Characterization of Age-Dependent Changes at the Anterior Cruciate Ligament (ACL) to Bone Enthesis

INTRODUCTION:
The anterior cruciate ligament (ACL) connects the femur to the tibia through two insertion sites, each a complex structure divided into four distinct yet contiguous zones (ligament, nonmineralized interface, mineralized interface, and bone), accompanied by region-dependent changes in both cellular and matrix composition[1-3]. Collagen fibrils originating from the ligament traverse the interface zones and insert into bone[3]. It has been observed that ACL tears commonly occur or begin at the ligament-to-bone enthesis[3-5]. An understanding of the ultrastructural properties of the native ACL-to-bone insertion is therefore essential for identifying the etiology of failure and optimizing treatment protocols for ACL injuries[3].

The objective of this study is to use transmission electron microscopy (TEM) to characterize collagen fibrils and mineral chemistry at the ACL-to-bone insertion site as a function of age. Knowledge of age-related changes at the insertion site is crucial because ACL injuries are largely reported in patients ranging from 15 to 35 years of age[6]. An in-depth evaluation of the region-dependent and age-related changes at the enthesis will also be important in advancing current efforts to regenerate the soft tissue-to-bone interface[3]. It is anticipated that both mineral crystal structure and collagen fibril characteristics (diameter, number of fibrils per unit area, and percent area occupied by collagen), will vary as a function of age.

METHODS:
Isolation of ACL-to-bone Insertion Samples: Bovine knee joints (n=4) were obtained from a local abattoir (Green Village Packing) and were divided into two age groups: immature (4-6 months old) and mature (2 to 5 years old). A straight midline longitudinal incision extending from the distal femur to the tibia in the bovine knee was performed under aseptic conditions and the femoral and tibial insertions were identified and excised. Each sample (10x5x5mm) was comprised of the ligament and intact insertion connected to bone. TEM Preparation: The samples were fixed with 0.5% glutaraldehyde and 2% paraformaldehyde in 0.05M cacodylate sodium buffer (pH 7.4) and post-fixed with 1% osmium tetroxide. Samples were dehydrated with serial concentrations of ethanol, embedded in spurr’s resin and cut into 70-90 nm sections using a microtome. Sections were stained with uranyl acetate and lead citrate, and then examined using transmission electron microscopy (TEM, JEOL, JEM-100CX, 80kV).

TEM Image Acquisition and Analysis: For each section, after first identifying the insertion site, images were collected starting from bone and progressed 50µm step-wise towards the ligament region. In short, the total distance imaged spans across the insertion from ligament to nonmineralized interface, mineralized interface, and bone. For each of these regions, a minimum of five images were collected and collagen fibril diameter, distribution, and total area were determined using Adobe Photoshop and Image-J software. For mineral structure, diffraction rings were indexed and analyzed using Image-J software.

RESULTS:
Collagen Fibrils: While no differences in average fiber diameter (Fig. 1A) or percent area occupied by collagen fibers (Fig. 1C) were found between the immature and mature groups, a significant difference in the number of collagen fibers per area or collagen fiber density (Fig. 1B) was detected. TEM images of collagen fibers found at the immature and mature ACL-to-bone insertion are shown in Figure 2, with each image representing an additional 50 µm step away from the ligament region (1 being the closest). Interestingly, no significant differences in collagen fibril diameter or distribution were found between regions (ligament, nonmineralized interface, mineralized interface, and bone) (Fig. 3). While differences in average fiber diameter and density were apparent between the immature and mature groups, these differences were however not significant. Mineral Crystal Structure: Electron diffraction patterns (Fig. 4) for the mineralized interface and bone of mature samples were indexed for hydroxyapatite crystal planes and found to be similar.

DISCUSSION:
Previous studies have shown that the collagen fibril diameter, as well as the mineral content and structure at the insertion site, plays an important role in ligament mechanical properties[8-12]. Interestingly, in this study a mean increase in collagen fiber diameter was observed in the mature group while fiber density decreased significantly with age as total area occupied by collagen remained the same. This dual change in collagen organization will likely contribute to age-related difference in interface mechanical properties, and is likely a result of adaptation to physiological loading. Furthermore, in this study, it was found that the calcium phosphate present in the mineralized interface exhibits the crystal structure of hydroxyapatite, with no difference in structure found between the insertion site and bone[13]. These observations suggest that calcium phosphate chemistry at the interface is similar to that of bone. Future studies will focus on elucidating the role of collagen organization and mineral distribution in the structure-function relationship of the ligament-to-bone insertion.

SIGNIFICANCE:
An understanding of the ultrastructural properties of the ACL-to-bone insertion is essential for identifying the etiology of ACL failure and developing integrative treatment strategies for ligament injuries.

REFERENCES:

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Figure 1: Average collagen fibril measurements across the region.

Figure 2: TEM images of (A) immature and (B) mature ACL insertion site (50,000X, 80kV, and scale bar = 500 nm).

Figure 3: Region dependent characterization of collagen fibrils. Each region is 50 µm from previous, 1 is the nearest to ligament.

Figure 4: Indexed electron diffraction pattern (camera length = 50nm) and corresponding TEM (10,000X, 80kV) of (A) mature insertion site and (B) mature bone.