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Cumulative Trauma Disorder: Skeletal Muscle Dysfunction

July 18, 2005

Grant Number: 5 R01 OH02918-10

6/1/2001 to 5/31/2005

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List of Abbreviations

b.w. = body weight

C = control

CTD = cumulative trauma disorder

deg = degrees

E-C = excitation-contraction

ELISA = enzyme-linked immunoassay

F = females

HPLC = high pressure liquid chromatography

Hz = Hertz

i.p. = intraperitoneal

M = males

mRNA = messenger RNA

ms = milliseconds

TGF-β1 = transforming growth factor beta 1

TGF-β2 = transforming growth factor beta 2

1. Final Performance Report:

A. Abstract:

Chronic pain originating from the musculoskeletal system is a dominant cause of sick leave in modern industry and often a very disabling and troublesome condition for the individual. Although the cause of this problem in skeletal muscle is unknown, one of the most frequent situations in which muscle pain is experienced is in industrial workers who have to move repeatedly and/or forcibly. The cumulative trauma disorder (CTD) which results from repetitive movements is of special interest because these repeat-motion injuries are one of the most difficult to anticipate and prevent.

Our studies in humans have shown that exposure to a single bout of repeated strains at can lead to myofiber and fascial rupture without bleeding but accompanied by muscle pain, restricted motion, and loss of strength and power. Little is known about the effect of repeated strains on muscles or why inflammation results from some but not all muscle strains. Since variations in human exposure and response, together with the necessity for repeated tissue sampling, make man unsuitable as a research subject, we have developed a rat model of repeated strain injury.

The remarkable similarity of our injured rat muscles and the extensor carpi radialis brevis taken from humans with long standing lateral epicondylitis requiring surgery [22] provides support for the rat as a good research model and muscles as important tissues in the development of pain and dysfunction. Since the extensor carpi radialis brevis is also very susceptible to strain injury [21], we believe that more studies using our chronic strain injury protocol in rats will reveal why repeated strain injury results in pain and not adaptation. Using our rat model of repeated muscle strains, the present study was designed: 1) to determine if muscle fatigue, in addition to mechanical damage from repeated muscle strains, results in increased myofiber pathology and inflammation, 2) to understand the mechanisms of increased collagen deposition (fibrosis) and pathology in skeletal muscles resulting from chronic strain injury and 3) to develop blood biomarkers for the assessment of muscle inflammation and pathology. This research consisted of experiments in which muscles were acutely or chronically injured by mechanical overloading (stretching) in deeply anesthetized rats. After removal from the animals, skeletal muscles were surveyed at various time intervals following injury by biochemical, immunohistochemical and histological techniques for specific cellular markers, components and mediators involved in tissue injury, inflammation and repair. The functional outcome of repeated injury was assessed by in vivo dynamometry of muscle performance (e.g. muscle strength).

From our studies, the mechanisms for muscle weakness (isometric force deficits) and histopathologic changes (inflammation) following repeated muscle strains can largely be dissociated from each other explaining why there is no correlation between weakness and inflammation. Apparently, injury leading to calcium accumulation that exceeds the buffering capacity of the cell is the most critical component in producing pathologic changes and inflammation in muscles. This calcium accumulation is a time-dependent process and long rest times attenuates the calcium accumulation. The individual variation in outcomes to repeated strains seen in humans probably results from differences in calcium buffering capacity or bioenergetics. Finally, functional testing in humans exposed to repeated muscle strains will not allow the assessment of the degree of muscle pathology or inflammation.

B. Significant Findings:

It is the focus of this work to provide sufficient understanding of the mechanisms and etiology of the development of muscle dysfunction and pain from repeated strain injury to establish early non-invasive monitoring, effective preventative programs and/or work-site modifications. The remarkable similarity of our injured rat muscles and the extensor carpi radialis brevis taken from humans with long standing lateral epicondylitis requiring surgery [22] provides support for the rat as a good research model and muscles as important tissues in the development of pain and dysfunction.

We have demonstrated that long rest times between strains are important in preventing histopathology in rat muscles. We initially believed that fatigue was a component in producing muscle histopathology that subsequently led to pain and dysfunction. However, careful analysis of the mechanism of histopathology has revealed evidence to support a time-dependent calcium overload, apparently influenced only slightly by cellular energetics, and is a novel finding of this research. This complex interaction of rest and cellular injury, verified in our rat models, could explain why, in groups of people doing identical work, some individuals do not report any musculoskeletal problems. Thus, it may not be individual variations in the biomechanics of the task that lead to dysfunction (CTD) but inherent differences in calcium handling perhaps due to divergences in energy balance [5].

In support of a role for individual variation in the production of histopathology, we examined another strain of rats, the Wistar Kyoto (WKY) rat. Histopathology was evident 48 hrs after a protocol of 30 stretches with 60 seconds or rest between stretches (n=4) which did not produce pathology in the same age Sprague Dawley rats (n=3). The use of protocols to produce muscle injury with and without histopathology should allow the assessment of biomarkers and the addition of different strain rats should enhance our understanding of the mechanisms of pathology and reveal methods for prevention.

When histopathology was present in strain-injured skeletal muscles, pain could also be demonstrated. Using a novel pressure-pain threshold analysis, we were able to document muscle pain following repeated strain injury. It would appear that muscle pain is a function of the number of injured muscle cells and not the decrease in isometric force (weakness) and the number of injured myofibers is a function of repetition number. From our studies, the mechanisms for muscle weakness (isometric force deficits) and histopathologic changes (inflammation) following repeated muscle strains can largely be dissociated from each other explaining why there is no correlation between weakness and inflammation. Therefore, functional testing in humans exposed to repeated muscle strains will not allow the assessment of the degree of muscle pathology or inflammation that precedes muscle pain.

Because inflammation could be prevented by increased rest times, pain and dysfunction should also be prevented in humans by alterations in work-rest times and other ergonomic interventions. Development of biomarkers for muscle specific inflammation would allow non-invasive monitoring of workers before muscle fibrosis became a problem and severe disability developed.

C. Translation of Findings:

Peak Stretch Force: Large strain forces produce the greatest force deficits and pathology so that reductions in loads during movements that can produce muscle strains seems appropriate.

Strength Testing: Isometric force deficits (weakness) appearing in standard strength tests of humans would not be indicative of strain injuries that would become pathologic leading to pain and dysfunction.

Dose: The number of repeated strains appears to correlate with the magnitude of inflammatory response even though a fatigue limit appears to result.

Risk Factors: Systemic diseases such as hypertension appear to increase susceptibility to adverse outcomes from repeated muscle strains.

Significance of Rest Times: Apparently inflammation can be prevented from repeated strains by appropriate rest periods even though loss of strength results.

Individual Variation: The innate ability to recover during rest appears to alter the outcome from muscle strain injuries. So defining susceptible individuals would allow job placements that would decrease the incidence of lost work and reduce potential negative outcomes from repeated strain injuries. If these inherent differences in

fatigability are not immutable, specific training protocols could be implemented to prevent some chronic strain injuries in some individuals.

D. Outcomes/Relevance/Impact

The number of injured myofibers correlates with the amount of inflammation but not the loss in muscle strength; the amount of inflammation should correlate with the pain experience in humans.

Strategies and interventions to prevent irreversible cell death such as rest times and identification of individuals at risk can be developed based on these findings.

E. Scientific Report:

This section will cover the period from 6/1/2001 to 5/31/2005. A description of the experimental results for each specific aim of the original proposal will be given below. Although we were on track, we have had some set-backs due to an electrical fire in our building from a transformer over-load requiring the building to operate on reduced power for some time and the loss of post-docs and the senior collaborator, Dr. Mark Willems. In April 2003, Dr. Willems, who had been working on this project since May 1995, left for a faculty position in England so we were short-handed and have had no additional funding to continue.

Specific Aim 1: The hypothesis was that muscle fatigue, in addition to mechanical damage from repeated muscle strains, results in myofiber pathology.

The relationship between fatigue and weakness. The relationship between fatigue and weakness is complicated. Since the force deficit (weakness) following repeated strains is dependent on the magnitude of the peak stretch force, reduced peak stretch force from fatigue would actually lessen the amount of damage. We have performed a few fatigue experiments using rest intervals of 10 seconds or less between the stretches that greatly reduced the histopathologic response. So fatigue might actually protect the muscle from further injury. However, if fatigue in humans was caused by a change in recruitment of more susceptible myofibers, then fatigue could exacerbate the problem. Likewise, if awkward postures are used to compensate for fatigue, muscle damage can result. We used tetanus toxin treatment to produce an awkward posture by causing the rat to walk with one hind-limb continually stretched out behind (3 legged walk) that resulted in histopathologic changes in the soleus muscle. Thus, submaximal loading resulting from awkward postures could result in muscle pathology [36].

Fatigue is often caused by decreased rest times between contractions so that rest time might be a critical intrinsic factor for muscle injury and pathology. In healthy female rats, histopathology and fibrogenic cytokine production occurred from repeated strains (RSI) in protocols with short inter-strain rest times (40 s) but not in protocols with long inter-strain rest times (180 s). This difference appears to be related to the time required to maintain calcium homeostasis during the strains but perhaps also related to bioenergetic capacity and connective tissue deformation. For example, long rest intervals during cyclic loading of human tendons resulted in significantly less strain accumulation [15].

1. Repeated strains with short rest times produced inflammation and pathology.

During occupational tasks, rest intervals are not set by the worker but by the assembly line or the work schedule but rest appears to be an important preventative measure. Since people differ in their ability to recover following even a short bout of activity [34], rest times need to be explored further especially in regard to muscle tissue during cyclic loading. Thirty repeated strains were performed with inter-strain rest times of 40 sec that resulted in force deficits of about 50% (Figure 1, S40) on activated plantar flexor muscles of female rats (n=12). Two days after the protocol, histopathology was clearly evident in these "short-rested" muscles (Figure 4)[37].

Histopathologic changes were identified and quantified by fiber counting. Using desmin and laminin double-labeled samples, histopathology was observed only in samples from the stretch protocol with short inter-strain rest times. Many desmin negative but laminin positive myofibers could be identified [37]. These

same fibers were positive for albumin and complement C3 but devoid of dystrophin staining indicating a lo plasma membrane integrity and entry of extracellular proteins. None of the samples from the stretch protc with the long inter-strain rest times or the isometric contraction protocol contained desmin negative fibers | In the pathologic myofibers, the loss of desmin and dystrophin was not accompanied by a loss of actin anc myosin. This remaining cellular material would be subsequently degraded by infiltrating W3/13 and ED1 positive cells (inflammatory cells) seen both surrounding the periphery and invading the pathologic myofib 48 hrs after injury consistent with a role in phagocytosis of cellular debris by mononuclear cells also seen i inflammatory myopathies [19]. Since phagocytes are known to release lysosomal hydrolases during phagocytosis, muscle proteins (e.g. myosin) could be degraded into fragments that could subsequently cr the capillary to enter the blood. The arrival of these inflammatory cells (Figures 4) was also accompanied muscle pain (Figures 11 & 12).

2. . Repeated strains with long test times prevented inflammation and pathology.

"Long-rested" muscles did not show evidence of histopathologic changes (Figure 3) in spite of a lo strength of more than 40% of the initial value (Figure 1, S180)[37]. Thirty repeated strains were performe inter-strain rest times of 3 min between strains (Figure 1). The force deficits following the protocol did not recover after one-hour of rest (Figure 2), a time normally sufficient for complete recovery from repeated isometric contraction protocols (i.e. non-damaging)(Figure 2, ISO40). Our system appears ideally suited t define the threshold rest time required for prevention of inflammation in rats and when using different strai rats will reveal intrinsic differences due to age, sex and strain. A single experiment could then be designe test applicability to humans and begin the design of work-place guidelines (Human experiments were not planned for this project).

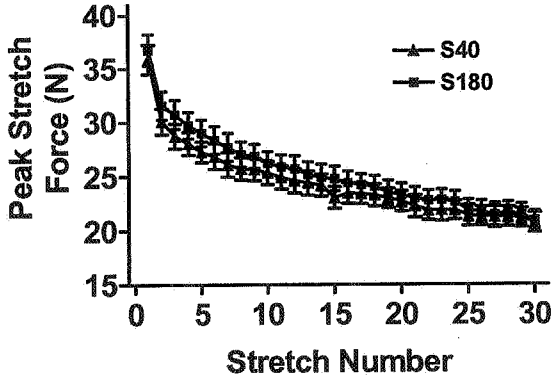


Figure 1. Force decline with stretch number.

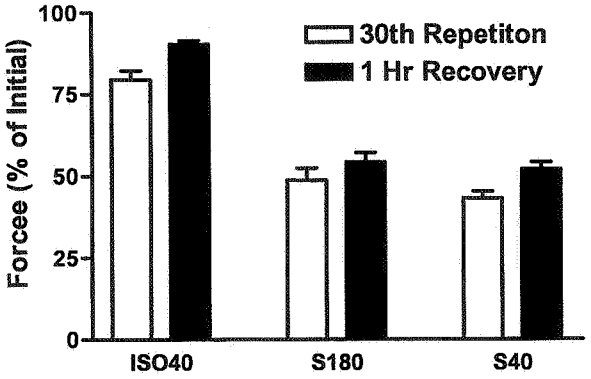


Figure 2. Force Recovery after 1 hr Rest.

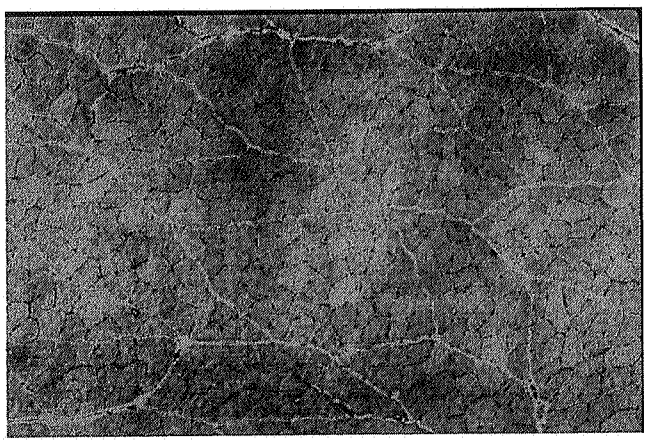


Figure 3. Gastrocnemius long-rest (S180)

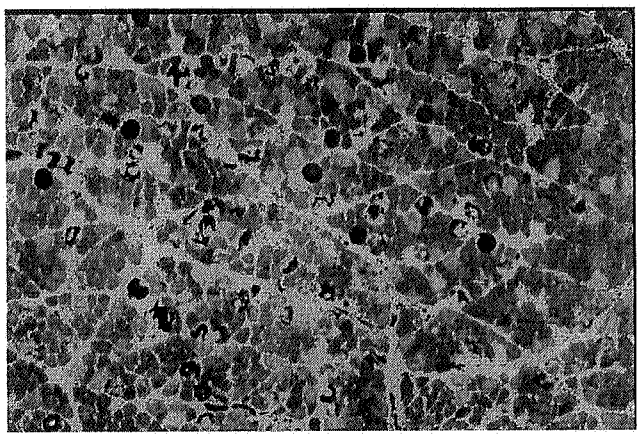


Figure 4. Gastrocnemius short-rest (S40)

3. Repeated isometric contractions with short inter-contraction rest times did not produce pathology. Histopathologic changes have been reported in the upper trapezius muscles of female cleaners reporting muscle pain but not in those performing the same task without pain [20] suggesting that long periods of sustained isometric contractions may also be detrimental under certain conditions. Repeated isometric contractions (30) with short inter-contraction times of 40 seconds were performed in rats. Even though the isometric force declined during the protocol, no evidence of histopathologic changes was observed [37]. Thus, the mechanisms of cellular injury appear to be different during repeated muscle strains (e.g. stretch-activated calcium entry) and not related solely to a drop in energy stores or decreased pH.

In contrast, repeated isometric contractions (1 per sec) for 3 hours did produce muscle damage in male rat muscles [13;14]. Using a variety of stimulation protocols ranging from 40 Hz (submaximal) to 120 Hz (supramaximal) and different rest times (5-90 secs), we were unable to produce histopathologic changes in female rats even though maximal isometric force was reduced dramatically (Willems & Stauber, unpublished observations). The lack of histopathologic changes from fatiguing isometric contractions (30-150) for over 2.5 hrs reveals that physiological fatigue mechanisms alone play a minor but undetermined role in muscle injury during repeated motions but may be different in male and female rats. However, longer trains of contractions and shorter rest intervals were not tested in female rats.

The relationship between injury and histopathology. While there is a relationship between repetition number and histopathology [16], no correlation exists for loss of strength (the objective measure of injury) and histopathology [41]. Repeated strains result in sarcomere damage [31] and shearing of t-tubules [38] leading to isometric force deficits (weakness). While some membrane rupture does occur, as evidenced by Trypan blue uptake immediately after the injury protocol (~2% of the fibers), most of the calcium required to produce a response that leads to inflammation comes from stretch-activated channels. Histopathology can be attenuated by blocking stretch-activated ion channels or inhibition of phospholipase A2 (a calcium-activated hydrolase) [42]. Furthermore, muscle injury from repeated strains can be present with or without histopathology [37]. These studies illustrate that the mechanisms for muscle weakness and histopathologic changes (inflammation) following repeated muscle strains can be largely dissociated from each other. For this reason, functional measurements are not predictive of outcomes and biomarkers are needed to identify muscle-specific inflammation.

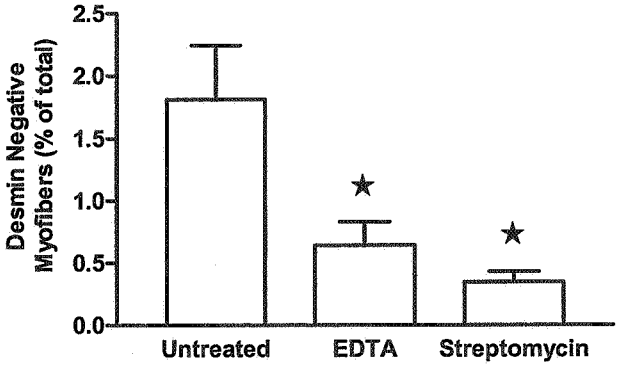


Figure 5. Percent injured myofibers

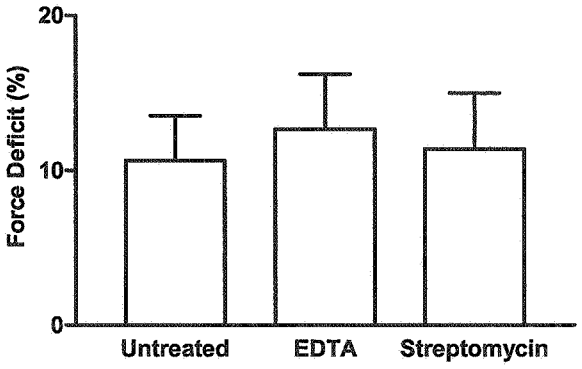


Figure 6. Amount of induced muscle weakness

4. Search for a mechanism for histopathologic changes: Pharmacologic manipulations.

a. Importance of extracellular calcium: Calcium overload of myofibers due to membrane breakdown is generally regarded as the major cause of cell death [40;44]. Using two pharmacologic interventions to reduce calcium influx: 1) removing extracellular calcium by chelation using EDTA [7](n=6); and 2) blocking stretch-activated calcium channels with streptomycin [25](n=6), we were able to attenuate, but not completely prevent, the development of histopathology (Figure 5) from strain protocols with similar amounts of weakness (Figure 6) in rat tibialis anterior muscles. To our knowledge, this is the first study to demonstrate that the mechanisms for

muscle weakness and histopathologic changes (inflammation) following repeated strains can largely be dissociated from each other with EDTA or streptomycin treatment. It also provides a rationale for the observation that the number of pathologic cells continually increases with stretch number but the loss of isometric force appears to reach a fatigue limit [16]. Finally, functional testing in humans exposed to repeated muscle strains would not allow the assessment of the degree of muscle pathology or inflammation.

Presently, we are using specific dyes to identify membrane perturbations in vivo immediately following the injury protocols. A small amount of membrane rupture occurs that increases with time during the subsequent rest period. This mechanical membrane damage, which is different from the processes resulting from calcium entry by stretch-activated channels, probably accounts for the small amount of histopathology remaining in the intervention studies above. For example, the number of Trypan blue (injured) fibers increased with time doubling during the first hour and further increasing by 6 hours following the injury protocol. Thus, further damage or death of muscle cells occurs during the recovery or rest phase following cyclic strain injury by unknown mechanism but probably due to the action of phospholipase A2.

b. Role of phospholipase A2: One possible explanation for the increase in number of injured cells during the recovery period is that enough calcium has entered the muscle cell to activate some calcium-activated hydrolases such as phospholipase A2 which can cause further cell membrane degradation [2]. Quinacrine, a known inhibitor of phospholipase A2, resulted in marked attenuation of histopathology in the S40 rats [42]. The number of desmin negative and albumin positive fibers in quinacrine-treated rats was 88% ($P<0.001$) and 84% ($P<0.005$) lower respectively compared to untreated rats. Taken together, these experiments reveal that the removal of additional calcium entering due to the strain protocol requires a time-dependent process. If calcium overload results from inadequate rest intervals from the demands of the workplace, then activation of phospholipase A2 could lead to further membrane damage with the subsequent production of chemoattractants leading to inflammation and cell necrosis.

c. Role of stretch-activated calcium channels: Because fatiguing protocols without stretch did not produce histopathologic changes, stretch-activated calcium (SAC) channels were implicated. Streptomycin, a known inhibitor SAC channels in muscle [24;26], was administered to rats (n=6) resulting in a significant decrease (>80%) in the number of injured fibers (Figure 5). Based on these results, it may be possible to rescue injured muscle cells before they enter the irreversible path to cell death and necrosis. Knowledge of how calcium enters the cell to produce cell death and how calcium is removed or buffered to prevent histopathologic changes is needed in order to develop preventative strategies and/or recovery techniques.

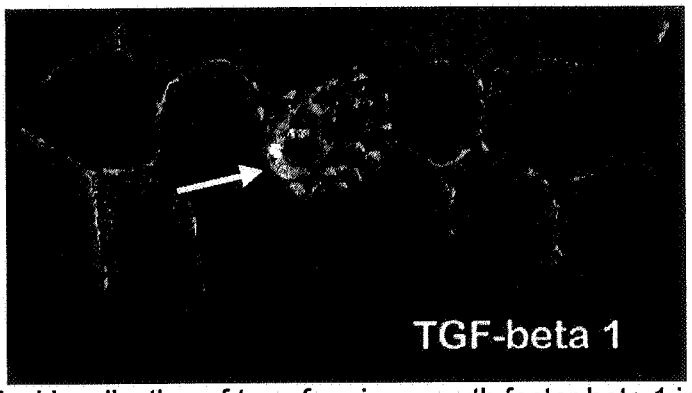


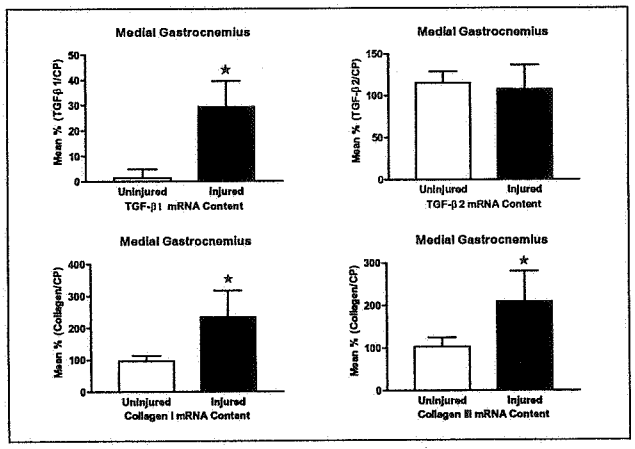
Figure 7. Immunohistochemical localization of transforming growth factor beta 1 in injured rat gastrocnemius muscle 48 hrs after 30 repeated stretches with short inter-stretch rest times. Arrow indicates localization around a necrotic myofiber.

Specific Aim 2: The hypothesis is that the number of pathologic cells produced by the contraction protocols in Specific Aim 1 will correlate with the distribution and quantity of TGF-β1, TGF-β2, and bradykinin.

We have developed a method of counting all the muscle fibers and the injured fibers in the medial gastrocnemius from rats injured by different protocols including the "short rest" protocol. In these injured

muscles, the loss of the intracellular protein, desmin, and the entry of the extracellular protein, albumin, were nearly identical – providing excellent markers for counting the number of pathologic muscle fibers. However, there are about 8,000 muscle fibers in the mid-belly region of the medial gastrocnemius that must be counted individually as the image analysis programs can not handle such large images. Even then, only a small percentage of fibers become pathologic following 30 cyclic strains (~2%) – more with 50 strains (~12%). Thus, if pain is proportional to the number of injured fibers, this method is useful to measure the relationship between inflammation and pain.

1. Collaborations: Collaboration with Dr. Srilanka Nadadur at the EPA, who has developed a custom array for screening over 30 inflammatory mediators, was initially encouraging but because of budget and time constraints he can not continue. However, preliminary studies did reveal that TGF-β and other pro-inflammatory markers were increased in the muscles from short rest interval but not the isometric or long rest interval protocols. These transcript studies have verified the elevation of TGF-β after injury and support our development of assays for TGF-β.



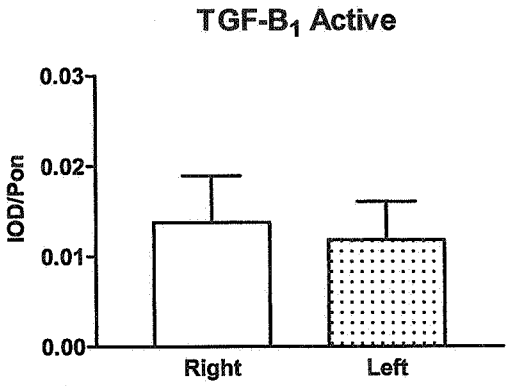


Figure 8. Transcript and protein content from control (right) and strain-injured (left) muscles.

2. TGF-β1 and Collagen: Because of the initial success noted above and the observation that TGF-β1, a fibrogenic cytokine, was localized around pathologic myofibers [32](Figure 7), new methods were developed to investigate the role of TGF-β1 in stimulating collagen synthesis and inhibiting muscle regeneration. As expected, TGF-β1 transcript (Figure 9) and latent TGF-β1 protein (LTGF-β1) content were both increased 48 hrs after injury. However, this increase did not correlate directly with the number of pathologic cells within the muscle. Although collagen I and III transcript content also increased, ~68% and ~79% respectively, there was no increase in active TGF-β1 (Figure 9) but collagen content increased. TGF-β1 may have been rapidly internalized by ligand binding to chimeric TGF-β receptors, thus stimulating collagen synthesis [1]. Additional studies are necessary to determine if specific TGF-β receptor populations (TβR-I, TβR-II, TβR-III) are upregulated and/or activated following muscle injury. Also the mechanisms of LTGF-β activation by proteolysis remain unclear, perhaps limited proteolysis occurs after the initial injury leaving a reservoir of LTGF-β1 in the extracellular matrix bound to betaglycan and/or decorin. Following chronic injury, this reservoir could become fully activated or released leading eventually to fibrosis.

3. Collagen ELISA Development: To document the proliferation of collagen in response to strain injury, we developed our own ELISA system for collagen Type I (Figure 9). We utilized quail muscle homogenates spiked with pure type I collagen to develop the assay system prior to evaluating rat muscles. Quail muscle was used to rule out non-specific binding of antibody to muscle specific proteins. The antibodies to rat collagen type I do not cross-react with avian collagens. We planned also to assay for collagen type III but the standards available were not free from contamination by other collagens. Using this assay system, type I collagen (Figure 10) was elevated in strain injured muscles 48 hours following injury indicating that increases in

collagen I transcripts correlate to increases in collagen I protein. Collagen I is found predominantly in fibrotic muscles and the ELISA assay will allow the assessment of muscle fibrosis following chronic RSI.

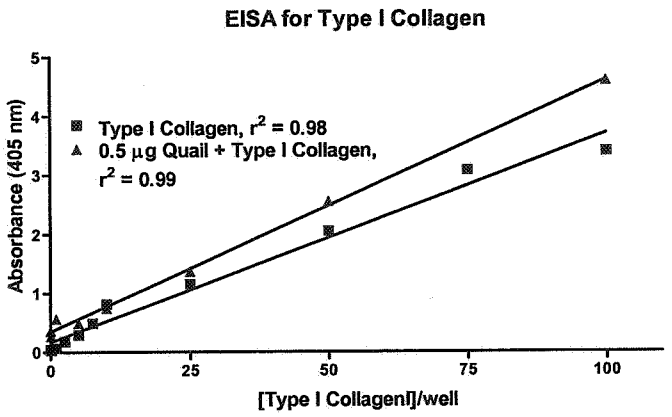


Figure 9. Standard curves for Type I collagen

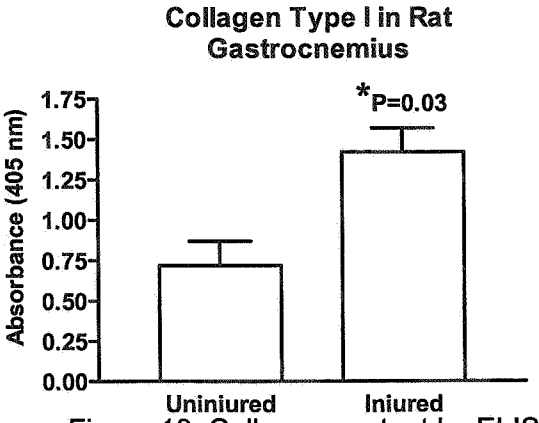


Figure 10. Collagen content by ELISA

4. TGF-β1 and regeneration: To test the biological effects of TGF-β1 on inhibition of muscle regeneration, myogenic regulatory factors (myogenin and MyoD) were measured. If TGF-β1 were to inhibit regeneration in vivo as it does in cell culture [30], then increases in MyoD and/or Myogenin associated with myogenesis following injury would be suppressed. MyoD protein and myogenin transcript abundance were not suppressed as both increased 48 hours following injury whereas myogenin protein levels were unchanged (Figures 18 & 19). Thus, muscle regeneration was inhibited at this time point.

5. Bradykinin and pain: Unfortunately, we were unable to measure bradykinin in muscle homogenates even though some can be observed in tissue sections. The ELISA assay system can detect exogenous bradykinin (spiked) added to the muscle samples but the quantity of bradykinin in the injured tissue must be below the limits of detection. The assay was quite variable even after column separation and concentration of bradykinin by SpeedVac have been applied indicating that bradykinin was just at or below the level of detection. These studies must wait for a more sensitive assay or other methods of detection applied and have been discontinued.

Specific Aim 3: The hypothesis is that chronic strain injury together with fatigue will produce increased collagen deposition (fibrosis) and pathology in skeletal muscles.

Based on observations in our lab, a continuum from adequate stimulus for increased muscle strength and hypertrophy to decreased function and fibrosis from chronic repeated strains exists depending on repetition number, rest interval, and frequency of exposure. A number of possible adaptations to daily chronic strains including isometric force, collagen and collagen crosslink content, and muscle fiber size were studied in female rat plantar flexor muscles following 6 weeks of daily exposure (except weekends). A decrease in strength following such large amounts of high intensity loading was expected. However, no decrement in maximal isometric force was observed for the plantar flexor muscles of rats exposed to 6 weeks of daily bouts of 50 strains to one leg. The lack of change in strength occurred together with a decrease in myofiber area of the gastrocnemius and soleus muscles. Since the gastrocnemius muscle is responsible for 80% of the isometric force of the plantar flexor muscles [6;43], a decrease in myofiber area of about 26% for the gastrocnemius muscle would be expected to decrease the maximal isometric force recorded.
Increased isometric force with decreased cross-sectional area of myofibers could arise if more force were produced per cross-sectional area or the specific tension of the muscle were to increase [9]. Two possibilities for increased specific tension would be: 1) an increase in the density of contractile proteins within each myofiber or 2) an increase in sarcomere force transmission to the skeleton [9]. We did not measure myofilament density of these muscles using electron microscopy. However, in humans, resistance training

increased the cross-sectional area of myofibrils but did not change the density of the thick filaments [23]. So changes in myofilament density probably did not contribute to the maintenance of isometric force.

For force to be transmitted to the skeleton, connective tissue is required in the form of tendon and fascial connections. In our chronic loaded rats, the Achilles tendon contained more collagen per unit dry weight implying that the collagen fibril density increased in order to accommodate the increased muscle force – a stronger tendon. Tendon adaptations to repeated eccentric muscle actions have not been studied except when used to produce a tendinosis in rats [28]. To produce a tendinosis, one hour of 1,800 repetitions 3 days a week for up to 4 weeks were used [28]. In our study, there was no evidence of pathology or overt inflammation in the Achilles tendons from the repeated eccentric muscle actions but increased collagen content was measured. The increase in collagen content could have resulted from a decrease in non-collagen components (e.g. proteoglycans) of the tendon. Further studies are needed to define the non-collagen changes that occurred. However, since collagen cross-link content did not increase in the Achilles tendons of resistance trained rats, tendon stiffness would not be expected to change even with an increase in collagen content.

In our previous studies of rat plantar flexor muscles, increased ECM and the formation of collagen struts between myofibers were reported following repeated strains. These collagen connections could serve to increase the lateral transmission of force from the sarcomeres to the tendon. For the anterior tibial compartment of the rat, extramuscular myofascial force transmission has been reported [17;18]. Therefore, the presence of increased collagen content of chronically strained muscles could have offset any decrease in force production by reduced myofiber area.

In summary, in spite of a decrease in myofiber areas of the soleus and gastrocnemius muscles, daily exposure to 50 strains for 6 weeks did not result in a loss of isometric strength. Indirect evidence for an increase in specific tension due to an increased proportion of connective tissue offsetting the decrease in myofiber area was supported by an increase in ECM area and collagen content. Increased collagen content of the Achilles tendon provided evidence that tendons may become stronger as well.

Specific Aim 4: The hypothesis is that, in response to strain injury that produces pathology, TGF-β2 will appear in the blood (Biomarker) in proportion to the number of pathologic muscle cells and can serve as a serum marker for the amount of on-going pathology whereas TGF-β1 will not.

While we were able to measure an increase in TGFβ2 transcripts and LTGFβ2 protein content 48 hours following injury, the increased TGFβ2 expression did not correlate with the number of injured myofibers (i.e. degree of injury). Therefore, we did not test serum. TGFβ2 may serve as a local mediator in muscle injury regulating myoblast fusion and/or motor neuron survival [27]. Since cells in vitro respond differently depending on other growth factors added to the culture medium, additional in vivo studies are needed to determine the physiological action of both TGFβ1 and TGFβ2 on muscle following strain injury.

Because of our preliminary work with a novel biomarker for muscle-specific inflammation, these experiments were ended. Instead, the PI took the liberty to alter direction and develop a method for pain assessment and muscle inflammation. These additional experiments were not listed in the original specific aims but are consistent with the long-term goals outlined for this research.

Additional Studies on Strain Injured Muscles: Pain Assessment.

The relationship between injury and pain. A poor correlation exists between the decrease in isometric force indicative of muscle strain injury and the increase in the sensation of pain [33]. However, the time-courses for myopain and macrophage accumulation are quite similar and consistent with an inflammatory-mediated pain response [33]. Since there is a relationship between repetition number and histopathology [16], muscle pain should be a function of the amount of histopathology (i.e. inflammation) and not loss of strength. Measurement of muscle pain could serve as an index of the amount of tissue inflammation and guidelines developed for treatment interventions or "light work days" to allow for recovery. No research has been conducted in this area. However, there may be an "inflammation" threshold for the sensation of pain as well as

an "inflammation" limit where further damage does not produce additional pain sensation because no further sensitization of the mechano-nociceptive pain system can occur.

1. Muscle Pain: Since pain, not weakness or muscle stiffness, is responsible for the loss of work, an objective measurement of pain was required for our strain-injured rats. In humans, the quantitative measure of myopain is pressure algometry [10]. We adapted pressure algometry to assess the pressure-pain threshold of skeletal muscles of rats (n=4)(Figure 11).

Muscle pain, which is exaggerated by pressure, provides evidence that mechanoceptors are involved in muscle pain following strain injury [3]. Mechanoceptors can be sensitized by bradykinin [12] resulting in hyperalgesia. Bradykinin has been shown to be elevated in humans following eccentric exercise [4] and bradykinin was observed in rats around damaged myofibers following repeated strain injuries (RSI) with short rest times. Bradykinin is produced from kininogens resident in the extracellular matrix and transported within infiltrating neutrophils [29] which are found in our injured rat muscles (n=9)[37;39].

2. Manual Injury Protocol: To investigate myopain, a manual stretching technique [35] was used to avoid the surgery required for nerve cuff implantation which itself produces hyperalgesia of the rat's lower leg. Two electrodes were placed under the skin on the medial and lateral side of the calf overlying the gastrocnemius muscles. Muscle activation resulted from electrical stimulation; the muscles were stretched by rotating the ankle through its physiological range of motion. The opposite leg was exposed to needles alone or equal numbers of repeated isometric contractions. Various combinations of repetition number and inter-stretch rest times, as pilot studies, were conducted prior to dynamometer experiments [37]. For example, using a 50 stretch protocol with an with inter-stretch rest time of 40 sec, 12% of the muscle fibers were albumin positive (injured) 48 hrs after the protocol indicating a loss of cell membrane integrity. These injured rat muscles were also painful with a decreased threshold for pressure-induced pain (Figure 12).

In pilot studies using a 50 manual stretch protocol, a residual, 38% isometric force deficit (weakness) was recorded 48 hrs after the protocol. The injured medial gastrocnemius muscle had an abundance of necrotic fibers (>12%) and marked inflammation. A new dynamometer is being tested and will assess the functional impairment from the manual stretching protocols on strength and power under submaximal loading.

3. Pressure-Pain Threshold: Algometry has become the objective measurement of choice for myofascial pain in humans [10;11]. In our hands, attempts using clinical algometers for testing rats were met with difficulties because the rat is more sensitive to pressure while recovering from anesthesia and the algometers tested were not sensitive enough to record the reduced pressure-pain threshold. Using sub-anesthetic concentrations of barbiturates, an exaggerated response to pain (hyperalgesia) can be observed but is not perceived as pain [8]. We exploited this response by using a short acting barbiturate, Brevital, for anesthesia and a modification of the Randall-Sellitto assay for measuring pain (Figure 11). We used a commercially available Paw Pressure Analgesia Meter with 2 weight discs and a pressure tip with a diameter of about 1-cm. A constantly increasing load was applied to the plantar flexor muscles while the rat is side lying. When a withdrawal reflex (jump response) was observed, the machine was stopped and the force recorded. Testing was reproducible with the coefficient of variance of 4.0 ± 3.0 %.

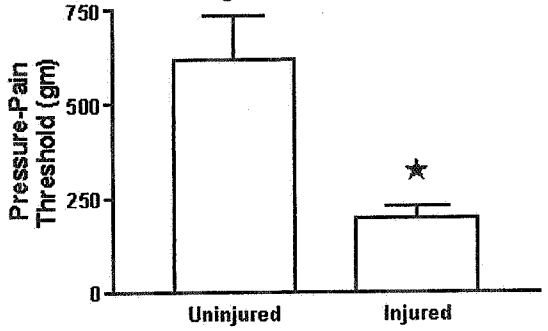


Figure 11. Modified Randall Sellitto assay for pain.

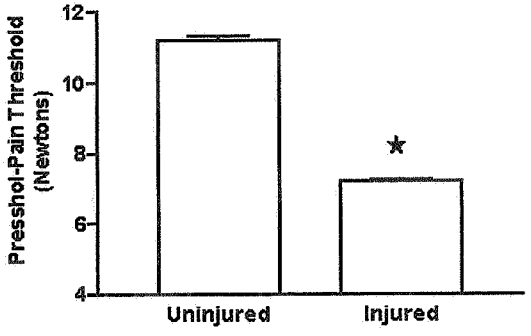


Figure 12. Custom-built Finger Dynamometer

We constructed a finger pressure device that allows us to quickly record a pressure-pain threshold by simply pinching the plantar flexor muscles between the thumb and first finger and stopping when we observe the "jump response"(Figure 12)(n=4). The objective measurement of myopain in rats using pressure-pain threshold determination (algometry) is a novel approach. Such myopain measurements will help illuminate the cause of acute and chronic pain in rats after exposure to different RSI protocols.

E. Acknowledgements:

The research was aided by the consultation and work of Drs. Steve Alway, Greg Cutlip, Gerry Hobbs, Cheryl Smith and Mark Willems. Technical assistance was provided by Francoise Stauber and Paul Harton.

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