Quantifying Mechanoreceptors with IHC Staining in an Intact Porcine ACL

Anika N. Breker,[1] Crystal J. Murray,[1] Benedikt L. Proffen,[2] Jillian E. Beveridge,[1] Braden C. Fleming[1]
[1]Dept of Orthopaedics, Brown University/Rhode Island Hospital, Providence, RI
[2]Dept of Orthopaedic Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA
anika_breker@brown.edu

Disclosures: BCF: MIACH Orthopaedics. American Journal of Sports Medicine, ORS Publications Committee. BLP: MIACH Orthopaedics. All other authors (N).

INTRODUCTION: The anterior cruciate ligament (ACL) provides mechanical stability and proprioception to the knee. Mechanoreceptors in the ligament and surrounding joint capsule provide afferent proprioceptive information to engage peripheral inhibitory reflexes and central somatosensory processing. The ACL mechanoreceptors include Ruffini corpuscles (40 x 100µm), Pacinian corpuscles (280 x 120µm), and Golgi tendon organ-like structures (GTOs, 600 x 100µm) [1]. ACL injury is particularly common in the active population and can disrupt these afferent neural signals that lead to neuromuscular deficits. Currently, ACL reconstruction (ACLR), in which the injured ligament is removed and replaced by a tendon graft, is the gold standard treatment. Although ACLR restores mechanical stability, the removal of native ACL tissue decreases the likelihood of reinnervation of the graft and restoration of proprioceptive function. Novel surgical procedures that preserve the ACL tissue in its entirety could potentially preserve the mechanoreceptors and facilitate reinnervation of the ligament [2]. Using a dual immunofluorescence histology protocol on a porcine ACL, we aimed to adapt the mechanoreceptor identification criteria of Freeman and Wyke [1] to establish the quantity and distribution of mechanoreceptors in the ACL and to prepare for a future study comparing mechanoreceptor preservation between competing ACL surgeries in the porcine model.

METHODS: An intact ACL was harvested from an adolescent Yucatan minipig. The ACL was fixed, decalcified, and embedded in paraffin. 7µm sagittal slices were then obtained at 100µm intervals across the ACL. The 100µm gap was selected to prevent double counting of mechanoreceptors [3]. The slices were treated and co-labelled with Neurofilament Protein (NFP) (Sigma AB5539) and S100-B (Abcam ab52642) monoclonal antibodies. Using viewing software (OlyVIA, Olympus), mechanoreceptors were identified using the following criteria: 1) positive co-staining with both antibodies; 2) the minimum size criteria of a Ruffini corpuscle (40µm), the smallest receptor, as reported by Freeman and Wyke [1]; 3) if a dual stained structure was >40µm in 1-dimension, adjacent slices were reviewed to help distinguish between a mechanoreceptor or a nerve, as nerves tend to travel through consecutive slices; and 4) confirmation using NFP and S100-B reference images that were previously published [4, 5]. The mechanoreceptors were identified, categorized, and mapped by two trained observers for the counting and distribution assessments (Fig. 1).

RESULTS: The ligament was partitioned into 68 individual slices (spaced 100µm apart). Due to the hourglass shape of the ligament and microtome alignment, the latter 43 slices showed only the proximal portion of the ACL including the femoral insertion. We identified 9 structures in 11 slices that fulfilled the criteria to be counted as a mechanoreceptor. Two of these structures resembled GTOs, which spanned multiple slices and the remaining structures resembled Ruffini corpuscles. Six mechanoreceptors were found in proximity to the femoral insertion and three mechanoreceptors were identified in association with the tibial insertion. No mechanoreceptors were identified in the mid-substance of the ligament. The staining appearance of the mechanoreceptors tended to be stippled (Fig. 2) compared to the solidly stained nerves (Fig. 3). Without the size constraint, approximately 600 structures were co-stained with NFP and S100-B but were excluded as being nerves or free nerve endings, which are responsible for sensing pain. As expected, most neural structures of the ACL were in proximity to blood vessels.

DISCUSSION: This study was performed to build a platform to histologically assess the mechanoreceptors of the ACL and ACL grafts in future studies. Immunostaining, which is highly specific for functional neural structures, and additional scoring criteria were added to existing approaches [1] to prevent overcounting. Our pilot results, using these new refinements, demonstrate the presence of mechanoreceptors in the porcine ACL, which were primarily located at the proximal and distal ends of the ligament and near blood vessels. The most common mechanoreceptor was the Ruffini corpuscle, which is consistent with other animal models [4] and plays a critical role in sensing static joint position and movement in the ACL. Our results also confirm the legitimacy of the co-labeling protocol with NFP and S100-B monoclonal antibodies and our criteria adapted from Freeman and Wyke [1] to identify functional neural structures. A limitation of our study is that the results are based on only one healthy porcine ligament. A study using multiple specimens is currently underway to formally evaluate the types and distribution of mechanoreceptors in the normal ACL. In future work, we will use the histology methods and identification criteria developed here to quantify the types, numbers, and distributions of mechanoreceptors in healing ligaments and grafts.

CLINICAL SIGNIFICANCE: The histological methods and post-processing protocol optimized in this study provide a foundation for future comparisons of the neural structures in the healing graft or ligament after ACL surgery.

ACKNOWLEDGEMENTS: The study was funded in part by the NIH (R01-AR083168), the Lucy Lippitt Endowment, and a Student Research Award (SRA) from the Warren Alpert Medical School of Brown University. Histology was performed under the direction of Peter Caradonna at the COBRE Histology and Imaging Core of University of New England (P20-GM103643).

REFERENCES: [1] Freeman and Wyke: J Anat. 1967; [2] BEAR-MOON Design Group: Orthop J Sports Med. 2022; [3] Young et al: Orthop J Sports Med. 2016; [4] Han et al: J Mol Histol. 2020; [5] Li et al: J Mol Histol. 2019.

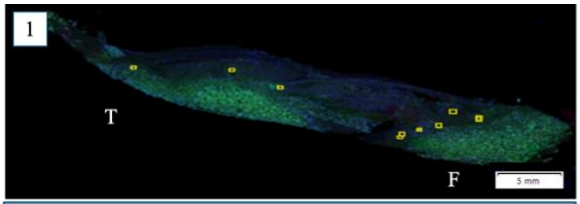
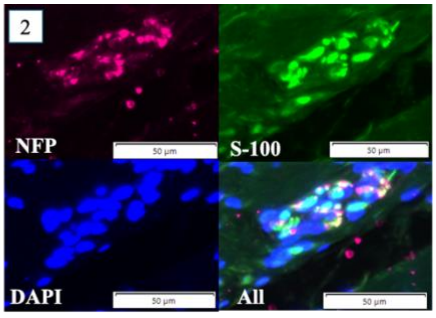


Fig. 1. Reference image of the relative distribution of mechanoreceptors within the ACL in a sagittal view.

Fig. 2. Mechanoreceptor found near the femoral insertion measuring 26.93 x 80.15 µm and co-stained with NFP, S100-B, and DAPI. Shown at 20x in OlyVIA.



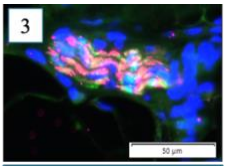


Fig. 3. Nerve near the tibial insertion measuring 84.84 x 24.52 µm and co-stained with NFP, S100-B, and DAPI. Shown at 20x in OlyVIA.