Early voluntary exercise does not promote healing in a rat model of Achilles tendon injury

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Godbout C, Ang O, Frenette J. Early voluntary exercise does not promote healing in a rat model of Achilles tendon injury. J Appl Physiol 101: 1720–1726, 2006.—Mechanical stress is an important modulator of connective tissue repair. However, the effects on tendon healing are very poorly defined, preventing optimal use of mechanical stress. We hypothesized that early voluntary exercise initially retards tendon repair but results in a faster recovery rate at longer term. Male Wistar rats were injured by a collagenase injection in the Achilles tendon, and exercise was voluntarily performed on a running wheel. We observed the persistent presence of neutrophils in injured tendons of rats that began exercise immediately after the trauma [injured + early exercise (Inj+EEx)]. Early exercise also increased the concentration of ED1⁺ macrophages in injured tendons after 3 and 7 days compared with ambulatory injured rats (Inj). Similar results were obtained with the subset of ED2⁺ macrophages in the tendon core 3 days after the collagenase injection. Furthermore, collagen content returned to normal values more rapidly in the Inj+EEx tendons than in the Inj group, but this was not associated with an increase in cell proliferation. Surprisingly, Inj+EEx tendons roughly displayed lower stiffness and force at rupture point relative to Inj tendons at day 28. Injured tendons of rats that began exercise only from day 7 had better mechanical properties than those of early-exercised rats 28 days postinjury. We speculate that the persistence of the inflammatory response and undue mechanical loading in the Inj+EEx tendons led to fibrosis and a loss of tendon function.

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MATERIALS AND METHODS

Experimental protocol. Male Wistar rats (Charles River, Saint-Constant, QC, Canada), ~8 wk old and weighing ~200 g, were housed two per cage before the operation and alone following the surgical procedure. Food and water were provided ad libitum throughout the experimental protocol, and the animals were housed under a 12:12-h light-dark cycle. The rats were divided into five groups: 1) noninjured + ambulation (controls; Ctr), 2) noninjured + early exercise (Ctr+EEx), 3) injured + ambulation (Inj), 4) injured + early exercise (Inj+EEx), and 5) injured + late exercise (Inj+LEx).
For the surgical procedure, the animals were anesthetized with an intraperitoneal injection of a ketamine-xylazine cocktail (87.5 and 12.5 mg/kg, respectively) and rehydrated with 5 ml of lactated Ringer solution injected subcutaneously. Injury was induced by injecting 30 μl of crude collagenase in PBS (10 mg/ml; Sigma, St. Louis, MO) in the right Achilles tendon using a 29-gauge needle as previously described by Marsolais et al. (23). The same volume of PBS without collagenase was injected in the left Achilles tendon (sham procedure). Ocular lubricant and lint-like material were used to prevent ocular drying and hypothermia, respectively. The Ctr and Inj groups were housed in standard cages and had normal activity. The early exercise groups were allowed to perform voluntary exercise immediately after tendon injury. A wheel (33.5-cm inside diameter) linked to an automatic counter was left in the cages during the entire protocol (Harvard Apparatus, Holliston, MA). The running distance was estimated by multiplying the periphery of the wheel with the number of turns executed. The rats were killed 3, 7, or 28 days after collagenase injection. In the protocol where mechanical testing was performed, the Inj-LEx group was also included. These injured rats had normal cage injection. In the protocol where mechanical testing was performed, the rats were killed 3, 7, or 28 days after collagenase injection. In the protocol where mechanical testing was performed, the Inj-LEx group was also included. These injured rats had normal cage activity for 7 days followed by 21 days of voluntary exercise. The Ctr and Inj groups were housed in standard cages and had normal activity. The early exercise groups were allowed to perform voluntary exercise immediately after tendon injury. A wheel (33.5-cm inside diameter) linked to an automatic counter was left in the cages during the entire protocol (Harvard Apparatus, Holliston, MA). The running distance was estimated by multiplying the periphery of the wheel with the number of turns executed. The rats were killed 3, 7, or 28 days after collagenase injection. In the protocol where mechanical testing was performed, the Inj-LEx group was also included. These injured rats had normal cage activity for 7 days followed by 21 days of voluntary exercise. The Laval University Research Center Animal Care and Use Committee approved all animal care and handling procedures.

Tissue preparation and immunohistochemistry. A first set of rats (n = 46) were anesthetized with the ketamine-xylazine cocktail and killed by neck dislocation. The Achilles tendons were harvested by sectioning the osteotendinous and musculotendinous junctions. The freshly excised tendons were embedded in optimum cutting temperature compound, frozen in isopentane precooled in liquid nitrogen, and stored at −80°C. Cryostat longitudinal sections (5 μm thick) were mounted on gelatin-chrome alum-coated slides and stored at −20°C overnight. Tissue sections were fixed in acetone-methanol (1:1), immersed in 3% H2O2 (diluted in methanol), and rinsed in PBS for 10 min after each step. Following 45 min in blocking buffer (100 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.2% gelatin, 3% bovine albumin serum, 2% horse serum, and 0.02% sodium azide), the tendon sections were incubated for 2 h at room temperature with the primary antibodies W3/13 (mouse anti-rat CD43, diluted 1:50 in blocking buffer), anti-ED1 (mouse anti-rat CD68, diluted 1:100) or anti-ED2 (mouse anti-rat CD163, diluted 1:100) from Serotec (Raleigh, NC) to label neutrophils, ED1+ and ED2+ macrophages, respectively. Control sections were overlaid with blocking buffer only. To evaluate cell proliferation, tendon sections were fixed in acetone for 10 min and overlaid with primary anti-proliferating cell nuclear antigen (PCNA) antibody overnight at 4°C (mouse anti-PCNA, diluted 1:100, Serotec). All sections were then consecutively incubated at room temperature with the biotinylated anti-mouse IgG (heavy and light chains) secondary antibody (diluted 1:200 in PBS; Vector Laboratories, Burlingame, CA) for 1 h and horseradish peroxidase-avidin D (diluted 1:1000 in PBS; Vector Laboratories) for 30 min. The sections were washed three times with PBS between each incubation period. The sections were then rinsed in PBS twice and in bdistilled water for 10 min, stained using an AEC kit (AEC substrate kit for peroxidase, Vector Laboratories), and mounted using glycerogel. Last, neutrophils, ED1+ macrophages and proliferative cells were counted using a Nikon Eclipse TE300 inverted microscope (Nikon, Mississauga, ON, Canada) by selecting three to five equidistant field columns, while ED2+ macrophage density was calculated by randomly selecting six quadrants from the paratenon and six from the tendon core. As the antibodies target specific antigens located on cell surface (ED2, ED1), they thus produce typical staining, allowing the investigator to count manually the number of labeled cells from the preselected or randomly selected fields. Finally, the areas were determined and multiplied by the thickness to express the number of cells per millimeters cubed.

Edema and hydroxyproline content. A second group of rats (n = 69) were killed by CO2 intoxication 3, 7, and 28 days after injury. Achilles tendons were excised and dehydrated for 16–18 h in a Speed Vac concentrator device (Savant Instruments, New York, NY). A ratio [[wet mass − dry mass]/wet mass] was calculated to obtain an index of water content. Dehydrated tendons were hydrolyzed in 6 N HCl at 130°C for 3 h, neutralized with NaOH, and diluted in water. The resulting solution was used to determine the hydroxyproline content (μg/mg of dry tissue), an index of total collagen content, by the method of Woessner (45).

Fig. 1. Immunohistochemical staining of neutrophils (A), ED1+ macrophages (B), ED2+ macrophages (C), and proliferative cells (D) on injured + early exercise (Inj+Ex) Achilles tendon sections at day 3 or 7 following injury. No labeling was observed when primary antibodies were omitted. Resident ED2+ macrophages were more concentrated in the paratenon (p) than in the core of the tendon (c). Dashed line separates the paratenon from the core. Bar = 50 μm.
Mechanical testing. In a third set of experiments, we evaluated the mechanical properties of the Achilles tendons at days 7 and 28 postinjury (n = 65). Briefly, anesthetized rats were killed by neck dislocation, and the tendons were excised with the calcaneum and the inferior portion of the gastrocnemius, soleus, and plantaris muscles. The specimens were immersed in PBS, and the muscle tissues were carefully removed. After the tendon was dabbed dry, the remaining aponeurosis was placed between a folded sheet of blotting paper and clamped in metal jaws covered with sandpaper (24, 35). The jaws were connected to the load cell of an 858 Mini Bionix II device (MTS Systems, Eden Prairie, MN). The calcaneum was placed in a conelike slot of ultra-high-molecular-weight polyethylene fixed on other metal jaws. The opening of the slot was closed by a custom-made metal part to retain the calcaneum in the slot. The initial length was manually set at a tension between 1 and 2 N. Using a TestStar II control system (version 3.2D), we obtained a tension-elongation curve at a strain rate of 10% of initial length per second until complete rupture. Tension (N) and elongation (mm) were monitored at a frequency of 10 Hz. Force at rupture point (FRP) and ultimate stiffness (US) were recorded. US was defined as the maximal slope of the linear portion of the tension-elongation curve with a time lapse of 1 s.

Statistical analysis. Statistical analyses were performed with StatView software (SAS Institute, Cary, NC). Differences between sham and Ctr tendons were detected using an unpaired t-test. The significance of other differences was evaluated with a one-way analysis of variance followed by a Fisher’s paired least significant difference test when a significant F ratio was obtained. All results are presented as means ± SE. The level of significance was set at P < 0.05 for all data collected.
RESULTS

Concentration of inflammatory cells. It should be mentioned first that, unless otherwise noted, exercise did not induce statistical changes in uninjured tendons. Globally, the sham procedure also did not produce significant effects compared with the Ctr group. Thus, for the sake of clarity, we have not included data from the sham-operated tendons in all figures. Inflammatory cell accumulations were analyzed by immuno-histochemistry 3 and 7 days postinjury. Neutrophil accumulation at day 3 was significantly higher in tendons from the Inj and Inj+EEEx rats compared with the Ctr animals (Figs. 1A and 2A). At day 7 postinjury, the concentration of neutrophils in the Inj tendons returned to Ctr values but remained significantly elevated in the Inj+EEEx tendons. The concentration of ED1+ macrophages increased roughly 42- and 82-fold in the Inj and Inj+EEEx groups, respectively, compared with Ctr at day 3 postinjury (Figs. 1B and 2B). The concentrations decreased by day 7 but were still significantly higher than in the Ctr tendons. Interestingly, early exercise caused a significantly greater accumulation of ED1+ macrophages in injured tendons at both time points. Because ED2+ macrophages are endogenously present in tendons and more specifically concentrated in the paratenon, we counted these cells separately in the paratenon and tendon core to avoid a misleading interpretation. No significant variations in ED2+ macrophage density among the groups were seen in the paratenon (Figs. 1C and 2C). Alternatively, a statistically larger accumulation of ED2+ cells was observed in the core of the Inj+EEEx tendons relative to the Inj tendons at day 3 (Figs. 1C and 2D). The same tendency was observed at day 7 postinjury (P = 0.09). Concentration of ED2+ macrophages was also significantly lower in Inj tendons 3 days after injury relative to Ctr group.

Edema, hydroxyproline content, and cell proliferation. Intratendinous water content was significantly higher in the Inj and Inj+EEEx tendons relative to the Ctr at days 3 and 7 (Fig. 3A). After 28 days, the edema persisted in tendons from the Inj+EEEx rats. As expected, the collagenase injection markedly reduced the collagen content in the Inj and Inj+EEEx tendons compared with the Ctr group at days 3 and 7 (Fig. 3B). Hydroxyproline content decreased by 37 and 28% in the Inj and Inj+EEEx tendons, respectively, at day 3 postinjury. A significant decrease was still observed after 28 days in the Inj tendons, whereas the Inj+EEEx ones had returned to Ctr values. More importantly, collagen content was significantly different between both injured groups at this time point. However, the
increase in collagen content was not associated with a higher rate of cell proliferation because PCNA immunolabeling performed during the proliferative phase (day 7) demonstrated a 6.2- and 7-fold increase of proliferative cells in the Inj and Inj+EEx groups, respectively, compared with Ctr tendons (Figs. 1D and 4).

**Mechanical Properties and Exercise Protocol.** Measurements of mechanical properties were taken to further characterize the effect of exercise on tendon repair. FRP values for Inj and Inj+EEx tendons represented only 46 and 50%, respectively, those of Ctr at day 7 postinjury (Fig. 5A). Similarly, US was significantly lower in Inj and Inj+EEx tendons relative to Ctr group (Fig. 5B). Furthermore, early voluntary exercise did not strengthen tendon structure at longer term (Fig. 5, A and B). Tendons from the Inj and Inj+EEx rats still had lower values at day 28 postinjury compared with Ctr. It is important to mention that FRP ($P = 0.06$) and US ($P = 0.09$) had a strong tendency to be higher in Inj tendons than in Inj+EEx tendons. Interestingly, Inj+LEX tendons had a significantly higher FRP and US than Inj+EEx group at day 28 but were not different from Inj tendons. Moreover, we observed that Ctr rats weighed more than any animals from other groups at 7 and 28 days (data not shown). This observation may explain in part why Ctr+EEx group had inferior mechanical strength than Ctr tendons at day 28.

Last, voluntary exercise was tied to the circadian rhythm because most physical activity was recorded during the dark period. We observed, as expected, that injured rats ran significantly less than Ctr+EEx rats in the first week postinjury (Table 1). Nevertheless, Inj+EEx values were similar to Ctr during the last 3 wk of the protocol, reaching roughly 118 km after 28 days. The Inj+LEX rats also ran significantly less than Ctr+EEx rats during the first week of exercise. We believe that the low level of physical activity performed initially is due not only to pain but also to adaptation to a new environment, because all groups displayed a significant progression during the first 3 wk of the exercise protocol.

**DISCUSSION**

Unlike growth factors, cytokines, and hormones, mechanical stress is often neglected as an important regulatory mechanism of the metabolism of connective tissues (10). Mechanical loading, like inflammatory cells, is obviously a double-edged sword for the extracellular matrix. It can, on the one hand, stimulate collagen synthesis and protect the tendon from degradation (10, 30, 38, 40, 47), but, on the other hand, it can promote metalloproteinase release and induce collagen rupture if repetitive strain exceeds the physiological capacity of the tendon (18, 19, 34, 42, 46, 48).

Voluntary exercise and inflammation. After acute tendon injury, an inflammatory reaction usually takes place, characterized by a regulated sequence of inflammatory cell accumulation and edema formation (23, 24). Neutrophils are the first cell type to invade the site of injury followed by phagocytic ED1+ macrophages. ED2+ macrophages then accumulate, which are generally considered as resident macrophages that do not participate in phagocytosis but preferentially release

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Values are means ± SE given in km; $n = 11$; 12; and 6 for the noninjured + early exercise, (Ctr + EEx), injured + early exercise (Inj + EEx), and injured + late exercise (Inj + LEX) groups, respectively. Distance includes weekly and total distance run by rats killed at day 28. *Significantly different from the Ctr + EEx group, $P < 0.05$. |
factors promoting cell proliferation (26, 27). The findings reported here show that early exercise induced the persistent presence of neutrophils and high concentrations of ED1⁺ and ED2⁺ macrophages. Our results suggest that early voluntary exercise may exacerbate the initial injury, resulting in the release of chemoattractant mediators that could further promote the recruitment of neutrophils and macrophages. The persistent presence of neutrophils may lead to a vicious cycle in which cytokines and proteolytic fragments of extracellular matrix proteins are released locally to amplify leukocyte accumulation and possibly tissue destruction (17, 44). In addition to modulating inflammatory cell accumulation, mechanical stress may influence neutrophil and macrophage activity in injured tendons. These cells are anchored to the surrounding extracellular matrix and can react to chronic or acute deformation due to variations in mechanical stresses through these attachments. For instance, macrophages respond to mechanical deformation by selectively increasing some metalloproteinases and by inducing immediate early response genes (49). Moreover, previous investigations have demonstrated that neutrophil depletion in mice accelerates skin wound closure, whereas macrophage depletion diminishes fibrosis and scarring (9, 25). While early voluntary exercise retards the resolution of inflammation, it still remains unclear to what extent the presence neutrophils and macrophages in injured tendons is beneficial or detrimental.

Effect of voluntary exercise on the recovery of mechanical properties. The present findings also demonstrated that early voluntary exercise promotes collagen deposition. Several previous reports have indicated that mechanical stress can induce cellular and molecular changes in stimulated fibroblasts in vitro. For example, cyclic stretching increases the expression of transforming growth factor β1 (TGF-β1), connective tissue growth factor (CTGF) and angiogenic factor VEGF in fibroblasts in vitro (31, 33, 39, 47). Interestingly, cyclic mechanical strain elevates collagen synthesis, which is linked to a greater production of TGF-β1 in fibroblasts and isolated tendon fascicles (31, 40, 47). Tensile stress can also modify the genetic program of fibroblasts in collagen lattices. The mechanically loaded fibroblasts upregulate their expression of fibrogenic cytokines and structural extracellular matrix molecules while repressing metalloproteinases (10). Last, tendon cells may communicate with one another via cell processes and gap junctions in tendons (28, 37). This intimate relationship between cell processes and the extracellular matrix suggests that there is a coordinated response to mechanical loading in normal and pathological tendons (6).

Our results that the increase in collagen content was roughly associated with a loss in mechanical properties following early voluntary exercise indicate that there is a fine line between the proper amount of exercise needed for adaptation and the excessive stress loading that causes tissue damage. We speculate that the persistence of the inflammatory response and early mechanical loading may lead to fibrosis and loss of tendon function. The differentiation of fibroblasts into myofibroblasts is particularly important in scar formation. Myofibroblast differentiation is a complex process that is regulated at least in part by TGF-β1, an extracellular matrix fragment (the fibronectin domain ED-A), and mechanical loading. To some extent, all of these regulatory elements may be present to promote excessive myofibroblast appearance and fibrosis following early mechanical loading (8, 13). In addition, the administration of decorin, interferon, suramin, or relaxin can reduce fibrosis and promote the recovery of functional strength (4, 11, 12, 32). Alternatively, the observation that a late voluntary exercise protocol provides greater mechanical benefits than early voluntary exercise indicates that the time to begin mechanical loading is of great importance. Previous studies have demonstrated that the mechanical environment is a crucial determinant for the remodeling and repair of connective tissues like bone (3, 5). Because tendons and bones share many characteristics, notably biochemical composition and reliance on mechanical forces, the principles that regulate bone structure may be similar for tendons (20). Cyclic strain applied periodically has been shown to improve healing following fractures (14, 15). Conversely, excessive loading or a prolonged absence of mechanical stimulation can adversely affect recuperation (1). Thus there is possibly a similar window of opportunity in tendons to initiate and promote healing through mechanical loading.

Perspectives and conclusions. In the present study, we attempted to obtain a global picture by analyzing components of the inflammatory, proliferative, and remodeling phases. As opposed to forced exercise, we believe that a voluntary exercise protocol is more physiologically relevant and certainly closer to clinical situations where pain, apprehension, fatigue, and circadian rhythm are part of the equation. Whereas early voluntary exercise accelerated collagen deposition in Achilles tendons, it amplified and prolonged the inflammatory reaction, which was roughly associated with a mechanically weaker tendon 4 wk posttrauma. Conversely, the later voluntary exercise protocol had no negative effects on tendon healing. Further studies are needed to determine whether early exercise stimulates fibrosis and the conversion of fibroblasts into myofibroblasts and to characterize the best mechanical loading protocols to achieve maximal tendon repair.

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