UNILATERAL, DAILY BOUTS OF MUSCLE LOADING LEADS TO MINERAL AND EXTRACELLULAR ADAPTATION OF THE IMMATURE ACHILLES ENTHESIS IN MICE

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INTRODUCTION

The tendon-to-bone attachment (i.e., enthesis) is a functionally graded tissue that translates muscle-generated force from tendon to bone. The enthesis matures during postnatal growth and is susceptible to overuse injuries. Repeated loading of the adult enthesis induces mineralization and causes abnormalities such as bony spur formation and calcification of the distal tendon. Increased activity during periods of rapid growth, such as in children and during adolescence, can lead to micromotion at the enthesis and apophysis, resulting in inflammation and the clinical presentation of Sever Disease [1]. However, the temporal effect of training on the growth and remodeling of the enthesis remains unclear.

The use of small animal models allows for mechanistic exploration of the effect of loading on the growth and structural remodeling of the enthesis. Previously, muscle unloading has been shown to disrupt the structural and mechanical properties of the growing postnatal enthesis [2-4]. In the present study, we aimed to determine the effect of repeated daily bouts of increased muscle loading (using optogenetically-controlled muscle contractions) on the growth and adaptation of the immature and mature enthesis. We hypothesized that the mechanoadaptation differs between the immature and mature enthesis, and we compared the mineralization and structural changes of the enthesis in response to *in vivo* loading-induced remodeling. To do this, we used non-invasive optogenetic control of muscle contraction, which relies on the expression of light-responsive microbial opsins (e.g., Channelrhodopsin-2, ChR2) in activatable cells to translocate ions across the cell membrane for cell-specific, fast optical control of the membrane potential [5]. We and others have previously used optogenetics to control muscle contraction *in vivo* in mice and we have demonstrated the feasibility of eliciting sustained muscle contractions in the triceps surae muscle group [6-8]. In this study, we used optogenetics to induce isometric muscle contraction for non-invasive loading of the Achilles tendon enthesis daily, for three weeks, in Immature (3-week old) and Mature (6-month old) mice. We used microcomputed tomography (microCT) and histology to evaluate the effect of repeated mechanical loading on the mineralization and structural changes of the Achilles enthesis between repeatedly loaded and contralateral entheses.

METHODS

All procedures were approved by our Institutional Animal Care and Use Committee. Doxycycline-inducible ACTA1-rtTA;tetO-cre mice (Acta1-Cre; C57BL6J background) were crossed with Ai32 reporter mice (C57BL6J background) to generate Acta1-Cre; Ai32 offspring that expressed ChR2 blue light-sensitive opsin (455nm sensitivity) in skeletal muscle when treated with tetracycline. Dams were treated with doxycycline chow at the time of mating until weaning. Offspring were genotyped using PCR (Transnetyx), and Acta1-Cre; Ai32 homozygous mice (Immature group: 3-week old, n = 6 ; 2 females and 4 males; Mature group - 6-month old: n = 4; 2 females and 2 males) were used for blue light-induced stimulation. In addition to contralateral within animal controls (right: stimulated, left: control), external age-matched littermates heterozygous and homozygous Ai32 offspring (n = 7; 4 females and 3 males) were used as non-stimulated controls for the Immature group. Mice were anesthetized with isoflurane, hair was removed from their right triceps surae muscles to expose the skin, and animals were placed on a heating pad at 37°C. Right limbs were stabilized at the knee joint and feet were placed on a foot pedal connected to a servomotor shaft (Aurora Scientific) to induce isometric muscle contractions. For light stimulation, a collimated LED light (455 nm, 900 mW, M455L3, Thorlabs) and a high-power LED driver (DC2200, Thorlabs) were used for pulse modulation [6]. Muscles were stimulated using 10Hz pulsed light (70ms on, 30ms off; 10 cycles)

followed by 4 seconds of rest for 20 minutes (240 consecutive loading cycles), for 5 consecutive days per week for (3 weeks). Ankle torque measurements were collected throughout loading bouts. Maximum ankle torque at the start of each bout of stimulation was compared after normalizing to body weight. Contralateral limbs of the Loaded group were used as paired controls for microCT. Knee and ankle placement was also repeated for non-stimulated control immature group for 20 minutes under anesthesia. After 3 weeks of unilateral loading, mice were euthanized using carbon dioxide asphyxiation. Hindlimbs were dissected and fixed overnight in 4% paraformaldehyde and then scanned using microCT (Bruker SkyScan 1276; 59kV, 175µA, 10.58µm voxel size, 930ms exposure, 0.5mm aluminum filter). Digital reconstructions were analyzed using CTAn (Bruker) and Dragonfly (Object Research Systems) for: calcaneal total and bone volume; apophyseal total and bone volume; apophyseal bone mineral density (BMD); and calcaneal length. After microCT, tissues were decalcified in 14% EDTA and paraffin processed for histology. Sagittal plane sections of the Achilles enthesis (6µm) were stained using Toluidine Blue and Hematoxylin and Eosin (for enthesis morphology) and DAB immunohistochemistry (IHC) for markers of enthesis tissue transition (Aggrecan, Collagen Type II, Collagen Type III). *Statistical analysis*: maximum measured torque was normalized to the measured body weight for each bout of loading and repeated measures ANOVA was used to compare the variation of normalized torque for each mouse for the duration of the experiments. Paired Student t-test was used to compare BMD, BV, TV, and BV/TV between contralateral limbs.

RESULTS

All mice tolerated the daily loading bouts well and were included in analyses. Normalized maximum torque did not fluctuate for the duration of the experiment for each mouse and between mice of each group (Immature and Mature) at each bout (data not presented here). After 3 weeks of unilateral loading in the Immature group, the Achilles enthesis showed increased vascular infiltration in the calcaneal apophysis and at the calcified fibrocartilaginous border of the enthesis in stimulated limbs compared to contralateral limbs (Fig. 1A, shown with arrows). In 50% of the Immature mice, repetitive loading resulted in the formation of an enlarged and irregularly shaped apophysis compared to unloaded controls (Fig. 1A). We also saw a smaller fibrocartilage zone, with less Collagen II staining, in the Loaded compared to control entheses of Immature mice and adaptation to compressive loading at the wrapping site of the enthesis (Fig. 1C).

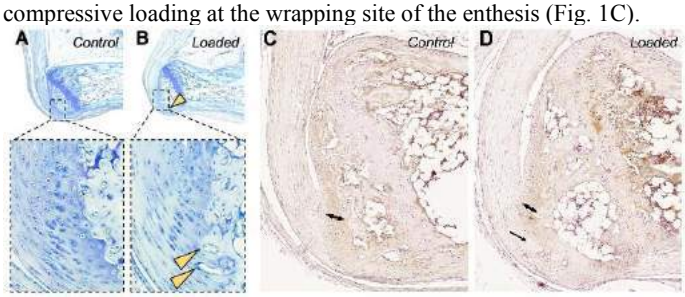

Figure 1: Morphology of the Achilles enthesis of (A) Control and (B) Loaded Immature mice after 3 weeks of daily bouts of optogenetic muscle contraction. Yellow triangles show misshapen apophysis near the enthesis and increased vascularity. (C, D) Loaded Immature mice have thinner entheses, indicated by less Collagen II (black arrow).

Bone loss visualized histologically in the loaded Immature group was confirmed following microCT analysis, both qualitatively and quantitatively. The apophysis was "pulled" in the posterior direction in the Immature group enthesis (dotted line, Fig. 2A), but not in the Mature group. Bone quality, measured as BMD, was lower in Loaded compared to contralateral Control apophyses in the Immature group (Fig. 2B). However, the total apophyseal volume did not differ between loaded and control groups in the Immature group. In the Mature group, the morphology of the Achilles enthesis and apophysis appeared histologically normal and there were no significant differences in BMD or apophysis volume between loaded and control sides (Fig. 2 E, F).

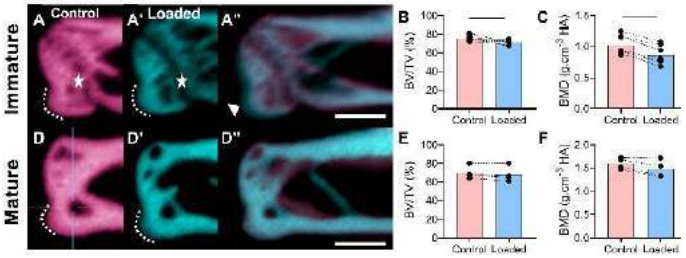

Figure 2: Sagittal microCT view of representative calcanei of Immature (A-A") and Mature (D-D") (Achilles attachment site outlined with dashed line). After 3 weeks of daily loading, Immature mice had posteriorly pulled apophyses (white triangle in A'') with growth plate opening (stars in A, A'). Apophyseal bone volume fraction and BMD (B, C) were reduced in the Loaded Immature group compared to contralateral apophyses. No structural differences were observed in the Mature group (D-F). Scale bar: 500 µm, bars indicate $p < 0.05$.

DISCUSSION

Using high-resolution three-dimensional imaging techniques (microCT) and histology, we found irregular apophyseal bone formation and increased vascularization, with smaller fibrocartilage and decreased bone quality, following repetitive loading in the Immature but not Mature enthesis. Vascular infiltration may be an indicator of bone and fibrocartilage remodeling in the immature entheses [9]. These findings model the structural and mechanical changes similar to those occurring during overuse activities in the young athlete and are generally associated with pediatric apophyseal pathologies [9-12].

Significance/clinical relevance: Pediatric sports-related injuries, such as Sever disease, Little Leaguer's elbow, and Osgood-Schlatter disease, all represent common clinical apophyseal pathologies in the young athlete. Increasing clinical evidence demonstrates the link between prolonged overuse activities and repetitive loading with other diagnostic (e.g., magnetic resonance imaging) and clinical abnormalities in skeletally immature athletes [10-13]. Our non-invasive, repeatable model for *in vivo* muscle loading enables mechanistic investigation of the effect of loading on the structure of developing and mature enthesis in order to improve clinical interventions for overuse injuries in children and adults.

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