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Characterization of the Mechanical Properties and Mineral Distribution of the Anterior Cruciate Ligament-to-Bone Insertion Site

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***Abstract* — The anterior cruciate ligament (ACL) connects the femur to the tibia through direct insertion sites and functions as the primary restraint to anterior tibial translation. The ACL-to-bone insertion sites exhibit a complex structure consisting of four zones of varied cellular and matrix components, consisting of ligament, non-mineralized fibrocartilage, mineralized fibrocartilage and bone, which allow for the effective load transfer from ligament to bone, thereby minimizing stress concentrations and preventing failure. The mineral content and distribution within the fibrocartilage region may be an important structural component of the insertion site which may influence the mechanical properties. The goals of this study are to characterize the compressive mechanical properties of the fibrocartilage region of the ACL-to-bone insertion site and evaluate how the mineral distribution at the interface relates to these compressive properties. In order to determine the compressive mechanical properties we have utilized a novel microscopic mechanical testing method combined with digital image correlation and employed Energy Dispersive X-ray Analysis (EDAX) in order to evaluate the mineral content and distribution across the femoral and tibial insertion sites. The results reveal that a regional mineral gradient is observed across the fibrocartilage which corresponds to depth-dependent variations in compressive mechanical properties. This depth-dependent mechanical inhomogeneity strongly correlates to the increase in mineral content of the mineralized fibrocartilage (MFC) region compared to the non-mineralized fibrocartilage (NFC). Additionally, the tibial NFC and MFC mechanical properties are greater than those of the femoral NFC and MFC which corresponds to a greater mineral content in the NFC and MFC regions of the tibial insertion. The findings of this study suggest that a structure-function relationship exists at the ACL-to-bone interface.**

I. Introduction

THE anterior cruciate ligament (ACL) connects the femur to the tibia (Fig. 1A) and functions as the primary restraint to anterior tibial translation [1]. The ACL is one of the most frequently injured ligaments of the knee with over 75,000 reconstruction procedures performed annually in the U.S. [2]. The ACL connects to bone through the femoral and tibial insertion sites and tears and ruptures frequently occur through or near the insertion sites [3].

The ACL-to-bone interface exhibits a complex structure consisting of several zones of varied cellular and matrix components [3]. The first zone is the ACL proper (**ACL**, Fig. 1B) with fibroblasts in a types I and III collagen rich matrix. The interface is dominated by fibrocartilage (**FC**) which is further divided into non-mineralized (**NFC**) and mineralized fibrocartilage (**MFC**) (Fig. 1C). The non-mineralized fibrocartilage is composed of chondrocytes and types II and I collagen, while hypertrophic chondrocytes and type X collagen are found only in the MFC. This MFC region then connects directly into bone tissue (**B**).

This linear variation in cellularity, mineral content and extracellular matrix composition is observed at both the femoral and tibial insertions. The controlled heterogeneity is believed to permit a graduated change in stiffness and allows for effective load transfer from ligament to bone, thereby minimizing stress concentrations and preventing failure [1],[3]. It is likely that the mineral content and distribution within the fibrocartilage region may be correlated to the material properties of the insertion site.

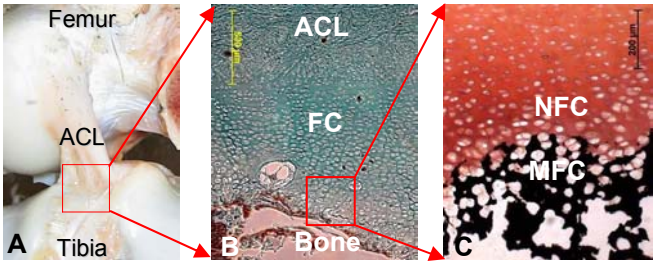

Fig. 1. *Neonatal bovine ACL-to-bone insertion site*

The mechanical properties of the ACL mid-substance have been investigated extensively however those of the ACL-to-bone insertion sites are not yet well understood. Since the fibrocartilage region contributes to load transfer between soft and hard tissue, it is critical to characterize the mechanical properties of this region. One of the major challenges associated with determining the mechanical properties of the fibrocartilage region is the physical scale of the tissue, which spans merely 100-300 μm [3]. Moreover, the width of the fibrocartilage interface decreases with increasing skeletal maturity [4]. Previous examinations of the interface utilizing conventional mechanical testing

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methods have only been able to provide an average mechanical response of all tissue zones at the insertion [3].

In this study, we have utilized a novel microscopic mechanical testing method combined with digital image correlation [5] to determine the mechanical properties of fibrocartilage. Fibrocartilage is often found in regions subjected to compressive forces, and the insertion site has been shown to be exposed to a complex profile of tensile, shear and compressive strains that develop as a result of joint loading [3],[6],[7]. Additionally, we have employed Energy Dispersive X-ray Analysis (EDAX) in order to evaluate the mineral content of the interface tissue. The objectives of this study are to 1) characterize the compressive mechanical properties of the fibrocartilage region, and 2) determine the mineral distribution at the interface.

II. Materials and Methods

A. Mechanical Testing

Intact ACL and insertion sites of neonatal (<1 week old) bovine knee joints were excised. The ligament midsubstance was removed and transverse cuts were made through both insertion sites thereby isolating rectangular samples containing regions of bone (B), fibrocartilage (FC) and ligament (L). All samples were frozen at -20°C prior to testing. After thawing, a smooth sample surface was achieved by cryosectioning.

The samples were tested in a custom uniaxial unconfined compression microscopy device as previously described [5]. Briefly, the samples were stained with Hoechst 33258 (Sigma) nuclear dye and loaded between two impermeable glass platens while the device was mounted on the stage of an epifluorescence microscope (Olympus). A tare strain of 10% was first applied and a sample image was acquired at equilibrium. Compression was then applied at ~1 µm/s in 5% increments (up to 20% strain). At equilibrium, sample images were acquired and the corresponding load was recorded for each increment.

Strain analysis was performed with optimized digital image correlation [5]. Axial strains (ε_{xx}) were calculated using linear regression of the displacement versus sample length. Incremental Young's modulus (E_Y) of the FC was calculated from the slope of axial stress (σ_{xx}) vs. strain (ε_{xx}) in that region.

B. Energy Dispersive X-ray Analysis (EDAX)

The distribution of mineral at the ACL-to-bone interface was characterized and quantified using scanning electron microscopy (SEM) coupled with Energy Dispersive X-ray Analysis (EDAX). Neonatal femoral and tibial insertion sites were excised, as described above, and immersed into and fixed with 100% ethyl alcohol for 24 hours at room temperature. After fixing, transverse cuts were made through the femoral and tibial insertion site thereby isolating samples containing regions of bone (B), non-mineralized fibrocartilage (NFC), and mineralized fibrocartilage (MFC). All samples were dehydrated in a vacuum desiccator for 1 week prior to analysis. On the day of analysis the samples were mounted on an aluminum post and sputter coated with palladium. Samples were examined and imaged with a SEM (FEI Quanta 600) using both the Everhart-Thornley secondary electron detector as well as the backscatter electron detector at an accelerating voltage of 20 kV. Energy dispersive x-ray analysis (EDAX, Phoenix Pro) was performed at 500x at an accelerating voltage of 20 kV and three spectra were collected for each region per insertion sample. Semi-quantitative analysis was performed to determine the Ca/P ratio for spectra within bone, NFC, and MFC.

C. Statistics

Statistical analysis was performed on the experimental results using one-way ANOVA and the Tukey-Kramer HSD test with statistical significance set at $p<0.05$. All data are presented as the mean ± standard deviation.

III. Results

A. Depth-Dependent Mechanical Properties

The results of this study reveal depth-dependent mechanical inhomogeneity in tissue displacement at the ACL-to-bone interface. The resulting displacement and strain in the NFC region is greater than that of the MFC region (Fig. 2) and this dependence is observed at both femoral and tibial insertions. The resulting stress and Young's modulus in the MFC is greater than in the NFC at all compressive strains for both the femoral and tibial insertion sites (Fig. 3).

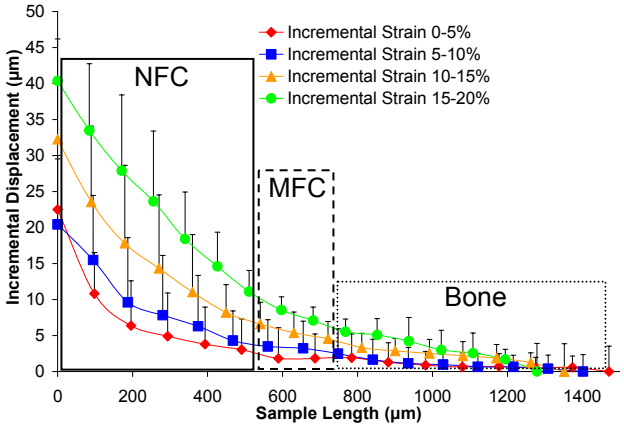

Fig. 2. *Representative graph of tissue displacement across the ACL-to-bone insertion site.*

B. Insertion Site Dependent Mechanical Properties

It is observed that the mechanical properties of tibial insertion site are greater than those of the femoral insertion site. Compressive stress and Young's modulus for both the NFC and MFC of the tibial insertion site are greater than those of the femoral NFC and MFC at all strain increments (Fig. 4).

C. Depth-Dependent Mineral Distribution

EDAX analysis revealed that a gradient of mineral distribution exists at both the femoral and tibial ACL-to-bone insertion sites. Peak-to-peak Ca/P ratios were determined by semi-quantitative analysis of the spectra within each tissue region which indicated that the Ca/P ratio decreases across the insertion site from mineralized bone to MFC to NFC for both femoral and tibial insertion sites (Fig. 5). Additionally, it was determined that the Ca/P ratio is significantly greater for mineralized bone than MFC and NFC (*,**p<0.05) and the Ca/P ratio is significantly greater for MFC than NFC (***p<0.05).

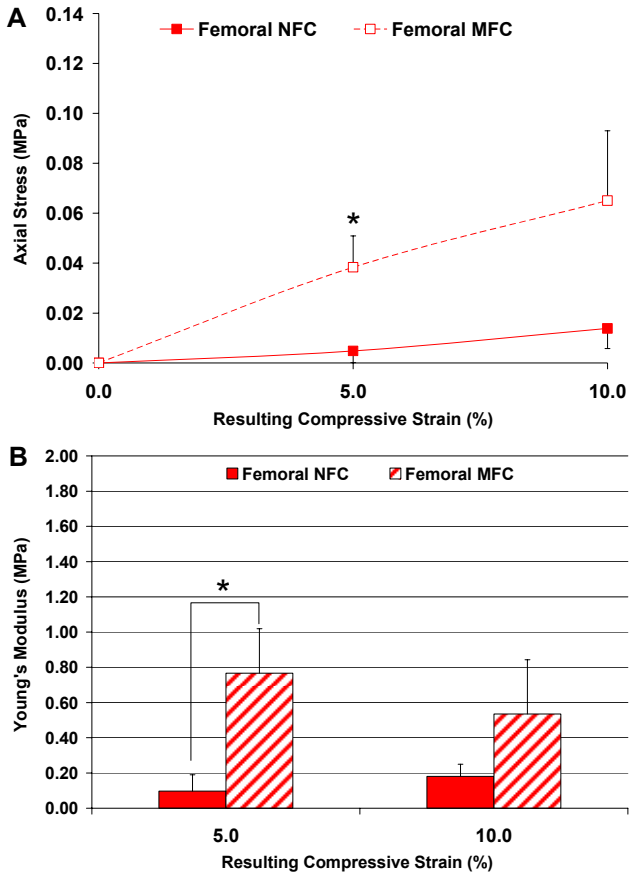

*Fig. 3. Mechanical properties of the femoral non-mineralized (NFC) and mineralized (MFC) fibrocartilage tissue (n=3); **A)** Stress-strain profile (*p<0.05), **B)** Young's modulus at 5 and 10% strain (* p<0.05).*

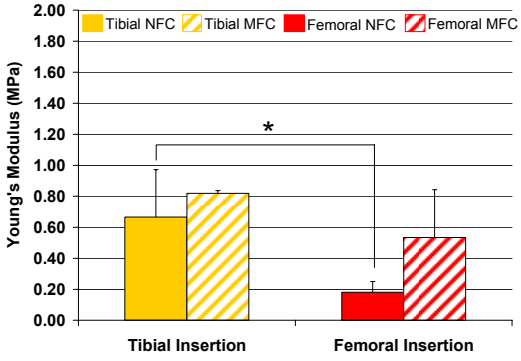

*Fig. 4. Compressive Young's Modulus of the femoral and tibial non-mineralized (NFC) and mineralized (MFC) fibrocartilage tissue at 10% strain (n=3, * p<0.05).*

D. Insertion Site Dependent Mineral Distribution

Semi-quantitative EDAX analysis indicated that the Ca/P ratio is greater within the mineralized bone, MFC and NFC regions for the tibial insertion compared to the femoral insertion. However, no significant difference is observed between the femoral and tibial groups with the exception of the NFC region (****p<0.05). It should be noted that the femoral NFC spectra do not contain calcium greater than background, therefore, the ratio is indicated as zero.

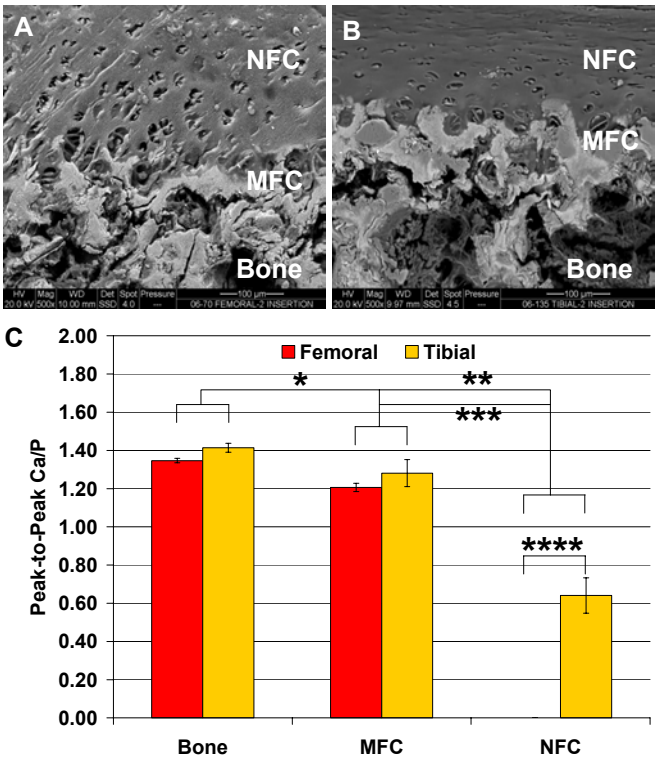

*Fig. 5. SEM images of ACL-to-bone interface **A)** femoral and **B)** tibial insertion site (Scale bar = 100 µm, 500x, 20kV); **C)** Semi-quantitative EDAX analysis of Peak-to-Peak Ca/P ratios for the femoral and tibial insertion site tissues (*,**,***,**** p<0.05).*

IV. Discussion

The results of this study demonstrate that the femoral and tibial ACL-to-bone insertion site fibrocartilage exhibits both depth-dependent and insertion site-dependent mechanical inhomogeneity and mineral distribution. Results of previous characterization studies and histological analysis of the bovine ACL-to-bone insertion site performed in our laboratory by Wang *et al.* [4] have revealed that an abrupt transition exists at the non-mineralized to mineralized fibrocartilage interface marked by the presence of mineral and hypertrophic chondrocytes within the matrix. Analysis of the Ca-P chemistry at the insertion site has revealed a gradient of calcium and phosphate content from the non-mineralized fibrocartilage matrix to mineralized fibrocartilage matrix, which is indicative of increase in mineral content of the ACL-to-bone insertion site fibrocartilage matrix. This analysis indicates that the transition from NFC to MFC is not abrupt, but, rather

gradual. The presence of mineral within the extracellular matrix of the MFC region of the femoral and tibial insertion may produce the effect of particulate strengthening which would support the observed decrease in axial strain, and increased axial stress and incremental Young's modulus for the MFC compared to the NFC region of the femoral and tibial insertion sites**.** Additionally, the tibial NFC and MFC mechanical properties are greater than those of the femoral NFC and MFC which corresponds to a greater mineral content in the NFC and MFC regions of the tibial insertion. The findings of this study demonstrate that an important structure-function relationship exists at the ACL-to-bone interface and should be considered in any efforts to regenerate the interface.

V. CONCLUSIONS

These results reveal that the depth-dependent variations in compressive mechanical properties correspond to the regional mineral gradient that is observed across the neonatal bovine ACL-to-bone fibrocartilage. This depth-dependent mechanical inhomogeneity strongly correlates to the increase in mineral content of the MFC region compared to the NFC. Additionally, the greater mechanical properties of the tibial fibrocartilage compared to the femoral fibrocartilage tissue may be related to the greater mineral content of the tibial fibrocartilage.

VI. ACKNOWLEDGMENT

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VII. REFERENCES

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