Anterior Cruciate Ligament Reconstruction With a Porcine Xenograft: A Serologic, Histologic, and Biomechanical Study in Primates


Purpose: This study proposes treatment methods to provide a mechanically competent, immuno-compatible, and sterile porcine graft for human knee ligament reconstruction. Methods: The anterior cruciate ligament (ACL) was reconstructed by using treated porcine patellar tendon grafts or controls of untreated porcine grafts or allografts in 20 rhesus monkeys. Animals were stratified into 2-, 6-, and 12-month postreconstruction cohorts. Serologic and histologic assessments were performed to evaluate host immunological and cellular response. Healing and functional integrity of the ACL reconstructions were assessed by tensile biomechanical testing. Results: Untreated porcine grafts were acutely resorbed and rejected, whereas treated porcine grafts and allografts were incorporated by the host as functional grafts. Temporal histologic assessment of treated porcine grafts and rhesus grafts revealed gradual host cellular infiltration and graft collagen remodeling through a similar mechanism of ligamentization. Biomechanical evaluations support graft functional integration with no difference between allograft and treated graft reconstructions. Conclusion: Rhesus allograft and treated porcine grafts presented with similar healing profiles in a long-term evaluation of ACL reconstruction. Clinical Relevance: Immunochemical modification and sterilization of porcine patellar tendon grafts may improve initial biocompatibility and long-term functionality of xenografts in musculoskeletal applications. Key Words: Anterior cruciate ligament—Ligament reconstruction—Knee—Remodeling.

Rupture of the anterior cruciate ligament (ACL) is a common traumatic injury to the knee in active people. The healing response of ruptured ACLs is poor, and without surgical reconstruction, the ACL deficient knee limits patient activities and can lead to future degenerative changes.1-4 Reconstruction of ruptured ACLs with autogenous patellar tendon or hamstring has become the standard of care, but use of these structures is not without complications. Although rare, clinical complications of autogenous grafts include donor-site morbidity, patellar fracture, anterior knee pain, neurologic deficits, quadriceps weakness, arthrofibrosis, and associated increases in rehabilitation time, concomitant medications, and loss of work.5,6 Long-term studies using patellar tendon allografts have shown the advanced clinical utility of allogeneic materials but source, supply, sterility, and structural integrity concerns have limited widespread clinical use.7,8 Furthermore, the potential risk of disease transmission from tissue allografts has been unfortunately shown and has heightened oversight of allograft sourcing, processing, and clinical use.7 Considering these autograft and allograft limitations, there exists an unmet clinical need for a safe and efficacious...
universal donor tissue for ACL reconstruction.\textsuperscript{4,8} This study describes functional preclinical evaluation of a novel immunochemically modified porcine bone–patellar tendon–bone construct as an ACL reconstruction device.

Previous attempts at the development of ACL reconstruction devices have been foiled by a combination of material and biological challenges. Synthetic devices, such as Dacron, Kevlar, and carbon fiber meet with some initial clinical success but ultimately failed because of inappropriate initial biomechanical properties, material fatigue profiles, and material shedding.\textsuperscript{9-12} The abraded synthetic particles collected in the joint space, lymph nodes, and other tissues and led to a chronic inflammatory response.\textsuperscript{13} An effort made in the 1980s to develop a bovine bone–patellar tendon–bone bioprosthesis was based on glutaraldehyde crosslink chemistries for porcine heart valves. This xenograft attempt also failed and was abandoned. Although the mechanism of failure for the bovine-based devices were mixed, poor biocompatibility attributed to excess glutaraldehyde, improper biomechanical properties preimplantation, and the lack of host integration were key variables leading to poor clinical results.\textsuperscript{14}

The major stumbling block for transplantation of xenograft animal tissues into humans has been immunological rejection. The primary cause of this rejection has been identified as a cell and matrix surface carbohydrate antigen called the \(\alpha\)-galactosyl (\(\alpha\)-gal) epitope with the structure Gal\(\alpha\)1-3Gal\(\beta\)1-4GlcNAc-R.\textsuperscript{15} Humans and Old World primates lack the \(\alpha\)-gal epitope, but all other mammals produce and incorporate many \(\alpha\)-gal epitopes into cellular and extracellular structures by using the \(\alpha\,3,\,\text{galactosyl transferase enzyme.}\textsuperscript{16}\) Humans, apes, and Old World monkeys continuously produce natural anti-Gal antibodies constituting about 1\% of circulating immunoglobulins and are not immunotolerant toward grafts expressing \(\alpha\)-gal epitopes. Consequently, transplantation of xenografts in these higher species results in acute rejection by anti-Gal antibodies.\textsuperscript{17} In this light, it is important to model host response to implant materials in an immunologically relevant model. Other than man, only higher primates produce and incorporate many \(\alpha\)-gal epitopes. Our recent development activities have focused on methods to treat porcine bone–patellar tendon–bone constructs to overcome anti-Gal–mediated rejection, attenuate immunological recognition on implantation in humans while assuring sterility, viral inactivation, and mechanical properties appropriate for an ACL reconstruction device.\textsuperscript{17-19,21,22} Optimized tissue treatments include rigorous decellularization, enzymatic cleavage of the \(\alpha\)-gal epitope with recombinant \(\alpha\)-galactosidase, low-level glutaraldehyde cross-linking, and terminal sterilization by irradiation. We hypothesize that these optimized treatments will provide an immunocompatible xenograft ACL reconstruction device that will provide initial structural support, serve as a dynamic scaffold with gradual cellular remodeling, and replacement by host tissue.\textsuperscript{1,2,3,26}

The current study used a rhesus monkey (Old World monkey) ACL reconstruction model to assess the safety, immunocompatibility, and efficacy of the xenograft device.\textsuperscript{27-29} Investigational objectives of this study were (1) evaluation of the safety, immunocompatibility, and biocompatibility of the xenograft as compared with untreated porcine control and rhesus patellar tendon allograft; (2) a temporal investigation of host cellular infiltration and integration of graft ligament and bone; and (3) assessment of device long-term efficacy through biomechanical testing of the regenerate femoral-anterior cruciate-tibial constructs.

**METHODS**

**Study Design**

A unilateral rhesus ACL reconstruction model was implemented with 2-, 6-, and 12-month sacrifice time points and clinical, serologic, histologic, and biomechanical evaluations. Twenty skeletally mature rhesus monkeys weighing 4.3 ± 0.3 kg were used in this evaluation. Three animals were reconstructed with treated grafts for the 2-month cohort, with an additional 5 at each 6 and 12 months. Controls consisted of 1 untreated porcine and 1 rhesus allograft at 2 months and 5 rhesus allografts at 12 months. Three animals from the 6-month time point and all 10 animals from the 12-month time point were allocated for biomechanical testing followed by histologic assessment. Although the rhesus knee is small, scaling to the human has proven merit and the tetraped gait of primates closely emulates human knee range of motion and flexion.\textsuperscript{29} Animal care and management were in strict adherence to current animal care and welfare guidelines.
Tissue Processing

Patellar tendons were harvested from fresh frozen stifles of 6- to 8-month-old pigs, thawed, and pulse lavaged to remove cellular components. Removal of the α-gal epitope by enzymatic treatment using recombinant α-galactosidase was verified by enzyme-linked immunosorbent assay (ELISA) with an α-gal-specific antibody and homogenized treated porcine tendon as solid-phase antigen as previously reported. Cross-linking of the devices was accomplished with 0.10% glutaraldehyde for 12 hours followed by a glycine endcapping to block unreacted glutaraldehyde molecules. The final packaged devices were terminally sterilized by electron beam irradiation at 17.8 kGy (validated sterility assurance of $10^{-6}$) and stored frozen at $-70^\circ$C until use.

Treated porcine bone–patellar tendon–bone grafts and fresh frozen rhesus bone–patellar tendon–bone control devices were thawed and each device cut into bone-tendon constructs. The test and control devices were prefabricated into 30-mm long by 4-mm wide tendon grafts with proximal 5 mm diameter by 7 mm in length bone plug ends. Final test articles were rinsed and wrapped in sterile gauze with 0.1% bacitracin until implantation.

Surgical Procedures

All animals were sedated by using intravenous ketamine HCl and diazepam (10 mg/kg and 0.5 mg/kg, respectively) as a preanesthetic with operative anesthesia maintained by intubation and administration of isofluorane gas. The surgical placement of the device parallels human ACL reconstruction techniques by using an open approach and unilateral ACL reconstruction. The knee joint was exposed by using an anteromedial incision and lateral displacement of the patella. The ACL was subsequently removed. The clinician acceptable outcome was normal or near normal as compared with the contralateral limb.

Serologic Methods

Blood samples were taken presurgically and on days 10, 14, 21, 28, 42, 56 and at 3, 6, 9, and 12 months for analysis of anti-Gal and anti–non-Gal antibodies (i.e., antibodies to pig tendon proteins). Standard clinical chemistries and hematologic and serologic panels were also performed preoperatively and quarterly.

Serum immunoglobulin (Ig) anti-Gal IgG and IgM activity was determined by ELISA by using standard methods and synthetic α-gal epitopes coupled to bovine serum albumin (α-gal-bovine serum albumin) as the solid-phase antigen. Primate sera were assayed in serial 2-fold dilutions with peroxidase coupled rabbit antihuman IgG or IgM used as secondary antibody. The titer was presented as the reciprocal of serum dilution displaying half-maximal binding in ELISA.

Serum anti–non-Gal activity was determined by using homogenized treated porcine patellar tendon as the solid phase antigen, as previously performed for porcine cartilage implants. This assay system monitors the production of antibodies to pig tendon antigens other than the α-gal epitope. Primate serum was depleted of any anti-Gal activity by adsorption to 30% rabbit red cells (vol/vol) for 1 hour at 4°C. Subse-
sequentlly, serum samples in 2-fold serial dilutions were measured for IgG or IgM binding to the treated tendon with horseradish peroxidase-conjugated rabbit antihuman-labeled secondary antibodies. Titer increase was calculated and reported as with anti-Gal titers.

**Histologic Methods**

After sacrifice, the naive and reconstructed limbs were partially dissected, exposing the knee capsule and ACL, photographed, and evaluated for gross pathological changes. The specifics of implant histologic areas of interest included intra-articular graft and bone tunnel insertion sites of the femur and tibia. Intra-articular specimens from all time points were dissected from femoral and tibial components, processed in paraffin, sectioned longitudinally at 4 μm thick, and stained with hematoxylin and eosin, toluidine blue, and Masson’s trichrome. Matching femoral and tibial specimens were processed undecalcified embedded in methacrylate and sectioned at a minimum of 2 levels either axially or longitudinally along bone tunnels through ground specimens. Alternately, two of the five 6-month specimens were sectioned in oblique sagittal plane as intact femoral-ACL-tibial constructs. Histology as separate femoral and tibial components on three 6-month and all 12-month specimens was performed postbiomechanical testing. Histologic analysis was aided by metabolic bone labeling with oxytetracycline (intramuscularly, 25 mg/kg) 2 weeks postoperatively and calcein (intramuscularly, 10 mg/kg) 13 and 3 days presacrifice. Plastic embedded sections were stained with toluidine blue, hematoxylin and eosin, Massons’s trichrome, or left unstained for metabolic bone label analysis and polarized light microscopy. All sections were evaluated qualitatively for signs of rejection, cellular response and type, graft to host bone integration, bone formation at the periphery of the tunnels, graft remodeling and maturation, and the presence of Sharpey-like fibers. Assessments were conducted in a blinded fashion by both veterinary and human transplant pathologists.

**Biomechanical Testing**

Tensile testing to failure of operated and unoperated femoral-ACL-tibial constructs from 6- and 12-month postoperative test groups was conducted according to previously established methods and fixtures. Testing used servo-hydraulic test frame (Shore Western Inc., Monrovia, CA). Specifics for tensile, axial testing to failure include storage of specimens at −80°C until testing; ambient air testing, load along the ligament axis at 30° of flexion, 100% ligament length strain rate per second, and data acquisition for load and displacement collected at 500 Hz.

Measured physical characteristics of reconstructed and control ligaments were collected and include intrarticular length of an average of anterior and posterior bundles (by micrometer) and average graft circumference midsubstance (by tensioned suture loop and micrometer). Testing yielded structural property values for ultimate load, yield load, ultimate displacement, yield displacement and stiffness, and standard derived material properties for ultimate strength, yield strength, ultimate strain, yield strain, and modulus. The study values are reported in mean and standard deviation. Descriptive statistics were compiled for all collected variables. Intergroup comparisons were conducted by an analysis of variance, with post hoc testing performed by a Tukey t test at $P \leq .05$.

**RESULTS**

**Clinical Observations**

The porcine grafts and surgical intervention were well tolerated with all animals returning to normal function at 7 weeks and placed in group housing with unrestricted cage activity by 8 weeks postoperatively. Range of motion and laxity at 6 and 12 months were considered to be clinically acceptable in all animals.

**Serologic Assessments**

There were no adverse findings from standard clinical chemistry, serologic, and hematologic panels in any animals. Titers for both anti-Gal antibody and anti–non-Gal antibody are reported in reciprocal dilu-
tion at 50% maximal binding. Figure 1 presents antibody titers for 1 untreated porcine engrafted animal (pPT-untreated) and all 13 treated graft (pPT-treated) reconstructed animals.

Anti-Gal titer in monkey engrafted with untreated porcine ligament increased by >200-fold within 2 weeks postimplantation. However, in monkeys grafted with the treated ligament (pPT treated), anti-Gal titers were attenuated by greater than 95% as compared with the monkey engrafted with untreated porcine graft. This result confirms previously published studies and supports the efficacy of α-galactosidase in epitope cleavage and attenuation of immunological recognition. The small increase in anti-Gal titers in monkeys engrafted with untreated porcine ligament is likely to be the result of an immune response to α-gal epitopes on the small number of porcine bone marrow cells remaining in cancellous bone interstices. Anti-Gal titers resolved to preimplantation range by 8 to 12 weeks postimplantation. The robust anti-Gal response to untreated porcine tissue, indicating acute rejection, resolves only in coordination with a rapid destruction and resorption of the graft. Anti–non-Gal titers were comparatively minimal and yielded no adverse hematologic or systemic changes. The decrease in anti–non-Gal antibody titers observed after 9 and 12 months, in comparison to those measured after 3 and 6 months suggest a decrease in the amount of pig tissue by ligamentization by the host’s cells and matrix. Thus, the amount of stimulation by porcine protein antigens is lower after 9 and 12 months. Additionally, anti-Gal and anti–non-Gal IgM titers were monitored, with only nominal changes observed (data not shown).

Gross Pathology

Treated xenograft and allograft reconstructions exhibited a structural appearance resembling native graft with similar healing profiles including re-establishment of synovial sheath and proximal attachment vascularization (Fig 2A and B). In contrast, the untreated graft was replaced by unorganized granular tissue. No degenerative articular changes or adverse synovial changes were present in any of the treated porcine engrafted or control allograft animals as assessed visually or by qualitative histopathology and ordinal scoring.

Implant Histopathology

Graft Histopathology: Two-Month Specimens

Rhesus Allograft: Grafts exhibited a fibroblastic reorganization of the collagen, more pronounced peripherally and less cellular in the central portion. New blood vessel formation was evident in focal fibrovascular regions. Sparsely distributed and limited regions exhibited a more histiocytic infiltrate and rare lymphocytes. Implant periphery exhibited additional blood vessel formation as well as synovial formation (Fig 3A). Bone tunnels exhibited graft integration with the host bone and peripheral revascularization.

Untreated Porcine Graft: Only remnants of the porcine graft were observed. This elimination of the xenograft is attributed to immunologically mediated
graft resorption. The proximal portion of the untreated porcine graft presented with granulation tissue containing lymphocytic infiltrate and immune-mediated destruction of the porcine ligament (Fig 3B). Bone tunnels exhibited advanced resorption of the graft, more pronounced in the tibial tunnel, and characterized by a mixed lymphocytic infiltrate and osteoclastic remodeling of graft at host bone junction.

**Treated Porcine Graft:** The macro-architecture of the treated grafts revealed a large central portion of unadulterated porcine graft with a periphery of reorganized collagen. The slight to mild infiltrate was primarily fibrohistiocytic with interspersed regions of fibrovascular reorganization and blood vessel formation. Sparsely distributed areas exhibited mild lymphohistiocytic infiltrates. Implant periphery exhibited additional blood vessel formation as well as synovial formation (Fig 3C). Bone tunnels exhibited tendon-to-host bone integration and consolidation as well as graft bone-to-host bone cancellous remodeling.

**Graft Histopathology: Six-Month Specimens**

**Treated Porcine Graft:** Graft histopathology revealed a contiguous reconstruction from femoral to tibial insertion sites characterized by remodeled and mature collagen, interspersed spindle-shaped fibroblasts, and viable tenocytes with minimal to no inflammatory cell infiltrates. Peripheral regions exhibited fibrovascular regeneration supporting periligamentous vascularization. The graft tendon to host bone interface showed direct apposition with host bone remodeling and Sharpey-like fibers at interface. Graft bone to host bone integration was mature with near seamless margin at some junctions and re-establishment of lacunae and marrow-filled interstices. Histopathology demonstrated increased remodeling and maturation of tendon graft collagen and advanced graft tendon and bone integration in host bone tunnels as compared with 2-month specimens (Fig 4A-C).

**Graft Histopathology: Twelve-Month Specimens**

**Treated Porcine Graft and Rhesus Allograft:** The intra-articular graft histopathology revealed a prominent peripheral synovium in both rhesus allograft- and xenograft-reconstructed specimens. The peripheral collagen of the graft matrix exhibited limited vascularization interspersed with spindle-shaped fibroblasts. The central portion of the graft was less...
cellular than the periphery and exhibited some focal regions of acellularity. Advanced remodeling and maturation of the peripheral and central portions of the grafts was evident by polarization birefringence. These findings were uniformly found in both treated graft and rhesus allograft groups (Fig 5A-D). A focal, mild, mixed plasmacytic infiltrate was found in 1 xenograft device and appears to be superimposed on the otherwise normal organizing ligamentous tissues. Bone tunnel histopathology for both treated graft and rhesus allograft presented with mature graft integration in bone tunnels as compared with 6-month specimens (Fig 6).

Biomechanical Testing

Failure mode for all specimens was consistently intra-articular graft midsubstance. Average reconstructed ligament lengths closely approximated control ACLs at both 6 and 12 months. Hypertrophy of the rhesus reconstructions was more pronounced at 12 months as compared with treated porcine grafts. Table 1 (online only; available at www.arthroscopyjournal.org) summarizes structural and material property results. Ultimate load increase 67% and corresponding stiffness increased 58% for porcine graft specimens in the 6- and 12-month interval. Changes in material property yield strength between 6- and 12-month time points was 64%, although nominal changes were found in ultimate strength. This finding highlights the transition between elastic and plastic regions and regenerate construct materials properties. Although not statistically significant, there were no relative differences in structural or material properties at 12 months between treated porcine grafts and rhesus allografts.

Figure 5. Treated porcine graft and rhesus allograft at 12 months: (A) peripheral section of the treated graft showing synovium (top) and different collagen organizations with interspersed linearly aligned fibroblasts (100× H&E), (B) same field as A under polarization showing collagen maturation and ligament-like collagen crimp periodicity (100× polarized), (C) rhesus allograft peripheral section showing vascularized synovium and maturing collagen with spindle-shaped fibroblast infiltrate (100× H&E), and (D) same field as C with peripheral polarization supporting collagen maturation (100× polarized).

Figure 6. Treated porcine graft at 12 months: (A) longitudinal intra-tunnel section with host bone (left) zone of attachment with Sharpey-like fibers and tendon fibers aligned along load transfer (100× H&E) and (B) tibial tunnel exit showing differential zones of graft tendon (lower) fibrocartilage and host bone (40× trichrome).
To allow comparison of our biomechanical results to published studies, the standard convention of reporting biomechanical properties in percent of contralateral, intact limb was applied to our data. A linear trend of return to intact ACL strength was observed and increased from 43% to 58% over the 6- to 12-month interval for treated grafts.

**DISCUSSION**

Although several studies have investigated the use of xenograft tissues in functional and orthotopic investigations, few have successfully evaluated implant site and host systemic response to untreated and modified materials in an immunologically relevant model of discordant xenotransplantation. Although the primary objectives of treated xenograft immunological acceptance and graft functional patency as compared with allograft were achieved, this investigation is not without limitations including small group sample size limiting rigorous statistical comparison both biomechanically and histologically and specimen artifact from postbiomechanical histologic assessment.

Histologic and serologic results at 2 months postoperatively support an acute humoral and local immunologically mediated rejection of the untreated porcine graft with normal healing showed comparing treated porcine graft and rhesus allograft. Postprocess analysis of treated grafts showed an effective removal of cellular debris, as assessed by embedding, sectioning, and photo-microscopy and a 2-log efficacy in α-gal epitope cleavage by α-galactosidase, as assessed by immunoassay. Although a discrete rejection threshold level of α-gal epitopes has not been derived for graft materials, a clear relationship exists between the presentation of α-gal epitopes, resulting immunological rejection, and limited functional integrity of xenograft materials.

Our second research question addressed the sequence of host cellular response and graft incorporation. Histologic results from 6- and 12-month time points show graft maturation and remodeling (i.e., ligamentization) from the graft periphery toward the central core, leading to a gradual replacement of the initially engrafted matrix with host collagen. Although there was a mild and transient anti-Gal and anti-non-Gal immunological recognition of the treated porcine graft material, local host response and cellular infiltration did not differ significantly as compared with rhesus allograft implants. In both treated graft and rhesus allograft reconstructions, sparsely distributed central regions of relatively unremodeled tendon were present even at the 12-month time point. These findings parallel human ACL reconstruction studies with biopsy histology and support a functional integration of graft tissue before complete graft remodeling.

The third research objective is an assessment of long-term efficacy through temporal mechanical evaluation and comparison of treated grafts to rhesus allograft. Six- and twelve-month time points were chosen to evaluate a return to normal ACL strength and results corroborated by comparison to native ACL and autograft primate characterization and reconstruction studies presented by Butler et al., Clancy 1981: vascularized autograft, and Butler 1989: composite vascularized and free autografts.

![Figure 7. Temporal comparison of percent strength of reconstructed to intact anterior cruciate reconstructions for non-human primate studies. Current study: treated xenograft, Clancy 1981: vascularized autograft, and Butler 1989: composite vascularized and free autografts.](image-url)
CONCLUSION

In summary, porcine bone–patellar tendon–bone constructs can be treated to reduce xenograft immunological recognition, enabling the natural process of ligamentization and functional graft incorporation. Treatments do not adversely affect the biomechanical properties of the graft. Immunochemically modified and sterilized porcine tissue may be substituted for allograft tissue and are currently under investigation in an approved human clinical trial at our facility.

Acknowledgment: The authors thank Thomas Hansen and Roy Gealer of Biomechanics LLC for biomechanical testing and Mei Shu-Shi of Skeletech Inc. for histopathological assessment.

REFERENCES

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Abbreviation: Ult., ultimate.