Effects of Gaps Induced Into the ACL Tendon Graft on Tendon-Bone Healing in a Rodent ACL Reconstruction Model

Vedran Lovric
Tomonoshin Kanazawa
Yoshinari Nakamura
Rema A. Oliver
Yan Yu
William Robert Walsh

Surgical and Orthopaedic Research Laboratories,
University of New South Wales, Prince of Wales Hospital, Sydney, Australia.

Corresponding author:
William R Walsh
Director, Surgical and Orthopaedic Research Laboratory
Prince of Wales Hospital
University of New South Wales
Tel: 9382 2657
Email: w.walsh@unsw.edu.au

Summary

Graft necrosis following ACL reconstruction is often associated with the use of autologous grafts. Host cells rather than graft cells contribute to the repair of the tendon-bone interface and the remodeling of the autologous graft. The native tendon-bone interface is not recreated and the biomechanical properties are not restored back to native values. We examined the effects of introducing gaps within the tendon graft prior to ACL reconstruction in a rodent model. We hypothesized that gaps will make physical way for host cells to infiltrate and repopulate the graft material post surgery thus prevent graft necrosis in the initial periods of the healing process and potentially improve biomechanical and histological properties of the tendon-bone interface.

Key words: ACL reconstruction, graft cells, graft necrosis, host cells, rodent model, tendon-bone healing

Introduction

The native insertional site of the Anterior Cruciate Ligament (ACL) is classified as a fibrocartilaginous enthesis and is characterized by four distinct zones. These regions gradually merge into one another and create a complex transitional structure that enables stress dissipation and smooth transition of forces across two materials with contrasting mechanical properties. The four zones that typify the fibrocartilaginous insertional site are morphologically separated into fibrous connective tissue, non-mineralized fibrocartilage, mineralized fibrocartilage and bone (1). Following surgical repair, particularly in the early postoperative periods, this highly specialized transitional zone is not recreated and normal mechanical properties are not achieved (2-4).

A strategy to improve the healing process and promote solid integration between tendon and bone in order to realize early biomechanical properties may hasten and improve rehabilitation and outcome.

The cell types that initiate and regulate the process of healing at this junction have not been concretely identified. It has been reported that in the first two weeks following ACL reconstruction in a rodent model, graft cells do not survive and the graft undergoes necrosis (5). It seems that the host cells rather than graft cells contribute to the repair of the interface and the remodeling of autologous grafts (6). These host cells could be of several types and the accumulation of these requires either recruitment of new cells from the circulatory system or mitogenesis of host cells found in the surrounding bone marrow, joint cavity, or the tendon graft (6). There is a need to facilitate the introduction of host cells into the grafted tendon in order to prevent early graft necrosis and initial weakness of the graft and further promote tendon-bone healing.

In this study we utilized a previously reported rodent ACL reconstruction model (6) to examine what effects the introduction of gaps within the tendon graft, prior to ACL reconstruction, may have on tendon-bone healing. We hypothesized that introducing gaps will make physical way for host cells to infiltrate and repopulate the graft material post surgery thus prevent graft necrosis in the initial periods of the healing process and potentially improve biomechanical and histological properties of the tendon-bone interface.

Materials and Methods

Study approval was obtained from the local Animal Care and Ethics Committee.

Study Design

A total of 42 female Sprague Dawley rats (11 weeks of age) were used in this study. Animals were randomly allocated to 2 equal numbered groups (control reconstruction or reconstruction with artificially induced gaps within the tendon graft) and surgery was performed. Animals were sacrificed at 1 week (n = 6), 2 weeks (n = 18) and 4 weeks (n = 18) following surgery. Six rats in each group were culled for biomechanical testing at 2 and 4 weeks while the other 6 specimens at each time point were prepared for histological examination (3 per
In order to test the hypothesis that gaps within the tendon do not compromise mechanical properties of the graft, an in vitro pilot study was completed prior to commencement of the animal study.

**Animal Model and Surgery**

A rodent ACL reconstruction model was adopted from Kawamura et al. (6) and slightly modified. Briefly, the animal was placed under general anesthesia and a longitudinal incision was made to the medial aspect of the distal leg and ankle. The flexor digitorum longus (FDL) tendon was identified and cut just distal to the ankle. A second incision was made over the knee a lateral parapatellar arthrotomy was performed and the native ACL excised. Using an 18-gauge needle (outer diameter 1.27 mm) a bone tunnel was made in the proximal tibia and the distal femur, entering the joint at the attachment sites of the ACL.

In the experimental group gaps were created between the collagen fascicles by the use of a 21-gauge needle (outer diameter 0.8 mm). 20 gaps were fashioned along two parallel rows (10 gaps within each) along the entire length of the graft (Fig. 1). The graft was left relaxed on top of a surgical swab while the gaps were created to prevent possible damage to the collagen fascicles. A 4-0 Ethibond suture (Ethicon, Somerville, NJ) was passed through each end of the previously-harvested tendon graft and then the graft was passed through the bone tunnels to replace the ACL. Both ends of the grafted tendon were secured to the surrounding periosteum at the extra-articular tunnel exit site at the distal femur and proximal tibia using 4-0 Ethibond suture. Muscle was closed with interrupted 4-0 Ethibond suture and skin was closed, as a separate layer to muscle, in a continuous fashion using 3-0 Vicryl suture (Ethicon, Somerville, NJ). Postoperatively, all animals were returned to their cage and activity was allowed ad libitum.

**Histology**

Following sacrifice, right hindlimbs (from the femoral head to the distal tibia) were harvested. The skin and soft tissues were dissected away from the bones, with care taken not to disturb the knee joint. The femur-ACL graft-tibia specimens were fixed for 48 hours in 10% neutral buffered formalin. Tissues were transferred into a 10% formic acid-formalin solution for decalcification. Once adequate decalcification had been achieved (generally 2-3 days), the femur-ACL graft-tibia complex was sectioned axially, perpendicular to bone tunnels, into 8 sections. The axial sections were processed for paraffin histology using a tissue processing machine (Shandon Excelsior ES®, Thermo Fisher Scientific, Kalamazoo, USA). The segments were embedded in paraffin wax, cut into 5µm thick serial sections using a hand-operated microtome (Leica RM2165, Leica Instrument GmbH, Nussloch, Germany) and mounted onto glass slides for histology. Sections were stained using Harris Haematoxylin & Eosin (H&E).

**Histomorphology**

Histomorphometric assessment of cellularity was quantified using Bioquant software (BIOQUANT Image Analysis Corporation, Nashville, Tennessee). One tibial section per specimen, 5 mm below the tibial plate, was used for quantification of cellularity. Quantitative analysis involved counting the number of fibroblasts within a corresponding tibial section of all the specimens prepared for histology. Firstly, images containing the entire graft area (×4 magnification) were digitally captured (for reference purposes) using a camera (DP72, Olympus, Tokyo, Japan) attached to the microscope. The magnification of the microscope was increased to ×20 and a further five equivalent regions of interest (ROIs) were captured for fibroblast cell counting. The ROIs namely ‘03’, ‘06’, ‘09’, ‘12’ (referenced to analogous hours on a clock), and ‘mid’ were placed in corresponding regions of the graft across all specimens examined (Fig. 2). The captured images were then imported to the Bioquant software and processed to select only the fibroblastic cells within the ROI (Fig. 3). The cells were quantified for each ROI and the number of fibroblasts within one section of one specimen was estimated by the total number of cells within all five ROIs of that specimen.

---

Figure 1. Schematic drawing illustrating the FDL tendon graft (A) and positioning of gaps fashioned between the collagen fascicles (B). Gaps were created using a 21-gauge needle (outer diameter 0.8 mm). 10 gaps were created along each of the two parallel rows along the entire length of the graft.
In vitro mechanical testing was performed on 16 rat FDL tendons (n = 8 control group & n = 8 experimental group). Gaps in the experimental group were created in identical fashion as the in vivo study. Mechanical testing parameters were kept constant. Mechanical testing of the specimens from the in vivo study involved harvesting the femur-ACL graft-tibia construct from each animal. The constructs were stripped of all soft tissue. Surgical loupes were used and extra care taken to ensure no damage was made to the ACL graft whilst dissecting the remaining knee ligaments. Sutures used to secure the graft were not removed from the specimens. Following complete dissection, the construct was embedded, in full extension, into custom made jigs using low point Wood’s metal to ensure secure fixation. Specimens were mounted onto a material testing machine 858 Bionix (MTS Systems, MN, USA). Mechanical testing was performed in force control with zero axial load. The construct was loaded to failure at a rate of 0.5mm/s. Failure mode of the specimen construct was recorded as the site of the graft failure; graft midsubstance, tibial tunnel, or femoral tunnel. Testing was performed at room temperature and tendon kept hydrated with Phosphate Buffered Saline (PBS) throughout.

Mechanical testing data was analyzed using MatLab 7.0 (The Math Works Inc, Natick, Massachusetts). Load-deformation curves were generated for each specimen and load and stiffness values obtained. The maximum load required for the construct to fail was defined as load-to-graft failure (Newton [N]). Stiffness
was defined as the linear part of the slope of the load deformation curve with units of N/mm.

**Statistical Analysis**
For all quantitative parameters (peak load, stiffness, and cellularity) statistical comparisons between the groups were performed by 1-way ANOVA. Significance was set to an alpha level of $p < 0.05$. Analysis was performed using SPSS version 18.0 for Windows (SPSS Inc, Chicago, Illinois, USA).

**Results**
The surgical procedure was well tolerated by all animals with normal cage activity resuming by the first post-operative day. At sacrifice, macroscopic dissection did not reveal any infection and all joint surfaces appeared normal. An intra-articular graft between the femur and tibia was present and appeared healthy in all specimens. Some thickened synovium was present at the anterior aspect of the intra-articular portion of the graft.

**Histology**
Slight visual differences were noted between the control and experimental groups at different time points. At week one post surgery microscopic evaluation of the healing tissue revealed formation of a loose and poorly organized fibrovascular interface layer between tendon and bone. Cellular composition of the interface consisted of neutrophils, fibroblasts, sparse osteoblasts on the outer edge of the bone tunnel and an influx of a high number of inflammatory cells. There were no differences noted between the groups. Graft necrosis was observed at the inner part of the tendon graft in the control group (Fig. 4a). Group with gaps within the tendon graft demonstrated an infiltration of host cells and a healthy live graft (Fig. 4b). Overall there was little or no evidence of structural incorporation of tendon into bone for either group. As time progressed, at the two week time period (Fig. 5), the fibrovascular interface layer demonstrated more collagen deposition and remodelling. It became more organized and dense. Differences were not noted between

![Figure 4](image1.png)
Figure 4. Histology images (H&E staining) of tendon graft at one week post surgery. Control group (a) showed a necrotic graft while treatment group (b) showed a live graft, populated with cells (original magnification x20). Significant difference was seen at one ($p < 0.01$) and two weeks ($p < 0.05$) post surgery.

![Figure 5](image2.png)
Figure 5. Histology images (H&E staining) of tendon-bone interface at two weeks post surgery B = Bone, I = Interface, T = Tendon, arrows denote the width of the interface layer. No major differences were noted between the interface layer and newly formed trabeculae surrounding the tendon graft across specimens from the control group (a) and treatment group (b) (original magnification x10).
the control and treatment groups. In both groups, there was evidence of newly formed trabecular bone circumferentially around the bone tunnel. Some early remodeling of the tendon graft was seen at the outer portion of the tendon. Although a higher number of cells were observed in the control group at two weeks compared to four weeks post surgery, graft necrosis was still noted. In the treatment group the grafts looked healthy with cell numbers increasing steadily.

At four weeks post surgery more new woven bone was deposited along the bone tunnel in the treatment group than the control group. There was some evidence of indirect type tendon-to-bone insertion in the group that had gaps induced into the grafted tendon as noted by fibre (Sharpy’s-like fibers) continuity between tendon graft and lamellar bone as denoted by thin arrows in Figure 6. In the control group there were distinct margins between tendon and surrounding bone. Cell numbers had increased in both groups and graft necrosis was not seen. Abundant number of small blood vessels (denoted by thick arrows in Fig. 6) indicating greater graft vascularity, was seen in the treatment group.

Histomorphology
Histomorphology confirmed histological findings of graft necrosis within the inner portion of the tendon graft in the control group at one and two weeks post surgery. Significant difference in cellularity count was noted between the two groups at both one week (* = p < 0.01) and two weeks (** = p < 0.05) while there was no significant difference found at four weeks (Fig. 7).

Mechanical Testing
In vitro mechanical testing of the rat FDL tendons with
and without gaps showed no significant difference in stiffness values between the groups (p = 0.74). Graphical representation of these results is depicted in Figure 8. Due to the size of the rat FDL tendon peak-load values were not recorded due to tendon slippage from the grips. During mechanical testing of the in vivo study specimens two samples failed at the tibial growth plate and were discarded from the results. Mechanical data is summarized below and presented in Figure 9 (load-to-graft failure) and Figure 10 (graft stiffness). All values are expressed as mean ± standard error. At fourteen days post surgery load-to-graft failure was higher for the control than the experimental group (3.68 ± 0.9 N and 2.98 ± 0.3 N, respectively) while the graft stiffness was almost identical (9.55 ± 1.28 N/mm and 9.75 ± 0.52 N/mm, respectively). These differences were not found to be significant. At four weeks post surgery the load-to-graft failure values increased in both groups with the percentage increase lower in the control group than the treatment group. The treatment group peak load to failure was higher than the control (4.12 ± 0.14 N and 3.76 ± 0.4 N, respectively) but the difference was not found to be significant. Graft stiffness decreased between two and four weeks in the control group (10.1 ± 3.04 N/mm) and was lower than that of the treatment group (12.67 ± 1.48 N/mm) which itself increased. This difference was not found significant.

No major differences were observed in failure modes between the groups. At two weeks in the treatment group, three specimens failed at midsubstance, one at the tibial tunnel, and two at the femoral tunnel while in the control group two specimens failed at each one of the failure sites. At four weeks, two specimens failed at midsubstance in the control group and four in the treatment group. No failures were seen at the tibial tunnel while three specimens failed at the femoral tunnel in the control group and one in the treatment group.

Discussion
It is widely acknowledged that the four zones typical of fibrocartilaginous type entheses, such as that present at the insertion of the ACL, are not regenerated following surgical repair (1, 7-10). This is thought to contribute to inferior mechanical properties of the interface, particularly in the early post-operative time periods, resulting in delayed rehabilitation. There is a need to hasten the early tendon-to-bone healing process and augment the mechanical strength of the interface between bone and tendon to facilitate an early return to competitive sport, work, and other regular daily activities. Tendon-bone interfaces are classified as direct or indirect based on the orientation of the collagen fibers at the interface (11). Considering the geometry of a tendon graft in a bone tunnel, it is impossible to form a direct interface since the collagen fibers are perpendicular to the bone. The introduction of a gap between the collagen fascicles without compromising the mechanical properties of the graft could allow for more robust tissue integration and potentially a more robust tendon-bone interface.

Our histomorphology analysis and histology observations indicate that graft necrosis usually observed in the initial two weeks of the healing process is averted by in-
ducing gaps into the tendon graft prior to ACL reconstruction. Furthermore, early infiltration of host cells and repopulation of the graft seems to initiate healing at an earlier stage, ultimately leading to a more profound tendon-bone interface as indicated by histology. These positive differences did not translate into superior mechanical strength of the interface. Biomechanical testing revealed similar load-to-graft failure and graft stiffness values across both groups at both two and four week time points.

The in vitro mechanical testing performed on the rat FDL tendons with and without gaps found no significant difference in graft mechanical properties. As a result we excluded any possibility that gaps may compromise the mechanical integrity of the graft in this study. Hence, it was assumed that the collagen fascicles were not damaged, and the mechanical properties similarly not compromised by creating gaps in the graft. To further reduce the risk of collagen fascicle damage, gaps were not created under tension i.e. the graft was left relaxed and

Figure 9. Mean load-to-graft failure of control and experimental groups at two and four weeks post surgery. No significant difference was noted between the groups or within groups across time points. Data is presented as mean ± standard error.

Figure 10. Mean graft stiffness of control and experimental groups at two and four weeks post surgery. No significant difference was noted between the groups or within groups across time points. Graft stiffness decreased between two and four weeks in the control group while an increase was seen in the experimental group. Data is presented as mean ± standard error.
Major differences were observed in terms of graft necrosis as measured by quantitative histomorphology and histological observations. Graft necrosis has previously been reported to occur following ACL reconstruction (14, 15). Kobayashi et al. (5) also studied the fate of host and graft cells in early healing of a tendon graft within a bone tunnel. They demonstrated that graft cells survived for the first week following transplantation but completely disappeared within two weeks while the host cells survived throughout all time periods. As there were only a few fibroblasts within the graft at one and two weeks post surgery the graft was deemed to be necrotic.

We found a significantly higher number of cells, progressively ingrowing into the grafted tendon, in the treatment group compared to the control group in the first two weeks. This correlated with our histological observation of graft necrosis in the inner portion of the FDL graft in the control group and a viable healthy graft in the treatment group. There were no differences in cell number and graft viability seen at four weeks post surgery and no differences in cell morphology across specimens within or across groups at any time point. We speculate that prevention of early graft necrosis is achieved by making physical way for the host cells to infiltrate and repopulate the grafted tendon. It is likely that local cells i.e. pluripotent cells of the bone marrow within the cancellous bone surrounding the intraosseous portion of the FDL tendon graft are the cells that infiltrate the grafted tendon. It has been demonstrated that these cells have the ability to differentiate in a variety of cell types which can subsequently form mesenchymal tissues, such as bone, tendon, and ligament (16-18). In this study the early infiltration of host cells proved to initiate healing at an earlier stage resulting in a more profound tendon-to-bone interface in the treatment group by the four week time period as indicated by histological characteristics observed. Rate of healing and remodelling was seen analogous in both groups at one and two weeks post surgery but differences in the tendon-bone interface were evident at four weeks. In the treatment group, more woven bone was seen circumferentially around and continuous with, the bone tunnel with some sporadic Sharpy’s-like fibers protruding from the bone to the tendon. These results indicate that the gradual remodeling process of tendon-to-bone healing can be hastened when gaps are introduced into the grafted tendon during an ACL reconstruction. Furthermore it can be deduced that the grafted tendon simply functions as a scaffold which supports the host mesenchymal cells that infiltrate and initiate the healing process.

Recently many researchers have reported improved tendon-to-bone integration utilizing various biomaterials as carriers for application of growth factors (19-21), mesenchymal stem cells (MSCs) (22, 23). The advantage of this study is that histological improvement at the tendon bone interface was achieved via mechanical means alone. In addition, graft necrosis that transpires following ACL reconstruction was averted. Clinically, inducing gaps within the tendon graft would be an easy step a surgeon could perform prior to graft placement. Furthermore the graft has the potential to be used as a scaffold and a carrier, and gaps could be injected with desired biomaterials to augment the interface between tendon and bone.

Future studies should focus on introducing biomaterials into the gaps created to further improve the healing of tendon-to-bone.

References


Muscles, Ligaments and Tendons Journal 2011; 1 (3): 91-99


Muscles, Ligaments and Tendons Journal 2011; 1 (3): 91-99