities, with biopsies showing evidence of chronic degeneration. This implies fatigue failure from chronic degeneration reducing the tendon’s mechanical properties to the point where it fails suddenly under a previously sustainable load. Rotator cuff tendon tears occur at the entheses on the humeral head and are common in the aging, with over 50% of those in their 80’s having a full thickness tear. Here the etiology appears to be a combination of factors including accumulated micro trauma, abrasion from under the acromion and age related degeneration [74].

The need for a strategy to prevent ACL rupture continues to drive research on understanding the mechanism of this injury. Proposed theories include; excessive force of quadriceps contraction, imbalance between hamstring and quadriceps forces, and even accumulated wear from abrasion by the intercondylar notch of the femur on the ACL. Female athletes participating in pivoting and jumping sports suffer a 2-8 fold greater rate of ACL injury than their male counterparts. This has generated theories implicating excessive knee valgus on landing causing knee abduction moments, joint laxity from hormonal effect, knee recurvation and reduced ACL size, to explain the increased rate in women [23].

Recent theories focus on impulsive compressive force generated on the knee joint during landing, which results in a motion of the tibia similar to the clinicians pivot shift test for ACL laxity. Here the resultant force and moment simultaneously pull the tibia forward while rotating it internally, thus stretching the ACL past its ultimate strain. Implicated also in this theory is a flat footed landing that reduces the ability of the calf muscles to absorb the ground reaction force and lessen its impulsive impact on the knee [23,75].
Native tendon and ligament healing process

Similar to dermal wound healing, healing in tendon and ligament consists of four sequential phases; coagulation, inflammation, proliferation, and a lengthy repair/remodeling phase. Initiation of the first phase requires bleeding – And while the tendon/ligament inner substance is an avascular bundle of collagen fascicles, rupture of the capillary network in the epitenon, (epi-ligament) its outer sheath, brings soluble blood proteins, normally shielded by endothelium, into contact with ECM collagen fibers and glycol-proteins. This triggers a complex, yet rapid series of reactions resulting in blood coagulation and inflammation. Progression through the subsequent healing phases requires release of chemokines and cytokines by activated platelets and leukocytes to recruit the necessary phagocytic, proliferative and remodeling cells from the surrounding vasculature and/or synovium [76–78].

Coagulation begins when platelets from the exuding blood coalesce at the wound site, their numerous surface receptors and integrins bound to collagen fibrils and adhesive glycoproteins among the exposed ECM, including laminin, fibronectin, thrombospondin and von Willebrand’s factor. Ligation of G-protein coupled surface receptors initiates an internal calcium mediated signaling path, stimulating two enzymes, phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3k), activating the platelet. [79,80] The platelet collagen receptor glycoprotein VI (GPVI), recognizes the repeated α-chain peptide sequence glycine, proline, hydroxyproline (Gly-Pro-Hyp), while Integrin α2β1 has affinity for several other collagen sequences including Gly-Phe-Hyp-Gly-Glu-Arg. Collagen ligation of GPVI alone is believed sufficient for platelet activation, although whether concurrent ligation of integrin α2β1 is also required is a matter of debate and ongoing research [79].

Activated platelets provide a catalytic surface (net negative charge with unsaturated acyl chains) for the blood coagulation system to restore hemostasis. Exposure of tissue factor on the ECM to circulating factor VII in blood initiates a cascade of reactions culminating in the conversion of
prothrombin to thrombin by activated factor X. Thrombin now cleaves fibrin from inactive fibrinogen. The resulting fibrin molecules readily bind together into fibers that crosslink into a reinforcing mesh across the platelet aggregate. Ideally this fibrin reinforced coagulation will hold the torn tendon/ligament fibrils together, facilitating repair by fibroblasts in the subsequent healing phases [79]. Coagulation however, must be regulated to prevent downstream ischemia. With negative feedback, tissue factor pathway inhibiter (TFPI), a protease inhibitor slows factor Xa’s conversion of prothrombin [81]. Plasmin, cleaved by thrombin from plasminogen, must eventually dissolve the fibrin coagulation and restore circulation, once the vessel endothelium is repaired [82].

Activated platelets release a burst of chemokines and pro-inflammatory cytokines from internal \( \alpha, \delta \) and \( \lambda \) granules. Platelet \( \alpha \) granules contain a large number (>280) of bio-active proteins including growth factors necessary to drive the proliferative and remodeling phases including; platelet derived growth factor (PDGF), transforming growth factor \( \beta \) (TGF-\( \beta \)) and fibroblast growth factor (FGF). Granule release of additional VWF and fibronectin amplifies the clotting cascade and furthers platelet aggregation. Recent evidence indicates that differential release of granules by platelets can selectively modify the wound site environment and regulate healing [80].

Inflammation begins with a loss of integrity between the normally tight junctions of blood vessel endothelial cells, allowing an efflux of cells and plasma proteins into an injured tissue to promote its repair. One inflammatory pathway occurs when circulating prekallikrien adheres to a negatively charged surface (platelet) facilitating activation by factor XII. Activated kallikrien cleaves high molecular weight kininogen, freeing bradykinin. Ligation of bradykinin to the B2 receptors on endothelial cells then activates their vasodilation [83]. Chemokine interleukin 8 (IL-8) attracts an influx of polymorphonuclear cells, or neutrophils (PMNs) from the surrounding blood vessels and synovium. These immune cells
begin by removing pathogens and wound debris by phagocytosis. PMNs arrive soon after platelet
degranulation, and reach a peak number early – as early as one day post injury in aseptically transected
rat MCLs [78], provided there is no bacterial infection.

Tissue damage can trigger activation of the complement cascade, a system of circulating innate
immune system proteins resulting in the release of potent chemoattractants, anaphylatoxins C3a and
C5a that recruit circulating monocytes to the wound bed where cytokines promote their differentiation
into mature tissue macrophages. These circulation derived monocytes/macrophages are identified by
their expression of antigen ED1 and are catabolic and phagocytic, clearing the wound bed of debris,
pathogens and apoptotic PMNs. A separate population of antigen ED2⁺ resident macrophages does not
participate in phagocytosis and is believed to have an anabolic role, stimulating cell mitosis and down
regulating `inflammation during the proliferation and remodeling phase. These resident ED2⁺
macrophages reside primarily in the epiligament/epitnon.

Macrophages follow PMNs and soon become the dominant immune cell type. One rat study
showed a peak population of both ED1 and ED2 types by day 5 after MCL transection in the rat.
Compared with a rat ACL-R study showing an absence of ED2 macrophages until day 11 postop, peaking
from day 14 to 28 reflecting time to migrate into the graft. Within several days after injury, tissue
macrophages have become the dominant cell type within the wound bed, and continue clearing it of
debris, pathogens and early arriving apoptotic PMNs by phagocytosis. Macrophages also produce
protease and metalloprotinase enzymes that facilitate the wound debridement process by cleaving
damaged collagen fibrils.

The presence of T-lymphocytes attracted by interleukin 1 (IL-1) marks the late inflammatory
phase and the transition to the proliferative phase. Now macrophages release TGF-β, PDGF and vascular
endothelial growth factor (VEGF), attracting an influx of fibroblasts into the wound bed. While the
fibroblasts fill the voids left from debridement by randomly extruding collagen fibrils and fibronectin,
VEGF prompts the infiltration of endothelial cells which form a capillary network to support this new granulation tissue.

The fourth, remodeling phase involves removal of the poor quality granulation tissue and its replacement and realignment along the lines of tensile stress with an organized matrix that approaches the mechanical properties of the original tissue [77].

Ligaments such as the medial collateral ligament (MCL) of the knee follow the above four phase process and are treated conservatively by clinicians, typically healing with rest and without the need for further intervention. This is because the MCL runs outside of the joint capsule for most of its length and has an ample blood supply from both surrounding tissues and its vascular tendon sheath, facilitating the arrival of platelets and the formation of a fibrin clot to hold the torn MCL ends together. The clot serves as a scaffold both for neovascularization to support the three phase healing process and for fibroblast cells to cross and lay down collagen fibers that restore the original matrix and its directional orientation [84].

Unfortunately the prognosis for the anterior cruciate ligament (ACL) is much poorer. Murray et al. examined 23 completely ruptured human ACL specimens recovered over a wide range of from ten days to three years after initial rupture when the patients underwent open knee surgery. Their study provides a unique histological documentation of all phases of the human ACL healing process. (Murray 2000).

The histology of those ACL specimens recovered up to three weeks post rupture appeared consistent with the inflammatory phase of healing. Staining of specimens revealed concentrations of immune cells around damaged capillaries near the break including neutrophils, lymphocytes and macrophages actively phagocytosing debris. Some clotting was seen on individual ACL remnants but never any bridging connection unifying both ends, instead the ACL remnants appeared swollen with torn
Friable ends resembling “mop ends”. Blood vessel density increased to a mean of 4 vessels / mm compared to intact control ACLs with 1.5 vessels / mm. Cell density had decreased slightly to a mean of 614 cells /mm compared with 701 /mm in controls.

Specimens from three to eight weeks post rupture showed a gradual overgrowth of synovial tissue covering the ligament remnants, eventually encapsulating them and giving them a mushroom like appearance. No evidence of any clotting or granulation tissue bridging the torn ACL remnants, nor any vascular proliferation had occurred. Clearly the second proliferative phase of healing had not begun. Murray coined the term “epiligamentous repair period” to describe this three to eight week encapsulation period specific to ACL healing. Within the synovial sheath growing over the ligament remnants, fibroblast proliferation and blood vessel density necessarily increased, with an abundance of these fibroblasts expressing α-smooth muscle actin, a protein with contractile properties.

Specimens from eight to twenty weeks after rupture was showed increasing blood vessel density and cell proliferation within the remnants characteristic of the proliferative healing phase, reaching a peak cell density of 2,244 /mm between 16 and 20 weeks. Most were fibroblasts arranged without any particular alignment to the ligament axis. The epiblast layer now completely covered both proximal and distal remnants and merged with the synovial capsule.

In ACL specimens from one to two years after rupture, the ligament ends remained separated, although remodeling had begun. Fibroblasts and collagen fascicles were now more aligned with the ligament remnant axis. Both blood vessel density and cell density had decreased from their maximum achieved between 16 – 20 weeks post rupture. The synovial tissue sheath over the remnants persisted but decreased in thickness. Clearly a healed functional ACL had not reformed.

The failure of a provisional scaffold to form bridging the two mop ends together; a necessary requirement for cells to invade and begin the proliferative and remodeling phases as in extra-articular
ligaments like the MCL, is likely due to the serine protease plasmin that rapidly degrades fibrin. Its precursor plasminogen, is present in the synovial fluid of the knee joint and is activated by a urokinase plasminogen activator produced by synovial cells after an injury. This prevents any coagulation from forming within the intra-articular environment that could restrict joint movement [82].

**Early attempts at primary repair of the ACL**

In 1972 before the advent of arthroscopic surgery, surgeons Feagin and Curl began studying ACL ruptures in 64 West Point Cadets [85,86]. All had undergone arthrotomy, visual verification of the ACL tear and an open primary repair. Each tear was at the proximal end of the ACL (at the femoral enthesis). Primary repair here meant a suture only repair, which consisted of a figure 8 stitch in the tendon to avoid pull out. The ends of the suture pulled through one or two holes drilled into the lateral femoral condyle, securing the severed end of the ligament back onto the femoral enthesis from which it had ruptured (Figure: 8). In their 1975 follow up study, Feagin and Curl [85,86] reported that of the 32 interviewed, 24 reported impaired athletic ability, and 12 required additional surgery. The second surgery revealed 6 with ACLs that were so lax that they were judged incompetent, two that were re-torn, and only 4 with adequate function. Further follow-up of these same cadets in 2009 revealed that greater than 50% reported impaired function in their repaired knees [86].

As a result of these and other studies, attempts at primary repair have been abandoned, in favor of complete ligament reconstruction, as this quote from Taylor et al eloquently states: “In the 30 years since the first West Point reports, ACL tears have become a well-recognized orthopedic injury. Surgical treatment has evolved from the initial attempts at primary repair to reconstructions that are now one of the most commonly performed orthopedic operations” [86].
ACL Reconstruction Procedure

The decision to undergo ACL reconstruction rests on the patient who must weigh managing recurrent knee instability, possibly with meniscal tearing and accelerated knee arthrosis, against the time lost undergoing ACL-reconstruction with 6 to 12 months of rehabilitation (along with the possibility of a failed repair). Reconstruction is recommended for athletes returning to play and most active patients opt to go ahead with it.

Currently most ACL reconstruction procedures are performed arthroscopically and consist of a tendon graft to replace the torn ACL that is pulled through a tunnel drilled in the anterior tibia, exits on the tibial plateau, crosses the joint space and terminates in a femoral socket drilled within the lateral side of the intercondylar notch. The goal is to place the tunnel exit and socket entrance within the footprints of the native tibial enthesis and femoral enthesis, respectively, with the graft appropriately pre-tensioned and anchored with interference screws, to approximate the anatomic position and function of the native ACL.

The decisions made on operative procedure, type of the tendon graft used and its source, autograft or allograft from a donor, are all currently controversial, each having pros and cons, with no clear evidence for the superiority of any one over another in the literature, with the final choice often depending more on the training, ability and experience of the surgeon.

Quadrupled hamstring, quadriceps tendon or bone-patella tendon-bone (BPTB) are commonly used for tendon autografts. The later BPTB, is harvested with bone blocks at each end, giving it the advantage of a friction fit into the femoral and tibial bone tunnels (with interference screws) and is recommended for athletes and those desiring a quick return to activity, in the hope that it will reduce graft slippage and prevent developing knee laxity. Autografts are free of concerns for disease transmission and immunologic rejection. Although they do result in longer operations, additional
harvest site wounds and are associated with increased risk for impaired flexion/extension function in the source muscle, and with increased donor site morbidity, particularly after the patellar bone block harvest for a BPTB graft.

Allografts are used in revision ACL-r when it is desirable to avoid harvesting from the contralateral limb, and in patients requiring a less demanding postoperative rehabilitation. Disadvantages of allografts include the possibility of immunological rejection, arthrofibrosis and higher costs for sterilization and storage. Sterilization is of necessity required for allografts, and unfortunately degrades the graft mechanical properties and is associated with higher failure rates.

An established ACL-r procedure is the transtibial (TT) technique where a portal is made and the tibial tunnel is drilled first. The drill then continues through the tibial tunnel into the joint-space and drills the femoral socket, without requiring an additional portal. Criticism of this technique comes from its tendency to position the femoral socket too high in the intercondylar notch, resulting in a non-anatomic vertically oriented graft that cannot constrain internal knee rotation [87]. An alternative technique is the anterior-medial portal (AMP) that requires an additional portal and increased knee flexion to drill the femoral socket within the native enthesis footprint. Proponents claim it results in a more obliquely oriented and anatomically correct graft [88]. A third technique known as the double bundle or double socket technique uses a doubled graft to replicate the distinct anterior-medial and posterior lateral fascicle bundles of the native ACL, where the anterior medial bundle resists anterior tibial translation, and the posterior lateral bundle resists tibial rotation [89]. Unfortunately a meta-analysis has failed to show the superiority of one technique over another [87].
Effect of Rehabilitation

No standardized rehabilitation program for ACL reconstruction exists and protocols vary among practitioners and institutions. Yet rehabilitation is essential to regain the muscle strength and knee range of motion (ROM) commonly lost with ACL rupture [14]. Unfortunately, surgical ACL-R brings additional swelling and effusion that further contribute to reduced ROM and muscle strength.

No universally effective method has been found to restore full pre-injury strength, and the muscle weakness associated with ACL injury persists with many patients having quadriceps muscle strength deficits in excess of 20% after 6 months of rehabilitation. Strength differences between the injured and un-involved leg can persist for years afterward and contribute to knee instability and early arthritis [90,91].

A reduced ability to voluntarily activate the quadriceps muscle known as arthrogenic muscle inhibition (AMI), along with already diminished strength, retards the progress of rehabilitation exercise. Diminished proprioception, the positional awareness lost with the stretch sensitive nerves within the original ruptured ACL, is a proposed etiology for AMI [90]. Unfortunately, since the quadriceps maintains dynamic knee stability through co-contraction with the posterior thigh muscles (hamstrings), any weakness or delayed activation places further strain on the healing tendon graft ACL-R. Weakened quadriceps and hamstring muscle groups leave the vulnerable ACL-R as the only taught tether restraining a femur sliding on the tibial plateau with the weight of the body above.

During the early postoperative weeks, the healing tendon graft ACL-R is secured only by interference screws or other fixation devices and can be dislodged if overstrained, therefore the patient must be guided through exercises that incrementally progress in ROM and intensity [14]. To protect it many surgeons prescribe a knee brace to be locked at or near full extension for walking and activities of daily living (ADL). However many studies show that brace use has little effect on long term knee stability,
and may even contribute to disuse muscle atrophy [92]. If additional repairs are performed during the same operation, longer term brace wear from four to eight weeks may be prescribed. These include; meniscal repairs, articular cartilage repair procedures including micro-fracture and OATS, an “over the top” ACL-R performed in skeletally immature youth (with a non-calcified growth plate), or a concomitant MCL injury that could further destabilize the knee [90,93].

ACL rehabilitation has evolved historically along with ACL-R techniques, often without objective evidence to support the protocols. In the 1970’s prior to the advent of arthroscopy, arthrotomies were made to access the knee joint and attempt either a sutured primary repair or primitive reconstruction. Afterwards the leg was placed in a plaster cast for up to 6 weeks of immobilization [85]. The practice of casting continued until it was observed surprisingly, that noncompliant patients who resumed activity early had superior functional results [94].

Quadriceps strengthening exercises were downplayed during the 1970-80’s to protect the graft from overstrain. In their place the use of Continuous Passive Motion (CPM) machines was copied from total knee arthroplasty practice to cyclically flex the knee without muscular activation. These have now been largely abandoned in favor of active motion exercises that help maintain muscle strength. Patients were not cleared for return to sports until the subjective criterion of six months post operation had passed [94].

A 1987 study hypothesized that early resumption of passive knee motion after ACL-R would not increase swelling and effusion. By week three a group that had begun CPM early on post-op day two lost an average of only 2 -3.5 cm of thigh girth. This compared favorably to a group which started CPM later on day seven and lost an average of 6.5 cm girth. However since the ACL-R in the early group was performed arthroscopically while the ACL-R in the late group was via an arthrotomy, it is difficult to say
whether time of onset or invasiveness of operative method more responsible in preserving thigh girth, although the authors vigorously advocated for an early resumption of motion in the conclusion [95].

A common goal of 6 months to return to sports activity is often given, although it can take as long as a year, the actual duration depends more on the patient’s compliance and achievement of performance milestones before advancing to the next phase of the protocol. Before clearance to return to any sport with pivoting, cutting and jumping activities the patient must demonstrate safe functional ability [94]. A generic protocol typically consists of one preoperative phase to reduce pain and swelling and four postoperative phases [14,92–94,96,97]:

Phase 0, Preoperative: Begins as soon as possible after injury. Object is to reduce pain and swelling and maintain ROM and forestall muscle atrophy.

Phase 1 Postoperative (Day 0 to 4 weeks): Begins on the gurney after ACL-R surgery with isometric quadriceps contractions and ankle pumps. Goals are to resolve postoperative swelling, regain full knee extension and up to 90° to 125° of flexion (depending on program), and restore ability to control the leg while weight bearing, all while protecting the tendon graft from overstrain.

Neuromuscular electrical stimulation may be added to increase quadriceps contraction during active motion exercises. Some programs end crutch use during the first week if gait is normalized with brace on, and end brace use soon after.

Phase 2 (weeks 5 – 11) Goals: Increase knee, hip and ankle strength, improve balance and proprioception. Regain full ROM at a pace of 15° per week. Less aggressive protocols aim to end crutch use by week six. By the eight week closed chain exercises including full squats and lunges are added to improve eccentric quadriceps and eccentric hip abductor strength.
Phase 3 (12 – 16 weeks): Goals regain 85% of the strength of, and reduce dependence on the non-operative leg. Develop eccentric muscle control to enable impact activities such as jogging. Before progressing to phase four the patient must be able to land from a hop showing good eccentric hip abductor control, quadriceps control and external rotation control.

Phase 4 (4 - 6 months): The overall goal is to return to pre-injury level of play or activity. The patient must develop neuromuscular control for rapid change of direction and quick stop movements.

Criteria for return to play are; demonstration of single leg impact control, full ROM, 85% of the strength of the noninvolved leg, less than 15% deficit in hamstring - quadriceps strength ratio and no pain or swelling.
Thesis Outline and Hypothesis Formulation

An essential goal of this chapter was to emphasize the complexity of tendon/ligament healing, a process of four sequential phases, each a prerequisite for the next and requiring temporal recruitment of specific cell types for completion. While connective tissues with adequate blood supply such as the Achilles tendon and the collateral ligaments normally heal through conservative management, tendon and ligament tears occurring within the synovial joint capsule often fail to heal due to dissolution of the initial coagulation, effectively preempting subsequent phases of the healing process.

A complete ACL rupture is a common and debilitating example of this failure to heal in the synovial environment. The gold standard for its care being surgical reconstruction via a tendon graft through bone tunnels, followed by a lengthy rehabilitation period at a large personal and societal cost, with over 120,000 per year in the US.

Clinical Problem

Immediately following ligament reconstruction the surgeon is faced with a dilemma, will the prescribed rehabilitation protocol adversely affect the healing tendon graft? A recent clinical trial of early aggressive ACL-R rehabilitation showed radiological evidence of bone tunnel widening [98]. Will the repeated tensile loading imposed during passive or active knee motion cause creeping elongation of the tendon graft? Increased knee laxity from either cause could result in failure of the reconstruction.

Yet active muscle contraction must begin immediately, preferably on postoperative day one, if the atrophic process is to be reversed, and the patient is to reacquire sufficient proprioception governing effective co-contraction of quadriceps and hamstring muscles, essential to stabilizing the knee joint. Otherwise the vulnerable tendon graft, secured primarily by interference screws (prior to bone tunnel ingrowth and remodeling), will be the sole restraint to relative tibial-femoral motion.
Ideally, to prescribe an optimum time point to initiate postoperative rehabilitation after ACL-R, the surgeon must balance the need for stress shielding allowing tendon graft to bone healing, with the active knee motion needed to retain/regain muscle strength and coordination. However little objective evidence exists to guide this prescription, and the time-point when tensile load ceases to be an obstacle to healing and instead a promotor of bone tunnel ingrowth and graft homeostasis remains unknown.

The long term goals of our research group are to better understand the intrinsic cellular and extrinsic mechanical factors that govern tendon to bone healing and to develop methods to recapitulate the native enthesis whether fibrous or fibrocartilagenous, in surgically repaired tendons and ligaments. The objective of this thesis is to identify the optimum time-point to initiate postoperative knee motion in a rat model of ACL-R. To do this we will test the following hypotheses:

**H₀**, we predict that immediately following ACL-R; cycles of tensile load on the tendon graft, applied daily for two to four weeks, will impair tendon graft to bone tunnel healing, compared with continuous postoperative load shielding of the graft for two to four weeks, as evidenced by changes in bone tunnel volume, cell populations and tensile strength of the ACL-R.

**H₁**, we predict that immediately following ACL-R; a load shielding period of 4 to 10 days, prior to the imposition of daily tensile graft loading, ending on postoperative day 14 or 28, will improve tendon graft to bone healing, compared to both immediate onset daily graft loading, and continuous load shielding.

**Rational:** We formulated these hypotheses based on our preliminary data showing a gradual, sequential infiltration of immune cell types into a transplanted tendon graft [10], entering first the new granulation tissue between tendon and bone tunnel wall. While populations of neutrophils engaged primarily in phagocytosis peaked by day four, those cells with a role in remodeling, such as ED2 antigen positive macrophages – believed capable of osteoclast differentiation, did not appear in the inner tendon graft until day 14, coinciding with peak vascular ingrowth. We posited that delaying rehabilitative motion until
these cells began repopulating the graft would have the most beneficial anabolic effects, while immediate motion would create a continuous influx of inflammatory cell types, with secretion of catabolic cytokines such as IL-1 and TNF-α, and production of metalloproteinases which while useful for debridement, would degrade and weaken the tendon graft.

**Significance:** May provide basis to contradict clinical trend toward immediate post-op mobilization. Improved rehabilitation protocols that improve the strength of ACL_Rs may help reduce the reportedly higher rates of failed reconstruction in young highly active patients.

**Aim 1:** Design a device to exert controlled tensile strains on a tendon graft healing into bone tunnels, in a rat model of ACL_R. Additionally, the device must also shield the tendon graft from strains induced by knee joint motion between experimental sessions. (Completion of aim 1 is detailed in chapter 3.)

**Aim 2:** Determine the effect of cyclic tensile load on tendon to bone healing. We applied protocols of either; a daily session of cyclic tensile strain, or continuous knee immobilization, to subjects on postoperative day one after receiving ACL_R, with durations of either 2 or 4 weeks. Healing was assessed through analysis of immune cell influx, bone tunnel ingrowth, mineral content, angiogenesis and failure load of the femur-tendon-tibia construct.

**Aim 3:** Determine the effect of varied immobilization periods prior to cyclic tensile loading, on tendon to bone healing. Post ACL_R we randomly assigned groups to four and ten day immobilization periods before beginning a daily protocol of cyclic tensile strain. Subject groups were sacrificed at either two or four weeks post ACL_R. Healing was assessed through analysis of immune cell influx, bone tunnel ingrowth, mineral content, angiogenesis and failure load of the femur-tendon-tibia construct.
Chapter Three:

A Novel In Vivo Joint Loading System to Investigate the Effect of Daily Mechanical Load on a Healing Anterior Cruciate Ligament Reconstruction.

Preface

Chapter three details fulfillment of the specific aim upon which all subsequent data collection depended, and was a published article in the ASME Journal of Medical Devices. It required a device to exert controlled tensile strains on a tendon graft healing into bone tunnels, modeling the effect of daily ACL-R rehabilitative sessions on the graft. Between experimental sessions the device was also required to function as a strain shield by blocking knee flexion and rotation.

To continue use of our established rat model of ACL-R, the most viable method for strain shielding the graft was via external mechanical knee fixation. Although the ACL experiences all strains; tensile, compressive and torsional, we decided to limit our model to uniaxial tension to simplify data collection and any future mathematical modeling. This decision required design of a complex external fixator to maintain the femoral and tibial bone tunnels in coaxial alignment, with the additional requirement that it split in two during experimental sessions, allowing its tibial half to be translated distally while constrained parallel to the tunnel axis, thus achieving uniaxial graft strain.

The methods section details design of this external fixator and its use with custom jigs during ACL_R surgery to facilitate coaxial drilling of the femoral and tibial bone tunnels (Figure 3.2). Unfortunately the thin 0.9 mm diameter threaded pins used to attach the fixator halves to the femur and tibia exhibited excessive bending under load, reducing both the stepper motor applied distraction of the joint space and the effective graft strain.
This called for an extensive characterization of mechanical compliance explained in section 3.1. Here optical tracking of reflective makers across the joint space measured the true knee joint distraction while load was applied through the fixator, in eight rat cadaver specimens of ACL_R. From each of the eight optically recorded displacement versus load relations, the original LVDT measured displacement-load relation was subtracted giving the total system compliance for each specimen. All eight tests exhibited a linear deformation characteristics, therefore a least squares approximation of 41.3 µm/N was calculated to use as a correction factor in the subsequent experiments.

Abstract

We designed and validated a novel knee joint fixation/distraction system to study tendon to bone healing in an in vivo rat model of anterior cruciate ligament (ACL) reconstruction. The system uses an external fixator to apply a cyclic distraction of the knee joint while monitoring the resultant force developed across the joint space, thus providing a temporal indication of structural changes during the healing process of the bone-tendon-bone reconstruction. Validation was performed using an optical kinematic tracking system to determine the local displacement of the knee. Average system compliance was determined to be 42.4 ± 8.8 µm/N with a coefficient of variation of 20.7%. The compliance was used to obtain a best fit correction factor which brought the total root mean square error of knee joint distraction to within 179 µm (16.1%) of the applied distraction. We performed a pilot study using 15 rats that had ACL reconstructions using a flexor digitorum longus tendon autograft and found that the animals tolerated the indwelling fixator and daily anesthesia over a 10 day loading protocol. Our knee joint fixation/distraction system provides a valuable tool to study how mechanical stimuli affect in-vivo bone-tendon-bone healing.
Introduction

A traumatic rupture of the anterior cruciate ligament (ACL) of the knee is a common athletic injury with as many as 100,000 occurring each year in the United States [99]. Loss of ACL function results in knee instability with the potential for further knee damage, and may predispose the individual to the early onset of osteoarthritis. Surgical treatment consists of ligament reconstruction using a tendon graft, placed in bone tunnels drilled into the femur and tibia, approximating the position of the native ACL and re-establishing its constraining link (Figure 3.1). This procedure requires secure tendon to bone healing to be successful [86].

The resumption of mechanical load on the reconstructed ACL normally begins during postoperative rehabilitation. Typically this rehabilitation involves some degree of passive and active joint motion, while avoiding excessive loading of the tendon graft as it heals to the bone. This joint motion places the tendon graft in tension, and produces shear stress at the tendon to bone tunnel interface [2]. Although it is well established that tensile strain has a positive effect on tendon and ligament physiology, its effect on the healing tendon-bone or ligament-bone junction is poorly understood. As a result, there is little consensus on the optimum time of onset of post-operative rehabilitation and the subsequent mechanical loading of the ligament graft [100,101].

The insertion site or entesis of the native ACL is a highly specialized tissue consisting of a progression through four distinct zones: ligament, fibrocartilage, mineralized fibrocartilage, and bone, that serves to resist stress concentrations and securely anchor the ligament to bone [54,102]. Mechanical loading is thought to play a role in the post-natal development of these four zones [103,104]. Animal models of ligament reconstruction via tendon graft demonstrate that the four zone morphology and composition of the native entheses is not restored during the healing process. Rather, a tendon graft heals to bone via formation of a fibrovascular scar tissue interface, with material properties inferior to the native entheses [2,16].
Our long-term goal is to study the effects of mechanical loading on the healing tendon-to-bone tunnel interface, and hopefully identify a mechanical load regime and time of application for optimal rehabilitation of post-surgical ACL reconstruction. We developed a novel mechanical system using a previously developed rat model of ACL reconstruction [105,106]. A custom designed external joint fixation device (ex-fix) is surgically placed during ACL reconstruction on the femur and tibia of each animal. This ex-fix is interfaced to our mechanical loading system to apply controlled cycles of distraction to the reconstructed knee joint, reproducing the relative graft motion of the tendon in the bone tunnel, or interface shear described above. Our novel system allows for repetitive, controlled loading of a healing tendon graft reconstruction an in-vivo animal model.

To effectively allow comparison of subject groups undergoing different loading protocols and to measure temporal changes in force as a function of joint distraction during healing, the system was designed to satisfy the following requirements:

1. Allow for cyclic distraction of the knee joint space in an anesthetized animal that has undergone ACL reconstruction using a tendon graft placed in bone tunnels in the femur and tibia.
2. Allow the experimenter to vary the amount of distraction and number of cycles.
3. Record the force generated across the knee joint space as a function of distraction, for each cycle, of each loading session.
4. Constrain knee joint motion between loading sessions to eliminate or minimize uncontrolled distraction during cage activity.
5. Avoid undue discomfort and allow normal cage activity and feeding.
Figure 3.1: Illustration of an ACL reconstruction using a tendon graft pulled through drilled bone tunnels in the femur and tibia. The graft is secured with sutures to the periosteum at the tunnel exits, outside of the joint.
Methods

System Overview. The system consists of two components: a metal, external fixator that spans the knee of each animal and a motorized Cyclic Distraction Mechanism (CDM). During daily loading sessions the ex-fix is attached to the CDM. The bar linking femoral and tibial parts of the ex-fix is removed, and controlled cyclic distraction of the knee joint is performed by the CDM, resulting in a cyclic elongation of the healing tendon graft.

Figure 3.2: Illustration of ex-fix placement immediately before ACL reconstruction.

Mounting jigs are temporarily supported on the bone tunnel drill bit. These jigs hold the ex-fix parallel to the tunnel axes, while two pins each, (0.9mm threaded k-wires) are drilled into the femur and tibia. After the ex-fix pin clamps are tightened down onto these pins, the drill bit and mounting jigs are removed. The graft tendon can now be pulled through the tunnels and secured with sutures to the periosteum at the tunnel exits, completing the ACL reconstruction.
Fixator Design.

The ex-fix provides for the controlled distraction of the knee joint and the elimination or minimization of knee motion when locked with a rigid bar between loading sessions (Figure 3.2). It consists of identical femoral and tibial “pin-clamps”, suspended by universal joints from the removable locking bar. The universal joints allow positioning of each pin-clamp so its jaws can be tightened onto two threaded 0.9 mm diameter pins (MicroAire 1600-635T Surgical Instruments, Charlottesville, VA) drilled into each bone, securing the ex-fix to the animal. This pin diameter was the maximum allowable without inducing an unacceptable incidence of fractures. After final adjustment, the screws on the universal joints and pin-clamps are tightened to maintain their relative orientation.

Initial prototypes of the ex-fix demonstrated excessive compliance caused by cantilever bending of the 0.9 mm diameter pins, which limited knee distraction. As a remedy a bilateral support of one pin from each bone was implemented (Figure 3.3). Tubular clamps supported from the CDM secured the free end of the threaded pin protruding through the skin on the medial side of the limb. This reduced pin deflection and compliance of the ex-fix to an acceptable amount relative to the distraction distance, as shown in the results.
Figure 3.3: Anesthetized rat during loading in the CDM with bilateral support of bone pins.
**Cyclic Distraction Mechanism.**

The CDM used a linear bearing (Nippon Bearing No. SEBS 12A2-145, Wood Dale, IL) consisting of two roller bearing blocks riding on a rigid linear rail (Fig.3). A stepper motor driven linear actuator (Oriel No. 18503 via Oriel controller No 20010, Oriel Corp., Stratford, CT. Accuracy: ± 5 μm, repeatability < 2 μm) moves one block while the other is held stationary by a load cell (Transducer Techniques MDB-5, Temecula, CA. nonlinearity < 0.05 % of rated output, nonrepeatability < 0.05 % of rated output) fixed to the frame of the CDM. A linear variable differential transformer (LVDT), measures the displacement of the moving block (Schaevitz LBB315 PA-100, Measurement Specialties, Inc., Hampton, VA. linearity < ±0.20 % of full range output, repeatability 0.10 μm).

During animal loading, the ex-fix is attached to the linear bearing with its femoral part secured to the stationary block and the tibial part secured to the motor-driven block. The ex-fix locking bar is removed to allow the actuator to displace the tibia from the femur. The load cell simultaneously reads the force developed through all soft tissues across the distracted joint space, including the graft tendon.

A custom program was developed in LabVIEW (National Instruments, Austin, TX) to control the CDM and record the load-distraction data. The program operated in closed loop mode and used displacement feedback from the LVDT to control the position of the stepper motor actuator. The force/distraction data were recorded to a file through a data acquisition card (No. 6036E, National Instruments, Austin, TX). The control program also provided for continuous correction of the displacement due to the mechanical compliance of the system.
Characterization of the System

Compliance. We determined the compliance of the entire system (CDM with ex-fix), rather than listing the specifications of its individual components, keeping in mind that mechanical stimulation of the graft depends on the accuracy and repeatability of joint space distraction. We also characterized the variability in the compliance, since numerous intra-operative factors may affect system compliance between animals.

System compliance was calculated indirectly in five animals with bilateral fixation in vitro by taking the difference between the displacement-load response of the ACL-reconstructed knee and the displacement-load response of the knee and system. The linear slope of the resulting displacement-load curve represented the compliance of the system alone. Displacement of the knee joint was measured by a kinematic tracking system as described in the section below (Measurement of Knee Distraction), while displacement of the knee and system was measured by the LVDT mounted on the CDM.

System compliance was also directly measured in three animals with bilateral fixation in vitro by securing a 1.2 mm diameter metal wire across the knee joint. After the wire became taught, it allowed no further distraction of the knee joint; therefore, the resulting joint distraction curve reflected deformation in the system. Slope of the linear portion of the displacement-load curve represented the compliance.

In both experiments, device compliance was calculated using the data from the sixth loading cycle. Maximum load for these experiments was limited to 39.2 N (4000 g) because higher levels caused increased incidence of bone fracture.

We also indirectly characterized the compliance of the system with unilateral fixation. This provided a basis for comparing the effect of adding bilateral support of the bones on compliance. The characterization was conducted using ten animals in vitro. Maximum load in these experiments was
limited to 12 N to ensure that the excursion range of our stepper motor or LVDT was not exceeded when using this lower compliance ex-fix.

Outcome measures for the data collected from the tests of each fixation method were: average device compliance, standard deviation and coefficient of variation (COV) to assess the inter-animal variability, and the average $R^2$ value and its COV as a measure of the goodness of fit of a linear model for the compliance of each individual ex-fix. Finally, a least squares linear approximation for all of the compliance data collected from both the bilateral and the unilateral groups and the associated $R^2$ value was calculated to determine the single compliance coefficient that most closely fit all of the experimental displacement-load responses in each group.

Accuracy of Knee Distraction.

For future experiments our primary independent variable will be distraction of the knee joint (i.e., separation of the joint space). Therefore, we characterized how accurately the device distracted the joint using five cadaveric animals. The knee distraction experiments were conducted using bilateral fixation, since this approach will be used in future studies.

Knee distraction was calculated using both the fixator compliance for each specimen and the least squares compliance for all specimens, and compared to an independent measurement of knee distraction as obtained by the kinematic tracking system. The load across the joint was then plotted against distraction as measured by: the kinematic system (our “gold standard”), the LVDT, and for both the animal-specific and the least squares compliance correction factors. We calculated the root mean square (RMS) error between the kinematic system and the displacements recorded by the LVDT and the displacements predicted using the animal-specific and the least squares compliance correction factors.
Measurement of Knee Distraction.

Measurements for knee distraction were obtained using a three-dimensional (3D) kinematic tracking system (ProReflex MCU, Qualisys Inc., Gothenburg, Sweden). This system recorded knee distraction by monitoring the 3D spatial position of reflective markers that were glued to the femoral and tibial tunnel exits of the reconstructed cadaveric knees (Figure 3.4). The tracking system was accurate to 53 µm RMS error (2.6%) with a COV of 3.2% for displacements of 2.00 mm using a digital caliper (Fowler Inc., Newton, MA) as our reference standard. The manufacturer-provided specifications stated a caliper resolution of 10 µm, an accuracy of 20 µm and a repeatability of 10 µm.

Three-dimensional position data of each marker were recorded for 6 distraction cycles at 10 Hz. The 3D spatial coordinates of each marker were post-processed using a fourth order Butterworth low pass filter with a 0.01 Hz cut-off frequency. The filter was implemented using the signal processing toolbox available in MATLAB (Mathworks, Natick, MA). Subsequently, the magnitude of the distance between markers on the tibia and femur was calculated over the six distraction cycles. Accuracy data was calculated from the data of the sixth loading cycle.
Figure 3.4: The kinematic tracking system measured the 3D spatial position of reflective markers glued to the femur and tibia, subsequently, displacement across the joint space was calculated. These data were used to develop a correction factor to account for the compliance of the ex-fix.
**Pilot Study**

The purpose of the in-vivo pilot study was to determine if the animals could tolerate the surgical procedure, continuous wearing of the ex-fix, and daily loading regimen under anesthesia. With approval of our Institutional Animal Care and Use Committee, fifteen male Sprague Dawley rats underwent surgical placement of the ex-fix, along with ACL reconstruction of the right knee using a flexor digitorum longus tendon (FDL) autograft. On post-operative day four, the animals began a ten day long loading regime, consisting of a daily fifty cycles of knee distraction to 2.5 mm within a load limit of 2400 grams. Distraction was performed in the CDM at 0.17 Hz, under 2% isoflurane anesthesia, resulting in total anesthesia time of approximately twenty minutes, allowing for set up and loading.

Since femoral or tibial fracture is a concern in this small animal model, we evaluated for fracture using high resolution anterior-posterior and oblique radiographs (Faxitron Model# MX-20 DC4, Faxitron X-ray Corporation, Wheeling, IL). Images of each rat were obtained prior to the first loading session to identify any peri-operative fractures, and then every two days over the course of the loading regimen. We also monitored the daily load-displacement curves as an indicator of slip in the mechanical components of the device, loosening at the pin-bone interface, or catastrophic failure of any of the tissues.
Results

Average system compliance for all specimens using bilateral fixation was 42.4 ± 8.8 µm/N (Figure 3.5). This yielded a COV of 20.7%. Average system compliance using unilateral fixation was 145.2 ± 44.9 µm/N (Figure 3.5) yielding a COV of 30.9%. The average $R^2$ value for fitting a linear equation to the compliance data for the bilateral and unilateral fixation were 0.989 ± 0.005 and 0.988 ± 0.007, respectively. This yielded a COV of 0.5% and 0.7% for bilateral and unilateral fixation, respectively.

**Figure 3.5:** Displacement-load responses of ten unilateral (left) and eight bilateral (right) external fixators obtained from our in vitro loading experiments. The least squares linear approximation (solid lines) for unilateral and bilateral fixation yielded a best-fit compliance of 143.4 µm/N and 41.3 µm/N with $R^2$ values of 0.72 and 0.92, respectively. The 95% confidence bands and the 95% prediction bands of the linear approximation are designated with long dashes (inner lines) and short dashes (outer lines), respectively.

The least squares compliance for all the specimens with bilateral fixation was 41.3 µm/N with an $R^2$ value of 0.92. The least squares compliance for all the specimens with unilateral fixation was 143.4 µm/N with an $R^2$ value of 0.72.
The total RMS error in knee distraction with bilateral fixation using the animal-specific compliance correction factor, the best fit correction factor, and compared to the LVDT were 43 µm, 179 µm and 746 µm, respectively (Figure 3.6). Maximum knee distraction in the experiments using bilateral fixation averaged 1.11 ± 0.32 mm; therefore RMS error of the animal-specific compliance correction factor, the best fit correction factor, and compared to the LVDT represents 3.9%, 16.1% and 67.2% of the average distraction of the knee, respectively.

![Graph showing load vs. displacement with markers for LVDT, Knee, Specific, and Least Squares.](image)

**Figure 3.6:** Sample data (specimen 6) comparing the knee displacements obtained from motion analysis (Knee) to: 1) displacements measured by the LVDT; 2) predictions of knee displacement obtained using the specific compliance correction factor for an individual animal (Specific); and 3) predictions of knee displacement obtained using a least squares fit to all the data.
The in vivo pilot study revealed that all animals survived surgical implantation of the external fixation device. The fifteen rats in the pilot study were immobilized until post-operative day four. Prior to the first loading session, three fractures (two femoral, one tibial) were detected with faxitron imaging. Over the test period, two of the remaining twelve animals experienced fracture of the femur. No tibial fractures were detected. One fracture occurred during the first loading session, and the other occurred during the second day of loading. Ten animals survived the duration of ten days of loading and were sacrificed on post-operative day fourteen. No fractures were detected on post-sacrifice images.

Examination of each animal’s load-displacement responses indicate that no catastrophic failure of soft tissue across the joint space occurred during the course of testing (Figure 3.7). Major slipping in the mechanical components or loosening at the pin-bone interface was also not evidenced by large drops in load over the course of loading.

Figure 3.7: Load displacement curves from 50 cycles of knee joint distraction in the CDM as recorded by the custom program from a single representative animal on day nine of the pilot study. The curves show no evidence of loosening of the mechanical components or failure of the soft tissue, which would be indicated by sudden drops in load.
Discussion

Injuries to ligaments and tendons compromise the stability and function of joints, which can ultimately lead to osteoarthritis. Ligament reconstruction procedures using tendon grafts to repair these injuries often require secure tendon-to-bone healing, yet little is known about the effect of mechanical load on healing of these repairs. To investigate this question we have designed and characterized a device that applies cyclic distraction to a reconstructed ACL in an in-vivo animal model. Unlike previous devices which only allow for load deprivation or static loading of tendons and ligaments, our system provides for the modulation of magnitude, frequency and onset of loading [107–109]. Our long-term goal is to identify the optimal rehabilitation protocol for patients who have undergone knee ligament reconstruction.

The CDM with bilateral fixation exhibited linear deformation characteristics under the loading conditions that it will experience in future experiments. This characteristic enables use of a first order correction factor to account for deformation of the device. Furthermore, the external fixator showed some variability in compliance (COV=21%) across animals. We are not surprised in this variability, however, since numerous intra-operative parameters must be controlled by the surgeon when placing the ligament graft and installing the fixator on the animal. These include location, length and angle of the intra-cortical pins used to secure the rat to the fixator [110].

We designed the ex-fix to maximize stiffness incorporating use of bilateral pin fixation to decrease fixator compliance relative to unilateral fixation. The results of this study revealed that bilateral fixation decreased compliance by factor 3.5, decreased variability in compliance by factor 1.5, and improved linearity of our best fit correction term by factor 1.3. Furthermore, the average compliance of our bilateral fixator (42.4 μm/N) was about 1.4 times more than a similar external fixator (29.5 μm/N) employing unilateral fixation and 1.1 mm diameter bone pins; however, the variability in the compliance of our device was about 1.8 times less than this design (COV=37%) [111]. Although use of larger diameter pins would further decrease compliance, this proved difficult in our small animal model because of
increased risk of fracture. Previous studies employing 1.1 mm pins in a rat femur loading model reported about a 30% complication rate [111].

The least squares linear approximation of fixator compliance decreased RMS error of predicted knee displacements by a factor of 4.2 compared to the LVDT-based measurements (decrease in RMS error from 67.2% to 16.1% of total knee distraction). Although the least squares approximation provided less accurate predictions of knee displacement compared to the animal specific compliance correction factor (3.9% error), it is impractical to include a specific correction for each individual animal, since measuring knee motions with a kinematic system in each animal is too time-consuming. Fortunately, using the least squares linear approximation of fixator compliance still yields acceptable error levels because target knee displacements in subsequent experiments (1 to 2 mm) will be 6 to 11 times greater than the error in knee displacement using the least squares correction factor (179 μm). For example, the ratio of maximum knee displacement as measured via motion analysis (1.1 mm) in our evaluation of the bilateral fixator to the error levels in predicted knee displacement using the best fit compliance correction factor (179 microns) is 6.2.

Overall, both the characterization of system compliance and the study of knee displacement accuracy provide us with important guidelines for designing studies based on our primary independent experimental variable, knee displacement. Specifically, these data provide us with basic information to choose discrete levels of knee distraction that are both statistically greater than zero, and different from each other. For example, since use of our least squares correction factor yielded 16.1% error in achieving target displacements, we must ensure that target displacements in future test groups differ by at least this much.

The novelty of the device stems from its ability to apply controlled distraction to a healing ACL reconstruction in a convenient and cost-effective rodent model. The device allows the investigator to vary
the time of onset of loading (immediately after surgery versus delayed), as well as the duration, frequency and magnitude of knee distraction. There are a wide variety of readily available biologic assays to examine the cellular and molecular response to different loading regimens. Additionally, the investigator can combine these mechanical effects with cytokines believed to modulate tendon to bone healing [112,113].

At the time of this writing, we have successfully performed ACL reconstructions and placed the external fixator on almost 200 animals, with an overall fracture rate of approximately 15%, and minimal other complications during loading in the CDM [9,114]. Our overall experience with this pilot study and other subject groups shows that rodents tolerate the external fixator and daily loading under anesthesia well, with no discernible adverse effects, and their cage activity is not unduly encumbered by the external fixator. Further study may be required to evaluate the physiological effects of daily anesthesia on tendon to bone healing, but is not the subject of this validation paper.

There are several limitations to our animal model and device. First, there is some slack length in the graft despite fixation under tension at the time of surgery, and there is likely variability in slack length between animals. Although determining the slack length of each individual rat would be ideal, it is impractical since the load contribution of other structures across the joint space masks the point at which the graft begins to take up load. Alternatively, we characterized the average slack length and its variability at time zero in vitro by dissecting down to the bone-tendon-bone construct. For subsequent studies, we can then use this to choose appropriate knee displacement levels that exceed this amount. This would ensure that most of the grafts experience mechanical stimulus at post-operative day zero. Since slack length may change beyond day zero due to both healing between graft and bone, and changes in graft mechanical properties, we focused on ensuring that our device could deliver known amounts of knee distraction. Our ultimate goal is to reproducibly distract the knee to a target displacement.
We also do not know the contribution of other soft tissues to the load-displacement response obtained from the daily loading regimens. To gain a better idea of the force through the graft tendon, we released the anterior joint capsule and all other major ligaments. However, the skin, the neurovascular structures, posterior capsule and several muscle groups (hamstrings, adductors, and gastrocnemius) cannot be transected because their elimination would compromise the viability of the limb. Variation in the load displacement response may be due to healing or adaptation of these surrounding structures. The unknown and changing contribution of the other soft tissues to the load is the reason we decided to use displacement control instead of load control. These limitations, however, are not relevant to the primary purpose of our loading system, which is to determine the impact of known levels of mechanical stimulus (i.e., knee distraction) on the biology of tendon-bone healing.

In conclusion, we have developed a new procedure and device for applying controlled mechanical stimulus to a healing ACL graft over time in a small animal model. Our evaluation of the device compliance and accuracy of knee distraction yielded important information that will guide us in defining groups with statistically different levels of knee distraction. Our in vivo assessment revealed that the animals tolerated the external fixator and daily anesthesia. Overall, this test system provides an important tool in achieving our ultimate goal of studying the effect of the timing and magnitude of motion on tendon-to-bone healing, and to identify and evaluate the mechanisms by which mechanical stimuli affects tendon-to-bone healing.

Acknowledgment

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Chapter IV:

Effect of short-duration low-magnitude cyclic loading versus immobilization on tendon-bone healing after ACL reconstruction in a rat model

Chapter four describes the initial use of the CDM device with external knee fixator in fulfillment of aim two: the determination of the effect of cyclic tensile loading on tendon to bone healing. Specifically we tested our first hypothesis that immediately following ACL_R, continual daily tensile loading of graft tendon, for up to four weeks will impair tendon to bone healing, compared with continuous strain shielding of the tendon graft [114].

This hypothesis predicts that compared with knee immobilization, daily graft loading via knee distraction for periods of two and four weeks will result in: (1) more inflammatory and osteoclast cells at the tendon-bone tunnel interface, along with increased angiogenesis; (2) less new bone formation; and (3) reduced failure load and stiffness of the femur-graft-tibia complex.

Materials and Methods

Surgical Procedure

With Institutional Animal Care and Use Committee approval, fifty two male Sprague-Dawley rats of weight 250-350 grams, received ACL_R of the lower right limb. In each, the flexor digitorum longus tendon was harvested from the same limb for use as the tendon graft. Through a medial knee incision, the patellar tendon, both collateral ligaments, original ACL and PCL were severed, to prevent these structure from shielding the tendon graft from tensile load during protocol sessions.
With a 1.2 mm diameter Keith needle, a tunnel was drilled through the medial diaphysis of the tibia, aiming carefully to exit through the original ACL enthesis on the tibial plateau. With the needle left protruding through the tibial tunnel, the knee was flexed until the needlepoint centered on the femoral enthesis within the intercondylar notch, and a coaxial tunnel was drilled through the lateral condyle.

The Keith needle was left temporarily in place within both tunnels, while temporary ex-fix mounting jigs were slipped over each end. These jigs held the femoral and tibial halves of the knee ex-fix parallel with their respective tunnels, while each was secured with two 0.9 mm diameter Kirshner threaded wires (1600-635T MicroAire Surgical Instruments, Charlottesville, Virginia), drilled into the diaphysis of the femur and tibia, (see chapter 3). All joints were securely tightened and the jigs removed, leaving the knee locked in approximately 60 degrees flexion.

The Keith needle was then used to shuttle the tendon graft through both tunnels. The graft was placed under light tension and sewn to the periosteum at the diaphyseal tunnel exits using 3-0 Ethibond non-degradable suture, by the same surgeon in all 52 animals. Average graft length was 15 mm from tunnel exit to tunnel exit. All wounds were then sutured closed and the rats given pain medications upon revival.

Figure 4.1: Placement of Ex-fix during ACL_R surgery. The Keith needle temporarily remains in place after drilling both bone tunnels, while used with jigs to guide ex-fix placement in relation to the tunnels.
Study Design

After surgery all rats were randomly assigned into treatment or control groups. The treatment group received daily sessions of cyclic knee joint distraction beginning on postoperative day one for a period of either 14 or 28 days. Control group subjects received postoperative knee immobilization via continuous wearing of the the ex-fix, again for periods of either 14 or 28 days. Subjects were sacrificed at the end of their assigned period.

Figure 4.2: Study Design

Determination Tendon Graft Strain Effected Through Knee Joint Distraction

Due to concerns over potential slack length left in the tendon graft after surgery reducing the effective strain applied during cycles of knee joint distraction, a pilot study was first completed using the same surgeon performing the identical ACL_R procedure described above on nine cadaver rats.

Using the optical tracking system described in chapter three [115], we measured the actual joint space distraction by tracking reflective markers placed on the femur and tibia. Although the cyclic displacement mechanism (CDM) moved the tibial portion of the ex-fix a commanded 1.5 mm distally, the mean joint distraction achieved in the nine cadavers (optically measured) was 0.88 ± 0.33 mm, due primarily to deflection of the 0.9 mm k-wires connecting the femur and tibia to the ex-fix.

Slack length in the graft was defined as the optically measured joint distraction distance reached when 30 grams of tension was measured through the isolated femur-graft-tibia complex. Therefore any
surrounding tissue that could contribute force across the joint space was dissected away in each of the nine specimens until only the femur-graft-tibia complex remained. The mean slack length at 30 grams of force across the joint space in the nine cadaver specimens was 0.55 ± 0.23 mm.

The mean total joint space distraction (effective graft elongation) for this study was defined as mean joint space distraction (0.88 mm) minus mean slack length (0.55 mm), and equal to 0.33 mm. Normalizing this by the average free initial graft length of 15 mm equates with an effective tendon graft strain of 2.2 %. This correlates with human studies estimating from 2 to 5 % elongation of both the native ACL and reconstruction grafts during light rehabilitation exercises.

**Daily Loading Protocol**

Beginning on postoperative day 1, rats in the treatment group received fifty cycles of cyclic knee distraction daily until the day before sacrifice at the end of their assigned period on day 14 or 28. After induction of 2 % isoflurane anesthesia, the rat was placed in the cyclic distraction mechanism (CDM) with the femoral and tibial components of the ex-fix connected to the load cell and actuator respectively. To establish a consistent starting position across subjects, the actuator first compressed the knee joint until -1.96 N (-200 grams) of force was read. The actuator then commenced fifty cycles of FDL tendon graft strain, each consisting of knee joint distraction to the target distance and return to the starting position, both at 0.24 mm/sec.

**Histological Analysis**

Twelve limb specimens were randomly assigned for histology, three for each treatment group and their time period subgroups. Each limb was divided at the graft mid-substance into separate tibial and femoral specimens, which were then fixed and decalcified. After embedding in paraffin the tibia was
cut into 5 µm thick coronal sections and the femur into 5 µm thick sagittal sections. These were then stained with hematoxylin and eosin, safranin-O, and picosirius red.

For immune-histochemical analysis, serial sections were treated with 3% H2O2 to quench endogenous peroxidase activity, and nonspecific antibody binding was blocked with 5% goat serum. Each primary antibody was applied to separate serial sections for sixty minutes at 37_°C. Bound antibodies were visualized with use of a goat avidin-biotin peroxidase system with 3,3’-diaminobenzidine (DAB; DAKO, Carpinteria, California) as a substrate.

Hematopoietic lineage cells were bound by the following antibodies: rabbit anti-rat neutrophil PMN (Accurate Chemicals, Westbury, NY), mouse anti-rat ED1-macrophage (ED1 antigen is a lysosomal glycoprotein expressed only by a subpopulation of macrophages and monocytes) and mouse anti-rat ED2-macrophage (ED2 antigen is a membrane glycoprotein found only on mature tissue macrophages) (Serotec, Raleigh, North Carolina). Osteoclast activity was evaluated by staining for tartrate-resistant acid phosphatase (TRAP; Zymed, San Francisco, California). Type-I procollagen stain (Santa Cruz Biotechnology, Santa Cruz, California) was used as an indicator of collagen synthesis by osteoblasts. Proliferating blood vessels were localized with rabbit antihuman factor VIII (DAKO). Negative controls were processed in an identical manner except for incubation with bovine serum albumin rather than the primary antibody.

Analysis of Histological Data

Digital images of the stained tissue sections were made through light microscopy (Eclipse E800; Nikon, Melville, New York), using a SPOT RT camera (Diagnostic Instruments, Sterling Heights, Michigan). An observer counted the number of positively stained cells in thirty randomly selected 40-power, 100 by 100-µm fields from the tibial bone tunnel, the femoral tunnel was excluded because of variability due to its thinner diameter.
**Micro-Computed Tomography Analysis**

Sixteen limb specimens underwent scanning by micro-CT (MS-8 Small Specimen Scanner; Enhanced Vision Systems, London, Ontario, Canada), four from each group at the two and four week time points, with a time zero scan of three specimens for a baseline comparison. Both tibial and femoral tunnels were scanned at 80 V and 80 mA and reconstructed at 22.5-µm resolution.

Bone formation and remodeling were evaluated with six quantitative measures: bone volume (mm³), tissue mineral content (mg), tissue mineral density (mg/mL), trabecular thickness (µm), trabecular number, and trabecular spacing (µm).

Total mineral content, mineral distribution and bone volume fraction (bone volume divided by total volume), were calculated for a cylinder of 2.2 mm diameter superimposed over the entire length of the graft tunnel. An SB3 standard of 1100 g of hydroxyapatite/cm³ was used to calibrate tissue mineral density. The mineral distribution was determined from the computed tomography value for each voxel of the micro-CT scan. The bone volume is the total of bone voxels within this cylinder.

**Biomechanical Testing**

Twenty-four ACL_R limb specimens, six for each time point and group, were stored at – 80° C. Several hours before testing they were thawed and all soft tissue dissected, leaving only the femur-graft-tibia complex. Both tibial and femoral ends were potted in cement to provide a secure gripping surface (Bondo; 3M Corp., Atlanta, Georgia). The potted ends of each specimen were securely mounted in the grips of a custom tensile test machine, with the graft parallel to grip translation.

Five cycles of preconditioning distraction preceded from 0 to 0.2 N load. The load to failure test followed with one potted end of femur-graft tibia complex displaced at 10 mm per minute until the recorded load peaked, and the specimen visibly failed. The stiffness (N/mm) and ultimate load (N) were calculated from the linear portion and peak, respectively of the recorded load-displacement relation,
using Microsoft Office Excel 2002 (Microsoft, Redmond, Washington). The site and of mode failure (tunnel pull-out or mid-substance graft rupture) were also recorded.

Statistical Analysis

One way analysis of variance with repeated measures was used to assess variations in the mean load at maximum knee distraction for each animal over the treatment period. A custom program was written in Matlab 6.1 (The MathWorks, Natick, Massachusetts) to read the daily CDM data and extract the load at maximum displacement from cycles 21 to 30 to calculate these averages. A student t-test compared the mean load averages between the two and four week daily knee distraction groups at various time points.

Additionally, on the day of sacrifice at either two or four weeks, the immobilization group underwent one sole knee distraction period of 50 cycles, solely to allow for comparison of maximum load, between both treatment groups. Systat Software (Chicago, Illinois) and SAS 9.1 for Windows (SAS Institute, Cary North Carolina) were used for the statistical tests, with a significance criteria of p < 0.05.

Results

Inflammatory Cells

Significantly more ED1+macrophages (Table 1) were counted in the two week-knee distraction group (3.0 ± 1.5 per field) than in the two week-immobilization group (2.4 ± 1.5 per field) (p=0.01). However there were no significant differences in ED1+ macrophages between the four week-distraction and four week-immobilization groups. Within the distraction treated rats there were significantly less ED1+ macrophages counted (1.9 ± 1.4 per field) in the four week group than in the two week group (p = 0.03). Counts of ED2+ macrophages showed no significant differences between treatment groups at either time point, nor within either group between time points. Few neutrophils were counted [114].
TABLE 4.1: Inflammatory Cell Histology Data [114]

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<th>Immediate - distraction</th>
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<td>3.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>1.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.8</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

*The letters a and b identify the two data points being compared in each case, with a indicating a p value of 0.01, and b indicating a p value of 0.03.*
Angiogenesis

Counts of Factor-VIII stained cells showed no significant difference (p = 0.08) between the two week-knee distraction group and the two week-immobilization group. Although within the distraction groups the two week group exhibited 1.6 cells per field while the four week group only 0.7 per field, the difference was not significant (p = 0.08).

TABLE 4.2: Angiogenesis Histology Data [114]

<table>
<thead>
<tr>
<th>Factor VIII</th>
<th>Cells per High-Power Field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Continuous - Immobilization</td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.8^a</td>
</tr>
<tr>
<td>Median</td>
<td>0.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.0</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.1</td>
</tr>
<tr>
<td>Median</td>
<td>0.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*The letters a and b identify the two data points being compared in each case, with both a and b indicating a p value of 0.08.
**Osteoclasts and Osteoblasts**

The was no significant difference in tartrate-resistant acid phosphatase stained cells between immobilized and knee distraction groups at either time point. Within the immobilized rats, the difference between the 2 week group (1.4 cells per field) and the four week group (0.7 cells per field) was not significant (p = 0.07). No significant differences in procollagen staining between groups or between time points were found.

**TABLE 4.3:** Bone Homeostasis Histology Data [114]

<table>
<thead>
<tr>
<th>Marker and Time Point</th>
<th>Cells per High-Power Field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Continuous - Immobilization</td>
</tr>
<tr>
<td><strong>TRAP staining</strong></td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Median</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.2</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Median</td>
<td>0.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Procollagen staining</strong></td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.7</td>
</tr>
<tr>
<td>Median</td>
<td>2.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.2</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2.6</td>
</tr>
<tr>
<td>Median</td>
<td>3.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*The letter a, identifies the two data points being compared, and indicates a p value of 0.07. TRAP = tartrate-resistant acid phosphatase.*
**Micro-CT**

The mean and standard deviation of data from the micro CT scans are shown in Table IV. Within the knee distraction group rats, the number of trabeculae after 4 weeks was significantly lower ($p = 0.02$) than at time zero. No other differences between treatment or time groups proved significant.

**TABLE 4.4: Micro-Computed Tomography Data [114]**

<table>
<thead>
<tr>
<th></th>
<th>Time Zero (N = 3)</th>
<th>2 Weeks</th>
<th>4 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Continuous - Immobilization (N = 4)</td>
<td>Immediate - distraction (N = 4)</td>
<td>Continuous - Immobilization (N = 4)</td>
</tr>
<tr>
<td>Bone volume (mm$^3$)</td>
<td>0.82 ± 0.04</td>
<td>0.85 ± 0.19</td>
<td>0.89 ± 0.30$^a$</td>
</tr>
<tr>
<td>Total mineral content (mg)</td>
<td>0.45 ± 0.03</td>
<td>0.48 ± 0.12</td>
<td>0.49 ± 0.15</td>
</tr>
<tr>
<td>Total mineral density (mg/mL)</td>
<td>543 ± 25</td>
<td>542 ± 32</td>
<td>536 ± 40</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
<td>61.6 ± 2.6</td>
<td>69.8 ± 13.1</td>
<td>67.5 ± 13.2</td>
</tr>
<tr>
<td>Trabecular number</td>
<td>3.71 ± 0.28$^c$</td>
<td>2.94 ± 0.33</td>
<td>2.94 ± 0.33</td>
</tr>
<tr>
<td>Trabecular spacing (μm)</td>
<td>246 ± 30$^d$</td>
<td>280 ± 45</td>
<td>297 ± 68</td>
</tr>
</tbody>
</table>

The values are given as the mean and standard deviation. The letters $a$, $b$, $c$, and $d$ identify the two data points being compared in each case; $a$ indicates a $p$ value of 0.07, $b$ indicates a $p$ value of 0.08, $c$ indicates a $p$ value of 0.02, and $d$ indicates a $p$ value of 0.09.
Biomechanical Testing

Data from daily knee distraction

Among the rats experiencing daily knee distraction, the mean load at maximum knee distraction increased over time: from 5.7 ± 2.3 N on postoperative day one to 9.8 ± 2.4 N on day fourteen (p = 0.04), and from 4.1 ± 0.9 N on postoperative day one to 9.4 ± 4.0 N on day twenty-eight (p = 0.03). For all groups loads measured on day 10 were significantly greater than day five (p = 0.01), loads on day 12 were significantly greater than those on day seven.

For the one time knee distraction of the immobilized groups immediately before sacrifice, the mean load measured on day fourteen was 10.7 ± 2.9 N and on day 28 was 10.9 ± 4.6 N.

Figure 4.3 Graph of the mean load achieved each day of the rats in the immediate-loading group [114].
Stiffness and Load to failure

The mean stiffness data for each group and time point are shown in figure 4.3. Within the immobilized rats the mean stiffness of the four week group $6.4 \pm 2.9 \text{ N/mm}$ was significantly greater than the two week group $3.8 \pm 1.4 \text{ N/mm}$. There was no significant difference between the knee distraction and immobilized groups at either time point.

Figure 4.4 Mean stiffness from the load to failure test of the femur-graft-tibia complex [114].
The mean Load to failure for each group and time point is shown in figure 4.4. At two weeks the mean load to failure was almost identical at 4.4 N in both the knee distraction and the immobilization groups. By four weeks the mean failure load increased significantly, almost doubling in both groups, 8.2 ± 3.6 N (p = 0.02) for the knee distraction group and 8.7 ± 2.3 N (p = 0.009) for the immobilized. No significant differences in load to failure were found between knee distraction and immobilization groups at either time point. The mode of failure was though pulling out of the bone tunnel in twenty-three out of twenty-four specimens, with one of the four week immobilization group failing through the mid-substance of the graft.

Figure 4.5: Mean load to failure of the femur-graft-tibia complex [114].
Discussion

This study is the first to apply controlled tensile load onto a healing tendon graft in-vivo, in a rat model of ACL_R, using a novel device. Previous studies of the effects of mechanical load on tendon to bone healing were limited to unquantifiable methods such as ad-libitum cage motion and treadmill running. We tested the hypothesis that immediate tensile graft strain, applied daily for periods of up to two and four weeks, would impair tendon graft to bone healing compared with continuous strain shielding of the graft.

Results showed that 2 % tensile strain applied to the tendon graft daily for two weeks post ACL_R surgery resulted in a significantly greater accumulation of macrophages within the tendon- bone interface at the two week time point. This partly supports our first prediction of a greater accumulation of inflammatory cells, although there was no significant increase in angiogenesis.

Regarding our second prediction of less new bone formation, histology showed no significant differences in cells stained for either TRAP or procollagen between groups at either time point, implying no differences in osteoclasts or osteoblast activity. However, the micro CT analysis counted fewer trabeculae at the 4 week-distraction group, when compared to a baseline analysis of three specimens at time zero, providing support for the second prediction.

Our third prediction of reduced stiffness and failure load was not supported, as both groups showed no significant differences in either measure, at either time point. Remarkably the mean load to failure for both groups was n nearly identical at 4.4 N in both groups at two weeks, and almost doubling to 8.7 N (immobilization) and 8.2 N (distraction) at the four week time point.

As with all studies there were limitations. The initial tension on the graft during surgical placement could not be reliably quantified, instead we relied on a single surgeon for repeatable graft placement. The duration and magnitude of mechanical stimulation required to affect healing may be greater than daily 50 cycles we applied or our average 2 % graft strain. However addition of greater
strains, more cycles, and additional daily sessions would increase the amount of time under anesthesia for each subject. Attempts at increasing strain through increased joint distraction distance met with diminished returns due to greater bending of the k-wires through the femur and tibia, compounded with an increased the risk of fracture.

Although only modest differences were shown between knee distraction and knee immobilization groups, this study will provide a baseline for future studies varying additional parameters such as strain magnitude and post-operative delay periods prior to the onset of the daily strain protocol.
Chapter 5

Effect of early and delayed mechanical loading on tendon-to-bone healing after anterior cruciate ligament reconstruction

Introduction

While Chapter Four compared the effect of the daily tendon graft loading versus continuous load shielding, Chapter Five fulfills aim three by adding an additional variable, *delay of treatment onset*, by imposing a period of immobilization to shield the tendon graft from mechanical load, prior to beginning the daily loading treatment on postoperative day four (group three) or day ten (group four).

This study will test hypothesis \( H_1 \), which states that an immediate postoperative period of load shielding lasting up to four or ten days, followed by a daily regime of tendon graft loading, ending on either postoperative day 14 or 28, will improve tendon graft to bone tunnel healing, compared to *both* continuous load shielding and immediate daily loading beginning postoperative day one.

Hypothesis \( H_1 \) predicts that in those animals where tensile loading was delayed, there will be: (1) Fewer inflammatory and osteoclast cells at the tendon-bone tunnel interface, along with reduced angiogenesis; (2) More new bone formation; and (3) Increased failure load and mechanical properties of the femur-graft-tibia complex.

Materials and Methods

Surgical Procedure

Animal Care and Use Committee approval was received for the use of 156 male Sprague-Dawley rats of weight 250-350 grams, approximately 11 weeks old, to receive ACL_R of the lower right limb. The identical surgical procedure described in Chapter Four, including placement of the ex-fix, was performed for all 156 subjects. Because each rat recovered from the surgery wearing the ex-fix, the knee joint of the right lower limb was immobilized at time zero.
**Study Design**

All rats were randomly assigned into one of the following treatment regimes (Figure 5.1):

1. Continuous Immobization.

2. Daily sessions of the loading treatment to begin on postoperative day one.

3. Immobilization through postoperative day three - daily loading treatment sessions begin on day four.

4. Immobilization through postoperative day nine - daily loading treatment sessions begin on day ten.

All subjects were sacrificed at the end of their study group length of 14 or 28 days.

![Figure 5.1: Study design](image)

**Tendon Graft Strain During Knee Joint Distraction**

As described in Chapter Four, a mean distraction distance of $0.88 \pm 0.33$ mm was calculated from the optically measured knee joint distraction distances achieved in nine rat cadaver ACL_R specimens. From this distance the mean graft slack length of $0.55 \pm 0.23$ mm, at 30 grams of tension was subtracted to give an average graft elongation of 0.33 mm. Normalizing this by the average graft length of 15 mm gives an effective graft strain of 2.2 %. 

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Daily Treatment Protocols

As in Chapter Four, those rats assigned “continuous immobization” (group 1) continuously wore a locked ex-fix for either 14 or 28 days. Those assigned “immediate loading” (group 2) received the loading treatment daily from postoperative day one until either day 14 or day 28, corresponding with the end of their study group. (Note, this study applied data from Chapter Four to groups 1 and 2, expanding these groups to 36 subjects each). Rats assigned to “delayed onset” groups continuously wore the locked ex-fix after surgery until the beginning of daily loading treatments, on either postoperative day four (group 3) or day 10 (group 4); see Figure 5.1.

Each daily loading treatment session (groups 2, 3 and 4) began with sedation of the subject rat via inhalation of a 2 % isoflurane - oxygen mixture. The rat was then placed in the loading mechanism (the cyclic distraction mechanism, CDM of Chapter 3) and the ex-fix locking bar removed. The CDM actuator initially compressed the knee joint until negative 1.96 N (-200 grams) of force was applied, establishing a baseline starting point for distraction control. Fifty cycles of tendon graft loading (elongation) commenced via the CDM alternately distracting and retracting the knee joint at 0.24 mm/sec. The distraction was applied in a reciprocating translation co-linear with the axis of both bone tunnels. After the fiftieth cycle, the locking bar was replaced in the ex-fix before removing the rat from the CDM for revival, immobilizing the knee until the following days treatment [115].

Histological Analysis

Histological and immunohistochemical analyses were performed identically as detailed in Chapter Four, with the details omitted here for brevity (see Chapter 4). However the following changes to the list of stains and antibodies were made:

Use of picrosirius red staining and type-I procollagen staining were not continued. Use of the antibody for neutrophils was not continued. The antibody used to detect proliferating blood vessels,
rabbit anti-human factor VII was replaced with a mono-clonal antibody to $\alpha$-smooth muscle actin ($\alpha$-SMA), a marker of vascular pericytes, (Sigma, St. Louis, Missouri). Histological staining continued using hematoxylin and eosin, and safranin-O.

Immunohistochemical analysis used the following antibodies: mouse anti-rat ED1-macrophage, mouse anti-rat ED2-macrophage, and a monoclonal antibody to $\alpha$-SMA. Chemical staining was used to detect tartrate resistant acid phosphatase (TRAP) secreted by osteoclasts.

**Analysis of Histological Data**

Digital images of the stained tissue sections were made through light microscopy (Eclipse E800; Nikon, Melville, New York), using a SPOT RT camera (Diagnostic Instruments, Sterling Heights, Michigan). An observer counted the number of positively stained cells in thirty randomly selected 40 power, 100 by 100-µm fields from the tibial bone tunnel, the femoral tunnel was excluded because of variability due to its thinner diameter.

**Micro-Computed Tomography Analysis**

Micro-CT scanning was performed with using the same procedure detailed in Chapter Four. However scanning was limited to tibial specimens because of their reduced variability in bone tunnel length. Two specimens were added at time zero scan to increase the baseline reference group to five.

Bone formation and remodeling were evaluated with six quantitative measures: new bone volume ($\text{mm}^3$), tissue mineral content (mg), tissue mineral density (mg/mL), trabecular thickness (µm), trabecular number, and trabecular spacing (µm).

Two-way analysis of variance was used to detect differences in the histological and micro CT data between the four treatment groups. Repeated-measures analysis of variance was also used to
detect changes with location along the same bone tunnel (articular aperture, mid-length and diaphyseal aperture), from the histology slides of a single specimen (Figure 5.5).

**Biomechanical Testing**

After sacrifice at the two and four week time points, the limb specimens were harvested and stored at –80° C. Several hours before testing they were thawed and all soft tissue dissected, leaving only the femur-graft-tibia complex. Both tibial and femoral ends were potted in cement to provide a secure gripping surface (Bondo; 3M Corp., Atlanta, Georgia).

The potted ends of each specimen were securely mounted in the grips of a custom tensile test machine, with the graft axis parallel to the grip translation. Five cycles of preconditioning distraction were given from 0 to 0.2 N load. The load-to-failure test followed with one potted end of femur-graft tibia complex displaced at 10 mm per minute until the load peaked, and the specimen visibly failed. The load-elongation data and failure site (mid-substance tendon graft rupture, graft pull-out from either femoral or tibial bone tunnels, or failure through the physis) were digitally recorded for each specimen.
Results

Inflammatory Cells

Significantly less ED1+ macrophages were counted in the two delayed loading groups than in the immobilized and immediate loading groups, at two and four weeks (p < 0.01, Table 5.1). Delaying knee loading appeared to reduce average ED1+ macrophages averages by half (Figure 5.2).

An opposite effect was found for ED2+ macrophages, significantly more (p < 0.02) were counted in both delayed loading groups than in the immobilized and immediate loading groups, at two and four weeks. No significant differences were found between the continuous immobilization group and the immediate loading group in the counts of either ED1+ or ED2+ macrophages [9].

| Table 5.1: Quantitative Analysis of Cells at the Tendon-Bone Tunnel Interface |
|--------------------------|-----------------|-----------------|-----------------|-----------------|
| Group                   | Cells per High Power Field |
|                         | Continuous Immobilization | Immediate Distraction | 4-Day Delayed Distraction | 10-Day Delayed Distraction |
| **ED1+ macrophages**    |                             |                             |                             |                             |
| 2 weeks                 | 2.42 ± 0.12 a | 2.96 ± 0.05 b | 1.49 ± 0.17 a,b | 0.63 ± 0.2 a,b |
| 4 weeks                 | 2.53 ± 0.82 c | 2.02 ± 0.91 d | 0.97 ± 0.21 c,d | 0.54 ± 0.13 c,d |
| **ED2+ macrophages**    |                             |                             |                             |                             |
| 2 weeks                 | 1.71 ± 1.5 e | 1.14 ± 0.47 f | 2.62 ± 0.54 e,f | 2.97 ± 0.7 e,f |
| 4 weeks                 | 0.83 ± 0.6 g | 1.36 ± 0.16 h | 3.37 ± 0.29 g,h | 3.35 ± 0.51 g,h |
| **Osteoclasts (TRAP)**  |                             |                             |                             |                             |
| 2 weeks                 | 1.44 ± 0.2 i | 1.02 ± 0.08 j | 0.58 ± 0.37 i,j | 0.35 ± 0.15 i,j |
| 4 weeks                 | 0.69 ± 0.08 k | 1.22 ± 0.17 l | 0.29 ± 0.14 k,l | 0.33 ± 0.15 k,l |
| **Neovascularity (SMA)**|                             |                             |                             |                             |
| 2 weeks                 | 0.78 ± 0.39 m | 1.54 ± 0.22 n | 0.3 ± 0.17 m,n | 0.27 ± 0.15 m,n |
| 4 weeks                 | 1.03 ± 0.62 o | 0.83 ± 0.46 p | 0.31 ± 0.16 o,p | 0.22 ± 0.1 o,p |

Data are reported as the mean and standard deviation. Values that share a letter were significantly different from one another, with a, b, c, d, i, j, k, and l indicating p < 0.01; e, f, g, and h indicating p < 0.02; and m, n, o, and p indicating p < 0.003.

Figure 5.2: Immuno-histochemical staining for ED1+ and ED2+ macrophages. (Upper chart) Significantly less ED1+ macrophages were counted in the two and four week delayed-knee loading groups than in the immobilized and immediate loading groups, at two and four weeks (p < 0.01). (Lower chart) Significantly more (p < 0.02) were counted in both delayed loading groups than in the immobilized and immediate loading groups, at two and four weeks. (Cells/HPF = Average number of cells counted per high-power field) [9].
Angiogenesis

In the two and four week delayed loading groups, staining for smooth muscle actin revealed less neovascularity, than in the continuous immobilization or immediate loading groups (Figure 5.3).

Figure 5.3:
Counting of cells staining for smooth-muscle-actin (SMA) showed reduced interface tissue vascularity in both the two and four week delayed-knee loading groups compared with that in the immediate-loading or continuous immobilization group at two and four weeks postoperatively (p < 0.003). (Cells/HPF = Average number of cells counted per high-power field) [9].
Osteoclasts

Significantly less cells staining positive for tartrate resistant acid phosphatase were counted in the two and four week delayed-loading groups than in the continuous-immobilization or immediate-loading groups (p > 0.01, see Table 5.1 and Figure 5.4). No significant differences were found between the continuous immobilization group and the immediate loading group in the counts of cells staining positive for TRAP [9].

![Osteoclasts (TRAP-staining)](image)

Figure 5.4 Significantly less osteoclasts were observed at the tendon-bone interface in the two and four week delayed-knee loading groups compared with the number in the immediate-loading or continuous immobilization group at two and four weeks postoperatively (p < 0.01). (Cells/HPF = Average number of cells counted per high-power field) [9].
Micro-Computed Tomography

The new-bone volume, tissue mineral content, and tissue mineral density observed in the tibial tunnels of the delayed-loading groups were significantly greater than those in the immediate-loading and continuous-immobilization groups at both two and four weeks postoperatively (new-bone volume, 1.47 ± 0.11 mm³ [postoperative-day-10 group] versus 0.89 ± 0.30 mm³ and 0.85 ± 0.19 mm³ [immediate-loading and immobilization groups, respectively] at two weeks; p < 0.003; Table 5.2 and Figure 5.6.

No significant differences in new bone volume, tissue mineral content, or tissue mineral density were observed between the continuous-immobilization and immediate-loading groups at any time point. Regional tunnel analysis revealed that the new-bone volume, tissue mineral content, and tissue mineral density at the intra-articular aperture (p < 0.01) and mid part of the tunnel (p < 0.01) were significantly greater in the delayed-loading groups than they were in the continuous-immobilization or immediate-loading group, but there were no differences between groups at the extra-articular aperture [9].
Figure 5.5: Sagittal micro-computed tomography images of the tibial tunnel. New-bone formation, tissue mineral content, and tissue mineral density were quantified for the entire tibial tunnel as well as for the intra-articular aperture A, mid-part of the tunnel B, and extra-articular aperture C [9].
Figure 5.6: Significantly greater new-bone volume (NBV) was observed in the tibial tunnels of both delayed loading groups compared with that in the immediate-loading or immobilization group at two and four weeks postoperatively (p < 0.003) [9].
In a preliminary analysis of the load to failure data by Bedi et al, the stiffness (N/mm) and ultimate load (N) were calculated from the linear portion and peak respectively, of the recorded load-displacement relation using Microsoft Office Excel 2002 (Microsoft, Redmond, Washington), however they did not separate those results by mode and site of failure. For the purposes of this dissertation a more in-depth analysis of the load-to-failure data was conducted as detailed in the following section.

**Statistical Analysis of Load-to-Failure Mechanical Tests**

To model the force-elongation response of the rat ACL-R, the load-to-failure data of the femur-graft-tibia specimens (N = 63) were fit to two response models by a least squares algorithm, using Matlab Release 15 software (The MathWorks Inc, Natick, MA). An exponential growth curve (Fung model); \( F = a(e^{bx} - 1) \) was first used as a model because it shows the slow initial nonlinear response of the tendon graft - the “toe region” typical of soft biological tissues, followed by a more linear response[116]. As it became clear that many data sets (n ≈ 26) exhibited a purely linear response to elongation, a second linear fit model; \( F = mx \), was used. Figure 5.7A shows an example of a successful exponential fit of one data set with the exponential growth model, while Figure 5.7B shows a successful fit for the linear model. A less effective fit using the exponential model is shown in Figure 5.7C, where the linear model was clearly more appropriate for a linear model.

For each data set, the yield load and yield elongation were visually chosen from the datum point at which force stopped increasing linearly with elongation. Both exponential and linear models were fit from the zero load - zero elongation point up to the individual yield point. The data from the exponential curve and linear models for each load-to-failure test are listed by row in Table 5.4 and include the following:
Coefficients $a$ and $b$ predicting the exponential curve fit $F = a(e^{bx} - 1)$ with their respective $p$-values for each test - together with the slope for the linear fit (columns six through ten).

The yield load (N), yield elongation (µm), maximum load (N) and maximum elongation (µm) (columns 11 through 14).

The site of failure for each femur-graft-tibia specimen (column 15):

- Femoral tunnel pull-out (Fem-Tnl) $n=16$
- Tibial tunnel pull-out (Tib-Tnl) $n=26$
- Pull-out via both femoral and tibial tunnels (Both Tnls) $n=12$
- Mid substance tendon graft failure (Mid-Subs) $n=4$
- Failure through the tibial physis (Tib-Physis) $n=2$
- Or as a combination of tunnel pull out and mid-substance failure (MdSB/T-Tn or MdSB/F-Tn; $n=3$).

Columns eight and nine of Table 5.4 show the respective $p$-values for coefficients $a$ and $b$ of the exponential curve model $F = a(e^{bx} - 1)$, calculated to determine the level of probability that this model, fits these data (SigmaPlot 10, Systat Software Inc, San Jose CA). Those specimens with $p$-values greater than 0.05 ($n=26$) are better represented by the linear fit slope shown in column ten.

We initially explored significant relationships between the data and experimental treatments using two-way ANOVA using Minitab17 software, (Minitab Inc. State College, PA.). However because ANOVA will only determine significant differences between treatment means, we chose to implement multiple linear regression to better reveal any relationships between delay periods, treatment durations, and study group lengths and the outcome variables (mechanical parameters).

The response or dependent variables assessed by regression included the coefficients of exponential curve model; $a$ and $b$, the slope of the linear fit, the yield load, yield elongation, maximum load and maximum elongation, as listed in columns 6, 7 and 10 – 14 of Table 5.4.

The first independent regression variable, “Delay” in column 2 of Table 5.4, reflects four levels of postoperative delay in days, prior to the onset of the daily loading treatment; with “four” indicating
daily loading commencing on postoperative day four, “ten” indicating daily loading commencing on postoperative day ten, “one” indicating loading commencing on day one (20 – 24 hours of true delay), and “zero” indicating the continuously immobilized control group. Factor two (Group days) included the treatment group lengths of either 14 or 28 days.

We included a third independent variable “Load (days)” in column 3 of Table 5.4, to account for differences in the total number of daily loading treatments received between groups (equal to onset Delay (days0 subtracted from Group (days)). However the regression model (in Minitab 17) failed to attribute any significant effect to Load (days) due to its dependence on and nesting within the levels of the Delay (days) variable. For example only the four-day delay group had a treatment length of either 10 or 24 days (2 weeks: 14 – 4 days delay, or 4 weeks: 28 – 4 days delay), and only the ten day delay group had a treatment length of either 4 or 18 days (2 weeks: 14 – 10 days, or 4 weeks: 28 – 10 days). Determining the significance of this factor would require a fully orthogonal study design with groups for all levels of each treatment factor, filling the empty spaces of the matrix shown in Table 5.3.

<table>
<thead>
<tr>
<th>Delay (days)</th>
<th>2 wk.</th>
<th>4 wk.</th>
<th>4 wk.</th>
<th>4 wk.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ten Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Four Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zero Delay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immobile</td>
<td>2 &amp; 4 wk.</td>
<td>2 wk.</td>
<td>4 wk.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>10</td>
<td>14</td>
<td>18</td>
</tr>
</tbody>
</table>

**Table 5.3:** Illustrates how each level of the third factor; Load (days) - total of daily loading treatments, is nested within a separate level of the first factor, Delay (days) - the delay in onset of the loading protocol.

Motivated by my expectation for a significant effect on tendon-to-bone healing of the large number of treatment days, 24 in total, incurred by the four-week, four-day-delayed group, I performed three separate single linear regressions to explore how maximum load, yield load and linear slope
changed in response to the seven levels of the single factor, total loading days (Load days), using SigmaPlot 10 software. Plots of these three regressions, including their significance, are shown in Figures 5.8, 5.9 and 5.10.

For correlations to the yield load and yield elongation the distribution of these two response variables was not normal (Figure 5.11A). However, distributions of the maximum load and maximum elongation variables were normally distributed (Figure 5.11B).

Multiple linear regression (MLR) was applied to the collective load-to-failure data from all 63 subjects (Minitab 17). The results are shown in Table 5.5. These 63 datasets were then sorted by their site of failure, to disclose any relationship between the mechanisms of failure and the experimental treatments. The sites of failure were separated as either; pull-out from femoral bone tunnel; pull-out from tibial bone tunnel, mid-substance tendon graft rupture, or failure through the physis.

A second MLR was performed using only subjects that failed via bone tunnel pull-out; either femoral, tibial or both tunnels (total N = 54), after removing mid-substance graft rupture and physis fracture, from the collective of datasets, Table 5.6.

The third MLR was performed after removing simultaneous failures from both tunnels from the collective, leaving only femoral and tibial tunnel failures (N = 42), Table 5.7.

The final and fourth MLR was performed after the removal all femoral tunnel failures leaving only tibial tunnel failures (N = 26), Table 5.8. The total of four mid substance failures was judged too small to provide any significant analysis.
**Figure 5.7A-C:** Typical load-elongation mechanical tensile tests for the rat bone tunnel-tendon-bone tunnel complex. Successful fits for load-elongation data with (A) an exponential growth model and (B) a linear model. (C) While neither an exponential or a linear model was a good fit, the linear model was chosen for final data analysis. The shape of the load-elongation response probably represents a combination of tendon and tunnel responses.
5.7B

R48-04-10.TXT: Curve Fit of Load to Failure Data

Fit: $y = a(e^{bx} - 1)$
$\text{a} = 232.1319$
$\text{b} = 4.1748e-05$
Yield Load = 14.505
yield x = 1450
Max Load = 15.15
max x = 1965
data pts: 45-94

Tib-Tnl. failure

105

5.7C

R53-10-10.TXT: Curve Fit of Load to Failure Data

Fit: $y = a(e^{bx} - 1)$
$\text{a} = 4607.9388$
$\text{b} = 1.522e-06$
Yield Load = 5.347
yield x = 863
Max Load = 7.805
max x = 3005
data pts: 29-58

Both Tnl.s failure
### Table 5.4 Exponential and Linear Curve Fit Results of Load to Failure Data

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<th>Linear Fit Slope</th>
<th>P-value</th>
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<th>Elongation Load</th>
<th>Max Elongation Load</th>
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<td>0.000</td>
<td>0.000</td>
<td>2.88E-03</td>
<td>7.84</td>
</tr>
<tr>
<td>Load</td>
<td>10</td>
<td>18</td>
<td>28</td>
<td>R34-10-10</td>
<td>5.79</td>
<td>6.04E-04</td>
<td>0.000</td>
<td>0.000</td>
<td>5.15E-03</td>
<td>9.16</td>
</tr>
<tr>
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<td>18</td>
<td>28</td>
<td>R38-10-10</td>
<td>19,098.50</td>
<td>1.88E-07</td>
<td>0.999</td>
<td>0.999</td>
<td>3.60E-03</td>
<td>4.76</td>
</tr>
<tr>
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<td>18</td>
<td>28</td>
<td>R55-10-10</td>
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<td>0.000</td>
<td>0.000</td>
<td>2.26E-03</td>
<td>5.26</td>
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<tr>
<td>Load</td>
<td>10</td>
<td>18</td>
<td>28</td>
<td>R56-10-10</td>
<td>14,074.33</td>
<td>2.22E-07</td>
<td>0.995</td>
<td>0.995</td>
<td>3.12E-03</td>
<td>2.64</td>
</tr>
<tr>
<td>Load</td>
<td>10</td>
<td>18</td>
<td>28</td>
<td>R35-10-10</td>
<td>15,821.83</td>
<td>5.70E-07</td>
<td>0.999</td>
<td>0.999</td>
<td>7.61E-03</td>
<td>2.83</td>
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<tr>
<td>Load</td>
<td>10</td>
<td>18</td>
<td>28</td>
<td>R27-10-10</td>
<td>2.25</td>
<td>1.28E-03</td>
<td>0.000</td>
<td>0.000</td>
<td>5.06E-03</td>
<td>4.52</td>
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<tr>
<td>Load</td>
<td>10</td>
<td>18</td>
<td>28</td>
<td>R41-10-10</td>
<td>5.23</td>
<td>5.87E-04</td>
<td>0.000</td>
<td>0.000</td>
<td>4.65E-03</td>
<td>8.85</td>
</tr>
<tr>
<td>Load</td>
<td>10</td>
<td>18</td>
<td>28</td>
<td>R42-10-10</td>
<td>92,699.47</td>
<td>6.97E-08</td>
<td>0.995</td>
<td>0.995</td>
<td>6.46E-03</td>
<td>13.46</td>
</tr>
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</table>
Figure 5.8: Linear regression of Maximum Load plotted against the number of loading treatment days. Note that the relationship of the two did not prove significant.
**Figure 5.9**: Linear regression of Yield Load plotted against the number of loading treatment days.

<table>
<thead>
<tr>
<th>Loading Treatments (Days)</th>
<th>Yield (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
</tr>
</tbody>
</table>

**Equation**: Polynomial, Linear $f = y_0 + a \cdot x$

- $R = 0.1447$
- $R^2 = 0.0210$
- $Adj \ R^2 = 0.0049$
- Standard Error of Estimate = 3.8202

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$y_0$</td>
<td>5.6954</td>
<td>0.7874</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$a$</td>
<td>0.0559</td>
<td>0.0490</td>
<td>1.1426</td>
</tr>
</tbody>
</table>
Figure 5.10: Linear Slope versus loading days. In either case the relationship did not prove significant.
Figure 5.11 A: Frequency of yield load data. B: Frequency of maximum load data. The responses for maximum Load approached a normal distribution while those for yield load did not.
## Mechanical Properties

<table>
<thead>
<tr>
<th>Factors</th>
<th>All failure modes, N = 63</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4,846.00</td>
</tr>
<tr>
<td>B</td>
<td>No Terms</td>
</tr>
<tr>
<td>Slope M</td>
<td>0.0035</td>
</tr>
<tr>
<td>Yield Load</td>
<td>2.16</td>
</tr>
<tr>
<td>Yield dist</td>
<td>1,169.00</td>
</tr>
<tr>
<td>Max Load</td>
<td>3.14</td>
</tr>
<tr>
<td>Max dist</td>
<td>2,762.00</td>
</tr>
<tr>
<td>P Delay (days)</td>
<td>0.239</td>
</tr>
<tr>
<td>P Study (days)</td>
<td>0.118</td>
</tr>
<tr>
<td>P Slope M</td>
<td>0.000084</td>
</tr>
<tr>
<td>P Yield Load</td>
<td>0.054</td>
</tr>
<tr>
<td>P Yield dist</td>
<td>0.000004</td>
</tr>
<tr>
<td>P Max Load</td>
<td>0.049</td>
</tr>
<tr>
<td>P Max dist</td>
<td>-33.7</td>
</tr>
<tr>
<td>P Constant</td>
<td>1096</td>
</tr>
<tr>
<td>P Study (days)</td>
<td>31.2</td>
</tr>
<tr>
<td>P Study (days)</td>
<td>0.083</td>
</tr>
<tr>
<td>P Yield Load</td>
<td>0.1547</td>
</tr>
<tr>
<td>P Yield dist</td>
<td>0.123</td>
</tr>
<tr>
<td>P Max Load</td>
<td>0.1602</td>
</tr>
<tr>
<td>P Max dist</td>
<td>0.065</td>
</tr>
</tbody>
</table>

### Table 5.5
Regression results for all 63 subjects, regardless of site or method of failure.

The first multiple MLR examined all 63 data-sets independent of failure site (Table 5.5). Stepwise regression at a significance level of $\alpha = 0.15$ for inclusion of terms produced the following equations predicting coefficient “a” of the exponential model and the slope of the linear model:

$$a = 4846 + 1096 \text{ Delay (days)}; \quad F_{2,61} = 2.52, \ p = 0.118, \ r^2 = 3.97\%,$$

$$\text{Slope} = 0.00335 + 0.000084 \text{ Study (days)}; \quad F_{2,61} = 3.12, \ p = 0.083, \ r^2 = 4.86\%,$$

However the effect of the independent variable was insignificant in each equation above, with (Delay (days); $\ p = 0.118$ and Study (days); $\ p = 0.083$.

For the maximum load, stepwise regression ($\alpha = 0.15$) resulted in the following equation:

$$\text{Max (N)} = 3.14 + 0.250 \text{ Delay (days)} + 0.1602 \text{ Study (days)}; \quad F_{3,60} = 4.47, \ p = 0.016, \ r^2 = 12.96\%$$

With significant effects for independent variables Delay (day); $\ p = 0.049$ and Study (days); $\ p = 0.028$.

Stepwise regression ($\alpha = 0.15$) also produced the following similar equation for the yield load:

$$\text{Yield (N)} = 2.16 + 0.226 \text{ Delay (days)} + 0.1547 \text{ Study (days)}; \quad F_{3,60} = 4.65, \ p = 0.013, \ r^2 = 13.43\%$$

Here the variable Delay (day) was almost significant, $\ p = 0.054$, while Study (days) was, with $\ p = 0.028$. 

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Table 5.6: Regression results reflecting tunnel failures only, N= 54, after removal of mid-substance, combined mid-substance/tunnel and physis failures from the collective of datasets.

For the second MLR (Table 5.6) all mid-substance tendon graft failures, combined mid-substance/tunnel and physis failures (n=9) were removed from the sample to focus solely on all sites of tunnel failure; femoral, tibial or both tunnels together. An α level of 0.15 prevented the inclusion of any of the independent variables in the attempts to build models predicting either the coefficients of the exponential model or the slope of the linear model.

The second MLR did produce a significant equation predicting maximum load:

Maximum (N) = 1.80 + 0.227 Delay (days) + 0.2209 Study (days); $F_{3,51} = 5.56$, $p = 0.006$, $r^2 = 18.15$

Here independent variable Delay (days) was not significant ($p=0.100$), while the variable Study (days) was significant ($p=0.004$) for the prediction of maximum load.

A similar equation was produced for the yield load:

Yield load (N) = 0.91 + 0.180 Delay (days) + 0.2164 Study (days); $F_{3,51} = 6.04$, $p = 0.004$, $r^2 = 19.15$

Again, Delay (days) was not significant ($p=0.150$), while Study (days) remained significant ($p = 0.002$).
### Table 5.7: Regression results reflecting femoral or tibial tunnel failures only, after removal of failures through both tunnels.

The third MLR (Table 5.7) removed failures through both tunnels from the sample to focus solely on femoral and tibial tunnel failures. Stepwise inclusion resulted in an equation predicting coefficient “a” of the exponential model, but failed to produce an equation for coefficient “b”, nor one predicting the slope of the linear model.

\[
A = 12909 + 838 \text{ Delay (days)} - 526 \text{ Study (days)}; \quad F_{3,39} = 3.46, \ p = 0.041, \ r^2 = 15.07\%
\]

Neither independent variable was significant in the above; Delay (days) \( p = 0.100 \), Study (days) \( p = 0.068 \)

The third MLR also produced significant equations for the maximum and yield loads.

\[
\text{Max (N)} = 1.24 + 0.278 \text{ Delay (days)} + 0.2352 \text{ Study (days)}; \quad F_{3,39} = 5.40, \ p = 0.009, \ r^2 = 21.68\%
\]

Variable Delay (days) \( p = 0.066 \) was insignificant, while variable Study (days) \( p = 0.007 \) was significant.

\[
\text{Yield (N)} = 0.72 + 0.239 \text{ Delay (days)} + 0.2120 \text{ Study (days)}; \quad F_{3,39} = 5.40, \ p = 0.009, \ r^2 = 21.67\%
\]

Variable Delay (days) \( p = 0.076 \) was insignificant, while variable Study (days) \( p = 0.007 \) was significant.

<table>
<thead>
<tr>
<th>Multiple Linear Regression</th>
<th>Femoral or Tibial Tunnels Only, ( N = 42 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Response</strong></td>
<td><strong>Factors</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Constant</strong></td>
</tr>
<tr>
<td>A</td>
<td>12,909.00</td>
</tr>
<tr>
<td>B</td>
<td>No terms</td>
</tr>
<tr>
<td>Slope M</td>
<td>No terms</td>
</tr>
<tr>
<td>Yield Load</td>
<td>0.72</td>
</tr>
<tr>
<td>Yield dist</td>
<td>678.00</td>
</tr>
<tr>
<td>Max Load</td>
<td>1.24</td>
</tr>
<tr>
<td>max dist</td>
<td>1,619.00</td>
</tr>
</tbody>
</table>
Table 5.8: Regression results reflecting tibial tunnel failures only.

The last MLR (Table 5.8) looked at tibial tunnel failures alone (N = 26). Again stepwise inclusion resulted in an equation for the coefficient “a” of the exponential model, but not for coefficient “b”, or the slope of the linear model.

\[
a = 29025 - 990 \text{ Study (days)}; \quad F_{2,24} = 5.44, p = 0.028, r^2 = 18.49%
\]

Variable Study (days) was significant with \( p = 0.028 \).

In the equations for yield load and maximum load, the \( \alpha \) level of 0.15 prevented stepwise regression from including a term for the number of Delay (days):

Max Load \( (N) = 2.01 + 0.248 \text{ Study (days)}; \quad F_{2,24} = 3.81, p = 0.063, r^2 = 13.69\%

However variable Study (days) was no longer significant \( p = 0.63 \).

Yield load \( (N) = 1.75 + 0.209 \text{ Study (days)}; \quad F_{2,24} = 3.85, p = 0.061, r^2 = 13.82\%

Independent variable Study (days) was no longer significant, with \( p = 0.61 \) in the above equation.
Discussion

The objective of Chapter Five was to identify the optimum time-frame to begin postoperative knee rehabilitation exercise in the rat model of ACL-R. This required the manipulation of several experimentally controlled variables including the delay period prior to beginning daily loading treatments (Delay (days)), the study length (Study (days)) and the actual number of treatment days (Load (days)). Multiple linear regressions were used to assess the effect of each independent variable in predicting the mechanical properties (maximum load, yield load and load-elongation model), of the reconstructed femur-tendon graft-tibia construct, and to tease out any association between these variables and the mode or site of failure.

The first MLR analyzed all data sets independent of failure site. It did not reveal any significant relationship between the experimental variables and the load-elongation relation (stiffness) of the construct, whether described by either the exponential “Fung” model or a linear model. This is not surprising considering the variety of failure modes across all 63 subjects (Figures 5.7 A-C), and the possible effect of friction between the tendon graft and tunnel as the tendon pulled through bone tunnels.

However both the Delay (days) and Study (days) variables had a significant effect as shown in the following regression model predicting the magnitude of the maximum load:

\[
\text{Maximum Load (N)} = 3.14 + 0.250 \text{Delay (days)} + 0.1602 \text{Study (days)}; \quad \text{Equation. 1}
\]

Note the effect of Delay (days) was roughly 1.56 times greater (N/day) than that of the number of study days. This supports the hypothesis that an initial period of load shielding prior to daily loading is more significant to increase the maximum load of the ACL-R. Considering that the factor Delay (days) remains
fixed from the start of the study, its predicted effect on maximum load could be visualized as three separate plots, one for each delay period group, of the maximum load at failure versus study length (Figure 5.12).

Figure 5.12: Plot of the expected effect of delay period on maximum load as predicted by equation 1.

Equation 1 partially explains the results of Bedi et al. (Figure 5.13), who in the initial publication of these data reported that the group beginning loading after a delay of 10 days achieved a mean maximum load of 9.6 (N) at the end of the 14 day study, more than double the 4.4 (N) means of the group loaded daily from day 1, and the immobilized group (p < 0.01).

However, while equation 1 would predict a greater maximum load for a 10 day delay over a 4 day delay period, Bedi et al. found that the 4 day delay group of the 28 day study achieved a mean maximum load of 13.5 (N), significantly greater than the 10 day delay and all other groups (Figure 5.13, p = 0.01). This implies there is a limit to the additive effect of the Delay (days) variable of equation 1,
which increases from zero to a peak in magnitude somewhere around the 4 day time-point, then begins to decline to day ten.

Figure 5.13: Plot of the maximum loads of all four treatment groups. At 2 weeks the 4 and 10 day delayed groups show increased maximum load as predicted by Equation 1. However, at 4 weeks, mean of the 4 day delayed group is significantly greater than all other groups, while the 10 day delay group is not significantly different from the others, contradicting equation 1. (From the first publication of these data by Bedi et al.) The asterisk indicates \( p = 0.01 \).

The second MLR removed all mid-substance/and or physis failures from the sample. As before neither the exponential coefficients nor linear slope had a significant relationship with the experimental variables. The Delay (days) factor was no longer significant in the regression equation for maximum load (\( p = 0.01 \)), leaving only study days as a significant factor (\( p = 0.004 \)).

Maximum Load (N) = 1.8 + 0.227 Delay (days) + 0.2209 Study (days); \hspace{1cm} Equation 2

The effect of Delay (days) has decreased to 1.03 times the effect of Study (days) in Equation 2, so removing mid-substance failures from the sample set has equalized the predictive effect of Delay
(days) and Study (days) on the maximum load. Failures by mid-substance indicate that the graft insertions have held up long enough for the load to exceed the strength of the tendon graft, their exclusion removes 9 data sets with a mean maximum load of 9.8 Ns from the sample and could explain the reduced maximum load predicted by equation 2.

Prior to the third MLR, we removed 12 failures via “both tunnels” from the sample set. This only slightly increased the effect of Delay (days) to 1.18 that of Study (days) in the regression equation for maximum load, although Delay (days) remained non-significant (p = 0.066). The “a” coefficient for the exponential model just missed significance (p = 0.056).

\[
\text{Max (N)} = 1.24 + 0.278 \text{ Delay (days)} + 0.2352 \text{ Study (days)};
\]  \text{Equation 3}

For the fourth MLR, femoral tunnel failures were removed to focus solely on tibial tunnel failures. This further reduced the significance of the Delay (days) term, bringing it above the \( \alpha \) level for inclusion in the model, and also resulted in the loss of significance of the Study (days) variable (p = 0.630), in the regression equation for maximum load

\[
\text{Maximum (N)} = 2.01 + 0.248 \text{ Study (days)}; \quad \text{Equation 4}
\]

Separation according to failure site into separate populations analyzed by subsequent MLR did not produce any significant models predicting the mechanical parameters of the femur-graft-tibia construct, for any specific failure type; whether mid-substance failure, femoral tunnel or tibial tunnel pull-out. Although the first MLR produced a significant equation for yield load, similar to that of maximum load, the non-normal distribution of the yield load sample population (Figure 511B) violates the assumptions of MLR, and invalidates further predictive modeling of the yield load.
Despite my expectation for an effect of the different number of loading days between treatment groups (the Load-days variable in Table 5.4), it failed to reach the $\alpha$-level of significance required for initial inclusion in any of the models produced by the MLRs. Future studies should consider equalizing this factor across groups; such as 4 and 10 day delay period groups, each receiving a subsequent 20 days of loading treatments, for a total study length of 24 and 30 days respectively. Six unloaded days could be added at the end of 4 day delay group, to equal the 30 day study length of the 10 day group, should prolonged study length prove to have an effect equal or greater to loading.

The fact that the 4 day delay group at 28 days achieved the largest mean maximum load, despite the Equation 1 predicting this for the 10 day group, suggests nonlinear behavior of the Delay (days) variable with a peak around four days. For a better estimate of the optimum immobilization period duration prior to post ACL-R knee re-mobilization, future studies could use non-linear regression, with the magnitude of the Delay (days) variable modeled as a bell curve centered close to four days. Ideally this would result in increased significance of the resulting regression equations predicting the mechanical parameters of the treated ACL-R.

A possible explanation of the mechanism behind the Delay (days) factor in equation 1, is the increased bone volume, mineral content and density of the tibial tunnel in both the 4 and 10 day delayed loading groups (micro-CT results of Table 5.2). The delay periods are associated with greater bone ingrowth into the tunnel, increasing contact with and friction on the graft tendon, resulting in greater loads required for failure of the femur-tendon graft-tibia construct.
Chapter 6

Effect of immediate and delayed high-strain loading on tendon-to-bone healing after anterior cruciate ligament reconstruction

The results of chapter five show that post ACL_R, adding a four to ten day immobilization period (load shielding) prior to the onset of the daily loading protocol (cyclic knee distraction), improves tendon to bone healing. Because the group receiving four days of immobilization prior to daily loading achieved a significantly higher failure load, we decided not to continue the ten day initial immobilization group. Instead, chapter VI will determine the effect of increasing graft strain to 10 %. We continued using the more effective (for this rat model) 4 day immobilization period prior to beginning daily loading on postoperative day 4, to a distance calculated to achieve 10% graft strain.

$H_2$, the modified hypothesis governing chapter VI states: post ACL_R surgery, after an initial four day period of load shielding, daily sessions of high-strain tensile graft loading will improve tendon graft to bone tunnel healing, compared to either continuous load shielding or immediate onset tensile graft loading.
Methods

With IACUC approval, seventy male Sprague Dawley rats weighing from 300 to 380 grams underwent the identical ACL reconstruction procedure and knee immobilizing ex-fix placement described in chapter IV and V. These rats were then randomly assigned among three experimental groups: (1) Continuous immobilization, (2) Immediate 10% cyclic tendon graft strain, daily beginning on postoperative day 1, and (3) Delayed onset 10% cyclic graft strain beginning on postoperative day 4.

As the study progressed thirty five animals needed to be sacrificed prematurely due to; infection (n=7), complications with anesthesia (n=4) and fracture of the limbs undergoing distraction (n=24). Upon dissection prior to load to failure testing, an additional nine specimens were discovered to have failed tendon grafts, with five occurring in the immediate loading group alone. The final group totals were; eight specimens of continuous-immobilization, seven of immediate-loading and eleven of the delayed-loading on day four, (figure 6.1).

Figure 6.1: Study Design
Daily Knee Distraction Protocol

The animals were anesthetized and placed in the CDM to receive fifty cycles of cyclic knee distraction as previously described in chapter four. For this study of high strain, 10% of the average graft length of 15 mm equated to 1.5 mm. Knee joint distraction to this distance was achieved using the compliance correction factor for the CDM with coupled ex-fix calculated in chapter III [115], and the average slack length from the cadaver study of chapter IV, in the following formula:

\[
\text{Knee Distraction} = \text{LVDT (mm)} - \text{Load (N)} \times \text{Compliance (mm/N)} - \text{average slack length (mm)}
\]

Here compliance = 42.4 µm/N, and average graft slack length = 0.55 mm

Upon study commencement an initial high incidence of fractures during joint distraction necessitated imposition of a load limit of 2,400 N on the CDM system. Thereafter the CDM distracted the knee joint at 0.24 mm/sec until the target distance of 1.5 mm, or the load limit was reached, then returned the joint to the starting position for the next cycle.

Micro-Computed Tomography Analysis

Micro-CT scanning of the tibia specimens was performed, focused on a cylindrical volume of interest of 3.4 mm diameter along the length of the tibial bone tunnel, using the procedure detailed in chapter four. Bone formation and remodeling were evaluated with five quantitative measures: new bone volume (mm³), tissue mineral content (mg), tissue mineral density (mg/mL), trabecular thickness (µm), and trabecular number.

Figure 6.2: Micro-CT image of the tibial tunnel; and its division into three cylindrical regions for analysis; intra-articular aperture, mid-tunnel, and extra-articular aperture [117].
Biomechanical Testing

The biomechanical testing procedure was previously detailed in chapters IV and V. Briefly, limb specimens were thawed and dissected until only the femur-graft-tibia complex remained. Both tibia and femur were potted and securely mounted in the grips of a custom tensile test machine, with the bone tunnel axes parallel to test machine translation.

After five cycles of preconditioning from 0 to 0.2 N load, elongation to failure of the femur-graft tibia complex proceeded at 10 mm per minute. The stiffness (N/mm) and ultimate load (N) were calculated from the load-displacement relation. The site and of mode failure (tunnel pull-out or mid-substance graft rupture) were noted.

Statistical Analysis

Two way analysis of variance followed by a post hoc Tukey test was used to find significant differences, (p < 0.05) among group data from the micro-CT and biomechanical data.

Results

Micro-CT

Both cyclic distraction loaded groups had significantly higher tissue mineral density in new bone through the entire tibial tunnel; with the four-day delayed-loading at 586 mg/ml and immediate-loading at 589.7 mg/ml, versus continuous-immobilization with 571 mg/ml, (p < 0.05). The four-day delayed-loading group showed significantly greater tissue mineral density around the distal-tunnel, extra-articular aperture (813.0 mg/mL), than either the immediate-loading (778.4 mg/mL) or continuous-immobilization group (784.9 mg/mL), (p < 0.05), (Table 6.I) [117].

The continuous-immobilization and the four-day delayed-loading groups had bone volume fractions (bone volume / total volume) that appeared greater than the immediate loading group; 0.24
and 0.23 compared to 0.20, (Table 6.1 and figure 6.2). The continuous-immobilization group also had a trabecular thickness in the mid-tunnel region that was significantly greater than the immediate-loading group, (106.5 versus 72.6 mm, p < 0.01) [117].

No significant differences were found for; bone volume (p = 0.241), new-bone volume (p = 0.331), trabecular spacing (extra-articular: p = 0.723, mid-tunnel: p = 0.331, intra-articular: p = 0.542), or trabecular number (extra-articular: p= 0.538, mid-tunnel: p= 0.272, intra-articular: p= 0.317) [117].

**Biomechanical Testing**

No significant differences in failure load (p = 0.366) or stiffness (p = 0.247) were found. The mean failure loads by group were (N):

Continuous-immobilization 7.8 ± 2.3; immediate-loading 6.5 ± 1.6; delayed-loading 6.4 ± 2.4

And mean stiffness by group (N/mm):

Continuous-immobilization 3.0 ± 0.3; immediate-loading 3.5 ± 1.3; delayed-loading 4.2 ± 1.6, [117]

![Biomechanical testing results showing no significant differences between groups in the failure load or stiffness of the femur-graft-tibia construct](image)

**Figure 6.3** Biomechanical testing results showing no significant differences between groups in the failure load or stiffness of the femur-graft-tibia construct [117].
Table 6.1: Summary of Micro-CT Data for the Tibial Tunnels

<table>
<thead>
<tr>
<th>Group</th>
<th>Continuous Immobilization</th>
<th>Immediate Distraction</th>
<th>4-Day Delayed Distraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue mineral density (mg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire tunnel (new bone)</td>
<td>571.0 ± 3.6</td>
<td>589.7 ± 14.8‡</td>
<td>586.5 ± 12.7‡</td>
</tr>
<tr>
<td>Entire tunnel (total bone)</td>
<td>819.4 ± 26.8</td>
<td>827.0 ± 17.2</td>
<td>837.9 ± 29.2</td>
</tr>
<tr>
<td>Extra-articular aperture</td>
<td>784.9 ± 26.4</td>
<td>778.4 ± 32.6</td>
<td>813.0 ± 24.9‡§</td>
</tr>
<tr>
<td>Mid-tunnel</td>
<td>794.7 ± 32.7</td>
<td>776.3 ± 35.0</td>
<td>792.0 ± 45.8</td>
</tr>
<tr>
<td>Intra-articular aperture</td>
<td>837.3 ± 65.6</td>
<td>845.4 ± 45.9</td>
<td>852.6 ± 52.8</td>
</tr>
<tr>
<td>Bone volume/total volume (total bone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire tunnel</td>
<td>0.24 ± 0.03</td>
<td>0.20 ± 0.05</td>
<td>0.23 ± 0.06</td>
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<tr>
<td>Extra-articular aperture</td>
<td>0.18 ± 0.06</td>
<td>0.22 ± 0.26</td>
<td>0.15 ± 0.08</td>
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<tr>
<td>Mid-tunnel</td>
<td>0.15 ± 0.07</td>
<td>0.13 ± 0.07</td>
<td>0.10 ± 0.06</td>
</tr>
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<td>Intra-articular aperture</td>
<td>0.27 ± 0.12</td>
<td>0.24 ± 0.07</td>
<td>0.21 ± 0.10</td>
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<td>Bone volume/total volume (new bone only)</td>
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<td></td>
<td></td>
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<tr>
<td>Entire tunnel</td>
<td>0.13 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Extra-articular aperture</td>
<td>0.069 ± 0.020</td>
<td>0.057 ± 0.022</td>
<td>0.069 ± 0.031</td>
</tr>
<tr>
<td>Mid-tunnel</td>
<td>0.069 ± 0.030</td>
<td>0.057 ± 0.023</td>
<td>0.066 ± 0.027</td>
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<tr>
<td>Intra-articular aperture</td>
<td>0.090 ± 0.030</td>
<td>0.071 ± 0.025</td>
<td>0.079 ± 0.021</td>
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<td>Trabecular number (avg./mm)</td>
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<tr>
<td>Extra-articular aperture</td>
<td>2.67 ± 0.47</td>
<td>2.49 ± 0.48</td>
<td>2.95 ± 1.20</td>
</tr>
<tr>
<td>Mid-tunnel</td>
<td>3.07 ± 0.65</td>
<td>2.47 ± 0.60</td>
<td>2.88 ± 0.80</td>
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<td>3.62 ± 0.78</td>
<td>3.16 ± 1.15</td>
<td>3.19 ± 1.11</td>
</tr>
<tr>
<td>Trabecular thickness (μm)</td>
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<td></td>
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<tr>
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<td>99.0 ± 21.8</td>
<td>83.8 ± 16.0</td>
<td>99.5 ± 24.0</td>
</tr>
<tr>
<td>Mid-tunnel</td>
<td>106.5 ± 23.0</td>
<td>72.6 ± 10.6#</td>
<td>89.7 ± 21.6</td>
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<td>Intra-articular aperture</td>
<td>143.8 ± 27.9</td>
<td>120.6 ± 25.4</td>
<td>127.8 ± 27.9</td>
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</table>

‡Significantly different from immobilized group (p < 0.05).
‡§Significantly different from immediate-loading group (p < 0.05).
§Significantly different from immobilized group (p < 0.01).
**Figure. 6.4:** Axial micro-CT images of the tibia tunnel; A) continuous-immobilization, B) immediate-loading, and C) delayed-loading. Three-dimensional reconstructions of the tibia tunnel, sagittal views; D) continuous-immobilization, E) immediate-loading, and F) delayed high-strain loading [117].

**Discussion**

Although this study shares several parameters with chapter five, including the use of immediate and four day delayed-loading groups, its goal was not to investigate temporal delays, but rather to determine the effect of a higher magnitude of strain on tendon to bone healing. High graft strains are clinically relevant, current ACL_R techniques such as trans-tibial drilling of the femoral bone tunnel result in non-anatomic and non-isometric graft placement, meaning that upon knee flexion the relative rotation and displacement of the tunnel apertures elongates the healing graft.
The micro-CT data partly supports our hypothesis, with the four-day delayed-loading group showing significantly greater tissue mineral density in the distal tibial tunnel. Both the delayed-loading and immobilization groups showed a trend toward greater bone volume and trabecular thickness. However neither the immediate nor four-day delayed-loading group showed improved biomechanical properties or bone structural properties than the continuous immobilization group.

This is in contrast with the findings of chapter V where a smaller 2% graft strain was applied. In that study the four day delayed-loading group showed superior results to the both the immobilized and the ten day delayed-loading groups. This comparison implies that the benefits of a four day delay before the onset of knee joint distraction have been offset by the order of magnitude increase in graft strain.

This study was particularly challenging as evidenced by the number of animals lost prematurely. Each of the 50 daily 1.5 mm distractions of the knee joint placed large bending loads on the k-wire pins used to secure the ex-fix to the bone, precipitating fractures, pin loosening and infections. Placement of the 2,400 gram load limit was necessary to reduce fractures, but as the soft tissue surrounding the graft healed over time, load across the joint space increased, resulting in some subjects not reaching the 1.5 mm target distance. Therefore we cannot guarantee reproducible graft strains across animals.

This study did show impairments in trabecular bone remodeling when comparing immediate postoperative onset high graft strain (immediate-loading) with both continuous immobilization and a four day immobilization period prior to the onset of high graft strain (delayed-loading). However this result did not correlate with failure load of the femur-graft-tibia construct. These results support our previous finding of the effectiveness of a four day period of pre strain immobilization and argue for human ACL rehabilitation programs consisting of a brief immobilization period followed initially by exercises that limit the amount of applied graft strain.
Chapter 7: Conclusion

To investigate the effect of tensile loading protocols on tendon graft to bone tunnel healing, we developed and validated a novel device to distract the knee joint in a rat model of ACL_R, thereby imposing tensile strain on the graft within. To our knowledge this is the first time a controlled amount of mechanical stimulation has been applied to an animal model of tendon graft implantation. Our goal is to enhance our understanding of tendon to bone healing and apply the knowledge gained to the design and development of improved rehabilitation protocols that effect more robust and functional ligament reconstructions and tendon repairs.

Exposure to tensile loading has been shown to maintain tendon/ligament homeostasis, with the tensile modulus of the tissue increasing in response to greater loading frequency or even decreasing in its absence. However after injury or surgical repair the effect of tensile load is less clear, with many studies implicating mechanical loading with impaired healing, particularly in the case of torn rotator cuff tendon. Clinicians need to know when they can safely reintroduce tensile strain on a healing repair, and ideally choose the time point when it can most benefit the remodeling process.

In Chapter IV we investigated the effect of beginning tendon graft strain immediately after ACL_R surgery, by starting daily sessions of knee joint distraction on postoperative day one (the immediate-loading group), and comparing the effect with strain shielded rats whose ACL_R knees were immobilized by continuous wearing of the ex-fix (the continuous-immobilization group). Although we did not see any significant differences in structural properties or new bone formation between the two groups there was a significant increase in inflammatory, ED1 antigen positive [118] macrophages from circulation in the two-week immediate-loading group. This result partly supported the hypothesis that
an immediate onset of tensile strain on postoperative day one would impair healing, as evidenced by an increased influx of inflammatory cells. Overall there was no measureable difference in the strength of the reconstruction between the immediate-loading group and continuous-immobilization group.

In Chapter V we explored the effect of delaying the onset of postoperative tendon graft strain by inserting either a four or ten day period of immobilization, enforced by the ex-fix, prior to beginning the daily knee distraction protocol (the four or ten-day delayed-distraction groups). These results supported the hypothesis that a four to ten day immobilization period prior to graft strain would improve tendon to bone healing, with both the four and ten-day delayed-loading groups showing more new bone formation in the tendon-tunnel interface, fewer inflammatory macrophages and a greater failure load and stiffness than the continuous-immobilization and immediate loading groups. The four-day delayed-loading group ending on postoperative day 28 had a remarkably high failure load of 13.5 N which compares relatively well after only four weeks of healing to the roughly 30 – 40 N failure load of the native ACL of this species reported in the literature and experienced in our laboratory.

For chapter VI we shifted our focus from varying the temporal onset to determining the effect of an order of magnitude increase in strain, by augmenting the joint distraction distance from two to ten percent of original tendon graft length (using the compliance correction of chapter III). Due to the superior performance of the four-day delayed-loading group in chapter V and an increased rate of fractures, we did not repeat the ten-day delayed-loading n group.

Both ten-percent strain knee distraction groups, the immediate-loading (postoperative day one) and the four-day delayed-loading group had a significantly greater tissue mineral density than the continuous-immobilization group. The four-day delayed-loading group also had a significantly higher tissue mineral density around the distal tibial tunnel exit, than either the immediate-loading or continuous-immobilization groups. However the continuous-immobilization group had a trabecular
thickness in the mid tunnel region significantly greater than the immediate-loading group. Overall the net effect of the increase in graft strain to 10% appeared negligible as there was no significant difference in the load to failure or stiffness of the femur-graft-tibia construct between all three groups. Apparently the effect of the four-day delay prior to daily cyclic knee distraction, which correlated with such a marked increase in the load to failure of the four-day delayed-loading group of chapter V, was somehow negated by the greater strain magnitude.

In a previous study our lab histologically documented the postoperative cell population within the tendon-bone tunnel interface, again using a rat model of ACL-R, but without immobilization or knee distraction. A rapid influx of neutrophils proceeded through the interface and into the tendon graft, peaking in population by postoperative day four and receding by day seven. This coincided with an increasing population of ED1+ macrophages in the interface that remained elevated from days 11 through 21. However resident ED2+ macrophages did not appear in the interface until day 11 where they reached a peak population by day 14 to remain at this level through the end of the study on day 28 [119].

Comparing the above with the current data from chapters IV and V one sees a significantly greater number of ED1+ macrophages in both the continuous-immobilization and immediate-distraction groups at both the 14 and 28 day time points. Presumably the three to four day lifespan neutrophil population receded long before sacrifice. It appears that these treatments are prolonging the inflammatory and proliferative phases of the wound healing process. In contrast, both the four and ten-day delayed-loading groups show a significantly greater number of ED2+ macrophages, appearing as if these groups had progressed into the remodeling phase of the healing process.
One explanation for the relatively poor performance of the immediate-loading group in chapter IV may be that the lack of an initial immobilization period never allows the inflammatory phase of wound healing to resolve. Instead initiation of knee distraction on postoperative day one continually ruptures coagulations before blood vessels heal, resulting in a prolonged recruitment of catabolic ED1+ macrophages from circulation. This is reflected in the higher levels of ED1+ cells counted in this group than in the four and ten-day delayed-loading groups. The catabolic nature of the ED1+ macrophage could result in its continual removal of ECM material from the interface and necrotic graft tendon tissue, possible accounting for the reduced failure load and stiffness in this group. In chapter VI the super-physiologic level of strain could also prolong the inflammatory-catabolic phase of heal and account for the lack of a difference in failure loads between groups.

The addition of four and ten day immobilization periods to strain shield the graft would allow time for healing to progress beyond the coagulation and inflammation phases and into the proliferation and remodeling phases. Accumulation of sufficient numbers of fibrocytes, and resident ED2 antigen positive macrophages with the potential to differentiate into osteoblast and osteoclast cells could result in the deposition of greater quantities of ECM material into the tendon-tunnel interface. Upon the beginning of cyclic knee distraction, the lower two-percent level of tensile strain could potentially guide these cells in remodeling the interface tissue along the predominant orientation of tensile stress, and account for the greater load to failure of the four- and ten-day delayed-loading groups of chapter V.

Out of all the rehabilitation protocols for the rat model of ACL_R described in this dissertation, the one judged most effective on the basis of greatest failure load and stiffness, most bone ingrowth, and lowest inflammatory cell population, is the four-day delayed-loading protocol of chapter V. It can be
equated to initial three to four days of postoperative knee immobilization, followed by a knee motion beginning on postoperative day four, that reliably imposes 2 % tendon graft strain, repeated for 50 daily repetitions until postoperative day 28.

Extrapolating the above parameters from the rat model may prove difficult. Perhaps the changeover in macrophage population from circulating to resident could provide a clue as to when to end the immobilization period and begin a knee mobilization/graft strain regime in a human clinical trial. A 2-4 % strain has been reported to occur in the human ACL from the activities of daily living, and exercises duplicating these activities applied in the trial. Flexion limiting knee braces are currently prescribed after ACL_R and could be used to impose a short period of knee immobilization. Therefore clinical trials of protocols utilizing initial immobilization periods are possible.

There are limitations in the transfer of knowledge acquired from the studies described herein. In-vivo loading of the native ACL is complex and consists of components of tension, compression, torsion and shear, while the knee distraction utilizes uniaxial tension alone, the most reliably controlled and quantified method available to us for this small animal model. Distraction could have potentially added to the resulting inflammation, particularly in the high strain study of chapter VI. Additional structures were severed during surgery to prevent their initial stress shielding of the graft tendon. Bleeding and release of cytokines and other bioactive factors from these structures would undoubtedly affect the healing response.

Future work in tendon to bone healing by our group has already begun. In response to concerns over the high rate of fractures engendered by the ex-fix bone pins while under load during knee distraction, I designed a second tendon graft loading system that elongates the graft via a subcutaneous pull wire. Here only a partial ACL_R is performed with one end of the graft secured in a lone femoral
bone tunnel, its opposite end terminates in a free floating bone block, from which a trans-cutaneous pull will applies tension to the graft during experimental sessions. A paper describing the initial results achieved with this device is expected to be published in this fall.

Need for a more clinically relevant rat model of ACL_R rehabilitation that more faithfully replicates common knee extension exercises resulted in my design of a rat knee flexion device [120].
References


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[60] 2001, Langman’s Medical Embryology, Lippincott Williams & Wilkins.


“acl_rehab_protocol.pdf.”


