Movement Regulation of Embryonic Tendon Mechanical Properties is Dependent on Developmental Stage

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INTRODUCTION: Embryonic and fetal movements are crucial mechanical cues for musculoskeletal development[1]. Reduced fetal movements are associated with tendon-related musculoskeletal abnormalities like clubfoot and arthrogryposis, which affect up to 1 in 100 live births per year[2]. Such birth defects are associated with formation of limb tendons with abnormal mechanical properties[3]. The chick embryo is an excellent animal model for musculoskeletal development as it shares molecular, biochemical, and structural similarities with mammals[4], but is more physically accessible as it is contained in its own eggshell. Additionally, chick embryo movements can be regulated through pharmacological treatments, including pancuronium bromide (PB, to induce flaccid paralysis) and 4-aminopyridine (4-AP, to induce hypermotility)[5]. In previous studies, paralysis during early chick development (D4-D10), when joint patterning is occurring, leads to joint fusion[6]. While active chick unilateral limb movements start around D7[7], calcaneal tendon functional (mechanical property) development is apparent after D12[8]. Notably, reduced movements can also occur later in development, after articulating joints have formed. Our previous study discovered that paralysis at later developmental stages (D17-D19) results in tendons with abnormally low elastic modulus, whereas hypermotility enhances the elastic modulus, without appearing to affect joint morphology and function[5]. Here, we aimed to investigate how movement frequency affects earlier stages of functional tendon development, after the articulating joint has formed. We hypothesize that reduced embryonic movement frequency inhibits mechanical development of the chick embryo calcaneal tendon as early as D13, and that mechanical properties can be rescued by induction of hypermotility.

METHODS: White Leghorn chick embryos ranging from D13-19 (chick embryos hatch at D20-21) were used for this study. All animal procedures received prior IACUC approval. Using our previously reported "marking protocol", calcaneal tendons were dissected and uniaxially tensile tested using an Instron 5542 tester[9]. *In ovo* injections of PB and 4-AP were performed every 24 h, as previously described[5], to reduce and increase limb movement frequency, respectively. Candling the eggs and counting embryo movements confirmed paralysis and hypermotility. Control embryos were injected with vehicle (saline). We used One-way ANOVA with Tukey's multiple comparison test to analyze pairwise statistical differences within multiple (≥3) groups, and Student's t-test to analyze statistical differences when comparing between only 2 treatment groups, with p-values indicated by the following *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Each data point is N=1 biological replicate. A minimum of N=3 (from 3 different chick embryos) was analyzed for each treatment group.

RESULTS: Paralysis during D13-D15, D15-D17, and D17-D19 each led to a significant difference in the tensile elastic modulus and peak stress, as compared to control tendons (**Fig. 1 A, B, C**), without effect on peak strain (not shown). Paralysis effects were embryo stage-dependent, with the highest difference seen at D13-D15 (**Fig. 1D, E**). To test the possibility of restoring the mechanical properties of paralyzed tendons by increasing embryo movement frequency, we induced natural motility (saline injection, Sal) or hypermotility (4-AP injection) for 48 h (D15-D17) following a 48 h paralysis period (D13-D15). Hypermotility completely restored the elastic modulus and peak stress of paralyzed tendons back to control mechanical properties (**Fig. 2A, B**), whereas natural motility only restored the elastic modulus to ~70% and peak stress to ~80% of controls (**Fig. 2A, B**). Interestingly, when embryos were paralyzed over a longer developmental period of D13-D17, neither natural motility nor hypermotility induction for a subsequent 48 h (D17-D19) had any restorative effect on the paralyzed tendon mechanical properties (**Fig. 3A, B**). Peak strain was not affected by sustained paralysis or rescue treatments for any groups.

DISCUSSION: This study revealed that reduced embryo movement frequency leads to significantly lower elastic modulus and peak stress in an embryo stage-dependent manner. We discovered that embryo movements are more crucial at earlier stages of functional tendon development: tendons paralyzed at D13-D15 had 75% lower elastic modulus and peak stress than normal tendons, whereas tendons paralyzed at D15-D17 and D17-D19 possessed ~30-40% lower modulus and peak stress than normal. We note that paralysis of flexor tendons from D15-D20 leads to tensile elastic modulus and peak stress that are respectively 25% and 35% lower than normal[11]. Based on previous studies, lysyl oxidase (LOX)-mediated collagen crosslinking, a primary regulator of developing tendon mechanical properties[8], begins increasing substantially by D14[10]. Our previous study discovered that paralysis during D17-D19 not only inhibited increases in elastic modulus, but also decreased LOX activity levels[5]. It is possible that movement also regulates LOX activity as early as D13, and that D13-D15 is a particularly critical period for LOX-mediated collagen crosslinking and associated mechanical property development. However additional experiments are needed to test this hypothesis. Previously, we demonstrated that 48 h of hypermotility resulted in calcaneal tendons with elastic modulus that was 160% of normal[5]. This motivated testing whether hypermotility can improve tendon mechanical properties after paralysis. Here, induction of hypermotility during D15-D17 fully restored the tendon mechanical properties of embryos that had been paralyzed during D13-D15. However, when the duration of paralysis was 96 h long from D13-D17, a subsequent 48 h hypermotility period from D17-D19 could not rescue elastic modulus or peak stress of paralyzed tendons. Others have reported that induction of hypermotility at very early stages of chick embryo development (D4-D10) can partially recover paralysis-induced abnormalities in knee and hip joints[6]. Notably, we did not observe any morphological abnormalities like joint fusion in either paralyzed or rescued embryos (data not shown). Taken altogether, our exciting results reveal embryo movements directly impact tendon mechanical properties during functional development and suggest mechanobiological pathways can be manipulated to rescue the effects of compromised fetal movement.

SIGNIFICANCE: Our results highlight a critical role for embryonic movements in the mechanical development of tendons and establish a model for future mechanistic studies that may lead to therapeutic solutions for tendon-related birth deformities.

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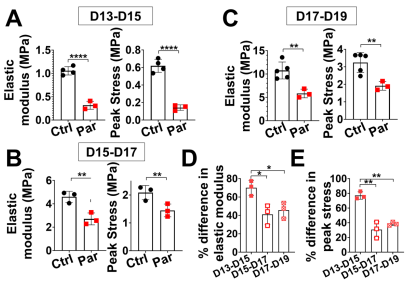

Fig. 1: Elastic modulus and peak stress of control (**Ctrl**) and paralyzed (**Par**) tendons at D13-D15 (**A**), D15-D17 (**B**), and D17-D19 (**C**) % difference in elastic modulus **(D)** and peak stress **(E)** of Par when compared to Ctrl.

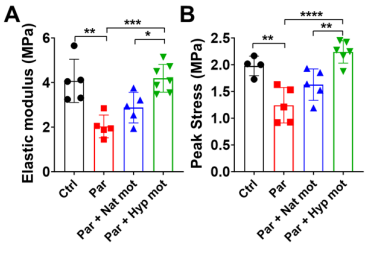

Fig. 2: Elastic modulus (**A**) and peak stress (**B**) of tendons after these treatments: **Ctrl** (saline at D13-D17), **Par** (PB at D13-D17), **Par+Nat mot** (PB at D13-D15, then Sal at D15-D17), and **Par→Hyp mot** (PB at D13-D15, then 4-AP at D15-D17).

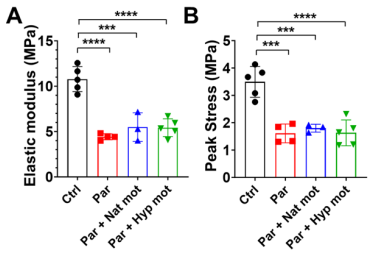

Fig. 3: Elastic modulus (**A**) and peak stress (**B**) of tendons after these treatments: **Ctrl** (saline at D13-D19), **Par** (PB at D13-D19), **Par+Nat mot** (PB at D13-D17, then Sal at D17-D19), and **Par→Hyp mot** (PB at D13-D17, then 4-AP at D17-D19).