Developing Jaw Tendon Mechanical Properties are Affected by Paralysis and Hypermotility

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INTRODUCTION: Little is known about the development of craniofacial tendons, including tendons that aid in jaw function. Jaw tendons are involved in vital movements such as talking, mastication, and swallowing[1]. The lack of knowledge surrounding the development of jaw tendons has led to a paucity of treatment options for pathologies, including temporal tendonitis, temporomandibular disorders, and physical traumas[1,2]. Mechanical loading, in the form of physical therapy, has been shown to treat temporomandibular disorders by increasing active pain-free jaw opening[2]. While the benefits of mechanical stimulation via physical therapy are compelling in the clinic, the mechanisms that drive this improvement and the effects on jaw tendon mechanical properties are unknown. We recently established the chick embryo as a model to study the formation of jaw tendons[1] and propose using this model to analyze how changes in mechanical loading affect tissue formation. In the present study, we examined movement frequency effects on the tendon attaching to the musculus adductor mandibulae externus (TmAM), which we previously identified as the key jaw-closing tendon in the chick embryo[1]. The TmAM is comparable in function and structure to human tendons involved in jaw-closing (masseter, temporalis, medial pterygoid)[1]. Our study is a significant first step in understanding how mechanical stimulation in the treatment of jaw tendon pathologies affects the mechanical properties of the jaw tendons.

METHODS: Fertilized White Leghorn chicken eggs were used for this study. All necessary animal procedures received prior approval from the University of Maryland Institutional Animal Care and Use Committee board. *TmAM mechanical property development:* Eggs were incubated until incubation days (D) 15, 17, and 20, at which time embryos were sacrificed and staged based on anatomical features. The TmAM was carefully harvested and imaged using Brillouin microscopy. *Paralysis and hypermotility induction:* Embryos were injected as previously described[3], with 100 µL of either 0.2585 mg/g embryo of pancuronium bromide (PB) to induce paralysis, 0.0517mg/g embryo of 4-aminopyridine (4-AP) to induce hypermotility, or sterile saline as normal development control, starting at D13-13.5 and again 24 h later. Candling the eggs and counting embryo movements confirmed paralysis and hypermotility. Tendons were harvested at D15-15.5 for Brillouin imaging. *Brillouin imaging:* Tendons were harvested for Brillouin imaging to assess longitudinal modulus, as previously described[4,5]. Each Brillouin shift map was taken as a 40 µm by 40 µm region with a 40x objective, with two Brillouin shift readings taken for each 1 µm region. At least three biological replicates (N=3), with a minimum of two distinct regions of interest (ROI), were imaged per treatment group. An average Brillouin shift was calculated by averaging all the Brillouin shifts taken over all the ROI of a biological replicate. The longitudinal modulus is proportional to the square of the Brillouin shift and thus a region of higher Brillouin shift indicates higher longitudinal modulus of the tissue[4]. *Statistics:* One-way ANOVA with Tukey's multiple comparisons was used to analyze the statistical differences between developmental stages. Unpaired Student t-test was used to analyze the statistical difference between the two treatment groups. A p-value < 0.05 was considered to be statistically significant.

RESULTS: The average Brillouin shift, indicative of tissue longitudinal modulus, remained constant during the developmental stages D15-D17, and then increased significantly between D17-D20 in untreated chick embryos **(Fig. 1)**. Reduced movement frequency (paralysis) for 48h (D13-13.5 to D15-15.5) resulted in a significantly lower average Brillouin shift, compared to the control (**Fig 2B**). Higher movement frequency (hypermotility) for 48h (D13-13.5 to D15-15.5) resulted in a significant increase in Brillouin shift, compared to the control **(Fig. 3B).**

DISCUSSION: Our study showed that the TmAM develops mechanical properties nonlinearly throughout development, with a constant Brillouin shift from D15-D17 and a significant increase between D17-D20. Brillouin imaging was used to assess TmAM mechanical properties due to the tendon's small size, which precluded the possibility of tensile testing. Furthermore, Brillouin imaging has the advantages of being a non-destructive and non-contact method of evaluating tissue mechanical properties[4,5]. We recently demonstrated Brillouin imaging to be an effective method to measure the mechanical properties of developing embryonic limb tendons, and showed a log-linear correlation between average Brillouin shift and elastic modulus measured using tensile testing[5]. Similar to the TmAM, we previously observed the embryonic calcaneal tendon also develops mechanical properties nonlinearly[5,6,7]. Our findings demonstrate that movement is necessary for the normal development of mechanical properties in jaw tendons, reflected by findings that paralysis of the embryo led to a significantly lower longitudinal modulus of the TmAM as compared to the control. Similarly, we have previously shown that the elastic modulus of calcaneal tendons is lower following paralysis, as compared to normal tendons[3]. Clinical evidence has shown that mechanical stimulation following injury or disease, such as temporomandibular disorders, can improve jaw function[2]. However, the specific effects of mechanical stimulation on jaw tendons remain unknown. Based on our previous study that discovered calcaneal tendon mechanical properties improve with embryo hypermotility[3], we induced hypermotility to study the potential benefits of mechanical loading on jaw tendon mechanical properties. Our findings excitingly show that an increase in mechanical stimulation led to a significant increase in the Brillouin shift of the TmAM. This study is a first report on characterizing development of embryonic jaw tendon mechanical properties, and also serves as a first step toward investigating the effects of mechanical stimulation on new jaw tendon formation, which has relevance to recovery from injury or disease.

SIGNIFICANCE: Understanding the effects of movement on the mechanical development of craniofacial tendons could inform future clinical approaches to treat jaw injuries and disorders.

REFERENCES: [1] Korntner et al., *Front. Cell. Dev. Biol.*, 2022; [2] Michelotti et al*., J. Orofac. Pain*, 2004; [3] Pan et al. ,*Phil. Trans. R. Soc. B*, 2018; [4] Scarcelli et al., *Nat. Photonics*, 2008; [5] Jana et al., *ORS abstract*, 2022; [6] Marturano et al., *PNAS*, 2013 [7] Navarro et al., *J. Biomech.,* 2022

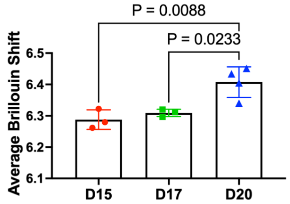

Fig 1. The development of mechanical properties of the TmAM in a chick embryo.

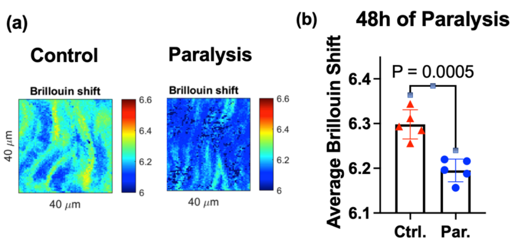

Fig 2. The effects of reducing frequency of movement for 48h on the mechanical properties of the TmAM.

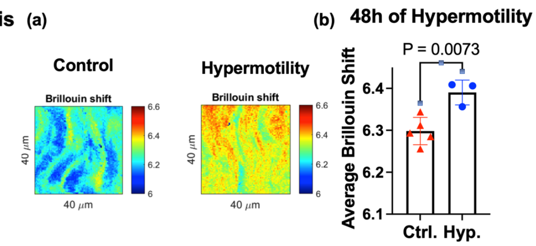

Fig 3. The effects of increasing frequency of movement for 48h on the mechanical properties of the TmAM.