Influence of *Staphylococcus epidermidis* Biofilm on the Mechanical Strength of Soft Tissue Allograft

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Abstract

We sought to determine the impact of bacterial inoculation and length of exposure on the mechanical integrity of soft tissue tendon grafts. Cultures of *S. epidermidis* were inoculated on human tibialis posterior (TP) cadaveric tendon to grow biofilms. A low inoculum in 10% growth medium was incubated for 30 minutes to replicate conditions of clinical infection. Growth conditions assessed included inoculum concentrations of 100, 1000, 10000 CFU. Tests using the MTS Bionix system were performed to assess the influence of bacterial biofilms on tendon strength. Load-to-failure testing was performed on the tendons, and the ultimate tensile strength was obtained from the maximal force and the cross-sectional area. Displacements of tendon origin to maximal displacement were normalized to tendon length to obtain strain values. Tendon force-displacement and stress-strain relationships were calculated, and Young's modulus was determined. Elastic modulus and ultimate tensile strength decreased with increasing bioburden. Young's modulus was greater in uninoculated controls compared to tendons inoculated at 10,000 CFU ($p=0.0011$) but unaffected by bacterial concentrations of 100 and 1,000 CFU ($p=0.054$, $p=0.078$). Increasing bioburden was associated with decreased peak load to failure ($p=0.043$) but was most significant compared to the control under the 10,000 CFU and 1,000 CFU growth conditions ($p=0.0005$, $p=0.049$). Presence of *S. epidermidis* increased elasticity and decreased ultimate tensile stress of human cadaveric tendons, with increasing effect noted with increasing bioburden.

Clinical Relevance: Understanding the impact of subclinical and chronic infection on mechanical strength following ACLR may reduce graft failure and prolong the integrity of the reconstruction.

Keywords: ACL reconstruction; infection; S. epidermis; graft failure

Introduction

Due to the high incidence of ACL injuries, increasing numbers of ACL reconstructions are performed each year making it one of the most common orthopedic prodcedures[1,2]. Reconstruction is the most common treatment for ACL rupture and many graft options are available, including different autograft and allograft tissues[3]. Autograft harvest is the most used technique for ACL reconstruction with potential graft sites including the hamstring tendons, patellar tendon, and quadriceps tendon. Allograft reconstruction is used in certain circumstances and includes the same types of tendons harvested from donors, as well as others, including hamstring, patellar, quadriceps, Achilles, and anterior/posterior tibialis tendons[4].

ACL reconstruction is generally safe and effective, and infection after ACL reconstruction is a very rare complication, with current literature suggesting a rate of approximately 0.14%-1.7%[5]. While rare, the occurrence of a significant postoperative infection after ACL reconstruction may directly compromise the mechanical strength of the graft. Infection rates are mostly attributed to members of the *Staphylococcus* species, with *S. epidermidis* being of particular clinical interest, as it has emerged as one of the most common organisms seen in prosthetic joint infections. Its predominance may be related to its ability to form biofilm infections on tissue grafts and implants, making antibiotic efficacy and bacteria removal more difficult[6].

Previous work by this research group has shown that bacterial DNA is commonly found on failed ACLR grafts in the form of biofilms, as its presence on failed graft tissue and monofilament suture was visually confirmed with fluorescence microscopy[7]. While bacterial DNA was detectable in torn graft tissue in most revision ACL cases, the degree to which biofilm formation affected the mechanical strength and stability of the ACLR graft remained unclear[8].

Furthermore, it is important to note that ACL failure due to subclinical infection may be underestimated as tissue and synovial cell cultures fail to accurately detect all biofilms. The tube method and congo red agar methods compared to tissue culture plates methods were associated with greater false negative rates when detecting clinical biofilm[9]. Also, while the presence of bacterial DNA does not necessarily cause clinically relevant infection symptoms, biofilm presence is known to be associated with other issues such as tunnel widening[8]. Previous studies have shown that increased tunnel widening could lead to graft failure, joint laxity, and increased revision surgery requirements[10].

Preliminary work in the lab has shown that *Staphylococcus epidermidis* can develop biofilms on human tendon grafts and that growth conditions for governing bioburden to mimic clinical infection can be controlled. Increasing incubation time is associated with greater bioburden and with increased exposure time, greater unraveling of the tendons making up the graft occur. Mechanical integrity seems to be impacted with increased exposure; however, inoculated allograft tendons have not been tested to confirm this hypothesis. The impact of bacterial colonization on the mechanical integrity of soft tissue ACL grafts as a potential cause of graft failure is concerning and deserves further investigation.

The purpose of this study was to assess the influence of *S. epidermidis* bacterial biofilm presence on tendon mechanical strength of soft tissue tendon allografts used in ACL reconstruction. We hypothesized that an increase in *S. epidermidis* concentration will compromise the mechanical strength of the soft tissue tendon allograft. Our approach to explore this hypothesis was to use mechanical testing protocols to observe a tendons ability to elongate and fail under axial loads. Quantitative measurements for tendon mechanical strength include an analysis of Young's modulus, peak stress and strain.

Materials and Methods

Strains and biofilm growth

Staphylococcus epidermidis ATCC® 35984™, a biofilm forming strain isolated from catheter sepsis, was cultured overnight in Brain Heart Infusion (BHI [Becton, Dickinson and Company; Sparks, Maryland]) at 37°C. Human graft tibialis posterior tendons were prepared and inoculated to form biofilm. Growth conditions assessed included inoculum concentration (100, 1000, 10,000 CFU), medium concentration (10%), and incubation time (30 minutes).

Determination of inoculation concentration and exposure duration

S. epidermidis ATCC® 35984™, isolated from catheter sepsis, was cultured overnight in BHI at 37°C. To determine the inoculation conditions a range of relatively low concentrations were used we considered would be more clinically relevant, human graft semitendinosus tendons were segmented into thin sections (~2.5 cm x 2 mm x 2 mm). Specimens were submerged in 5-mL of culture in 6-well plates and incubated statically at 37°C with growth conditions of 105 and 104 CFU/mL, medium concentration (10 and 50%), and incubation time (0.5, 3, 6, and 24-hours). Increasing incubation time was associated with greater bioburden. In contrast, starting inoculum concentration had minimal impact on bioburden (Figure 3). No unraveling was observed in control BHI specimens. Thus, low inoculum, 10% growth medium, incubated for 30 minutes was recommended to replicate conditions of clinical infection.

Tibialis posterior graft preparation

Cadaveric human posterior tibialis tendons were surgically released from surrounding tissues, maintaining the proximal end of the tendon to its muscular attachment. Fresh frozen specimens were stored in a -20°C freezer and removed the day prior to mechanical testing. For

tendon preparation, tendons were submerged in 50-mL of sterile water in a clear tempered glass pie dish (dimensions, 9” 23) and trimmed to remove any remaining muscular attachment. Following graft composition, tendons were imaged, and calculation of surface area was approximated using the analysis function of NIH ImageJ (https://imagej.nih.gov/ij/). The length of the tendon was measured and six data points along the width of the tendon were averaged, and cross-sectional area was approximated assuming an elliptical shape (Figure 1).

Inoculation protocol

For biofilm growth, the posterior tibialis tendon allograft specimens were submerged in 75-mL of culture in an airtight, leak resistant tempered glasslock storage container (capacity, 485-mL). Once prepared, tendons underwent submersion in inoculum concentrations of 100, 1000, 10000 CFU with a nutrient medium concentration of 10%. Grafts then underwent shaking at 37°C and 60 rpm incubation for 30 minutes. A control was run in 75-mL of 10% BHI following the same experimental conditions in the absence of bacterial inoculation. After shaking, to remove planktonic cells, samples were washed 3x in 30-mL of sterile Dulbecco's phosphate-buffered saline (DPBS) and immediately placed in a sterile 50-mL conical centrifugal tube over ice for transport.

Mechanical testing and data collection

Following biofilm growth, the allograft tendons underwent mechanical testing using a servohydraulic materials test frame [MTS 858 Bionix, MTS Corp. Eden Prairie, MN]. Testing parameters were set to apply an axial load at a defined strain rate of 10mm/min. The tendon specimen was gripped by two custom-fabricated, corrugated cryo-clamps, reinforced using dry ice (to improve adhesion between the tissue and clamp), and further clamped using two C-

clamps to mitigate the risk of slipping (Figure 2A). The texture of the corrugated surface gives a good method of monitoring slippage of the specimen; displacement was demonstrated to be tissue extension and not specimen sliding in the grip. Force-deformation curves were generated during elongation to failure of the tendon.

An assessment for graft displacement and load were recorded across the length of the experiment. To find the greatest stress the tendon could withstand, the ultimate tensile strength, the maximum load on the tendon was divided by cross-sectional area to obtain stress values. The displacements of the allograft tendon origin to maximal displacement were normalized to tendon length to obtain strain values. From the data obtained, the tendon force-displacement and stress-strain relationships were calculated, and Young's modulus were determined.

Interpretation of stress-strain curves

A total of 40 allograft tibialis tendons (10 with each inoculum concentration) underwent biomechanical testing undergoing uniaxial loading to failure (rate of 10 mm/sec) using the MTS 858 Bionix Test system. This displacement rate was chosen because the recorded quantitative data on the characteristics of soft tissue was most favorable to observe tissue behavior prior to tendon failure, usually about 2 minutes of applied axial load.

A general stress-strain relationship for tendons was modelled and included calculations for ultimate tensile strength, peak tendon strain, and Young’s modulus. Young’s modulus of tendon tissue was calculated by dividing yield stress by the corresponding tendon strain.

Environmental Scanning Electron Microscopy (ESEM) preparation

All tendon components were soaked in prefixing agents containing 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.4) for 24 hours at room temperature in a 12-well plate. These

components were then rinsed with cacodylate buffer twice. After the final rinse, the specimens were dehydrated by placing in increasing concentrations of ethanol series (70%, 90%, and 100%) two times each for 5 min. Components were lastly dehydrated in 100% HMDS for 5 minutes, twice, then depressurized before a sputter coating procedure. These components were dried at room temperature before imaging via Quanta 200 imaging system.

Statistical analysis

Data were analyzed using standard statistical software on Microsoft Word. The association between bacterial presence and modulus of elasticity (Young's modulus) and peak stress to failure (sigma) were assessed by ANOVA analysis. To assess for an association between bacterial concentration and changes in both elasticity and peak stress, paired t-tests assuming equal variance were conducted across each inoculum concentration. Differences were considered significant for $P<0.05$.

Results

Interpretation of stress-strain curves

The recorded data were observed as a force-displacement curve and a stress strain curve was calculated. Ultimate tensile strength was dependent on level of inoculation and cross-sectional area (CSA) of the tendon. Across 10 replicates, the uninoculated experimental control displayed an increased peak load to failure among tendons. Peak load to failure values ranged from 1300 N-3000 N. After adjusting to account for the variable CSA of the tendons, stress values averaged to 20.197 N/mm^2 in the control group. Comparatively, peak load to failure values for the 10,000 CFU inoculation ranged from 900N-2800N, and stress averaged 14.79 N/mm^2. Averages for the 100 and 1,000 CFU inoculations were 17.25 N/mm^2 and 16.93 N/mm^2

respectively. Tendon stiffness was approximated using the slope of the stress strain curve at its most linear segment. Across all conditions, bacterial inoculation did not significantly alter tendon strain ($p = 0.73$). Tendon stiffness averaged .33-.36 N/mm^2. Data confirmed that due to varying levels of crimp within tendon fibrils, the tendon exhibited the expected non-linear stress-strain response (Figure 4).

Association between bioburden and mechanical strength

Young's elastic modulus and ultimate tensile strength decrease with increasing bioburden. Young's modulus was greater in the uninoculated control group compared to tendons inoculated at 10,000 CFU ($p=0.0011$) but was unaffected by bacterial concentrations of 100 and 1,000 CFU ($p=0.054$, $p=0.078$) as detected by T-Test: two-sample assuming equal variances (Figure 5). Increasing bioburden was also associated with a decreased peak load to failure ($p=0.046$, Welch ANOVA). The difference was most significant when compared to the control under 10,000 CFU and 1,000 CFU conditions ($p=.0005$, $p=0.049$). No significant differences were observed regarding the experimental control compared to the 100 CFU inoculum concentrations as assessed by a paired t-test assuming equal variance ($p=0.072$). Therefore, no adjustment or stratification was performed based on these variables in subsequent analyses. There was no association between bacterial concentration and strain as measured by the MTS Bionix testing system.

Discussion

While rare, infection after ACLR exists and subclinical bacterial colonization of orthopaedic graft material has proven to be clinically relevant[11]. Subclinical biofilm formation

and its impact on the mechanical integrity and success of soft tissue grafts has rarely been studied.

The most important finding of the present study is that increasing levels of bacterial colonization are associated with a weakening of the mechanical properties of the ACL soft tissue allograft. In particular, despite short-term exposure and relatively low inoculation time, the presence of increasing bioburden showed evidence of increased elasticity and decreased ability to tolerate axial load.

Despite short-term exposure and relatively low inoculation time during our experiments, we believe that post-surgical infection can harbor an appropriate environment for bacterial growth and biofilm formation regardless of starting inoculum concentration. Perez et al. found that *S. epidermidis* was capable of invading osteoblasts and fibroblasts in vitro[12]. It is known that bacterial colonization is associated with transosseous tunnel widening after ACL reconstruction, most likely due to the same mechanism observed above[8]. There is evidence that allograft tendons are safe and effective with no higher risk of infection and equivalent failure rates compared to autografts[13]. These findings suggest that while initial exposure may contain little bioburden, *S. epidermidis* and other infectious species may persist intracellularly and grow to reach experimental conditions assessed in this study. If bioburden is sufficient for biofilm growth, surrounding tissues including bone and soft tissues may be impacted by bacterial presence regardless of operative graft technique.

Clinically, failures of primary ACL reconstructions are mainly due to poor technique leading to graft laxity, traumatic re-injury, and poor biology that manifests as either failure of graft ligamentization or subclinical infection[14]. A loose graft at 6 months after ACLR has been shown to increase the risk of later ACL revision surgery, reduce patient return to sport and

ADLs, and cause permanent increased anterior laxity[15]. Evidence from our studies suggest that an increase in tendon laxity and a decreasing load to failure in the presence of infection may play a role in graft failure. Increased tendon laxity can lead to re-injury either traumatically or non-traumatically by altering the biomechanics of the knee.

Because of low ACLR infection rates, there is no clear consensus regarding the appropriate management of infection related complications. Current research suggests that pre-soaking grafts in vancomycin may lead to decreased deep infection rates[16,17,18]. Some studies, including a systematic review by Xiao et al, found that soaking ACL tendon grafts with vancomycin before implantation was associated with a nearly 15 times decrease in infection compared with grafts not soaked[19]. Another study suggested that through reducing hematoma occurrence post-surgery using a Hemovac drain, deep surgical site infections could be reduced when using hamstring tendon allografts[20]. Given the results of our study, it is important to consider the role of ACL infection prevention given the favorable outcomes presented in several studies performed.

Inoculated tendons stretched to failure show a decrease in their ultimate tensile stress with an increasing bioburden. Our experiments with varying inoculum concentrations showed that even a low presence of *S. epidermidis* increased the elasticity of human cadaveric tendons. It is unclear how decreases in tensile stress before graft rupture affect the kinematics of the reconstructed knee. Further research is needed to determine how biofilm infiltrates the tendons structural integrity and if biofilm formation can be reduced using decontamination protocols.

Future Directions

Mechanical integrity of the tendonous materials seems to be impacted with increased exposure; however, inoculated tendons will need to be tested to confirm this hypothesis. The

exact physiologic mechanism mediating a reduction in mechanical strength remains to be elucidated. Future studies will aim to assess bioburden quantitatively using CFU counts (possible contamination during this experiment) and observe unravelling of soft tissue specimens by Staphylococcus epidermidis. On a subset of tendons, we plan to complete SEM imaging to get finer resolution of the unraveling and if it is related to the presence of biofilm.

Limitations

There are several limitations to the current study. There is no prior reported evidence of impaired mechanical strength of soft tissue allografts inoculated with *S. epidermidis*; due to a lack of available data on the topic, we could not perform a power analysis. The current study could potentially be underpowered which would increase the risk of beta error (i.e., failure to detect a relationship that does exist). The current study demonstrates a link between bacterial colonization and a decrease in mechanical strength of soft tissue allografts, but it does not prove causation between bacterial colonization and graft failure. Our choice of control included a cadaveric posterior tibialis tendon which effectively controlled for an absence of bacterial colonies, mimicking ideal surgical conditions. However, the possibility of contamination and bacterial colonization during mechanical testing was not controlled for on the surface of the MTS clamps. Whether bacterial colonization occurred outside of the inoculation protocol cannot be determined by the current study design. Additionally, although the measurement of peak stress was recorded, the measurement of cross-sectional area needed to be approximated to account for differences in tendon thickness. This could have an impact on the calculated values for stress and thereby Young's elastic modulus.

Conclusions

The presence of *S. epidermidis* increased the elasticity and decreased the ultimate tensile stress of human cadaveric tendons, with increasing effect noted with increasing bioburden.

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Legend for Figures

Figure 1. Tibialis posterior graft preparation. For tendon preparation, tendons were submerged in sterile water in a clear tempered glass dish and trimmed to remove any remaining muscular attachment.

Figure 2. Mechanical Testing of the tibialis posterior tendon. The tendon specimen was gripped by two clamps, reinforced using dry ice, and further clamped using two C-clamps to mitigate the risk of slipping (A). Specimens underwent an axial strain until peak load was obtained or until the graft snapped (B). To account for tendon size differences, initial length and tension values were included in calculations (C). Tendons underwent mechanical testing using the MTS 858 Bionix Test system (D).

Figure 3. Semitendinosus bioburden by incubation time and initial inoculum concentration. Increasing incubation time was associated with greater bioburden. In contrast, initial inoculum concentration had minimal effect on bioburden.

Figure 4. Stress strain curves. Tendons underwent an axial load at a defined strain rate of 10mm/min until tendon failure. Representative stress-strain curves were generated across each experimental replicate. Peak load to failure of the control specimen was significantly greater than that of the 10000 CFU inoculum. This trend was observed when comparing the control to the 100, 1000, and 10,000 CFU concentration but was statistically significant in comparison to both the 1,000 and 10000 CFU inoculum.

Figure 5. Box and whisker plots of calculated values for Young's modulus (5A) and peak stress (5B). A There was no difference in bacterial concentration and change in elasticity when comparing the experimental control to 100 and 1,000 CFU. There were statistically lower values for Young's modulus among tendons inoculated under 10,000 CFU. **B** There was no statistical difference between increasing bioburden and peak load to failure when comparing the experimental control to 100 CFU. However, there were statistically lower values for peak stress among tendons inoculated under 10,000 and 1,000 CFU.