Exploring Mechanism of Calcific Tendonitis Using a Novel Turkey Model

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INTRODUCTION: Calcific tendonitis is a common clinical condition characterized by an ectopic pathological deposit of minerals within the tendon, resulting in chronic pain and dysfunction at the affected site such as the rotator cuff. This condition usually occurs in athletes and people with repetitive strain injuries. However, the underlying mechanism and causes of tendon calcification in humans remain unknown**.** Unlike humans, most of the tendons in turkey legs are naturally calcified during development, but this does not occur in young turkeys or chickens. Therefore, we hypothesized that loading applied to the tendons during turkey development would play a critical role in tendon calcification. Thus, this project aimed to investigate the biomechanical and biological factors involved in the tendon calcification process during the turkey growth period. To test our hypothesis, we used a different aged turkey model to analyze both biological and biochemical parameters to better understand the possible mechanism behind turkey tendon calcification, leading to interpreting the possible etiology of human calcific tendonitis.

METHODS: *Animal Model:* Hindlimb tendons from captive-bred wild turkeys (Meleagris gallopavo) were collected, Turkeys of varying ages, ranging from 1 to 16 weeks, were included (1,2,3,4,5,6,7,8,12,14, and 16 weeks), The bird mass measurement in 1 week: 0.16 kg till 16 weeks, weight: 10.65 kg. The flexor tendons studied(n=4) included Flexor digitorum longus (FDL), flexor perforans digiti (FPD), flexor perforans perforatus digiti (FPPD), and flexor hallucis longus (FHL). *qPCR* After sacrifice, lower extremity flexor tendon tissue was isolated for gene expression analysis. Total RNA was extracted using TRIzol Plus RNA Purification Kit (Invitrogen). *Histology and Image Analysis:* Tendon samples were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned (~7 µm) for staining (H‐E, safranine O, Van Kosa, and Picrosirius red) following protocols. *Immunohistochemistry*: The specimens were incubated with primary antibodies against osteocalcin, Runx2, and Osteopontin (1:200 dilution). *Scanning Electron Microscopy (SEM)/TEM:* We imaged tendon structure using SEM to visualize the ultrastructure and confirm histological observations. Element EDS System was used to characterize the mineral's elemental composition of calcific structures present within the tendon collagen bundle. *Biomechanical Analysis:* 34 turkey tendons were tested from 7 (n=7), 8 (n=6), 12 (n=7), 14 (n=7), and 16 (n=7) weeks to investigate mechanical behavior in the tendons as the turkeys aged for distribution of percent stress relaxation, Young's modulus, stiffness, and cross-sectional area values in each age group.

RESULTS SECTION: According to our data, calcification-related gene markers were significantly higher in old groups (8,12,14, and 16 weeks) compared to young turkeys (1,2,3,4,5,6,7) (Fig 4)**.** We identified the major genes responsible for tendon calcification osteogenic markers (Osteopontin, Osteocalcin, and Runx-2) which accumulate in turkey leg while increasing in bird age and weight. Transmission electron microscopy (TEM) ***(Fig. 1C)***: At 7 weeks, shows the first observable dense deposits of mineral phase are found in association with vesicular structures in the interfibrillar collagen spaces deposits of calcium-phosphate grains on the surfaces of interfibrillar plates, along with mineral deposits on collagen subfibrils (arrows). These results indicate that tendon calcification begins early in 7 weeks and then reach fully calcified by the 8 weeks. Histology Analysis ***(Fig. 3)***: Calcific tendon was evaluated by using Alizarin red stain, Van Kossa stain (calcium deposit), Picrosirius red stain (collagen fiber arrangement), and safranin O stain (detection of cartilage) showing a chondroid-like cell change in calcific tendon thus explain that under compressive or excessive load especially in turkey tendon adapt to the bone. Regarding biomechanical results, the calcific tendon group showed higher measurement in stiffness, stress relaxation, Young's modulus in comparison to the non-calcific young turkey tendon group (***Fig 5.)***.

DISCUSSION: Our preliminary results, indicate osteogenic differentiation of old tendons relative to the increased weight and age of the turkey bird which blocked the effect of the anti-calcific protein Fetuin B in the flexor tendon area closely responsible for tendon calcification. The stiffness and Young's modulus increased with the advancement in tendon age due to the presence of mineral deposits in the calcified older tendon. This report provides for the first time a spatial survey and comparison between calcified and non-calcified turkey leg tendons in relation to weight and age differences and defines turkey leg tendons as a model for tendon calcification research. A limitation of this study is the small sample size. Thus, we will continue this study using a larger sample size and defining the biochemical and biomechanical alterations following tendon calcification.

SIGNIFICANCE/CLINICAL RELEVANCE: The next phase of the study is to validate the loading effect theory behind the upregulation of calcification markers by de-loading tendon from muscle attachment in a 5-week in vivo study. The birds will be sacrificed at 16 weeks, along with in vitro components including applying high, moderate, and low dynamic tenocyte tendon loading strain using a Flex Cell subjected to cyclic compressive loads through the FX-6000C™ Flexer-cell dynamic culture system. The outcomes will enable us to better understand not only the natural process of tendon calcification in turkeys but also the potential pathological pathway of calcified tendonitis in humans. This knowledge may facilitate the development of possible interventions targeting the factors involved in tendon calcification.

REFERENCES: (1) Zhaoyong Zoua et al. Three-dimensional structural interrelations between cells, extracellular matrix, and minerals in normally mineralizing avian leg tendon 2020. DOI: 10.1073/pnas.1917932117. (2) Andrea H Lee, Dawn M Elliott J Anat 2019 DOI: 10.1111/joa.12913

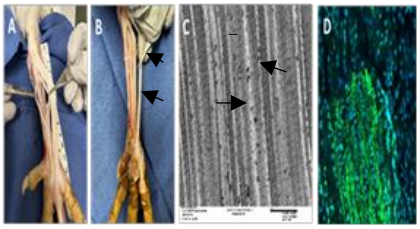

Figure 1: Image (A) and (B) Showing calcific non calcific change in tendon. (D) IHC shows positive DAPI /Osteocalcin (an osteogenic marker) for calcified tendon

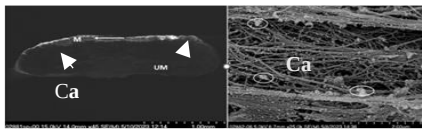

Figure 2: SEM/TEM FDP A 8-week trumps/CPD showing tendon calcification.

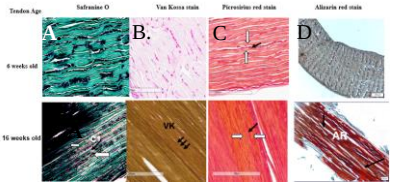

Figure 3: Histology stain section (A) SO, (B) VK, (C) PR, And (D) AR for comparison difference between 6-week-old tendon to 16-week-old

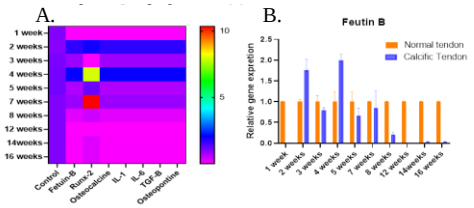

Figure 4: (A) qPCR result and (B) Fetuin B gene expression profile differences between calcific and non-calcific tendon area from 1 week up to 16 weeks of turkey age.

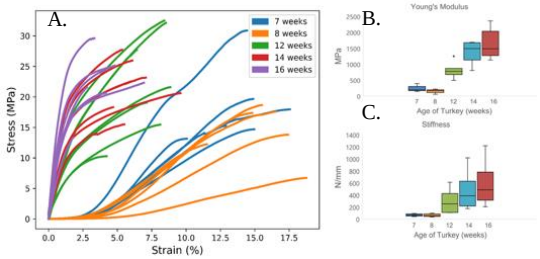

Figure 5: (A) Overlaid stress-strain plots from the load to failure test for each tendon. Tendons are color-coded by age to highlight characteristic differences in the curves as a function of age. (B) distribution of Young's Modulus and (C) stiffness in each age group.