ABSTRACT: Recovery from chronic strain injury (50 strains daily, five times weekly for 6 weeks to hyperactive soleus muscles) was followed for 3 months in female rats after cessation of chronic hyperactivity induced by pretreatment of the plantar flexor muscles with tetanus toxin. After 6 weeks of repeated strains, muscle mass decreased by 62%, myofiber areas were reduced by 87%, and noncontractile tissue expanded dramatically by 222%. Collagen content increased by almost ninefold (control 40 ± 3 μg/mg, chronic injury 392 ± 53 μg/mg), whereas the molar ratio of collagen (pyridinoline) crosslinks to collagen remained the same (control 0.20 ± 0.01, chronic injury 0.16 ± 0.01). After 3 months of ambulation, muscle mass returned to normal but myofiber areas remained smaller by 21%, noncontractile tissue was still markedly elevated by 18% with increased collagen content (107 ± 15 μg/mg), and the molar ratio of crosslinks to collagen increased by 75% during recovery. Thus, rat soleus muscles recovered very slowly and incompletely from chronic strain injuries that produced muscle fibrosis, highlighting the necessity of devising preventative strategies for repeated strain injuries.
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RECOVERY FROM 6 WEEKS OF REPEATED STRAIN INJURY TO RAT SOLEUS MUSCLES

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The combination of repeated strain[21] and overuse[28] has been implicated in the development of many repetitive strain injuries involving skeletal muscles. In human muscles exposed to repetitive loading and muscle strains (eccentric muscle actions) during occupational tasks, repeated cycles of injury and regeneration have been proposed to account for muscle pathology.[21] Although it appears from published micrographs of affected muscles that the extracellular matrix (ECM) is expanded,[20,21] no analysis of the ECM has been reported. An expanded ECM and fibrotic changes around selective muscle fibers could play a role in impaired regeneration leading to clinical symptoms and dysfunction.

When rat muscles were strained repeatedly three times per week for 1 month in healthy rats, myopathic changes were observed.[30] Myofibers were reportedly smaller and nonmyofiber content increased,[30] indicative of fibrotic changes.[29] Other models of chronic muscle injury[6] and chronic myopathy[17] have led to the same result of an expanded ECM accompanying muscle pathology.

Except for studies on myotoxin-injured muscles,[6,27] recovery after chronic muscle injury has not been investigated. In myotoxin-injured muscles, muscle fiber branching and endomysial fibrosis were still present after 1-month recovery[6]; muscle weights and areas remained reduced after 6 months of recovery even though the number of fibers was no different than controls.[6] Thus, recovery of skeletal muscle following chronic injury appears to be slow and incomplete in animals.

We have developed a model of repeated strain

Abbreviations: ANOVA, analysis of variance; ECM, extracellular matrix; EDL, extensor digitorum longus; HPLC, high-performance liquid chromatography; OCT, optimal cutting temperature; one T, 6 weeks of tetany without injury plus 1-month recovery; PBS, phosphate-buffered saline; TA, tibialis anterior; zero T, 6 weeks of tetany without injury

Key words: collagen; fibrosis; high-performance liquid chromatography (HPLC); morphometry; muscle; pyridinoline

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injury in rats to study the outcome of chronic strain injury in hyperactive muscles. Chronic strain injury[12,19] and hyperactivity of muscles[15] have been associated with occupational disorders of overuse. Hyperactivity refers to the continuous activation of skeletal muscles for long periods without rest, but not of the magnitude expected to produce muscle cramps. We used tetanus toxin treatment to induce chronic hyperactivity in rat triceps surae muscles. The present study tested the hypothesis that hyperactive soleus muscles of rats would become fibrotic after chronic strain injury and would not recover fully after 3 months of normal ambulation when the hyperactivity had subsided.

MATERIALS AND METHODS

Female Sprague-Dawley rats (220–250 g, 73 days old) were used. The care and use of the animals in this study was approved by and followed the guidelines of the West Virginia University Animal Care and Use Committee in compliance with the Animal Welfare Act and Department of Health and Human Services guidelines governing laboratory animals. The rats were administered tetanus toxin (60 μg/mL in 0.9% NaCl; Conaught Laboratories, Toronto, ON, Canada) on one occasion by injecting 0.2 mL into both the lateral and medial gastrocnemius of the left hindlimb. After 4 days without further treatment, the hindlimb muscles became hypertonic[8] and were held in a position similar to an extension synergy (i.e., hip extension, knee extension, and ankle plantar flexion), but they were not rigid. Rigidity can be produced by using higher doses of tetanus toxin.[25] Hyperactivity and muscle strain are commonly seen in workers where occupational disorders are common.[28] The rationale for using tetanus toxin was to produce a state of chronic hyperactivity (tetanic spasm) that would later recover spontaneously. Although much more exaggerated than expected in humans, the hyperactive muscles would mimic overactivity seen during occupational tasks.[28] Hyperactive muscles could then be strained repeatedly without using nerve cuffs or needle electrodes to activate the muscles. The spontaneous cessation of the hyperactivity (tetanic spasm) allowed recovery to be followed, as would occur in a setting of rest without specific rehabilitation.

For 6 weeks, five bouts of ten repeated strains were performed daily to the triceps surae muscles of the left hindlimb of ether-anesthetized rats with a 30-s rest between bouts (chronic injured groups). Each strain involved stretching the hyperactive muscles quickly from a position of plantar flexion (140° to about 40°) by manually moving the foot.[30] At the end of 6 weeks, the tetanus had ceased, as evidenced by return to normal ambulation, and the rats continued caged activity without any rehabilitation (recovery groups). Rats treated with tetanus toxin and no strain for 6 weeks (tetanus-toxin controls = zero T) were used to evaluate the effect of the tetanic spasm (hyperactivity). Other age-matched controls were injected with saline with no injury (control). Body weights were recorded three times per week. The animals were followed for 1 month after cessation of the tetanic spasm for the tetanus-toxin–treated control group (tetanus recovery = one T) and for 1, 2 and 3 months following 6 weeks of chronic strain without specific exercise or rehabilitation for the chronic injury group.

At the respective times, groups of animals were weighed and exsanguinated under anesthesia (sodium pentobarbital at 8 mg/100 g body weight), and the soleus muscles removed. After dissection, the muscles were cleaned and weighed, and the midbelly region placed on a cork with optimal cutting temperature (OCT) compound and frozen in isopentane cooled by liquid nitrogen. The samples were stored at −80°C until sectioning. The muscle samples were sectioned at 8 μm and the sections collected on glass slides coated with poly-L-lysine and stored at −80°C until use.

Morphometric Analysis. Slides were stained with Azure A for evaluation of morphology, documentation of pathology, and measurement of the total cross-sectional area of the soleus muscles. For morphometric analysis, the extracellular protein, fibronectin, was used to visualize the noncontractile tissue[23] (Fig. 1) and serve as a boundary marker for myofibers, similar to methods reported previously.[24] Fibronectin was visualized using indirect immunohistochemistry,[23] which resulted in myofiber areas similar to those reported using other boundary markers.[24,30] After a 200-fold dilution was prepared in phosphate-buffered saline (PBS), rabbit anti-rat fibronectin (AB 1942, Chemicon International Inc., Temecula, CA) was applied to the slides, which were incubated for 30 min and then rinsed in PBS. Secondary antibody (rhodamine-labeled donkey anti-rabbit immunoglobulin G; AP 182R, Chemicon) was diluted 100-fold and applied for 30 min. The slides were rinsed in PBS, a drop of glycerin/PBS solution was applied, and a cloverslip placed on the slide. Four muscle samples were used from each group.

Four photographs were taken from each soleus muscle representing a random medial, lateral, dorsal, and ventral area of the muscle. Color photo-

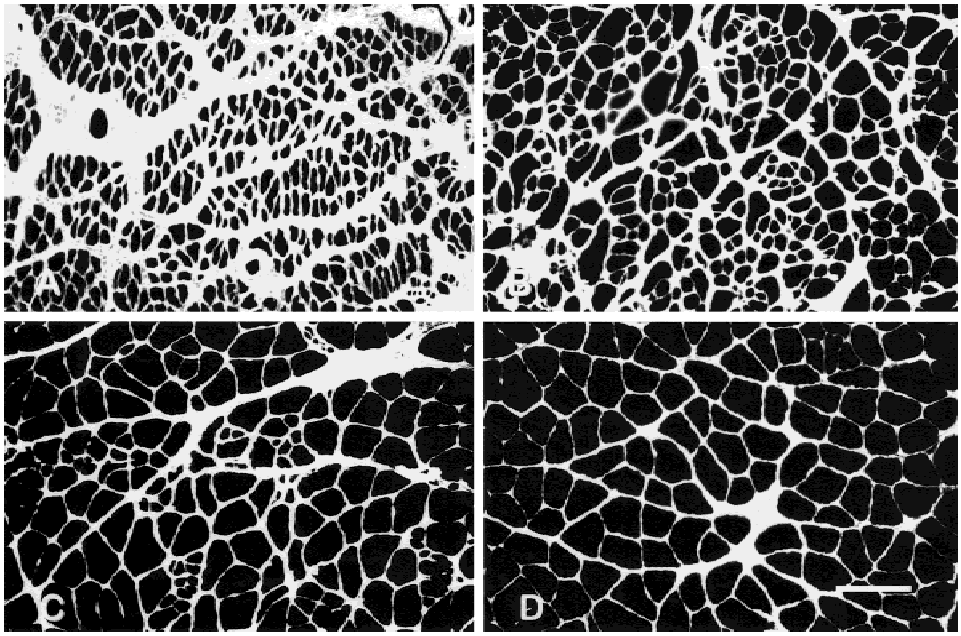

FIGURE 1. Immunohistochemical localization of fibronectin in rat soleus muscles after 6 weeks of chronic strain and recovery. **(A)** Six weeks of strain, no recovery; **(B)** 1 month of recovery; **(C)** 2 months of recovery; and **(D)** 3 months of recovery. Bar = 100 μm.

graphs (35-mm slides, 50×) were produced from a 20× primary magnification.

The 35-mm slides for each soleus muscle were scanned using a Nikon LS-1000 35-mm film scanner into PHOTOSHOP 5.0 (Adobe Systems Inc., San Jose, CA) by an individual who had no knowledge of the experiments and performs only image analysis. The images were measured using OPTIMAS 6.2 image-analysis software (Media Cybernetics, Silver Spring, MD) for myofiber areas and percent noncontractile content using a custom calibration sample. Because blood vessel proliferation is common in the injured muscle, attempts were made to exclude arteries and veins from the measurement of noncontractile content. This exclusion reduced the noncontractile material in some samples. For calculation of the relative frequency distribution of fiber areas, all areas below 50 μm^2 were excluded from analysis. For statistical analysis, the mean value for each 35-mm slide myofiber area or percent noncontractile material was used ($n = 16$).

High-Performance Liquid Chromatography Analysis. Analysis of hydroxyproline (collagen) and pyridinoline (collagen crosslinks) was performed on the same biopsy samples used for morphometric analysis.[23] Frozen sections (250 μm) were cut from the soleus muscles adjacent to those used for morphometric analysis. After removal of OCT compound by rinsing, the samples were dried, weighed, and hydrolyzed (6N HCl). A portion of the hydrolysate was analyzed for hydroxyproline employing reverse-phase high-performance liquid chromatography (HPLC) and fluorometric detection with rat collagen type I (C-8897, Sigma Chemical Co., St. Louis, MO) as the standard.[23] The content of collagen is expressed as collagen units (micrograms per milligram dry weight) as there were no differences in hydroxyproline content between type I, type II, type III, and type IV collagens (95.4 μg hydroxyproline per milligram collagen or about 10% of the weight of collagen is hydroxyproline). From the remainder of the hydrolyzate, nonreducible pyridinoline crosslinks of collagen were separated by partition chromatography (CF1 cellulose chromatography; recovery 87%) and analyzed by reverse-phase HPLC.[9] Both absolute amounts of collagen and crosslinks were measured to calculate the molar ratios of pyridinoline to collagen using 300,000 Da for collagen and 429 Da for pyridinoline.[9]

Data Analysis. The data were entered into an analysis program (PRISM 2, GraphPad Software, Inc., San Diego, CA) in which means, standard deviations, standard errors, one-way analyses of variance (ANOVAs), Dunnett's posttest differences, and frequency distributions were performed. Differences were accepted as significant at $P < 0.05$. Because the variance for the "zero" samples (zero group = 6 weeks of chronic strain and no recovery) was extremely large for the percent noncontractile material and collagen content, $\log_{10}$ was computed for all

data sets. The calculated values were then used for statistical analyses (Table 1).

Because the fiber areas were bimodal for some of the samples, two populations of normally distributed fibers were assumed to exist. The small-fiber population (A) was differentiated from the larger fiber population (B) by estimating a best-fit curve to the left face of the histogram for population B and calculating the difference between the derived curve and the experimental data to construct the right face of the population A histogram. Two data sets were produced and analyzed from the histograms of the experimental data.[30]

Table 2. Calculated fiber areas for soleus muscles.

Experimental group	Population A fiber areas (μm^2)	Population B fiber areas (μm^2)	Percent Population B fibers
Control	—	3184	>99.8
Zero recovery	399	—	0
1 m recovery	315	1712	31.3
2 m recovery	289	1887	84.5
3 m recovery	256	2545	95.0
Zero tetanus	—	3485	>98.5
One tetanus	—	3100	>99.1

Animal groups are: control; zero, *6 weeks of strain;* 1 m, 2 m, *or* 3 m recovery, *6 weeks of strain plus 1, 2, or 3 months of recovery;* zero tetanus, *6 weeks of hyperactivity (tetanus-treated) without injury;* one tetanus, *6 weeks of hyperactivity (tetanus-treated) without injury plus 1 month of recovery.*

RESULTS

The effects of chronic strain to tetanus-toxin–treated rats were compared to tetanus-toxin–treated rats without strain injury and to saline-injected control rats without injury (Tables 1 and 2). After 6 weeks of repeated strains, the animals did not lose weight but the soleus muscles were much smaller on gross examination with evidence of intermuscular adhesions. There was a significant decrease of 62% in the mass of the soleus muscles compared with the saline-treated controls or the tetanus-toxin–treated controls (Table 1). The muscle mass recovered after 3 months.

Morphometric Analysis. The decrease in muscle mass could be accounted for by the marked reduction in myofiber area of 87% (Table 1) in spite of an increase in noncontractile material (Fig. 1). Myofiber area, although increasing throughout the 3-month recovery period (Table 1), never fully recovered (reduction by 21%; Fig. 2). The muscle mass and myofiber areas (Table 1) of the tetanus-treated (uninjured) control rats were not different after 6 weeks or after 1-month recovery.

At the end of the period of chronic injury, the muscle fibers formed a single population of very small fibers (Fig. 2A, Table 2); a bimodal distribution of small and large fibers was observed during recovery (Fig. 2B–D, Table 2). The small-fiber population (population A) diminished with time and the large-fiber population (population B) increased with respect to the relative number of fibers and fiber area during recovery (Table 2).

Proliferation of noncontractile material was seen in fibronectin-stained slides (Fig. 1). After 6 weeks of chronic strain injury, the percent noncontractile content was markedly elevated by 222% (Table 1) and, although diminishing with time, remained elevated by 18% even after 3 months of recovery (Fig. 2D, Table 1). There was no difference in percent noncontractile content in either of the tetanus-toxin–treated control groups (Table 1).

Following chronic strain injury, alterations in myofiber size and increases in noncontractile content remained for 3 months, whereas muscle mass returned to control levels. In contrast, the tetanus-toxin–treated (uninjured) controls did demonstrate some pathological changes with a small, but insig-

Table 1. Measured variables in control and strained injured rats.

	Control (n = 6)	Zero (n = 5)	One (n = 6)	Two (n = 6)	Three (n = 6)	Zero T (n = 4)	One T (n = 5)
Body mass (g)	268 ± 9	254 ± 6	266 ± 4	281 ± 7	291 ± 10	254 ± 10	267 ± 4
Soleus mass (mg)	129 ± 9	49* ± 6	72* ± 4	103 ± 7	101 ± 6	107 ± 15	116 ± 11
Myofiber area (μm^2)	3170 ± 126	410* ± 26	1060* ± 145	1630* ± 167	2520* ± 117	3550 ± 118	3200 ± 140
Percent noncontractile content	15.7 ± 0.4	50.5* ± 2.0	29.1* ± 1.6	23.0* ± 1.2	18.6* ± 0.7	16.3 ± 0.5	15.8 ± 0.3
Collagen (μg/mg dry wt.)	40 ± 3	392* ± 53	164* ± 13	115* ± 22	107* ± 15	56 ± 24	61 ± 26
Molar ratio	0.20 ± 0.01	0.16 ± 0.04	0.29* ± 0.02	0.35* ± 0.02	0.35* ± 0.02	0.17 ± 0.02	0.21 ± 0.03

Values are means ± SEM; n *represents number of animals.*
Animal groups are: control; zero, *6 weeks of strain, no recovery;* one, *6 weeks of strain plus 1 month of recovery;* two, *6 weeks of strain plus 2 months recovery;* three, *6 weeks of strain plus 3 months of recovery;* zero T, *6 weeks of hyperactivity (tetanus-treated) without injury;* one T, *6 weeks of hyperactivity (tetanus-treated) without injury plus 1 month of recovery.*
**Significantly different from controls at* $P < 0.05$.

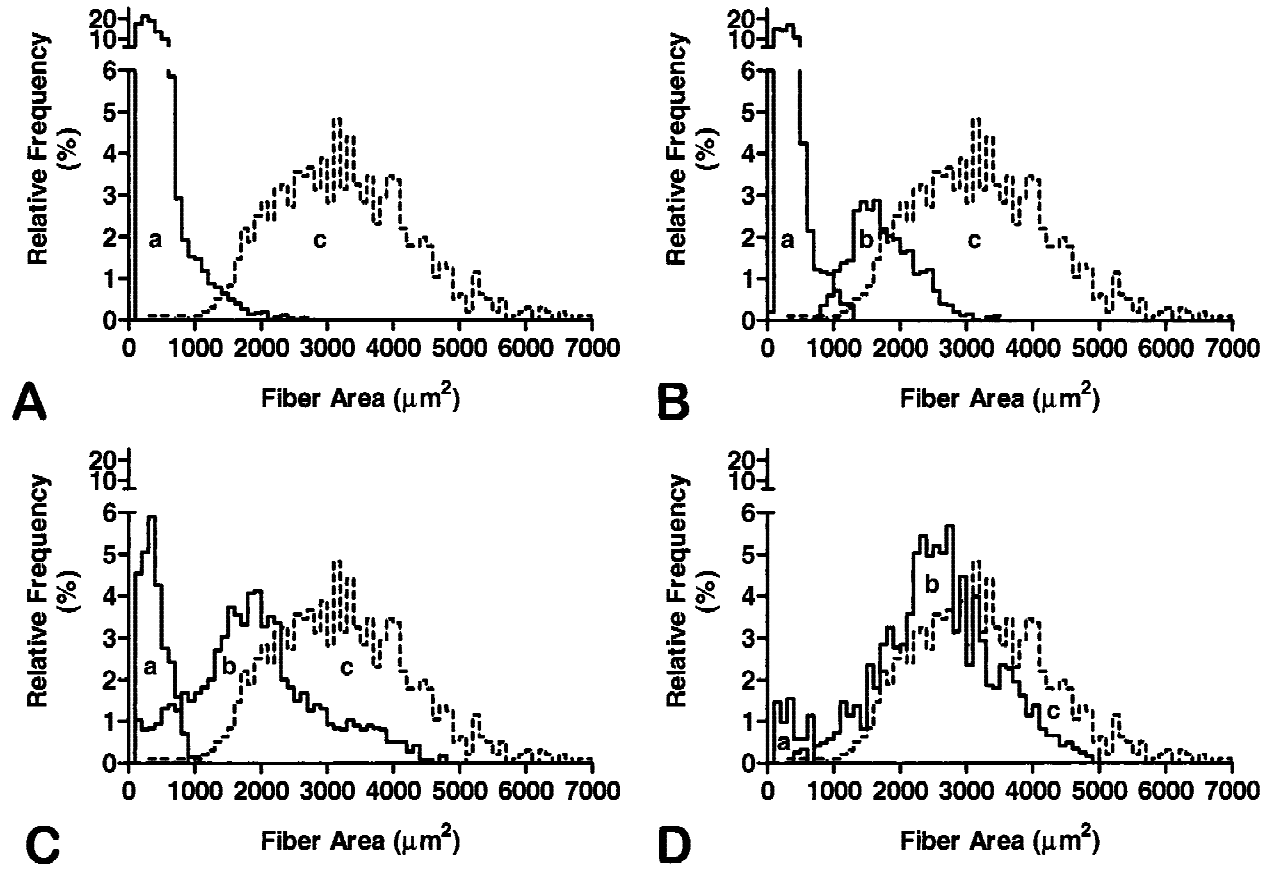

FIGURE 2. Frequency distribution histograms of soleus muscle fibers during recovery after 6 weeks of chronic strain. Solid lines: experimental muscles; dashed lines: control muscle, no injury. **(a)** Population of small fibers; **(b)** population of atrophic fibers; and **(c)** population of control fibers. **(A)** Six weeks of chronic strain, no recovery; **(B)** 6 weeks strain, 1-month recovery. **(C)** 6 weeks of strain, 2-month recovery; and **(D)** 6 weeks of strain, 3-month recovery.

nificant, number of small fibers and occasional central nuclei. The tetanus-toxin–treated rats recovered completely after 1 month (Table 1).

To estimate the number of myofibers in the soleus muscles, the total cross-sectional area of the muscle, the fractional content of total myofiber area, and the average myofiber area were used. The number of apparent myofibers estimated increased significantly by 33% from 2377 ± 277 to 3152 ± 222 after 6 weeks of chronic strain, but returned to control values of 2416 ± 357 following 3 months of recovery.

HPLC Analysis. Proliferation of noncontractile material was confirmed by HPLC determination for collagen and collagen (pyridinoline) crosslinks.[22] Collagen increased by 880% at the end of the period of chronic injury, and decreased to 167% after normal ambulation for 3 months. The molar ratio of crosslinks to collagen was unchanged after the period of chronic injury, but increased during the recovery period to level off after 2 months (75% increase; Table 1).

DISCUSSION

Skeletal muscle pathology that accompanies repetitive strain injuries in humans has not been investigated in detail due to the necessity to perform muscle biopsies. From the limited studies available, evidence of muscle necrosis (degeneration) and regeneration have been reported for a wrist extensor muscle in patients with lateral epicondylitis[21] and the upper trapezius muscle of forest machine workers with myalgia.[16] With the exception of some hypertrophy in type I fibers, many of the same pathological changes reported for human muscles can be seen in rat soleus muscles subjected to repeated strain injuries or overexertion.[31]

Little is known about the response of the ECM of human muscles to repeated strains. The published reports suggest that the ECM is expanded in painful muscles from humans,[20,21] but no morphometric analysis of ECM has been reported. An expanded ECM and fibrotic changes around selective muscle fibers could induce mechanical distortion of the interstitial space where the pain receptors are located,

resulting in painful movements.[28] Increased connective tissue could also play a role in impaired regeneration leading to delayed recovery.

Because chronic strain injury[30] and repeated toxin-induced muscle injury[6] produce pathology and alterations in connective tissue similar to those seen in some muscles from occupational disorders, studies can be designed to evaluate whether muscle fibrosis and muscle pathology are reversible when the injury stimulus is removed. Skeletal muscle has the remarkable ability to regenerate completely following a single injury,[1] including after complete disruption of myofibers following treatment with toxins[4,14] or transplantation.[7] This process is usually complete within 21 days in the rat if the basal lamina remains intact,[22] but may take longer if blood vessels are disrupted.[7] In our model of repeated strain injury, the blood supply is not disrupted and bleeding never occurs; complete recovery (i.e., return to control morphology) from a single episode of strain injury can occur within 14 days.

In contrast, following chronic toxin-induced muscle injury, recovery appeared slow and incomplete. Following repeated muscle injury produced by injections of bupivacaine into rat extensor digitorum longus (EDL)[6] and tibialis anterior (TA) muscles,[27] extensive fiber branching, increased internal nuclei, endomysial fibrosis, and much variation in myofiber size have been reported, similar to our findings. Bradley[6] allowed rats to recover for 6 months after repeated injury and found that EDL muscle weights and muscle areas remained less than control values.

Similar morphological changes were seen in chronically strain-injured[30] or overloaded[31] rat soleus muscles. In soleus muscles that were strained three times per week for 1 month, two populations of fibers were observed,[30] similar to what was seen in the 1-month recovery group in the present study (Fig. 2B). Only one population of small fibers was found at the end of our injury protocol with daily repeated strains for 6 weeks (Fig. 2A). Because both the number of injury days per week and number of weeks of injury were greater in our study, the shift to small fibers would appear to be more complete. We found that the number of fibers increased by 33% after 6 weeks of chronic strain injury and returned to control levels by 3 months of recovery, perhaps due to the branching of existing fibers into small fibers[6] and subsequent fusion of small, branched fibers to form normal-sized myofibers. For fusion of adjacent small fibers to occur, the connective tissue between fibers has to be removed; this is a slow process because collagen turnover is slow.

Slow return to normal fiber size and connective tissue content has also been reported for muscles of rats immobilized for 3 weeks at relaxed (i.e., short) muscle lengths and allowed to recover for 3 months.[18] The soleus muscles initially lost about 30% of their fiber cross-sectional areas and did not return to control values following caged activity. Connective tissue increased by about fivefold due to immobilization and remained elevated after recovery.[18] Interestingly, if high-intensity treadmill running for 8 weeks was imposed, many of the changes approached control levels.[18]

During the repair of injured muscles, two processes are competing: the regeneration of disrupted muscle and the production of connective tissue scar. Collagen participates in muscle regeneration by forming a sheath around fusing myoblasts during myotube formation.[3] When diseases such as systemic sclerosis[5] or repeated trauma[6] produce excessive scar tissue in muscle, a dense mechanical barrier to regenerating muscle fibers may be produced.[2] Thus, the removal of excessive collagen produced during the early stages of healing seems essential for optimal tissue repair.

Connective tissue proliferation following chronic injury and recovery, estimated using morphometric analysis of the noncontractile components, was verified by chemical (HPLC) analysis of collagen (hydroxyproline) and the evaluation of collagen crosslinks (pyridinoline). After 6 weeks of chronic strain injury, collagen concentrations increased almost ninefold. Although collagen concentrations decreased with recovery time, the molar ratio of crosslinks to collagen actually increased during recovery. This ratio reached a peak at 2 months of recovery and remained elevated even at 3 months.

Increased collagen crosslinks have been reported during development (birth to maturity),[26] with aging[13] and with avian muscular dystrophy.[11] The crosslinking of adjacent collagen fibrils would increase both the tensile strength of collagen and muscle stiffness. The formation of collagen crosslinks would also result in the resistance of collagen to degradation by proteases,[32] further reducing the ability of the ECM to remodel. Thus, the slow removal of collagen during 3 months of recovery in our study can be attributed in part to the increased molar quantity of crosslinks, which, similar to avian dystrophic muscle,[10] would be resistant to proteolytic attack.

The similar slow recovery process seen after chronic injury[6] and long-term immobilization at short muscle lengths[18] is significant to therapists and rehabilitation specialists, who have often noted that muscles injured and held in shortened positions

later take months of rehabilitation before complete restoration of function could be accomplished, if at all. Further work is needed to design optimal interventions and prevention procedures for muscle fibrosis due to chronic muscle injury or myopathic disease.

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