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Fast, non-eccentrically loaded exercise worsens tendinopathic healing responses in a murine model

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OBJECTIVE
To advance the understanding of how alterations in exercise speed and grade (flat vs 17° incline or decline) affect the quality of tendon healing, and to determine if a biomarker relationship exists between serum levels of a ColX breakdown product (CXM) and animals exposed to treadmill running protocols.

ANIMALS
35 male mice (C57BL/6J), 8 weeks of age.

PROCEDURES
Mice were preconditioned on a treadmill for 14 days. Tendinopathy was then induced by 2 intra-tendinous TGFβ1 injections followed by randomization into 7 exercise groups. Exercise capacity and objective gait analysis were measured weekly. Mice were euthanized and histopathologic analysis and evaluation of serum CXM levels were performed. Statistics were conducted using a 2-way ANOVA (exercise capacity), Mixed Effects Model (gait analysis, effect of preconditioning), and 1-way ANOVA (gait analysis, the effect of injury, and rehabilitation normalized to baseline; CXM serum analysis), all with Tukey post hoc tests and significance set to $P < .05$.

RESULTS
Exercise at a fast-flat speed demonstrated inferior tendinopathic healing at the cellular level and impaired stance braking abilities, which were compensated for by increased propulsion. Mice exposed to exercise (at any speed or grade) demonstrated higher systemic levels of CXM than those that were cage rested. However, no ColX immunostaining was observed in the Achilles tendon or calcaneal insertion.

CLINICAL RELEVANCE
Exercise at a fast speed and in absence of eccentric loading components (incline or decline) demonstrated inferior tendinopathic healing at the cellular level and impaired braking abilities that were compensated for by increased propulsion.

Achilles tendinopathy remains a prevalent and frustrating sports injury for human athletes and aging populations.[1–3] A 52% lifetime risk of developing Achilles tendinopathy has been reported for elite runners.[4] Similarly, animal species suffer debilitating levels of tendinopathy, with common calcaneal injuries being 1 of the top causes of lameness in working dogs,[5] and superficial digital flexor tendon (SDFT) injury being the primary reason for retirement of Thoroughbred racehorses over a 12-year epidemiologic study period.[6] Despite surgical and therapeutic advancements in any species, few interventions have proven superior to controlled exercise alone.[7] A clear explanation for the response of tendinopathy to exercise is lacking,[8] but 1 proposed therapeutic mechanism of action includes the beneficial effects on collagen homeostasis.[9] Specifically, an increase in collagen synthesis measured via microdialysis was demonstrated after 12 weeks of eccentric exercise in Achilles tendinopathy.[9] Eccentric loading

refers to the lengthening of the muscle-tendon unit as a load is applied that can be accomplished through either incline or decline exercise.[7] This is in contrast to concentric loading for which the muscle-tendon unit shortens as a result of the applied load.[7] Eccentric loading magnitudes[10] and intra-tendinous sinusoidal oscillations occurring specifically during the eccentric phase of therapeutic exercises have been proposed to stimulate tendon remodeling,[7] consistent with other reports that collagen synthesis may be affected by exercise loading magnitude.[11] Despite these findings, the optimum "dose" and "type" of therapeutic exercise with respect to speed and grade of exercise remains unknown.

Murine studies have demonstrated that induction of tendinopathy via injection of TGF-β1 directly into the tendon body allows for the consistent reproduction of chronic tendinopathic changes including hypercellularity, collagen disorganization, chondroid deposition, and decreased material properties.[12] Specifically, the accumulation of a chondroid matrix in this model—characterized by elevated levels of sulfated glycosaminoglycans,[12] aggrecan, and hyaluronan[12,13] within the tendon body—replicates pathologic changes appreciated in human tendinopathic studies.[7] When a mechanical loading component was applied to this murine model via daily short-fast (32 cm/s for 25 minutes) treadmill running, improved biomechanical properties and histopathology (lessened chondroid deposition and collagen disorganization) were evident at 2 weeks post-injury. However, long-term (4 weeks post-injury) continued treadmill running was detrimental.[14] Due to alterations in the tendinopathic response to varied exercise regimes, controlled studies investigating exercise variables such as speed, duration, and incline or decline (eccentric load) are necessary to tease out parameters for the clinical effectiveness of rehabilitative protocols.

Additionally, we were curious to determine whether ColX turnover may be used to quantitatively assess tendon healing through serological assessments. Collagen X has been historically considered in the context of cartilage, as its roles in chondrogenic differentiation and chronic osteoarthritis have been previously described.[15,16] Specifically, an association between elevated levels of collagen X and osteoarthritic cartilage has been demonstrated,[15–19] but its role as it pertains to tendons is minimally explained.[20,21] Expression of ColX in the enthesis of the rat Achilles tendon has been described as being produced by cells in transitional zones between calcified and non-calcified tissue (the interface between articular cartilage and subchondral bone).[20] Similarly, ColX was localized at the insertion site of the bovine Achilles tendon, suggesting it may be a resident of mineralized fibrocartilaginous zones of tendon or enthesis junction that may aid in the anchoring of tendon or ligament to the bone.[21] As hind limb exercise effects the entire calcaneous-Achilles–gastrocnemius (eg, bone-tendon-muscle) unit, the study of ColX locally and systemically may assist in the evaluation of exercise protocol effectiveness on the ankle joint as it relates to tendon zonal enthesis attachment and healing after mid-substance tendon injury.

Given this, the objective of this study was to advance the understanding of how alterations of speed (slow vs fast) and grade (incline vs decline) of exercise-based rehabilitation affect (1) the quality of tendon healing and (2) local ColX and systemic CXM levels, using a translational murine model. It was hypothesized that gradual (slow) exercise at an incline (eccentric loading) after tendon injury would demonstrate superior kinematic and histopathologic healing compared with no activity, more intense (fast) exercise, or exercise at a decline. Overall, further understanding the relationship between exercise protocols and tendinopathic remodeling may provide valuable translational insight that could help focus further species-specific investigations.

Materials and Methods

Study design

This study was a prospective, controlled, blinded experiment. All study methods were conducted in compliance with Colorado State University's Institutional Animal Care and Use Committee (IACUC) standards (Protocol #16-6927A). The proposed experimental sample size (5 mice per group) was calculated using GPower Version 3.1.1. Specifically, an a priori power analysis utilized pilot gait data (min dA/dT, a measure of the maximal rate of change [cm^2/s] of paw area in contact with the treadmill belt during the propulsion phase that assesses how rapidly the animal is able to propel itself into the next step).[22] This power analysis resulted in an effect size of 1.25 and a power of 97.3%, using a standard deviation of 10 for all groups.

Murine population

C57BL/6J male mice were purchased at 8 weeks of age from a commercial vendor (Jackson Laboratories). All mice were allowed to acclimate for 14 days before experimentation. Standard preconditional treadmill running[12,14] was initiated for 14 days before injury (days −14 to 0; fast protocol; 32 cm/s for 25 minutes) to establish conditioned animals or tendons for enhanced clinical translation to athletic patients. Therefore, the injury occurred when mice were 12 weeks of age to match previously published reports.[12,14] All mice were weighed weekly, housed at 22 °C to 24 °C, and maintained on a 12-hour light/dark cycle with water and food available ad libitum during the experiments. Thirty-five total animals were utilized for this study with 5 mice in each exercise group **(Figure 1)**.

Murine model of tendinopathy and exercise groups

To induce tendinopathy, 6 uL of 100 ng active human recombinant TGF-β1 was injected into the mid-substance of the right Achilles tendon as previously described.[14] Two injections were performed, 2 days apart, using a 28G needle with mice under anesthesia with 2% to 3% isoflurane.[14] To ensure

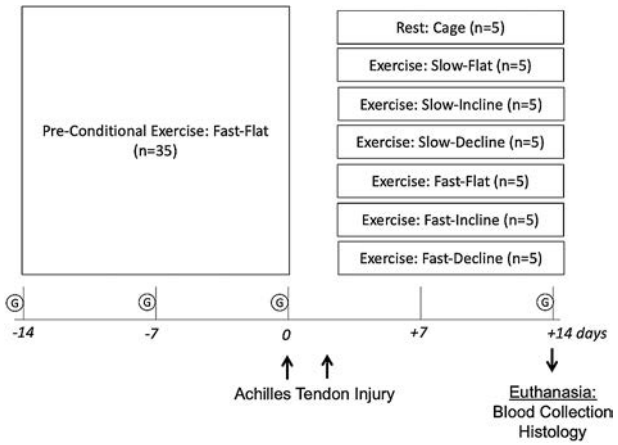

Figure 1—Experimental timeline and groups: After pre-conditional treadmill running and induction of Achilles tendon injury via 2 injections (2 days apart) of TGF-β1, mice were randomized into 7 treatment groups with the healing response assessed at 14 days post-injury. Gait analysis (G) was measured on days −15, −8, −1, and 13 with weekly maintenance in between measurements.

reproducibility of injection location (central tendon mid-substance), the same person (K.J.S.) performed all injections. After the injury, mice were randomized into the following groups: (1) rest (normal cage activity) and exercise protocols of (2) slow-flat (20 cm/s with no incline), (3) fast-flat (32 cm/s with no incline), (4) slow-incline (20 cm/s at 17° incline), (5) fast-incline (32 cm/s at 17° incline), (6) slow-decline (20 cm/s at 17° decline), and (7) fast-decline (32 cm/s at 17° decline). All animals underwent exercise protocols for 25 minutes per day starting on day 3 of the experimental protocol for 5 days per week with 2 days of rest each week.[12,14] All exercise protocols were performed on a 5-lane trea, and electrical stimulation (0.1–0.3 mA) was utilized for compliance during both pre-injury (pre-conditional) and post-injury (exercise) treadmill running (PanLab).

Exercise capacity

To relate the tolerance of exercise protocols for clinical applicability to quadruped patients, the exercise capacity of animals undergoing treadmill running was measured. Exercise capacity was based on the number of faults per animal that was recorded during protocols, where a fault qualified as falling behind the treadmill onto the stimulation device. A high number of faults and, therefore, increased required stimulation during standardized exercise was considered consistent with general exercise intolerance. The specific pain or behavioral reasoning for exercise intolerance was not able to be delineated in the current study. Exercise capacity was then expressed as the ratio of required stimulation after injury relative to pre-injury for comparison between exercise groups. The coefficient of variation was calculated for each animal and then averaged for each group to describe the spread as a representation of the animal-to-animal variability in the completion of exercise protocols.

Gait

Gait measurements were taken weekly before the injury and before sacrifice at 14 days post-injury (Figure 1). Mice were acclimated to a treadmill-based gait analysis system (DigiGait™, Mouse Specifics) over 1 week before data collection. Paw statistics, automatically calculated by associated software, were collected for all mice at 35 cm/s on a flat treadmill. Key gait parameters, including stride length and swing speed, were collected for all mice at each of the exercise protocols (all parameters measured can be seen elsewhere; **Supplementary Tables S1 and S2**). Maximum dA/dT (braking) was defined specifically as the maximal rate of change (cm^2/s) of paw area in contact with the treadmill belt during the braking phase consistent with how rapidly the mouse was able to decelerate.[22] Minimum dA/dT (propulsion) was defined as the maximal rate of change (cm^2/s) of paw area in contact with the treadmill belt during the propulsion phase consistent with how rapidly the mouse was able to propel itself into the next stride.[22]

CXM serum analysis

Blood was collected via an intra-cardiac stick while under general anesthesia (2% to 3% isoflurane) on day 14 of the experimental protocol immediately before euthanasia (Figure 1). Blood serum concentrations of CXM for each experimental group were measured via ELISA using custom components as previously described.[17]

Tissue collection and histopathology

Mice were sacrificed on day 14 of the experimental protocol. After intra-cardiac puncture for blood collection, euthanasia occurred via CO_2 asphyxia with cervical dislocation before tissue collection. After euthanasia, both hind limbs were removed, fixed, embedded in paraffin, sectioned (5μm; sagittal), and stained with SafraninO/Fast Green (SOFG) and Hematoxylin and Eosin (H&E). Specimens were qualitatively interpreted by a board-certified pathologist (K.S.S.) based on the following criteria: (1) tissue swelling; (2) hyper-cellularity; (3) collagen disorganization; and (4) chondroid deposition. Additional parameters evaluated included: peritenon (1) calcification, (2) edema, (3) neovascularization, and (4) inflammation. Additionally, modifications to the adjacent retrocalcaneal fat pad[23] (mononuclear cell infiltration) and bursae[24] (degree of synovial hyperplasia) were noted due to recent studies highlighting the importance of both structures in human Achilles tendon pathology. Of note, samples were not quantitatively graded due to variable tissue sectioning that precluded full morphologic evaluation of all criteria.

Immunohistochemistry

Given that a subset was evaluated, a subjective impression of relative fluorescence of the distal hindlimb Achilles tendon and calcaneal insertion was provided (normalized to positive fluorescence for ColX in the growth plates). For immunohistochemistry, formalin-fixed paraffin-embedded sections were

deparaffinized and pretreated for antigen retrieval. Briefly, antigen retrieval involved treatment with 1 mg/mL protease (Roche) in 1XPBS for 30 minutes at room temperature, followed by 1 mg/mL hyaluronidase (Sigma-Aldrich) in 1XPBS for 30 minutes at 37 °C. All sections were blocked with 5% bovine serum albumin (BSA) in 1X PBS for 45 minutes and incubated overnight at 4 °C with primary antibodies to collagen X (1:1000; purified chicken pAb to mouse rNC1 sequence as described previously (Coghlan, 2017; PMID: 29212713). The collagen X antibody was a kind gift from William A. Horton, Shriners Hospitals for Children, Portland, OR. Sections were washed with PBS and incubated for 45 minutes at room temperature with Alexa Fluor 555-conjugated goat anti-chicken (Life Technologies), diluted 1:500 in 1% BSA in 1XPBS. Slides were counterstained with 300 nM DAPI (Life Technologies) for 15 minutes and mounted with ProLong™ Diamond Antifade Mountant (Life Technologies).

Statistical analyses

Statistical analysis was performed using commercial software (GraphPad Prism 7). Exercise capacity was analyzed using a 2-way ANOVA incorporating grade, speed, and grade or speed interaction factors with Tukey's post hoc comparisons. To evaluate 2 scenarios of changes in gait parameters including (1) before and after preconditioning and (2) between exercise groups (post-op values normalized to baseline pre-op), paw statistics were analyzed using either a Mixed Effects Model (with the mouse acting as the random effecter) or 1-way ANOVA, respectively, with Tukey's post hoc comparisons. Time points for which various paw statistics were not able to be accurately calculated on the Digigait program were treated as missing data and omitted for statistical analysis. For CXM serum analysis, a 1-way ANOVA, with Tukey's post hoc comparisons was utilized for comparisons between groups. Statistical significance was considered $P < .05$ for all comparisons.

Results

Exercise capacity

Exercise capacity expressed as the ratio of required stimulation after injury relative to pre-injury was not significantly different between groups for speed ($P = .07$), grade ($P = .28$), or speed or grade interaction ($P = .25$); however, trends and qualitative observations during exercise protocols suggest that animals required more simulation for maintenance over running for all fast exercise protocols **(Figure 2)**. Interestingly, this was animal specific as demonstrated via the coefficients of variations reported. Notably, all mice showed similar pre-injury stimulation values during fast-flat pre-conditional running.

Gait

Preconditioning effects—All mice demonstrated a trended increase in max dA/dT when comparing day −15 to day −1 (before vs after the pre-conditioning period but before injury induction) ($P = .09$) and when comparing day −8 to day −1 ($P = .06$; **Figure 3**). No significant differences in min dA/dT, or any other gait parameters, were appreciated when comparing across the pre-injury time course. All evaluated gait parameters and statistical differences are reported elsewhere (Supplementary Table S1).

Exercise protocol effects—The mice in the fast-flat group demonstrated significantly increased max dA/dT (propel capacity) values compared with those in the slow-incline, slow-decline, fast-incline, and fast-decline groups relative to pre-injury ($P < .0001$ for all comparisons; Figure 3). Mice in the fast-flat group also demonstrated a significantly decreased min dA/dT (braking capacity) compared with those in the slow-incline ($P = .005$), slow-decline ($P = .005$), fast-incline ($P = .02$), and fast-decline ($P = .0009$) groups relative to pre-injury. All evaluated gait parameters and statistical differences are reported elsewhere (Supplementary Table S2).

(A)

(B)

Group		COV (%)
Slow	Flat	177.7%
	Incline	43.02%
	Decline	66.40%
Fast	Flat	151.6%
	Incline	100.8%
	Decline	51.56%

Figure 2—(A) Exercise capacity (% stimulation during running) of mice after injury relative to pre-conditioning levels (A). No significant differences were found between groups for speed ($P = .07$), grade ($P = .28$), and interaction ($P = .25$) factors. (B) Coefficient of variation for exercise capacity after injury relative to pre-conditioning levels. A wide variation in the percent stimulation ratio was appreciated in the fast-flat exercise group compared with other groups, consistent with detrimental tendinopathic healing characterized by impaired braking, impaired propelling, and histopathologic evidence of chondroid metaplasia.

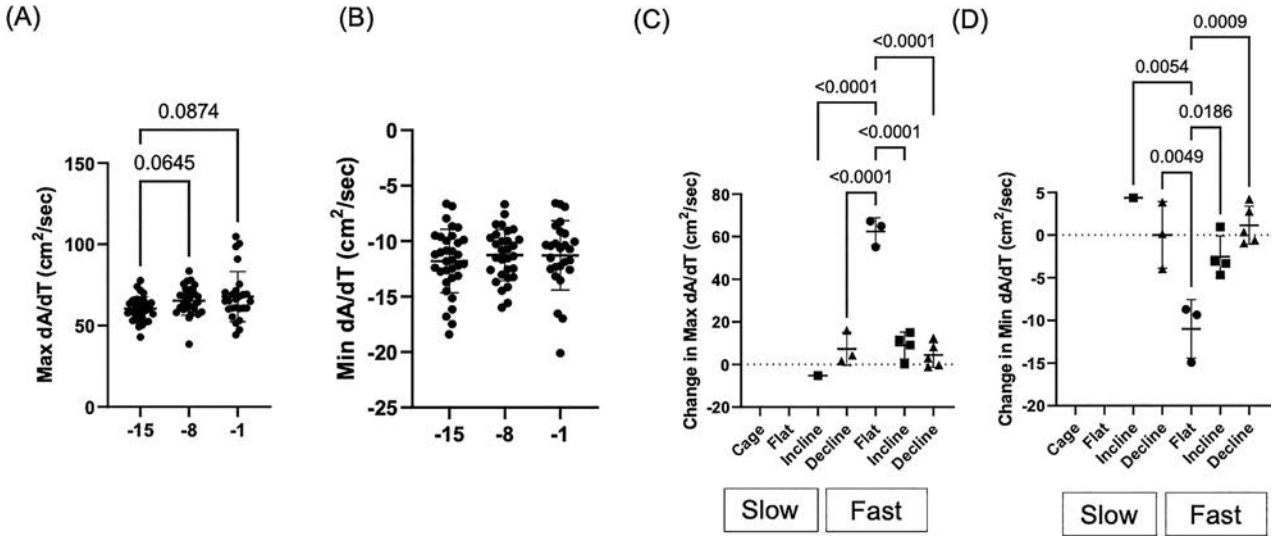

Figure 3—Effect of pre-conditioning on (A) max dA/dT and (B) min dA/dT gait parameters. Data represented as average ± STD for all mice for each time-point. Significant *P*-values marked. Effect of injury and rehabilitation treatment on the change in (C) max dA/dt and (D) min dA/dT relative to pre-injury levels. Data represented as average ± STD with individual animals marked and *P*-values listed.

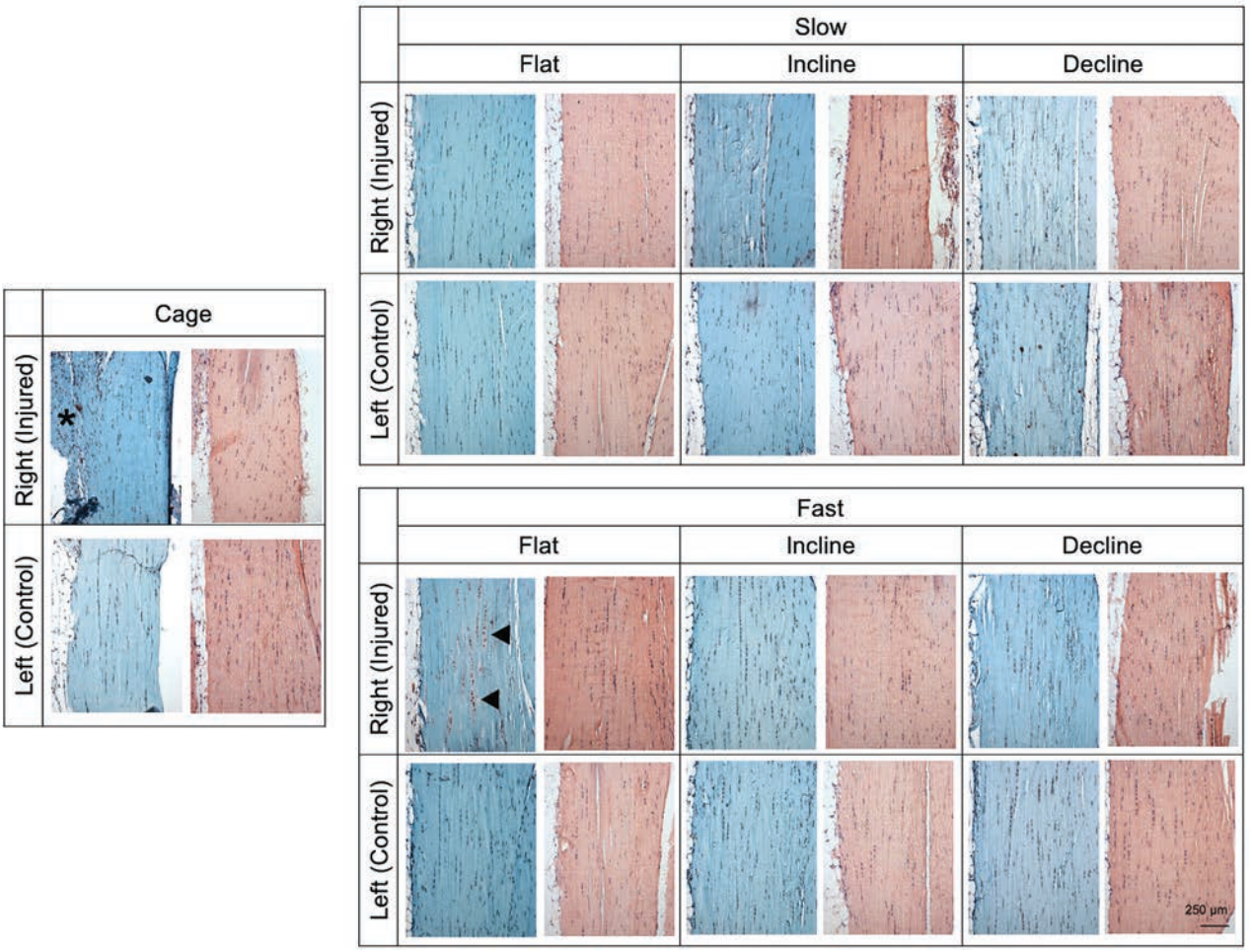

Figure 4—Representative SafraninO/Fast Green (SOFG) and Hematoxylin and Eosin (H&E) stained injured (right limb; top panels) and control (left limb; bottom panels) murine Achilles tendons for each experimental group. Each image is oriented as follows: top (proximal), bottom (distal), left (anterior), right (posterior). Arrows denote chondroid metaplasia and * denote peritenon hyperplasia.

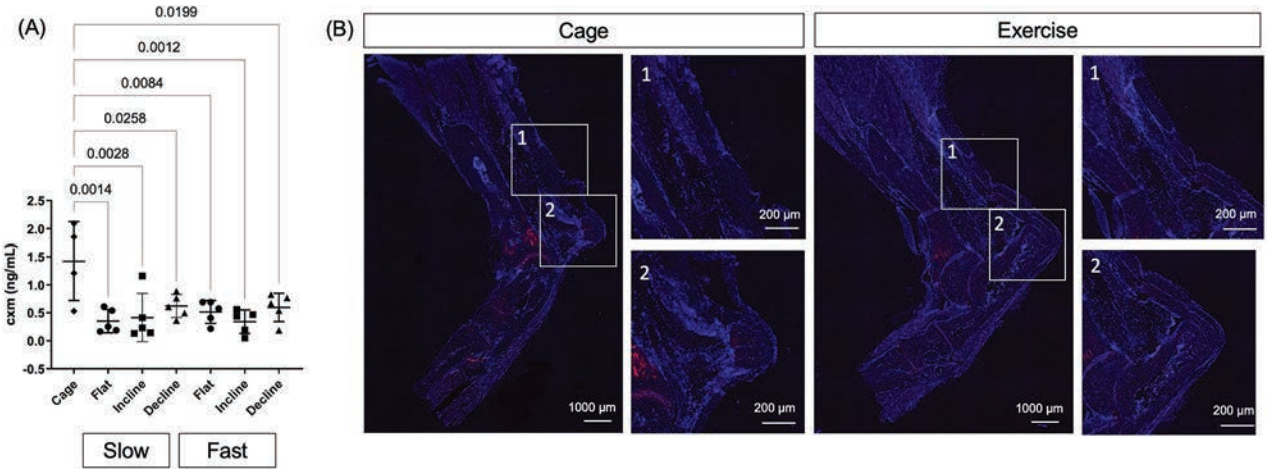

Figure 5—(A) Blood serum concentrations of CXM (a collagen X breakdown product) for each experimental group. Data is presented as mean ± STD with individual data points marked. Statistical significance between groups is marked and *P*-values listed. (B) Representative ColX IHC stained mouse limbs for cage and exercise (slow-incline) groups (blue: DAPI; red: ColX). Zoomed in areas represent (1) Achilles tendon and (2) calcaneal insertion.

Histology

Minimal histologic differences were seen in the Achilles tendons between the injured limb and the contralateral limb **(Figure 4)**. In the fast-flat group, the injured limb showed increased tendon mid-substance chondroid metaplasia relative to the contralateral control limb. Specifically, chondroid metaplasia ranged from mild to moderate in the fast-flat group. Notably, some minor peritenon hyperplasia was evident in the injured limb of the cage active and fast-flat groups. Lastly, both the cage active and fast-flat exercise groups showed mild to moderate modifications to the adjacent fat pad and bursae in the right injured limb relative to the left contralateral control limb, with minor changes seen in all other groups. Specifically, these changes included bursal synovial hyperplasia and increased presence of perivascular mononuclear cells.

CXM serum analysis

All exercise groups showed significantly ($P < .05$) less serum CXM than mice who did not receive exercise post-injury (cage activity; **Figure 5**). Within the exercise groups, CXM was lowest in the slow-flat group and highest in the decline groups irrespective of speed.

ColX immunohistochemistry

For all groups, no positive ColX staining was observed in the Achilles tendon (Figure 5) or calcaneal insertion. Representative images of ColX IHC stained limbs from all groups can be seen elsewhere **(Supplementary Figure S1)**.

Discussion

Given that few therapeutic interventions have proven superior to controlled exercise alone in the treatment of Achilles tendinopathy,[7] the objective of this study was to advance the understanding of how alterations of speed and grade (flat vs incline vs decline) of exercise-based rehabilitation affect the quality of tendon healing using a translational murine model. Specifically, further understanding the relationship between exercise protocols and the tendinopathic response would help provide evidence-based recommendations for generalized exercise regimes as a physiotherapeutic modality to augment currently used tendon therapies for all clinical species.

The results of this study demonstrated that a fast-flat (non-eccentrically loaded) exercise protocol was the most detrimental to tendinopathic healing. This was substantiated by impaired braking (min dA/dT) and propelling (max dA/dT) gait abilities and histopathologic evidence of chondroid metaplasia. Additionally, serum (systemic) CXM levels were found to be highest in the cage-active group, with no appreciable IHC ColX levels in the Achilles tendon or calcaneal insertion. Cumulatively, this study investigated exercise capacity, morphology, and functional outcomes to provide further insight into tendinopathic healing responses in relation to various exercise variables of exposure itself, speed and grade that are currently unknown.

An initial unexpected finding of this study was histopathologic evidence to suggest that preconditioning (exercise exposure itself before lesion induction) had a beneficial, protective effect in this murine model. Historically, a single injection of TGF-β1 into the body of the murine Achilles tendon has resulted in acute tendinopathic features of tendinous swelling, hyperplastic aggrecan-rich deposits, and collagen disorganization accompanied by loss of tensile properties.[12] This early loss of biomechanical properties and matrix disorganization, however, was largely reversed by extended treadmill running, limiting its utility as an acute tendinopathic model only.[12] Subsequently, a 2-injection lesion induction protocol (2 injections of TGF-β1 performed 2 days

apart, as used in the current study) was utilized that successfully simulated more severe injury, capturing acute, intermediate, and chronic tendinopathic characteristics.[14] Previous histopathologic observations at day 14 post-lesion induction demonstrated strong evidence of tendon swelling, collagen fiber disorganization, tendon mid-substance hypocellularity, and peritendinous edema with hypercellularity.[14] This same 2-injection TGF-β1 model was utilized herein, but interestingly, less chondroid metaplasia deposition, minor peritenon hyperplasia, and less severe alterations in the adjacent fat pad and bursae in the injured limb compared with the uninjured contralateral limb were appreciated. In contrast to the previous investigation,[14] the use of pre-conditioning to develop conditioned tendons before lesion induction may have subjectively reduced these more severe histologic findings. This finding, in conjunction with a trended increase in propelling capacity (max dA/dT) that was appreciated for day −15 relative to day −8 and day −1 comparison (before vs after the pre-conditioning period but before injury induction) offers some evidence that preconditioning exercise to augment tendon "fitness" may be protective against subsequent injury. These findings are in accordance with extensive literature that has documented the beneficial effects of exercise on tendon remodeling.[10,25–27] Specifically, athletic loading has been documented to increase collagen turnover rates in naturally occurring Achilles tendinopathy as evidenced via serial microdialysis sampling.[9] Similarly, exercise has also been postulated to subsequently modify mechanical properties making tendons more load-resistant,[25] and in a meta-analysis of exercise intervention studies on healthy adults, stiffness adaptation significantly depended on loading intensity.[10] Further work to delineate the mechanisms contributing to this protection is warranted, but similar to historic findings, the results described herein corroborate a beneficial, protective effect on tendinopathy when primed with exercise. When considering naturally occurring tendon injuries in veterinary species, it is interesting to note that in the authors' experience, tendinopathy occurs in both physically primed athletes and in prospects preparing for higher-level sports. Noteworthy, however, is that despite reportedly high re-injury rates in horses,[28] many tendinopathic patients do return to athletic use, leading authors to speculate if that may be in part due to the preconditioned nature of their damaged tendons (in comparison with athletically naïve tendons). Additionally, it may be worth considering the incorporation of judicious, standardized exercise protocols involving eccentric loads in absence of speed for juvenile athletes before the commencement of formal training in an attempt to physiologically load or prime more naïve tendons. This information is certainly worth noting as this tendinopathic model continues to be utilized in future studies pertinent to the practicing veterinary clinician.

The considerate, patient-specific prescription of controlled exercise remains a fundamental aspect of veterinary rehabilitation programs after tendon injury, but specific exercise recommendations regarding specific factors such as speed, duration, intensity, and frequency are largely based on clinical subjective impressions and anecdotal guidelines.[29–30] In horses, exercise recommendations after naturally occurring tendinopathy involve a period of rest followed by gradual increases in exercise to promote collagen remodeling to ultimately improve the mechanical properties of the damaged tendon,[29] but specific recommendations in terms of incline or decline exposure are largely lacking. Similarly, canine rehabilitation guidelines after tendon injury generally consist of initial activity restriction followed by rehabilitation (controlled jumping, changing direction, running, etc) and lastly return to work.[30] Murine experimentation as described herein, despite not being directly applicable to veterinary patients such as canine and equine athletes, seeks to advance our understanding of how alterations in these exercise factors may affect tendon healing, with evidence to suggest that exercise at a fast-flat speed is the most detrimental to tendinopathic remodeling. With the recent motivation to mobilize veterinary patients more quickly once clinical comfort is achieved to mitigate water and proteoglycan content loss that occurs with prolonged tendon immobilization,[31] these findings may help the practitioner frame controlled eccentric loading opportunities into guided, professional rehabilitation programs with more confidence while minimizing exercise prescription at speed on a flat surface. Further recommendations can be expected as subsequent investigations continue to deepen the understanding of how exercise may be utilized to beneficially affect tendinopathic remodeling.

When considering treatment regimens, mice exposed to fast-flat treadmill running demonstrated significantly decreased min dA/dT (braking capacity), increased max dA/dT (propelling capacity), and the most evidence of chondroid metaplasia compared with other exercise groups (rest or cage activity, slow-flat, slow-incline, fast-incline, slow-decline, and fast-decline). When considered together, these findings suggest that the fast-flat exercise group has the most significantly impaired gait and deteriorated cellular qualities, respectively. Potential explanations for this include the absence of eccentric loading, the consistent exposure to fast speed itself, or a combination thereof. Given that similar findings were not appreciated in the other fast (fast-decline or fast-incline) or flat (slow-flat) exercise groups, authors speculate it is likely a combination of an impaired braking capacity (decreased min dA/dT) that resulted in compensatory propelling capacity (increased max dA/dT) due to the forced compulsory gait cycle that occurred with treadmill running post-lesion induction. It may also be attributed to the fact that mice exposed to fast treadmill running, in general, had difficulty maintaining speed and more variable exercise tolerance post-injury, which may have altered loading and potentially caused additional tendon micro-damage. Specifically, mice in the fast-flat group had the most variation in exercise

capacity, despite no change in grade or speed from pre-conditional exercise. While the exact reasoning for altered exercise tolerance (pain vs behavioral) could not be elucidated in the current study, the exercise capacity measurement seeks to relate the tolerance of exercise protocols for clinical applicability to veterinary patients. Biomechanical characteristics of in vivo eccentric exercises in human Achilles tendons have been previously described, for which tendons were found to vibrate at higher frequencies during the eccentric phase than during the concentric phase.[32] Potential downstream effects that may subsequently reduce the fortitude of tendons not exposed to eccentric exercise include incomplete activation of the motor neuron that occurs during concentric-only phases of exercise or the lack of fibroblast stimulation amongst other various neural activation strategies that occurs with eccentric loading only.[32] Similarly, a systematic review concluded that tendon structural changes are not solely responsible for the positive effects of eccentric rehabilitation regimes, but rather neural, biochemical, and myogenic changes may be involved.[8] While this investigation did not focus on mechanistic explanations, the appreciated gait deficits and histopathologic characteristics of mice specifically exercised at a fast-flat speed highlight a future potential avenue to assess interventional therapeutics.

In the present study, serum (systemic) CXM levels were found to be highest in the cage-active group. In contrast, no positive ColX staining was observed in the Achilles tendon or calcaneal insertion, suggesting that systemic changes may be associated with exercise induced changes to other structures. These findings are intriguing because the relationship between collagen X and exercise exposure specifically has not previously been reported. The results suggest that cage activity alone (rest) leads to elevated systemic levels of CXM not appreciated in mice that were exercise exposed. Given that cage activity mice demonstrated inferior morphologic and biomechanical gait patterns in comparison with those receiving exercise, and that rest in general is detrimental to tendinopathic healing,[33–38] longitudinal serum CXM levels may represent a future exercise-specific biomarker to explore during tendon healing. Given that a systemic biomarker relationship to non-invasively assess the status of mid-body tendinopathy has not been previously described, further work is certainly warranted.

Acknowledged study limitations include the use of male mice, only; future work would consider sex a biological variable. Further, consideration of age at time of injury would provide an interesting comparison to understand how adolescents vs adults vs aged populations respond to tendinopathic injury and exercise rehabilitation protocols. Of note, mice were 12 weeks old at the time of injury for historical comparison with previous studies utilizing this model. Additionally, 1 predetermined timepoint was investigated based on outcomes utilized in previous studies. The inclusion of both earlier and later harvest days would allow a longitudinal assessment of relevant outcomes. The authors would also like to acknowledge that this investigation did not evaluate postmortem tendon biomechanics that would have further complimented the histopathologic analysis described herein. Lastly, this study represents an investigative first step to determine how alterations in exercise speed and grade (flat vs incline or decline) affect the quality of tendon healing, but results cannot be directly extrapolated to clinical application in veterinary patients. A logical next step would be to validate these findings in a larger species animal model using similar, yet extrapolated exercise exposures.

In summary, mechanical loading through graduated, controlled exercise continues to be a fundamental aspect of tendon rehabilitation; however, there is a lack of studies investigating the specific thresholds of frequency, intensity, and duration in relation to the effect on healing. The major benefit of the proposed study design was the ability to relate exercise capacity, morphology, and functional outcomes to exercise through the use of longitudinal tracking of individual animals. Through this investigation, exercise initiated at a fast speed and in absence of eccentric loading components demonstrated inferior tendinopathic healing at the cellular level and impaired braking abilities that were compensated for by increased propulsion. Mice exposed to exercise demonstrated higher systemic levels of CXM than those receiving cage rest, with no positive ColX IHC staining in the Achilles tendon or calcaneal insertion. These findings ultimately advance the understanding of how the intensity of exercise affects tendinopathic healing in a chronic tendon injury model, for which there currently is no peer-reviewed evidence to support.

Acknowledgments

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The authors declare that there were no conflicts of interest.

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Supplementary Materials

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