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A Model for Wrist and Elbow Musculoskeletal Disorders

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TABLE OF CONTENTS

Abstract

The prevention of work-related tendionopathies, a group of common and debilitating disorders associated with hand intensive work, is hampered by the limitations of epidemiologic studies to clearly identify specific and generalizable risk factors, especially biomechanical risk factors. The purpose of this research was to use a rabbit model to investigate the effects of long-term, repetitive, digit loading on structural and cellular changes of degeneration on the tendon at the epicondyle. The goals were to study the pathophysiologic mechanisms of tendon damage and to determine the relative contributions of various biomechanical characteristics of loading, such as repetition rate and applied force to injury.

An in vivo rabbit model was developed to expose the tendon to repetitive loading for 2 hours per day, 3 days per week for 12 weeks (80 cumulative hours). Three combinations of peak forces (High: 0.42N; Low: 0.14N) and repetition rates (High: 60 reps/min; Low: 10 reps/min) were selected: High Force/High Repetition (HFHR), High Force/Low Repetition (HFLR) and Low Force/High Repetition (LFHR). At the end of the exposure period, changes to tendon microstructure and biology were measured.

Microtears were quantified using photomicroscopy and image analysis methods. Biological changes were measured by immunohistochemical staining of cells for growth factors associated with angiogenesis and matrix repair (VEGF, VEGFR-1 and CTGF).

Microtears were increased under HFHR loading. There were moderate increases with HFLR loading and no changes associated with LFHR loading. Dose-response relationships were observed for both force and repetition with tear measures being affected more by peak force than repetition rate.

Cell densities of VEGF, VEGFR-1 and CTGF staining cells were increased under HFHR loading. No changes were found under the other loading conditions. VEGF staining cell density correlated to microtears (tear density) regardless of the loading pattern. VEGFR-1 cell density, the main receptor for VEGF, was associated to tear density only under HFHR loading.

This is the first study to systematically document the relationship between repetitive loading of the upper extremity and the formation of microtears in tendon. Microtears have been hypothesized as being a mediator or the initial route of damage in tendon leading ultimately to chronic tendinosis, but the evidence has been missing until now. The results of this study provide evidence at the basic science level for a relationship between repetitive loading of the upper extremity and injury to tendons. As the tendon experiences repetitive loading, microtears may accumulate causing a cellular response, such as an up-regulation of VEGFR-1. This suggests a pathway for inflammatory and angiogenic mediators further downstream in tendon degeneration.

The study also provides evidence that during repetitive loading, the peak force of the load contributes more to microtear damage to the tendon than the repetition rate. These findings suggest that in order to decrease risk of tendon related injuries among workers due to hand intensive tasks, greater benefit may be obtained by reducing the peak hand loads than by reducing repetition rates.

Significant Findings

The structural and cellular responses of tendon to three different patterns of repetitive loading were investigated in this in vivo model using the rabbit digit flexor muscle (Flexor Digitorum Profundus). The loading patterns included a high and low repetition rate (60 vs. 10 repetitions/min) and high and low peak force (0.42N vs. 0.14N) in the following three patterns: high force high repetition (HFHR), high force low repetition (HFLR) and low force high repetition (LFHR). Structural outcomes included the formation of microtears as measured by the tear area as a percent of tendon area (%), the tear density (tears/mm^2) and mean tear size (μm^2). Cellular outcomes included evaluating the densities of cells staining for Vascular Endothelial Growth Factor (VEGF), its receptor, Vascular Endothelial Receptor 1 (VEGFR-1), and Connective Tissue Growth Factor (CTGF). These growth factors are involved in inflammation and angiogenesis.

1. Repetitive loading caused an increase in all measures of microtears in tendon

High Force High Repetition loading (HFHR) generated large increases in measures of microtears in the loaded tendon compared to the unloaded tendon. All measures of tears significantly increased ($p < 0.05$, RMANOVA) in the loaded tendon compared to its unloaded counterpart. This is the first study to systematically document a relationship between repetitive loading and the formation of microtears in tendon.

2. Peak force was a greater contributor to microtears than repetition rate

While HFHR loading caused large changes in microtear measures, no change in microtear measures between loaded and unloaded tendon were observed with the lower peak force and high repetition loading pattern (LFHR). With the high force low repetition rate loading pattern (HFLR), differences in microtear measures were observed,

but were not as great as the HFHR loading pattern. For HFLR, the tear area as a percent of tendon area ($p = 0.01$) and the mean tear size ($p = 0.03$) were significantly greater in the loaded tendons. These findings suggest that peak force is a greater contributor to tendon injury than repetition rate.

3. High force high repetition loading also caused an increase in growth factors

The densities of tendon cells staining for the growth factors VEGF, VEGR-1 and CTGF were increased in the loaded tendon under the HFHR loading pattern. These findings support the microtear findings that the HFHR loading leads to injury to the tendon.

4. Low force high repetition loading caused a decrease in growth factors

The HFLR loading pattern caused no changes in growth factor density between limbs. Surprisingly, the LFHR loading led to a decrease in VEGF-1 cell density in the loaded tendon compared to the unloaded tendon. These findings suggest that the LFHR loading pattern may promote a healing response (reduction in growth factors).

5. Growth factor density in tendon was related to microtear density

The linear relationships between tear density and VEGF, VEGFR-1 and CTGF were investigated by calculating correlation coefficients and slopes. VEGF cell density was correlated to tear density regardless of load status or loading pattern. The slopes between VEGF and tear density was significantly greater than zero for all cases and ranged from 0.22 to 0.35. VEGFR-1 cell density did not correlate to tear density for HFLR and LFHR loading in both the loaded and unloaded tendons. REDO###### However, the slope was significantly greater than zero under loading in the HFHR loading pattern and was significantly greater than the unloaded slope. The increase in

VEGFR-1 cell density when experiencing HFHR loading may directly impact the downstream effects of neovascularization because it is the main receptor for VEGF, which has major impacts on angiogenesis.

6. Microtears primarily occurred in the outer region of the tendon

Regional differences in tear measures were observed within the tendon. Regardless of loading status (loaded vs unloaded limb) or the loading pattern, tear measures were greater in the outer regions of the tendon. Similar regional variations were observed for growth factors, but the variations were not as pronounced as they were for microtears. The regional variations were likely due to the inhomogenous transmission of load through the tendon. The outer regions experience greater tensile stresses while the inner regions experience a combination of tension, compression and shear loading.

Translation of Findings

Chronic injuries to tendons of the hand and arm, such as epicondylitis or wrist tendonitis, are an important cause of disability among workers who daily perform hand intensive tasks. Nationwide, these disorders account for significant workers' compensation costs. This is the first basic science study to systematically investigate the effects of the risk factors peak force and high repetition rate on the underlying mechanisms of injury in the development of chronic tendon disorders. The formation of microtears in the tendon was found to be more sensitive to differences in force than in repetition rate. A similar finding was observed for growth factors, but, interestingly, high repetition rate with low force led to lower growth factor levels suggesting a beneficial effect.

These results may be applied to high risk occupational setting in order to prevent or manage tendon disorders. The study findings suggest that for jobs requiring repetitive gripping or pinching at high forces, decreasing the required grip or pinch forces will have a greater effect on reducing the risk of tendon degeneration than decreasing repetition rate. In other words, reducing the weights of objects or tools or selecting different hand tools may critical interventions to make in the workplace to prevent tendon injuries. Examples of workplace changes that can decrease grip force are selecting pipettors in the laboratory that require less thumb force to use; picking nut drivers with clutches or other modifications that reduce the required grip force; sharpening tools so that they require less force to cut (e.g., knives, scissors, dental scalers, pruners, etc.); and purchasing tools that are powered (e.g., caulking guns, nail guns, cake decorators).

Relevance

Safety engineers, ergonomists, industrial hygienists, occupational medicine physicians, employers and employees need clear guidelines to follow for the prevention of chronic musculoskeletal disorders in the workplace, such as tendonitis and epicondylits. These disorders account for approximately 20% of all workers' compensation dollars and significant pain and disability in the US workplace. The biomechanical risk factors for these disorders include repetition rate, force, and posture. Unfortunately, the findings from the epidemiologic literature are not specific enough to identify dose-response relationships or thresholds of injury for these important risk factors. On-the-other-hand, these same risk factors are also important for producing products. Without force, and motion, and posture changes work could not be done. This study begins to prioritize the contributions of these risk factors to tendon injuries. Specifically, the research finds that peak applied force contributes greater to markers of tendon injury than repetition rate. These findings can help safety professionals and others better focus resources on the workplace changes that will be effective in preventing tendon related injuries in the workplace.

Publications from this Research

Journal Articles (published articles attached)

Rempel D and Diao E. Entrapment neuropathies: pathophysiology and pathogenesis. J Electromyography and Kinesiology 2004, 14(1): 71-75.

Nakama L, King K, Abrahamsson SO, Rempel D. Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. J Orthop Res 2005, 23(5):1199-1205.

Nakama L, King KB, Abrahamsson SO, Rempel DM. VEGF, VEGFR-1 and CTGF cell densities in tendon are increased with cyclical loading: An *in vivo* tendinopathy model.. J Orthop Res 2006, 24(3):393-400.

Nakama L, King KB, Abrahamsson SO, Rempel DM. The effect of repetition rate on the formation of microtears in tendon in an *in vivo* cyclical loading model. J Orthop Res 2006 (in press)

Books

Musculoskeletal Disorders and the Workplace. (Panel Member) National Research Council and Institute of Medicine. National Academy Press, Washington, D.C., 2001.

Proceedings

Nakama L, King K, Rempel D. The effect of repetition rate on the formation of microtears in tendon in an in vivo repetitive loading animal model. Human Factors and Ergonomics Society 2006, San Francisco.

Marecek GS, Opel C, Rempel D, King KB. Repetitive finger joint flexion without external load leads to articular cartilage thinning in an in vivo rabbit model. Orthopaedic Research Society, 2006, Chicago.

Nakama LH, Amano K, King KB, Rempel DM. The effect of repetition rate on blood vessel formation in the paratenon of a repetitively loaded tendon in vivo. Orthopaedic Research Society, 2006, Chicago.

Nakama LH, King KB, Rempel DM. The effect of loading rate on VEGF, VEGTR-1, and CTGF production in an in vivo cyclically loaded tendon. Orthopaedic Research Society, 2006, Chicago.

Opel C, Rempel D, King KB. In vivo cyclical joint loading decreases unmineralized cartilage thickness in the rabbit metacarpophalangeal. Orthopaedic Research Society, 2005, Washington.

Nakama LH, King KB, Rempel DM. The localization of connective tissue growth factor in a cyclically loaded tendon at the epicondyle in an in vivo model of tendinosis. Orthopaedic Research Society, 2005, Washington.

Rempel D. Effects of repetitive loading on nerve: basic science update. American Occupational Health Conference 2004, Kansas City, MO

Rempel DM, Nakama LH, Barr A. Tendon microtears in an animal model of epicondylitis caused by cyclical loading. Orthopaedic Research Society, 2004, San Francisco

Nakama LH, King KB, Rempel DM. The localization of vascular endothelial growth factor in a repetitively loaded tendon in vivo: An immunohistological study. Orthopaedic Research Society, 2004, San Francisco

Rempel D. An in vivo model for entrapment neuropathy due to repeated finger loading. Orthopaedic Research Society 2001, San Francisco, California.

Dissertation

Nakama L: [Ph.D., 2006] Tendon Degeneration Due to Repetitive Loading: an In Vivo Tendinopathy Model, Ph.D. Thesis, Bioengineering Graduate Group, University of California at Berkeley and University of California at San Francisco.

Materials Available for other Investigators

The research protocols and forms used for data collection for the study are available for other investigators by contacting Dr. David Rempel at david.rempel@ucsf.edu.

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CHAPTER I

Background and Significance

The annual incidence of musculoskeletal disorders of the upper extremities associated with repetitive work has been increasing over the last 15 years and these disorders now account for a majority of work-related illnesses reported by employers (Bureau of Labor Statistics (BLS) 1998). Examples of these problems are non-specific pain, tendon-related disorders (e.g., rotator cuff tendonitis, epicondylitis, stenosing tenosynovitis), and nerve entrapments (e.g. carpal tunnel syndrome). In some industries (e.g. office sector, meat packing) these are the most common occupational health problems. These disorders are also associated with high levels of disability (Pransky et al. 2000). In the BLS survey (1998), disorders related to repetitive motion resulted in the longest absences from work compared to other events and exposures. A tendon injury model in the rat confirms this slow healing process (Carpenter et al. 1998). The supraspinatus tendon demonstrated incomplete or very slow healing up to 12 weeks after the injury. Estimates for the total annual US costs for work-related neck and upper extremity disorders range from 563 million dollars to 3 billion dollars (Webster et al. 1994, NIOSH 1996).

Prevention of these disorders is hampered by the limitations of epidemiologic studies to clearly identify specific and generalizable risk factors, especially biomechanical risk factors. This is due partially to limitations in accurately assessing outcomes and exposures. These soft tissue disorders are difficult to classify; the most objective tools are invasive (e.g., biopsies) and therefore not often used. Some studies

rely exclusively on symptoms or do not use consistent physical examination criteria (e.g., Faucett et al. 1994, Marras et al. 1993, Moore et al. 1994). Quantifying exposure is problematic due to the constant changes in tasks and jobs that occur in the modern work place and the resultant changes in exposure. Some studies use self-report for exposure classification (DeKrom et al. 1990) and others use schemes that are difficult to duplicate (Nathan et al. 1988). Quantifying biomechanical factors over time is nearly impossible. Obtaining adequate sample sizes in specific exposure categories is difficult (Moore et al. 1994, Osorio et al. 1994). Because of differences in work practices, even the results of a well executed prospective study, which are rare, may be difficult to generalize to other industries. Although some gross biomechanical factors (e.g., repetition, load) repeatedly emerge as risks (Stock et al. 1991, Bernard et al. 1997) the relationships between the specific biomechanical factors and disease remain unknown.

Specific information regarding biomechanical factors would greatly assist in the prevention of these disorders. It is not adequate to simply recommend reducing repetition rate or load levels at work because these same factors are critical to productivity. What is needed is more precise knowledge of the exposure-disorder relationships between specific biomechanical factors and disease. Is the primary biomechanical problem repetition rate (Roquelaure et al. 1997, Silverstein et al. 1986, 1987), load (Moore and Garg 1994, Luopajärvi et al. 1979, Chiang et al. 1993, Roquelaure et al. 1997, Silverstein et al. 1986, 1987), acceleration, cumulative tendon travel (Marras and Schoenmarklin 1993), or other factor? Is the problem that an initial very high load event is followed by continued low loads? If load is the problem, is it peak load or mean load? Or, if all of these factors are involved, what are their relative contributions? Are the exposure-

disorder relationships linear, or are there threshold levels based on healing? Answers to these questions could assist industrial engineers, ergonomists, and other heath professionals in the design of safe work without unduly interfering with productivity. For example, if high loading rate is the major biomechanical risk factor, tasks and tools could be designed to minimize impact loading, peak accelerations, or sudden torque changes without limiting productivity. If repetition is the major risk, tasks and tools could be designed to eliminate extra repetitions, but maintain similar mean force patterns.

For the reasons cited above, epidemiologic studies are unlikely to answer these questions; however, these questions have the potential to be answered by animal models. Unfortunately, few animal models have been developed to explore these questions. There are no animal models for epicondylitis associated with repetitive loading. But there is a relevant overuse model for shoulder tendinosis in the rat (Carpenter et al. 1998), and Achilles tendinosis in the rabbit (Backman et al. 1990). Rotator cuff tendinosis was produced in the rat with treadmill running and led to an increase in cellularity and collagen disorganization in tendon compared to controls (Carpenter et al. 1998). The biomechanical changes were an increase in tendon cross-sectional area and a decrease in tissue modulus. Limitations were lack of characterization and control of the biomechanical loads – no force or repetition data was reported by the authors.

Backman et al. (1990) exposed the Achilles tendon of 13 New Zealand white rabbits to repetitive loading by percutaneously stimulating the gastrocnemius muscle while applying a load to ankle flexion. Loads of 150 flexions per minute were applied for 2 hours per day, 3 days per week for 5-6 weeks. Although not published, and not precisely controlled, the peak load is estimated at 15% of maximum. A four point, semi-

quantitative scheme was used to evaluate histopathologic changes in the tendon. In comparison to the control leg, the exposed leg morphology demonstrated a thickened paratenon with edema and increased capillaries, and increased fibroblasts and lymphocytes. The central portion of the tendon exhibited degenerative changes. The model established that repetitive loading can lead to an inflammatory-like reaction at the paratenon, but it is limited in its relevance to human work situations and in its lack of characterization of internal tissue loads. The repetition rate was high compared to what might be expected for humans, and no other loading conditions were explored. Furthermore, this hind leg model does not allow for the investigation of nerve entrapments or tendon related disorders common to the upper extremity (e.g., epicondylitis).

Our poor understanding of the cellular and biochemical processes underlying epicondylitis is formulated from the indirect evidence of histological findings from surgical specimens in humans. The pathology is characterized by regions of dense hypertrophy of fibroblasts, vascular hyperplasia, and disorganized collagen (Kraushaar and Nirshl 1999, Merkel et al. 1982, Regan et al. 1992, Doran et al. 1990, Chard et al. 1994). There are no biochemical studies of this tissue and these studies, while valuable, are merely histologic 'snap shots' of the poorly repaired end result and do not give insight into the initial events and subsequent progression of overuse pathology. However, the biochemical events may be partially inferred from studies of chronic achilles tendinosis in humans. Tissue samples from 11 athletes with chronic achilles paratenonitis were compared to 4 male cadavers (Kvist et al. 1988). This tissue is also characterized by neovascularization, fibrinous exudations, and thickened and edematous

paratenon. Immunohistologic findings revealed increased staining for fibrinogen and fibronectin in adhesion areas and in the edematous paratenon.

The observation that fibrocartilage-like regions form on the tendon at sites where the tendon is subjected to compression may have some relevance to injury (Merrilees and Flint 1980, Abrahamsson et al. 1989, Vogel et al. 1989, Malaviya et al. 2000). These regions are morphologically and biochemically different from the tendon proper, and contain a high density of rounded instead of elongated tenocytes. The collagen fibers are not generally oriented along the axis of the tendon but are irregular, loose and of thinner diameter. These regions contain few or no vessels (Lundborg et al., 1977). The proteoglycans are large, aggragating and rich in chondroitin sulfate (e.g., aggrecan, versican) whereas within the tendon proper the proteoglycan is small, dermatan sulfate rich (e.g., biglycan, decorin)(Vogel et al. 1986). These regions have some similar features to the histology observed in human tendinosis but they also differ in that they are vessel poor and contain few inflammatory cells.

Changes to the ECM components, the quantity and quality of molecules, are relevant to soft tissue injury and repair. Plaas et al. (2000) show in their rabbit model of ligament injury that small proteoglycans (e.g., biglycan) and two large proteoglycans (likely versican and aggrecan) accumulate following injury. Another ECM and cell surface protein, tenascin, is increased in the area surrounding rounded cells found in the *disorganized* fibrocartilage and around infiltrating blood vessels (Riley et al. 1996) of injured or degenerated tendons.

In both the development and repair of soft tissues the increase in matrix production by the resident cells is often attributed to the presence of cytokines or growth

factors. Interleukin 1 (IL-1), IL-4 and tumor necrosis factor alpha (TNF-α) stimulate tenascin synthesis and matrix deposition by fetal conjunctival fibroblasts (Rettig et al. 1994). TNF-α may also be involved in peripheral pain receptor activation. Transforming growth factor beta (TGF-β) also stimulates fibroblasts from some tissue sources to produce ECM, possibly due to the ability of TGF-β to modulate other signaling pathways. In another example of complex TGF-β effects, van Beuningen et al. (2000) demonstrate that injection of TGF-β into the knee of their mouse osteoarthritis model results in an increase in matrix synthesis and cell proliferation. Paradoxically, prolonged exposure to TGF-β led to local proteolysis and osteoarthritis-like events rather than proper repair. Cells of tendon tissue cultured in the presence of TGF-β are stimulated to increase aggrecan and biglycan production (Robbins et al. 1997).

The role of basic fibroblast growth factor (bFGF) is also relevant. This cytokine induces angiogenesis by creating capillary networks and granulation tissue to transport cells to the injury site for inflammation and collagen synthesis (Folkman and Klagsbrun, 1987). In their rabbit tendon healing model, Chang et al. (1998) show that following tendon transection and repair, bFGF is upregulated (increased bFGF mRNA) in tenocytes and sheath fibroblasts. A further complication to cytokine regulation of the ECM is that certain cytokines bind one or more ECM molecules. TGF-β is bound by the small proteoglycans decorin, biglycan, and fibromodulin (Yamaguchi et al. 1990, Hildebrand et al. 1994) while bFGF binds to heparin sulfate proteoglycans (Shing et al. 1984). Thus, matrix proteins can modulate the modulators of their own synthesis.

Physiological loading may lead to increased ECM synthesis and deposition in tendon yet does not lead to proliferation, inflammation or angiogenesis as seen in

injury/overuse loading (Woo et al. 1980; Woo et al. 1981). The key difference could be the involvement of cytokines such as TGF-β and bFGF. Minor repair of connective tissues occurs continually on a limited basis. The necessary breakdown of flawed matrix components, prior to rebuilding, is performed by enzymes including the family of matrix metalloproteinases (MMPs). Since MMPs break down the matrix, an imbalance in this process could release cytokines bound by the matrix and thus increase cell stimulation. Where loading is increased to the point of injury, a cycle of repair, release of cytokines, and more repair could amplify a deleterious imbalance of break down and repair of matrix. It is not clear, however, how this process is regulated nor at which point in the cycle it is readily reversible. Understanding when these proteins and cytokines are expressed relative to the injury and each other will help unravel the injury mechanism and expand the possible interventions for prevention of permanent injury.

The purpose of this proposal is to use a model of repetitive stimulation of the large finger flexor muscle of the rabbit to investigate pathophysiologic mechanisms and exposure-disorder relationship of musculoskeletal disorders such as epicondylitis. . Knowledge of the pathophyisiologic mechanisms are not only useful in ultimately unravelling the biomechanical factors important in injury and healing but may also lead to early identification of biochemical markers of injury in humans. In the future, the model may also be used to investigate healing and medical treatments and the effects of load modification on recovery. The model has the potential to be developed in the future to also investigate the pathophysiology of trigger finger, tenosynovitis at the wrist, osteoarthritis, and skeletal muscle injury.

The proposed research is directly responsive to the NIOSH Announcement PA-99-144 which requests proposals that address NORA (1996) priority research areas. One of these areas is musculoskeletal disorders of the upper extremity. Proposals should develop "knowledge that can be used in preventing occupational diseases and injuries and to better understand their underlying pathophysiology". This proposal also directly addresses recommendations for future research outlined at a conference titled Repetitive Motion Disorders of the Upper Extremity, sponsored in 1994 by NIAMS, AAOS, and NIOSH (Gordon et al. 1995). This proposal also addresses several research priorities of the National Research Council's report (1999) on Work-related Musculoskeletal Disorders: (1) research is needed on the models and mechanisms that underlie the established relationship between causal factors and outcomes and (2) research is needed to improve our understanding of the mechanisms that produce tissue failure.

References

Abrahamsson SO, Gelberman RH, Lohmander SL. Variations in cellular proliferation and matrix synthesis in intrasynovial and extrasynovial tendons. An *in vitro* study in dogs. J Hand Surg 1994; 19A(2):259-265.

Abrahamsson SO. Similar effects of recombinant human Insulin-like Growth Factor I and II on cellular activities in flexor tendons of young rabbits: Experimental studies *in vitro*. J Orthop Res 1997; 15:256-262.

An KN, Ueba Y, Chao EY, Cooney WP, Linscheid RL. Tendon excursion and moment arm of index finger muscles. J Biomechanics 1983; 16:419-425.

An KN, Askew L, Chao E. Biomechanics and functional assessment of upper extremities. In Trends in Ergonomics/Human Factors III. Korowoski W (ed), Amsterdam, Elsevier, 1986. p350.

Armstrong TJ, Foulke JA, Joseph BS, Goldstein SA. Investigation of cumulative trauma disorders in a poultry processing plant. Am Ind Hygiene Assoc J 1982; (43)2:103-116

Backman C, Boquist L, Friden J, Lorentzon R, Toolanen G. Chronic achilles paratenonitis with tendinosis: An experimental model in the rabbit. J Orthop Res 1990; 8:541-547.

Benjamin M, Qin S, Ralphs JR. Fibrocartilage associated with human tendons and their pulleys. J Anat 1995; 187: 625-633.

Benjamin M, Ralphs JR. The development and functional anatomy of tendons and ligaments. In Repetitive Motion Disorders of the Upper Extremity (eds. Gordon S et al.). Park Ridge, Illinois, AAOS, 1995.

Bernard B (Ed). Musculoskeletal disorders and workplace factors: A critical review of epidemiologic evidence for work-related musculoskeletal disorders of the neck, upper extremity, and low back. DDHS Publication No. 97-141. 1997. National Institute for Occupational Safety and Health, Cincinnati, Ohio.

van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB. Osteoarthritis-like changes in the murine knee joint resulting from intra-articular transforming growth factor beta injections. Osteoarthritis Cartilage 2000; 8:25-33.

Bureau of Labor Statistics. Annual Report of Occupational Injury and Illness, US Department of Labor, Washigton, D.C., 1996.

Butler DL, Grood ES, Noyes FR, Zernicke RF, Brackett K. Effects of structure and strain measurement technique on the material properties of young human tendons and fascia. J Biomech 1984; 17:579-596.

Carpenter JE, Flanagan CL, Thomopoulos S, Yian EH, Soslowsky LJ. The effects of overuse combined with intrinsic and extrinsic alterations in an animal model of rotator cuff tendinosis. Am J Sports Med 1998; 26:801-807.

Carpenter JE, Thomopoulos S, Flanagan CL, DeBano CM, Soslowsky LJ. Rotator cuff defect healing: A biomechanical and histologic analysis in an animal model. J Shoulder and Elbow Surg1998; 7: 599-605.

Chang J, Most D, Thunder R, Mehrara B, Longaker MT, Lineaweaver WC. Molecular studies in flexor tendon wound healing: the role of basic fibroblast growth factor gene expression. J Hand Surg 1998; 23A:1052-1058.

Chard MD, Cawston TE, Riley GP, Gresham GA, Hazleman BL. Rotator cuff degeneration and lateral epicondylitis: a comparitive histological study. Ann Rheum Dis 1994; 53:30-34.

Cheadle A, Franklin G, Wolfhagen C, Savarino J, Liu PY, Salley C, Weaver M. Factors influencing the duration of work-related disability: a population-based study of Washington State Workers' Compensation. Am J Pub Health 1994; 84:190-196.

Chiang HC, Ko YC, Chen SS, Yu HS, Wu TN, Chang PY. Prevalence of shoulder and upper-limb disorders among workers in fish-processing industry. Scand J Work Environ Health 1993; 19:126-131.

Cohen MJ, Kaplan L. Histology and ultrastructure of the human flexor tendon sheath. J Hand Surg 1987; 12A:25-9.

DeKrom M, Kester A, Knipschild P, Spaans F. Risk factors for carpal tunnel syndrome. Am J Epi 1990, 132:1102-1110.

Dennerlein J, Miller J, Mote CD, Rempel D. A low profile tendon force transducer: The influence of tendon thickness on calibration. J Biomechanics, 1997, 30:395-397.

Dennerlein JT, Diao E, Mote CD, Rempel D. Tensions of the flexor digitorum superficialis are higher than a current model predicts. J Biomechanics 1998; 31:295-301.

Dennerlein JT, Diao E, Mote CD, Rempel DM. *In vivo* finger flexor tendon force while tapping on a keyswitch. J Orthop Res; 17:178-184.

Doran A, Gresham GA, Rushton N, Watson C. Tennis elbow. A clinicopathologic study of 22 cases followed for 2 years. Acta Orthop Scand 1990; 61:535-538.

Fahey JJ, Bollinger JA. Trigger-finger in adults and children. J Bone Joint Surg 1954; 36A:1200-18.

Faithful DK, Moir DH, Ireland J. The micropathology of the typical carpal tunnel syndrome. J Hand Surg 1986; 11B:131-2.

Faucett J, Rempel D. VDT-related musculoskeletal symptoms: Interactions between work posture and psychosocial work factors. Am J Ind Med, 1994, 26:597-612.

Folkman J, Klagsbrun M. Angiogenic factors. Science 1987; 235:442-447.

Fuchs P, Nathan P, Myers L. Synovial histology and carpal tunnel syndrome. J Hand Surg 1991; 16A:753-8.

Gordon SL, Blair SJ, Fine LJ (editors) Repetitive Motion Disorders of the Upper Extremity. American Acad Orthop Surgeons, Rosemont, Il. 1995.

Goldie I. Epicondylitis lateralis humeri (epicondylalgia or tennis elbow). A pathological study. Acta Chir Scand, Suppl 339, 1964.

Harriman DG. Ischemia of peripheral nerve and muscle. J Clin Pathol, Suppl 30 1977; 11:94.

Hildebrand A, Romaris M, Rasmussen LM, Heinegard D, Twardzik DR, Border WA, Ruoslahti E. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J 1994; 302:527-534.

Kraushaar BS, Nirschl RP. Tendinosis of the elbow (Tennis Elbow). J Bone Joint Surg 1999; 81A:259-278.

Larsen JO. Stereology of nerve cross sections. J Neuroscience Methods 1998; 85:107-118.

Loitz BJ, Zernicke RF, Vailas AC, Kody MH, Meals RA. Effects of short-term immobilization versus continuous passive motion on the biomechanical and biochemical properties of the rabbit tendon. Clin Orthop and Rel Research 1989; 244:265-271.

Luopajärvi T, Kuorinka I, Virolainen M, Holmberg M. Prevalence of tenosynovitis and other injuries of the upper extremities in repetitive work. Scand J Work Environ Health 1979; 5:48-55.

Mackinnon SE, Dellon AL, Hudson AR, Hunter DA: Chronic Human nerve compression - a histologic assessment. Neuropathology and Appl Neurobiology 1986; 12:547-565.

Malaviya P, Butler DL, Korvick DL, Proch FS. *In vivo* tendon forces correlate with activity level and remain bounded: evidence in a rabbit flexor tendon model. J Biomech 1998; 31:1043-9.

Malaviya P, Butler DL, Boivin GP, Smith FNL, Barry FP, Murphy JM, Vogel KG. An *In vivo* model for load-modulated remodeling in the rabbit flexor tendon. J Orthop Res 2000; 18:116-125.

Markel KHH, Hess H, Kunz M. Insertion tendopathy in athletes: A light microscopic, histochemical and electron microscopic examination. Path Res Pract 1982; 173:303-309.

Marras WS and Schoenmarklin RW. Wrist motions in industry. Ergonomics 1993; 36: 341-351.

Moore JS and Garg A. Upper extremity disorders in a pork processing plant: relationships between job risk factors and morbidity. Am Ind Hyg Assoc J 1994, 55:703-715.

Moore JS and Garg A. The strain index: A proposed method to analyze jobs for risk of distal upper extremity disorders. Am Ind Hyg Assoc J 1995; 56:443.

Nathan PA, Meadows KD, Doyle LS. Occupation as a Risk Factor for Impaired Sensory Conduction of the Median Nerve at the Carpal Tunnel. J Hand Surg 1988; 13B(2):167-170.

National Research Council. Work-related musculoskeletal disorders. National Academy Press, Washington, DC. 1999. p21.

National Occupational Research Agenda (NORA). DDHS Publication No. 96-115. 1996. National Institute for Occupational Safety and Health. Cincinnati, Ohio.

Neary D, Eames RA. The pathology of ulnar nerve compression in man. Neuropathol Appl Neurobiol 1975; 1:69.

Ochoa J. Nerve fiber pathology in acute and chronic compression. In GE Omer, M Spinner (Eds): Management of peripheral nerve problems. W.B. Saunders Co., Philadelphia, 1980, pp 487.

Osorio AM, Ames RG, Jones JR, Rempel D, Castorina J, Estrin W, Thompson D. Carpal tunnel syndrome among grocery store workers. Am J Ind Med 1994; 25:229-245.

Plaas AHK, Wong-Palms S, Koob T, Hernandez D, Marchuk L, Frank CB. Proteoglycan metabolism during repair of the ruptured medial collateral ligament in skeletally mature rabbits. Arch Biochem Biophys 2000; 374:35-41.

Pransky G, Benjamin K, Hill-Fotouhi C, et al. Outcomes in work-related upper extremity and low back injuries: results of a retrospective study. Am J Ind Med 2000; 37:400-409.

Quinn T, Allen R, Schalet B, Perumbuli P, Hunziker E. Matrix damage and cell injury caused by ramp compression of adult bovine articular cartilage explants: effects of strain

rate and peak stress. Proceedings of the 2000 Annual Meeting of the Orthopaedic Research Society, p0106.

Regan W, Wold LE, Coonrad R, Morrey BF. Microscopic histopathology of chronic refractory lateral epicondylitis. Am J Sports Med 1992; 20:746-749.

Rempel D, Lundborg G, Dahlin L. Pathophysiology of nerve compression syndrome: response of peripheral nerves to loading. J Bone Joint Surgery 1999, 81-A(11):1600-1610..

Rempel D, Abrahamsson S-O. The effects of reduced oxygen tension on cell proliferation and matrix synthesis in synovium and tendon explants from the rabbit carpal tunnel: An experimental study *in vitro*. J Orthop Res 2000 (in press).

Rettig WJ, Erikson HP, Albino AP, Garin-Chesa P. Induction of human tenascin (neuronectin) by growth factors and cytokines: cell type-specific signals and signalling pathways. J Cell Sci 1994; 107:487-497.

Riley GP, Harrall RL, Cawston TE, Hazleman BL, Mackie EJ. Tenascin-C and human tendon degeneration. Am J Pathol 1996; 149:933-943.

Robbins JR, Evanko SP, Vogel KG. Mechanical loading and TGF-beta regulate proteoglycan synthesis in tendon. Arch Biochem Biophys 1997; 342:203-211.

Roquelaure Y, Mechali S, Dano C, Fanello S, Benetti F, Bureau D, Mariel J, Martin YH, Derriennic F, Penneau-Fontbonne D. Occupational and personal risk factors for carpal tunnel syndrome in industrial workers. Scand J Work Environ Health 1997; 23:364-369.

Rufai A, Ralphs JR, Benjamin M. Structure and histopathology of the insertional region of the human achilles tendon. J Orthop Res 1995; 13:585-593.

Sampson SP, Bedalamente MA, Hurst LC, Seidman J. Pathobiology of the human A1 pulley in trigger finger. J Hand Surg 1991; 16A:714-21.

Scelsi R, Zanlungo M, Tenti P. Carpal tunnel syndrome: Anatomical and clinical correlations and morphological and ultrastructural aspects of the tenosynovial shealth. J Orthop Traumatology 1989; 15:75-80.

Schoenmarklin RW, Marras WS, Leurgans SE. Industrial wrist motions and incidence of hand/wrist cumulative trauma disroders. Ergonomics 1994; 37:1449-1459.

Schuind F, Ventura M, Pasteels JL. Idiopathic carpal tunnel syndrome: Histologic study of flexor tendon synovium. J Hand Surg 1990; 15A:497-503.

Schuind F, Garcia-Elias M, Cooney WP, An KN. Flexor tendon forces: *In vivo* measurements. J Hand Surg 1992; 17A:291-298.

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived endothelial cell growth factor. Science 1984; 223:1296-1299.

Silverstein BA, Fine LJ, Armstrong TJ. Hand wrist cumulative trauma disorders in industry. Br J Ind Med 1986. 43:779-784.

Silverstein BA, Fine LJ, Armstrong TJ. Occupational factors and carpal tunnel syndrome. Am J Ind Med 1987; 11:343-358.

Snedecor GW and Cochran WG. Statistical methods. Iowa State Univ Press, Ames, Iowa. 1989

So YT, Olney RK, Aminoff MJ. Evaluation of the thermography in the diagnosis of selected entrapment neuropathies. Neurology 1989; 39:1-5.

Stock SR. Workplace ergonomic factors and the development of musculoskeletal disorders of the neck and upper limbs: A meta-analysis. Am J Ind Med 1991; 19:87-107.

Stromberg T, Dahlin LB, Brun A, Lundborg G. Structural nerve changes at wrist level in workers exposed to vibration. Occup and Environ Med 1997; 54:307-311.

Terada N, Bjursten LM, Papaloizos M, Lundborg G. Resorbable filament structures as a scaffold for matrix formation and axonal growth in bioartificial nerve grafts: long term observations. Restorative Neurology and Neurosciences 1997; 11:65-69.

Webster BS, Snook SH. The cost of compensable upper extremity cumulative trauma disorders. J Occup Med 1994; 36:713-727.

Wehbe MA, Hunter JM. Flexor tendon gliding in the hand. Part I. *In vivo* excursion. J Hand Surg 1985; 10A:570-4.

Woo SL, Ritter MA, Amiel D, Sanders TM, Gomez MA, Kuei SC, Garfin SR, Akeson WH. The biomechanical and biochemical properties of swine tendons – long term effects of exercise on the digital extensors. Conn Tissue Res 1980; 7:177-183.

Woo SL, Gomez MA, Amiel D, Ritter MA, Gelberman RH, Akeson WH. The effects of exercise on the biomechanical and biochemical properties of swine digital flexor tendons. J Biomech Eng 1981; 103:51-56.

Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature 1990; 346:281-284.

CHAPTER II:

Evidence of tendon microtears due to cyclical loading in an *in vivo* tendinopathy model

2.1 Abstract

Tendon injuries at the epicondyle can occur in athletes and workers whose job functions involve repetitive, high force hand activities but the early pathophysiologic changes of tendon are not well known. The purpose of this study was to evaluate early tendon structural changes, specifically the formation of microtears, caused by cyclical loading. The Flexor Digitorum Profundus (FDP) muscle of 9 New Zealand rabbits was stimulated to contract repetitively for 80h of cumulative loading over 14 weeks. The contralateral limb served as a control. The tendon at the medial epicondyle insertion site was harvested, sectioned, and stained. Microtears were quantified, using image analysis software, in four regions of the tendon, two regions along the enthesis and two distal to the enthesis. The tear density (loaded: 1329 ± 546 tears/mm^2; unloaded: 932 ± 474 tears/mm^2) and mean tear size (loaded: 18.3 ± 6.1 μm^2; unloaded: 14.0 ± 4.8 μm^2) were significantly greater in the loaded limb ($p < 0.0001$) across all regions compared to the unloaded limb. These early microstructural changes in a repetitively loaded tendon may initiate a degenerative process that leads to tendinosis.

2.2 **Introduction**

Tendon injuries due to overuse a common problem in athletes and workers and account for 30 to 50% of all sports-related injuries (11, 12) and almost half of occupational illnesses in the United Sates (29). Epicondylitis, a tendinopathy at elbow, is a common disorder in adults, the incidence in general practice is approximately 4 to 7 per 1000 patients per year with an annual incidence of 1% to 3% in the general population (1, 7). Although epicondylitis is related to forceful and repetitive hand activities, little is known about the early mechanisms of injury that ultimately lead to tendinopathy. Elucidating the early structural, cellular, and molecular changes in the tendons exposed to cyclical loading may ultimately improve prevention and treatment options.

Lateral epicondylitis is an injury of the common extensor tendon at the lateral epicondyle while medial epicondylitis is an injury to the common flexor tendon at the medial epicondyle. Epicondylitis presents as localized pain, tenderness and occasionally swelling (32). Biopsies of the tendon and surrounding scar tissue in patients with epicondylitis reveal fibrovascular and cellular proliferation, intratendinous calcification and cartilage formation, loss of parallel tendon fibers, fibrofatty degeneration and partial tendon rupture (6, 13, 20-22, 25). The absence of inflammatory cells has led some authors to propose the term tendinosis instead of tendonitis (13, 15, 23).

Large tears (on the order of cm^2) have been observed in tendons of humans with tendinosis using high-resolution ultrasound (10),(14), MR imaging (8, 25, 33), and 3D volume-rendered images from multi-detector computer tomography (MDCT) (19). However no animal or human studies have investigated the tendon for smaller structural

defects or microtears, on the scale of 1-500 μm^2, that may occur early following cyclical loading.

The purpose of this study was to evaluate microstructural changes, specifically microtear formation in the FDP tendon at the medial epicondyle following cyclical finger loading using a rabbit model. Rabbits were used because the FDP muscle could be isolated for electrical stimulation using small percutaneous needles. In spite of some anatomical differences, the general structure of the tendon insertion site at the epicondyle, the composition and the biology of tendon healing in rabbits are similar to that in humans (3, 4, 9, 16, 26).

2.3 Methods

2.3a Animal Model

Nine female, young adult, New Zealand White rabbits weighing 3.49 kg (± 0.30) were used. Under general anesthesia, the FDP muscle of one forelimb was electrically stimulated to contract repetitively for 2 hours per day, 3 days a week, for a total of 80 cumulative hours of loading. The contralateral limb, although supported in the same posture as the loaded limb during loading, did not receive a stimulus and served as the control. This study was approved by the University of California, Berkeley's Committee on Animal Research.

After inducing anesthesia with isoflurane, the rabbit was placed in a supine position with the forearms loosely secured to supports (Figure 1). A muscle stimulation needle (33G) was inserted subcutaneously in the middle of both forearms so that the needle barrel was in contact with the surface of the FDP muscle and the needle tip was pushed back through the skin. A lightweight, brass glove was slipped over digit 3 of the

stimulated limb and connected to a load cell by a wire in order to measure the flexion force of the digit due to FDP contraction. The muscle was stimulated (S48 and SIU5, Grass Instruments) with 1Hz pulse trains with train durations of 200ms, pulse widths of 2ms, and pulse rates of 100 pulses/s. The stimulation voltage was adjusted [6-12V] to maintain a mean peak fingertip force of 0.42N (15% of peak tetanic force). The resultant load was selected to be within the physiologic range of the muscle and the number repetitions and the duration of loading are less than that experienced workers who perform repeated tasks (18).

After two hours of cyclical loading, the stimulation electrodes and finger glove were removed, the anesthesia was discontinued, and the rabbit was returned to its cage. This process was repeated 3 days per week for a total of 80 hours of stimulation. Weekly examinations of the paw, forearm and elbow revealed no tenderness, limping, nodules, swelling, limitation in range of motion, reduction in gross claw flexion strength, or skin breaks.

2.3b Tissue and Histological preparation

After 80h of cumulative loading, the animals were weighed (3.89 ±0.19kg) and euthanized. Evaluation of the subcutaneous area at the stimulation needle insertion site revealed minimal scar tissue localized within 5 mm of the needle insertion site; the scar tissue did not extend to the FDP tendon. Both medial epicondyles were dissected free with the FDP tendon and muscle attached, fixed in 10% formalin for 24h, decalcified in EDTA for three weeks, paraffin embedded and sectioned 7μm longitudinally.

Nine serial sections from the center of the tendon block were deparaffinized, rehydrated, stained (Iron Hematoxlin, Safranin-O and Fast Green), dehydrated and cover

slipped. Safranin O and Fast Green staining was used as a contrast to distinguish tears (non-staining regions) from intact tendon tissue. Histological preparation and staining was completed at the same time for tissue from both limbs. Histologists were blinded to specimen loading status.

2.3c Image Acquisition

Four regions of interest (ROI) (Figure 2) were digitally photographed under 200x magnification using Axiovision software v3.1 and an Axioskop2 microscope with an Axiocam digital camera (Carl Zeiss, Germany). Prior to image acquisition, the camera was white balanced to ensure a uniform background color. The microscope's light intensity was maintained at a constant level to ensure the background mean gray values of the images were similar throughout the image acquisition process.

2.3d Image Analysis

The images were cropped to contain only the ROI. The boundaries of all the non-staining areas in the tendon (e.g. tears) were identified using custom software (IMAQ, National Instruments Vision Builder 6) to threshold gray values (Figure 3). Thresholded values were selected based on mean gray values of tears present in each ROI and thresholded images were compared to the original ROI image to ensure all tears were captured in the thresholding process. All tears of sizes 3 to 300μm^2 were quantified, smaller or larger tears could be considered artifacts and were not included in the analysis.

Summary measures of all of the tears for each ROI were calculated (tear area as a percent of tendon area, tear density and mean tear size). The distribution of tear sizes was determined by sorting tears from 3-10μm^2 in size into intervals of 1μm^2, 3-100μm^2

in size into intervals of $10\mu m^2$, and 3-$300\mu m^2$ in size into intervals of $100\mu m^2$. Image acquisition and analysis was performed blinded to specimen loading status.

2.3e Statistical Analysis

A mixed model repeated measures ANOVA was used to analyze differences in tear measures by region (inner enthesis, outer enthesis, inner distal or outer distal) and by limb loading status (loaded or unloaded). Post hoc analysis was performed using the Tukey method for multiple comparisons. The distribution of tears by tear size were transformed into normal distributions using a log transformation preceded by the addition of the smallest value to each data point to avoid taking the logarithm of a zero, then the transformed tear density was compared between loaded and unloaded limbs with the paired t-test with $\alpha \leq 0.01$ to adjust for multiple comparisons.

2.4 Results

Across the four ROIs, the mean tear area as a percent of total tendon area ranged from 0.8% to 4.5% in the loaded tendon compared to 0.4% to 3.1% in the unloaded tendon (Figure 4A). The limb by region interaction term in the ANOVA was significant ($p < 0.007$). Using the Tukey follow-up test, significant differences between regions and exposure status were found. The same letter indicates significant differences between regions and is shown in figure 4A. The loaded limb had a higher percent of tear area than the unloaded limb in the outer regions of the tendon, both at the enthesis ($p < 0.0001$) and distal to the enthesis ($p = 0.001$). In contrast to this finding, the inner regions of the tendon, at the enthesis ($p = 0.85$) and distal to the enthesis ($p = 0.40$), did not have significantly larger tear area percents in the loaded limbs when compared to the unloaded limbs.

Mean tear density ranged from 650 to 1788 tears/mm^2 in the loaded tendon and 358 to 1491 tears/mm^2 in the unloaded tendon across the four regions of interest (Figure 4B). The limb by region interaction term was not significant ($p = 0.22$). Loaded limbs had significantly greater microtear densities than the unloaded limbs ($p < 0.0001$), regardless of region. Based on the Tukey follow-up tests, there were regional variations; the inner enthesis region had a significantly lower microtear density than the other three regions ($p < 0.003$). The inner distal region had a significantly lower microtear density than both the outer enthesis ($p = 0.003$) and the outer distal region ($p < 0.0001$).

The mean tear sizes ranged from 13 to 26μm^2 in the loaded tendon and from 9 to 21μm^2 in the unloaded tendon, across the four regions of interest (Figure 4C). Limb by region interaction term was not significant ($p = 0.27$); the loaded limbs had significantly larger tears ($p < 0.0001$), regardless of region. There were also significant regional differences based on the Tukey follow-up tests. The outer region distal to the enthesis had significantly larger mean tear sizes than the other regions ($p < 0.02$). The outer enthesis had significantly larger tears than the inner enthesis ($p = 0.0001$) and the inner distal region ($p = 0.019$).

The distribution of tears by size varied by region and loading status. Across all tear sizes the tear density is higher in the loaded tendon than the unloaded tendon (Figure 5 and 6). At the enthesis, significant differences were observed primarily in the outer region, almost evenly distributed across tear sizes. Distal to the enthesis, significant differences were observed in the outer region, primarily in the larger tear sizes.

2.5 Discussion

This is the first study to examine tendons for microtear (3-300μm^2) formation in response to cyclical loading in an *in vivo* animal model. All three measures of tear, tear area as a percent of tendon area, tear density and mean tear size, were significantly greater in the cyclically loaded tendon compared to the unloaded tendon. In addition, there were variations in tear measures by region. The outer regions of the tendon, both at the enthesis and distal to the enthesis, had a greater tear density and a larger mean tear size than the inner regions. The observed regional differences in tear distribution may be due to differences in stress distributions in the tendon. As the FDP tendon is loaded, the region adjacent to bone (inner enthesis) experiences compression as well as tension resulting in fibrocartilage formation (16, 30). Fibrocartilage has different mechanical and biological properties that allow it to absorb compressive stresses (31). Therefore the inner and outer enthesis are structurally different and may have different modes of failure under repetitive loads.

Wakabayashi et. al (31) used finite element analysis to estimate the stress distribution in the supraspinatus tendon, which attaches to bone in a similar arrangement as the FDP attaches to the medial epicondyle. The stresses in the tendon are not uniformly distributed throughout a loaded tendon and this differential stress distribution is likely to be present in the FDP tendon and may explain the increase in tear density in the outer enthesis and mean tear size in the different regions of the FDP rabbit tendon in our animal model.

Previous *in vivo* cyclical tendon loading studies offer varying findings. Backman et al. (4) used rabbits in a chronic Achilles tendinosis model (N = 13, 30 to 36 hours of

cumulative loading) and found changes in the paratenon and tendon, most notably tendon fibrillation and an increased number of inflammatory cells and blood vessels after repetitive eccentric exercise. The semiquantitative results showed changes to the entire tendon and paratenon but did not focus on specific areas within the tendon, such as near the tendon-bone junction or the tendon-muscle interface. Archambault et al. (3) also used a rabbit to model Achilles tendinosis (N = 4, 66h of cumulative loading) but found no changes in tendon histology in terms of degeneration or density of inflammatory cells but some suggestion of an increase in mRNA expression of collagen III and IL-1β and decrease in expression of IGF-II. Backman et al. used a loading frequency of 2.5Hz, which was twice that used in Archambault's study. The loading frequency was decreased in the Archambault study because it was considered a slow hopping rate for rabbits and within physiological limits. Mean tendon force was 26N in the Archambault study; tendon load data was not available for the Bachman study. These studies did not evaluate the tendon for microtears.

Other investigators studying the effect of overuse injuries in human biopsy specimens and animal loading models have reported a disruption in collagen fiber organization, tendon fibrillation and tendon thickening (4, 5, 17, 24, 27). These structural effects may alter the tissue's gross mechanical properties. Soslowsky et. al (24) found a decreased maximum tensile load on loaded tendons in their study after 20h of cumulative loading in rats undergoing a repetitive exercise protocol that consisted of treadmill running. At longer loading periods, they reported larger cross-sectional tendon areas, decreased moduli, smaller allowable maximum stresses, increased cellularity, collagen disorganization, and changes in cell morphology in a loaded tendon compared to

nonloaded cage control rats. Barbe et. al (5) reported tendon fibrillation and an infiltration of macrophages in their rat tendinosis model after 18h of cumulative repetitive loading that included rats reaching for food. Although the animals had a preferential limb to use for the task, they did not compare these results to the nonloaded limbs of the same animals, but rather to cage controls. Macrophages are known to release pro-inflammatory cytokines and metalloproteinases following repetitive loading injury of tendon fibroblasts and these may induce the degradation the collagen matrix (2, 28). Matrix degradation may lead to mechanical instability and the formation of microtears. Some weaknesses of the aforementioned studies include a lack of characterization and control of the biomechanical loads.

Limitations of this study should be considered. First, the tissue preparation includes formalin fixation and paraffin-embedding, which require heat treatment and dehydration of the tissue, both of which may disrupt tissue architecture and could cause tendon fibers to separate. While FDP tendons of both limbs may experience outside loads during normal animal activity, the high microtear densities of both the unloaded and loaded limbs may have been amplified by the histological preparation. However, the tissues were prepared and analyzed simultaneously, histology technicians were blinded to limb exposure, and the loaded tendon was compared to the matched, non-loaded limb of the same animal. Therefore, there may be disruptions in tissue architecture caused by the histology preparation method, but the differences observed between limbs are due to the effects of loading. Generalizing from the rabbit to the human should be done with caution. In rabbits, the flexor muscles (FDP and FDS) originate at the medial epicondyle but in humans, the FDS originates at the medial epicondyle while the FDP originates

along the proximal ulna close to the medial epicondyle. However, the tendon biochemical composition, healing processes, and mechanical properties of rabbit tendon are similar to human (3, 4, 9, 16, 26).

The pathophysiology of tendinopathy due to overuse likely involves an accumulation of microstructural damage, generated by mechanical fiber failure and/or biological mediators, that exceeds the healing capabilities of the tendon. One possible degenerative pathway may involve microtear formation that may induce tendon cells to release matrix metalloproteinases or cytokines, which may further degrade the tendon's matrix either directly or indirectly by initiating a degradation pathway that may lead to the formation of additional microtears.

This in vivo animal model demonstrates that all three measures of microtears, tear area as a percent of tendon area, tear density and mean tear size, are increased in tendons cyclically loaded at physiological loads for 80 cumulative hours. In addition, microtear density and size are greater along the outer regions of both loaded and unloaded tendon. Regions with large microtears or increased density of tears are likely to be the nexus of tendinosis and ultimately undergo the changes typical of epicondylosis such as degenerative changes with new capillary formation and fibrillation.

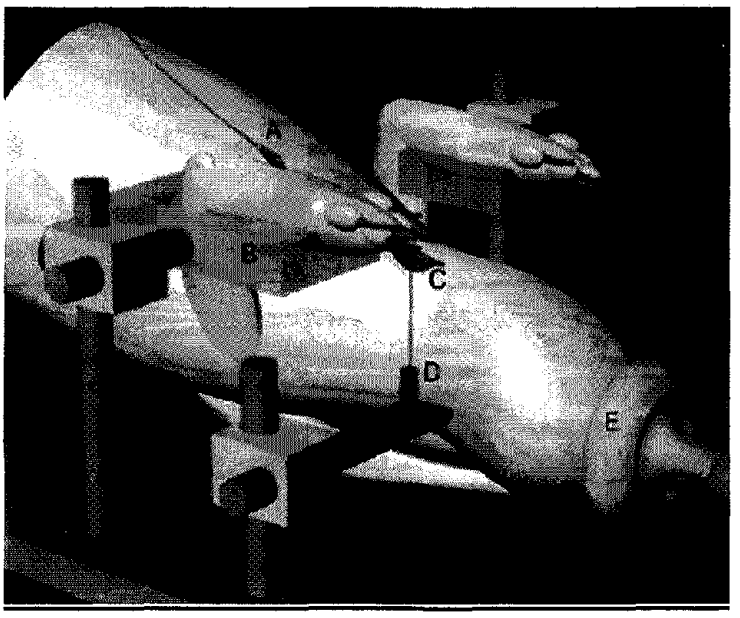

Figure 2.1. Cartoon of the loading apparatus with rabbit in a supine position with head to the right and forearms supported. A) stimulation needle, B) forearm support, C) third digit with metal glove, D) load cell, E) anesthesia mask.

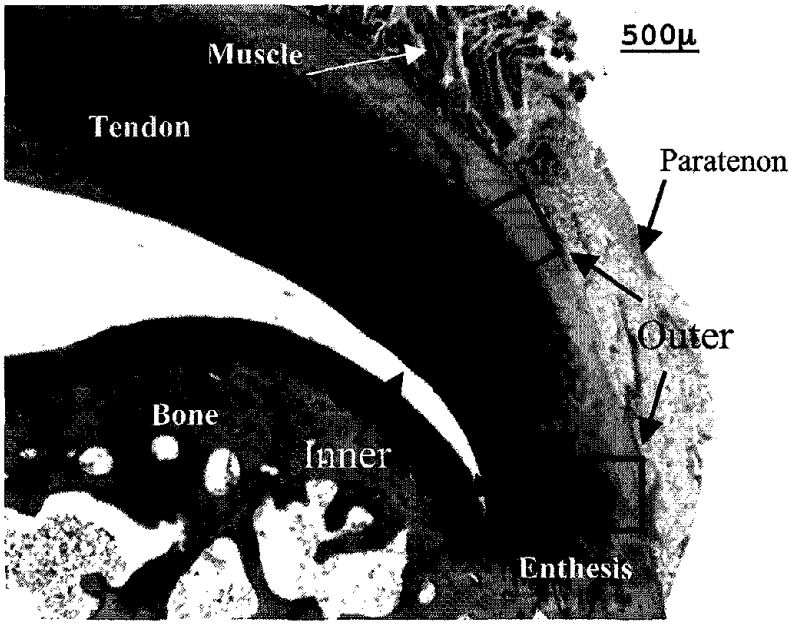

Figure 2.2. Epicondyle with bone, tendon, paratenon, and muscle. Four regions of interest are highlighted, two along the enthesis, and two 1500µm distal to the enthesis. The regions of interest are 200µm by 400µm.

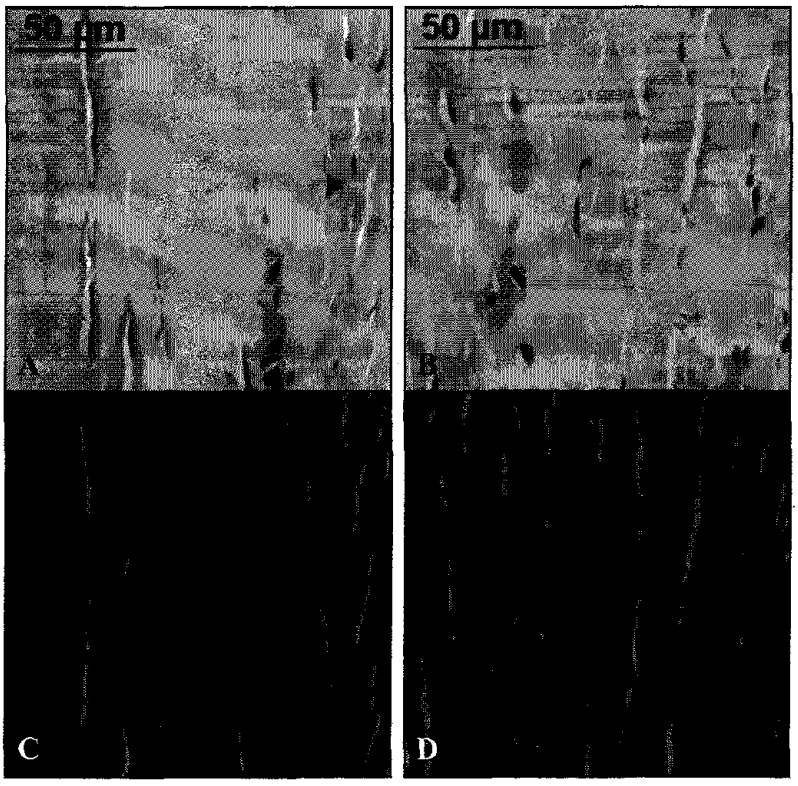

Figure 2.3. Unloaded (A) and loaded (B) tendon stained with Iron Hemotoxlin, Safranin O and Fast green. Thresholded images in same unloaded (C) and loaded (D) tendon identify microtears as red regions.

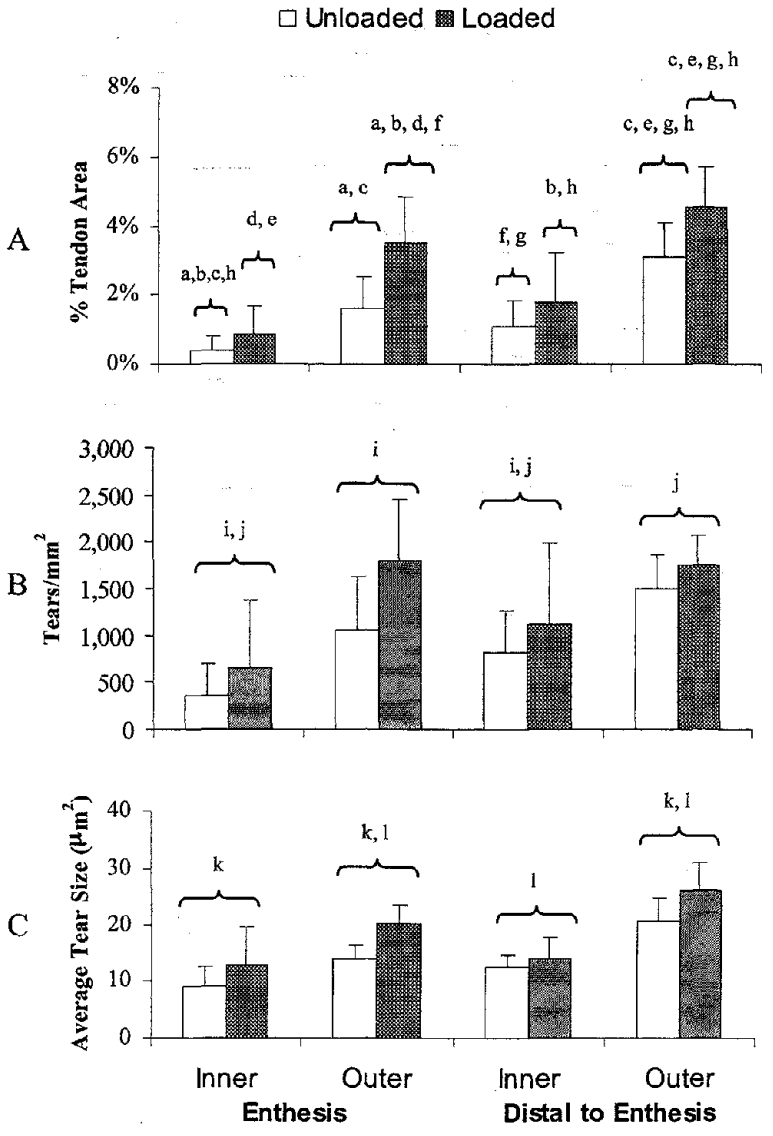

Figure 2.4. The tear area as a percent of tendon area (A) has a significant limb by region interaction term, whereas the interaction term for the tear density (B) and average tear size (C) was not significant. These endpoints were significantly higher in the loaded limbs compared to unloaded limbs regardless of region. The same letter indicates significant differences between regions, based on the Tukey follow-up test. Columns are mean ± s.d.

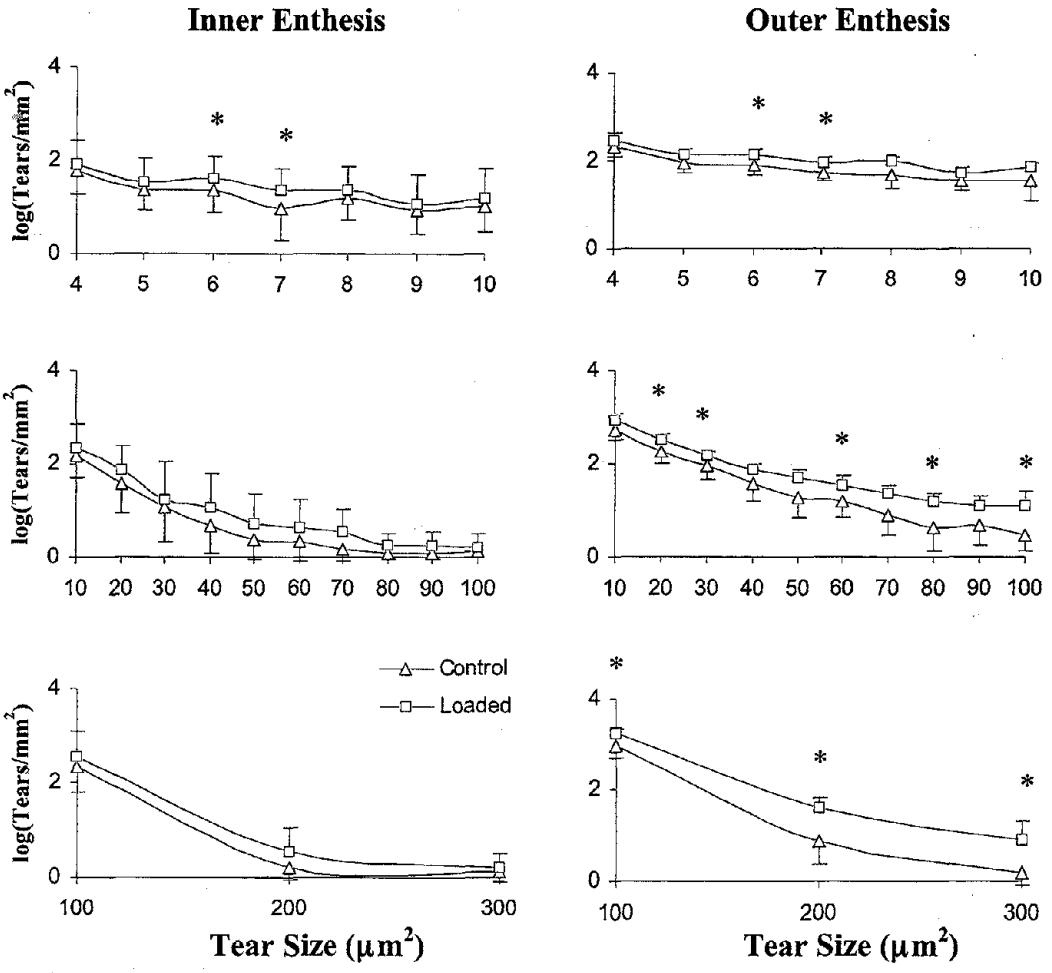

Figure 2.5. The log of the distribution of microtears by tear size is shown (± s.d.) for the inner and outer regions of the tendon along the enthesis (n = 9). Significant differences between unloaded and loaded limbs for each microtear size are indicated with an * (paired t-test, $p < 0.01$).

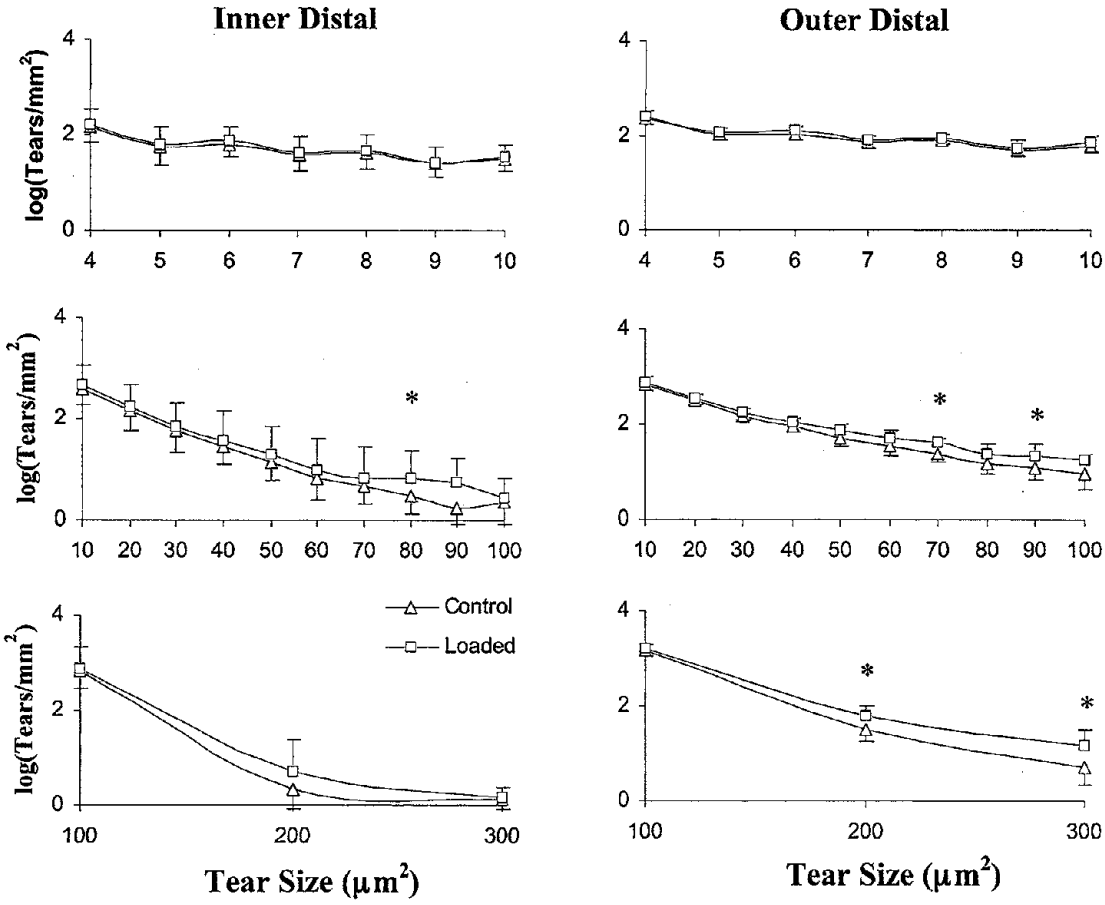

Figure 2.6. The log of the distribution of microtears by size is shown (± s.d.) for the inner and outer parts of the tendon distal to the enthesis (n = 9). Significant differences between unloaded and loaded limbs for each microtear size are indicated with an * (paired t-test, $p < 0.01$).

REFERENCES

1. Allander E: Prevalence, incidence, and remission rates of some common rheumatic diseases or syndromes. *Scand J Rheumatol* 3:145-53, 1974
2. Almekinders LC, Banes AJ, Ballenger CA: Effects of repetitive motion on human fibroblasts. *Med Sci Sports Exerc* 25:603-7, 1993
3. Archambault JM, Hart DA, Herzog W: Response of rabbit Achilles tendon to chronic repetitive loading. *Connect Tissue Res* 42:13-23, 2001
4. Backman C, Boquist L, Friden J, Lorentzon R, Toolanen G: Chronic achilles paratenonitis with tendinosis: an experimental model in the rabbit. *J Orthop Res* 8:541-7, 1990
5. Barbe MF, Barr AE, Gorzelany I, Amin M, Gaughan JP, Safadi FF: Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *J Orthop Res* 21:167-76, 2003
6. Chard MD, Cawston TE, Riley GP, Gresham GA, Hazleman BL: Rotator cuff degeneration and lateral epicondylitis: a comparative histological study. *Ann Rheum Dis* 53:30-4, 1994
7. Chard MD, Hazleman BL: Tennis elbow--a reappraisal. *Br J Rheumatol* 28:186-90, 1989
8. Cvitanic O, Henzie G, Skezas N, Lyons J, Minter J: MRI diagnosis of tears of the hip abductor tendons (gluteus medius and gluteus minimus). *AJR Am J Roentgenol* 182:137-43, 2004
9. Enwemeka CS: Inflammation, cellularity, and fibrillogenesis in regenerating tendon: implications for tendon rehabilitation. *Phys Ther* 69:816-25, 1989
10. Gibbon WW, Cooper JR, Radcliffe GS: Sonographic incidence of tendon microtears in athletes with chronic Achilles tendinosis. *Br J Sports Med* 33:129-30, 1999
11. Jozsa L, Kannus P: Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 7:113-8, 1997
12. Kannus P: Tendons--a source of major concern in competitive and recreational athletes. *Scand J Med Sci Sports* 7:53-4, 1997
13. Kraushaar BS, Nirschl RP: Tendinosis of the elbow (tennis elbow). Clinical features and findings of histological, immunohistochemical, and electron microscopy studies. *J Bone Joint Surg Am* 81:259-78, 1999
14. La S, Fessell DP, Femino JE, Jacobson JA, Jamadar D, Hayes C: Sonography of partial-thickness quadriceps tendon tears with surgical correlation. *J Ultrasound Med* 22:1323-9; quiz 1330-1, 2003
15. Maffulli N, Khan KM, Puddu G: Overuse tendon conditions: time to change a confusing terminology. *Arthroscopy* 14:840-3, 1998
16. Malaviya P, Butler DL, Boivin GP, Smith FN, Barry FP, Murphy JM, Vogel KG: An in vivo model for load-modulated remodeling in the rabbit flexor tendon. *J Orthop Res* 18:116-25, 2000
17. Messner K, Wei Y, Andersson B, Gillquist J, Rasanen T: Rat model of Achilles tendon disorder. A pilot study. *Cells Tissues Organs* 165:30-9, 1999

18. National Research Council (U.S.). Panel on Musculoskeletal Disorders and the Workplace., Institute of Medicine (U.S.): *Musculoskeletal disorders and the workplace : low back and upper extremities,* pp xv, 492. Washington, D.C., National Academy Press, 2001
19. Ohashi K, El-Khoury GY, Bennett DL: MDCT of tendon abnormalities using volume-rendered images. *AJR Am J Roentgenol* 182:161-5, 2004
20. Pfahler M, Jessel C, Steinborn M, Refior HJ: Magnetic resonance imaging in lateral epicondylitis of the elbow. *Arch Orthop Trauma Surg* 118:121-5, 1998
21. Potter HG, Hannafin JA, Morwessel RM, DiCarlo EF, O'Brien SJ, Altchek DW: Lateral epicondylitis: correlation of MR imaging, surgical, and histopathologic findings. *Radiology* 196:43-6, 1995
22. Regan W, Wold LE, Coonrad R, Morrey BF: Microscopic histopathology of chronic refractory lateral epicondylitis. *Am J Sports Med* 20:746-9, 1992
23. Soslowsky LJ, Thomopoulos S, Esmail A, Flanagan CL, Iannotti JP, Williamson JD, 3rd, Carpenter JE: Rotator cuff tendinosis in an animal model: role of extrinsic and overuse factors. *Ann Biomed Eng* 30:1057-63, 2002
24. Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter JE: Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *J Shoulder Elbow Surg* 9:79-84, 2000
25. Steinborn M, Heuck A, Jessel C, Bonel H, Reiser M: Magnetic resonance imaging of lateral epicondylitis of the elbow with a 0.2-T dedicated system. *Eur Radiol* 9:1376-80, 1999
26. Stone D, Green C, Rao U, Aizawa H, Yamaji T, Niyibizi C, Carlin G, Woo SL: Cytokine-induced tendinitis: a preliminary study in rabbits. *J Orthop Res* 17:168-77, 1999
27. Tallon C, Maffulli N, Ewen SW: Ruptured Achilles tendons are significantly more degenerated than tendinopathic tendons. *Med Sci Sports Exerc* 33:1983-90, 2001
28. Tsuzaki M, Guyton G, Garrett W, Archambault JM, Herzog W, Almekinders L, Bynum D, Yang X, Banes AJ: IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. *J Orthop Res* 21:256-64, 2003
29. United States. Bureau of Labor Statistics.: *Occupational injuries and illnesses--counts, rates, and characteristics,* p v. Washington, DC, The Bureau : For sale by the U.S. G.P.O. Supt. of Docs., 1995
30. Waggett AD, Ralphs JR, Kwan AP, Woodnutt D, Benjamin M: Characterization of collagens and proteoglycans at the insertion of the human Achilles tendon. *Matrix Biol* 16:457-70, 1998
31. Wakabayashi I, Itoi E, Sano H, Shibuya Y, Sashi R, Minagawa H, Kobayashi M: Mechanical environment of the supraspinatus tendon: a two-dimensional finite element model analysis. *J Shoulder Elbow Surg* 12:612-7, 2003
32. Wetzel BJ, Nindl G, Swez JA, Johnson MT: Quantitative characterization of rat tendinitis to evaluate the efficacy of therapeutic interventions. *Biomed Sci Instrum* 38:157-62, 2002

33. Yu JS, Popp JE, Kaeding CC, Lucas J: Correlation of MR imaging and pathologic findings in athletes undergoing surgery for chronic patellar tendinitis. *AJR Am J Roentgenol* 165:115-8, 1995

CHAPTER III:

VEGF, VEGFR-1 and CTGF cell densities in tendon are increased with cyclical loading: An *in vivo* tendinopathy model

3.1 Abstract

Tendon injuries can occur in athletes and workers whose tasks involve repetitive, high force hand activities, but the early pathophysiologic processes of tendinopathy are not well known. The purpose of this animal study was to evaluate the effects of cyclical tendon loading on the densities of cells producing growth factors such as vascular endothelial growth factor (VEGF), its receptor, vascular endothelial growth factor receptor 1 (VEGFR-1) and connective tissue growth factor (CTGF) in the Flexor Digitorum Profundus (FDP) tendon at the epicondyle. The FDP muscle of 9 New Zealand rabbits was electrically stimulated to contract repetitively for 80h of cumulative loading over 14 weeks. The contralateral limb served as a control. The tendon at the medial epicondyle insertion site was harvested and sections were immunostained with antibodies directed against VEGF, VEGFR-1 or CTGF. Positive-staining cells were counted in six regions of interest: three along the enthesis and three corresponding regions 1500μm distal to the enthesis. VEGF ($p = 0.0001$), VEGFR-1 ($p = 0.046$) and CTGF ($p = 0.0001$) cell densities were increased in the tendon of the loaded limb as

compared to the nonloaded limb. In addition, there were regional differences in VEGF, VEGFR-1 and CTGF cell densities. VEGF, VEGFR-1 and CTGF are increased in tendon experiencing cyclical loading and may play a role in the early vascular changes in the progression to tendinosis.

3.2 Introduction

Tendon injuries due to overuse are a common problem in athletes and workers and account for 30 to 50% of all sports-related injuries (20, 21) and almost half of the occupational illnesses in the United Sates (52). Epicondylitis, a tendinopathy at the elbow, is a common disorder in adults, the incidence in general practice is approximately 4 to 7 per 1000 patients per year with an annual incidence of 1% to 3% in the general population (2, 14). Although epicondylitis is related to forceful and repetitive hand activities, little is known about the early mechanisms of injury that ultimately lead to tendinopathy. Identifying the initial biological changes in tendons exposed to cyclical loading may ultimately improve prevention and treatment options and further expand our understanding of the etiology of tendinosis and its pathogenesis.

Epicondylitis presents as localized pain, tenderness and occasionally swelling (54). Biopsies of the tendon and surrounding scar tissue in patients with epicondylitis reveal fibrovascular and cellular proliferation, intratendinous calcification and cartilage formation, loss of parallel tendon fibers, fibrofatty degeneration, partial tendon rupture, and the formation of capillary buds (13, 23, 38, 39, 41, 48). The absence of inflammatory cells has led some authors to propose the term tendinosis instead of tendonitis (23, 27, 46).

Vascular Endothelial Growth Factor (VEGF), also known as Vascular Permeability Factor (VPF), is one of the most important angiogenic components of tissue healing. VEGF has been found in human biopsies of degenerated tendons, e.g. Achilles (1, 36, 40), and in cyclically strained fibroblast cell cultures (37) indicating that it may play a role in overuse injuries leading to tendon degeneration. VEGF stimulates the

proliferation of microvascular endothelial cells, inducing angiogenesis and rendering the microvasculature hyperpermeable (17, 45). In the tendon, expression of VEGF can be up-regulated by both mechanical, e.g. cyclic strain (37), and biochemical stimuli, e.g. hypoxia (35) and the presence of other growth factors (12, 16, 35). Recent studies have shown that in an acutely injured tendon, the highest concentrations of VEGF occur after inflammation when it acts as a potent stimulator of angiogenesis (10). The growth of new blood vessels towards the repair site from within the healing tendon appears necessary for healing to occur.

Several receptors for VEGF play important roles in pathological conditions involving angiogenesis. VEGFR-1, also known as Flt-1, and VEGFR-2, also known as Flk-1/KDR, are tyrosine kinase receptors for VEGF. VEGFR-1 has the highest affinity for VEGF165, one of the several isoforms of VEGF, with a dissociation constant (K_d) of approximately 10-20 pM (15). VEGFR-2 has a lower affinity for VEGF, with a K_d of approximately 75-125 pM (49). VEGFR-1 and VEGFR-2 have been observed in ruptured human Achilles tendons, but not in healthy adult tendons (36). . VEGFR-1 expression has been shown to be up-regulated during angiogenesis and hypoxic conditions, while VEGFR-2 is not (19).

Connective Tissue Growth Factor (CTGF) has recently been investigated in wound healing and scar formation studies. CTGF is increased in the synovial sheaths of rats trained to do repetitive reaching (7) but its role in tendon pathophysiology has not be well characterized. CTGF is a cysteine-rich secretory protein and belongs to the CCN family, which consists of six distinct members, CYR61, CTGF and NOV ('CCN') and the Wnt-induced secreted proteins-1, 2 and 3 (11). The members of this group are

known to be involved in many fundamental biological processes such as cell proliferation (18), attachment (55), migration (11), differentiation (31), wound healing (9, 25, 26), matrix production (18) and angiogenesis (4, 12) as well as in the development of several pathologic conditions including fibrosis and tumorigenesis (24). The role CTGF plays in tendon repair or degeneration is not yet known, but may involve stimulating angiogenesis and matrix production (18, 25). Its interaction with VEGF has not been investigated in the tendon; however, like VEGF, CTGF is known to increase in fibroblasts with mechanical loading (42, 43). Identifying the role or roles CTGF plays in tendon overuse injuries is important in understanding the underlying mechanisms involved in tendinopathy.

Clarifying the cellular and molecular pathways that occur during early periods of cyclical loading may lead to a better understanding of mechanisms associated with tendon injury and remodeling. A rabbit model of epicondylosis was used in which the Flexor Digitorum Profundus (FDP) muscle is repeatedly stimulated against a load (29). The purpose of this study was to evaluate the regional variation of cells producing VEGF, VEGFR-1 and CTGF in the FDP tendon at the epicondyle in response to cyclical loading. We hypothesize that our *in vivo* loading model will increase the number of cells producing the aforementioned growth factors in the loaded limbs compared to nonloaded limbs of the same animal. The presence of these growth factors may play a significant role in the beginning phases of tendinosis.

3.3 Methods

3.3a Animal Model

The animal loading model was described previously to establish microtear formation in a cyclically loaded tendon (29). To summarize, nine female, young adult, New Zealand White rabbits weighing 3.49 kg (± 0.30) were used. Under general anesthesia, the FDP muscle of one forelimb was electrically stimulated (Figure 1) to contract repetitively for 2 hours per day, 3 days a week, for 80 hours of cumulative loading. The stimulation train was adjusted to maintain a mean peak digit flexion force of 0.42N (15% of peak tetanic force). The contralateral limb, although supported in the same posture as the loaded limb during loading, did not receive a stimulus and served as the control. This study was approved by the University of California, Berkeley's Committee on Animal Research. Weekly examinations of the paw, forearm and elbow revealed no tenderness, limping, nodules, swelling, limitation in range of motion, reduction in gross claw flexion strength, or skin breaks.

3.3b Tissue and Histological preparation

After 80h of cumulative loading, animals were weighed (3.89 ±0.19kg) and euthanized. Evaluation of the subcutaneous area at the stimulation needle insertion site revealed minimal scar tissue localized within 5 mm of the insertion site; the scar tissue did not extend to the FDP tendon. Both medial epicondyles were dissected with the FDP tendon and muscle attached, fixed in 10% formalin for 24h, decalcified in EDTA for three weeks, paraffin embedded and sectioned 7μm longitudinally.

Nine serial sections from the center of the tendon block were deparaffinized and rehydrated. Sections to be stained for VEGF and VEGFR-1 were pre-treated with a hyaluronidase (600 units/ml, Sigma-Aldrich) and sections to be stained for CTGF were

pre-treated with trypsin (No. 00-3008, Zymed Laboratories), for 10 minutes at 37°C. Samples were then treated with a 1% H_2O_2 in a phosphate buffered solution (pH = 7.4) for 15 minutes to block endogenous peroxidase activity. Tissue sections were blocked with normal horse serum for 45 minutes at room temperature then incubated for 1 hour with a mouse monoclonal antibody directed against VEGF (2μg/ml) (No 350-P0, NeoMarkers Fremont, CA), VEGFR-1 (15μg/ml) (No MAB321, R&D Systems Minneapolis, MN) or CTGF (15μg/ml) (No MAB660, R&D systems, Minneapolis, MN). Sections were then incubated with a biotinylated horse anti-mouse 2° antibody (Vector Laboratories) at room temperature for 30 minutes. Sections were stained with the Vectastain ABC system, and developed with 3, 3'-diaminobenzidine (DAB), then dehydrated and coverslipped.

3.3c Image Acquisition

Six regions of interest (ROI) were digitally photographed at 200x magnification using an Axiocam digital camera and Axiovision software v3.1 (Carl Zeiss, Germany). Prior to image acquisition, the camera was white balanced to ensure a uniform background color. The microscope's light intensity was maintained at a constant level to ensure the background mean gray values of the images were similar throughout the image acquisition process. The six ROIs (Figure 2) include the three areas along the enthesis distinguished by a tidemark (classified as inner, center and outer) and three corresponding areas 1500μm distal to the enthesis. The inner area is that part nearest the bone. Positive staining cells were manually counted in each region (200x400μm^2) and normalized by the area observed to calculate density. Tissue and histological preparation

and cell counting was completed at the same time for tissues from both limbs and was performed blinded to limb loading status.

3.3d Statistical Analysis

A mixed model repeated measures ANOVA was used to analyze differences in cell density by region (6 regions) and by limb loading status (loaded or unloaded). Post hoc analysis was performed using the Tukey method for multiple comparisons.

3.4 Results

3.4a VEGF cell density

Across the six ROIs, the density of VEGF (Figure 3AB) labeled cells ranged from 372 to 774 cells/mm^2 in the unloaded tendon and from 539 to 1011 cells/mm^2 in the loaded tendon (Figure 4). The limb by region interaction term in the repeated measures ANOVA was not significant ($p = 0.99$). Loaded limbs had significantly greater VEGF-staining cell densities than the unloaded limbs ($p = 0.0001$), across all regions. Based on the Tukey follow-up tests, there were also significant regional differences. The outer regions of the tendon, both at the enthesis and distal to the enthesis, had significantly higher VEGF cell densities than the other four regions (Figure 4).

3.4b VEGFR-1 cell density

Across the six ROIs, the density of VEGFR-1 (Figure 3CD) staining cells ranged from 440 to 611 cells/mm^2 in the unloaded tendon and 514 to 744 cells/mm^2 in the loaded tendon (Figure 4). The limb by region interaction term in the repeated measures ANOVA was not significant ($p = 0.87$). Loaded limbs had significantly greater VEGFR-1 staining cell densities than the unloaded limbs ($p = 0.046$), across all regions. Based on the Tukey follow-up tests, there were also significant regional differences. The outer

region of the tendon at the enthesis had a significantly greater VEGFR-1 cell density than the inner ($p = 0.019$) region distal to the enthesis (Figure 4).

3.4c CTGF cell density

Across the six ROIs, the density of CTGF (Figure 3EF) staining cells varied less by region (Figure 4) and ranged from 397 to 570 cells/mm^2 in the unloaded tendon and from 584 to 778 cells/mm^2 in the loaded tendon. The limb by region interaction term in the ANOVA was not significant ($p = 0.48$). The density of CTGF-staining cells was significantly greater in the loaded tendon than the unloaded tendon ($p < 0.0001$), across all regions. Based on the Tukey follow-up tests, there were regional differences. Both the inner ($p = 0.02$) and outer ($p = 0.008$) regions of the tendon at the enthesis had significantly greater CTGF cell densities than the outer region of the tendon distal to the enthesis (Figure 4).

3.5 Discussion

Studies (8, 50) have shown that VEGF is involved in the tendon's healing response in acute tendon injuries. This is the first study to regionally quantify VEGF, VEGFR-1 and CTGF-staining cells using an *in vivo* overuse animal model. The densities of VEGF, VEGFR-1 and CTGF staining cells are increased in the rabbit flexor tendon at the epicondyle as cyclical loads are applied *in vivo* for a total of 80 hours over a period of 14 weeks. Regional variations were also present and mainly occurred between the outer region at the enthesis and other regions in the tendon. The highest cell density occurred at the outer region at the enthesis in the loaded tendon for all three proteins.

The cell densities of VEGF and VEGFR-1 are inhomogeneously distributed in the tendon, having a tendency to be lower at the inner and center regions, both at the enthesis

and distal to the enthesis, and higher at the outer regions. This inhomogeneous distribution is similar in both the loaded and unloaded tendon. At this type of tendon bone junction there is a differential stress or strain distribution throughout the loaded tendon (53). Cyclic strains up-regulate VEGF synthesis in tendon fibroblast cell cultures (37) while hydrostatic pressure inhibits VEGF production in cultured tendon cells (33). The greater compressive forces experienced in the inner region of the tendon may inhibit VEGF production, while the higher strains experienced in the outer region may lead to increased VEGF production.

The densities of CTGF staining cells were also increased (25% to 70%) in the loaded tendon in comparison to the unloaded tendon regardless of region. However, the regional distribution of CTGF staining cells varied less than that of VEGF staining cells. The highest concentration of CTGF cells was in the outer region of the loaded tendon at the enthesis, while the lowest concentrations were along the outer region distal to the enthesis. Recent studies have demonstrated a pronounced up-regulation of CTGF expression in fibroblasts by contractile mechanical stresses (42, 43) a finding which may partially explain the elevated number of CTGF staining cells at the inner regions, where compressive loads dominate (53). No studies have examined the effect of cyclical loading on CTGF expression in tendons where both compressive and tensile loads are present. The results presented here may indicate that both compressive and tensile stresses play a role in CTGF up-regulation in tendons exposed to cyclical loads.

These changes in VEGF, VEGFR-1 and CTGF are similar to changes in microtear density that we previous reported in this model (29). We found increased microtear densities with loading, plus the microtear density was greater at the outer region at the

enthesis compared to other regions. We have not examined earlier time points but the regional overlap in findings suggests that either the microtears alter local tissue stress patterns and signal cells to express these growth factors or that the growth factors lead to regional alterations in tendon structure and these regions become more susceptible to structural damage. VEGF is increased in response to an acute injury and plays an important role in healing (8, 50). It is likely that the time course and levels of expression of VEGF are different for the acute tendon injury than the injury due to overuse. The prolonged elevation of VEGF with overuse may be involved in a process leading to degeneration.

Previous *in vivo* cyclical tendon loading studies offer varying findings. Backman et al. (5) loaded rabbit Achilles tendon with repetitive eccentric exercise (30 to 36 hours of cumulative loading) and found fibrillation and an increased number of inflammatory cells and blood vessels in the tendon and paratenon. The semiquantitative results showed changes to the entire tendon and paratenon but did not focus on specific areas within the tendon, such as near the tendon-bone junction or the tendon-muscle interface. Archambault et al. (3) also used a rabbit to model Achilles tendinosis (N = 4, 66h of cumulative loading) but found no changes in degeneration or density of inflammatory cells but some suggestion of an increase in mRNA expression of collagen III and IL-1β and decrease in expression of IGF-II.

Other overuse injury animal models have demonstrated an increase of VEGF (32, 40) and CTGF (7) with loading. In the rat supraspinatus tendon Perry et al. (32) reported elevated VEGF mRNA expression after 3 days of treadmill running. These levels dropped at 1 week, only to increase at later time points. The regional variation of VEGF

was not examined in this study. The same model demonstrated decreased maximum tensile load in loaded tendons after 20h of cumulative loading (47)and after longer loading periods, larger cross-sectional tendon areas, decreased moduli, smaller allowable maximum stresses, increased cellularity, collagen disorganization, and changes in cell morphology in a loaded tendon compared to non-exercised cage control rats. Barbe et al. (6) reported tendon fibrillation and an infiltration of macrophages in their rat tendinosis model after 18h of cumulative repetitive loading that involved rats reaching for food. Although the animals had a preferential limb to use for the task, they did not compare these results to the nonloaded limbs of the same animals, but rather to cage controls. Some weaknesses of the aforementioned studies include a lack of characterization and control of the biomechanical loads.

The biological activity of VEGF is mediated by binding to and being activated by its receptors. Activated VEGF in human umbilical vein endothelial cells (HUVEC) can lead to an induction of interstitial collagenase (51). If the mechanism in tenocytes is similar this pathway may lead to the modification of the mechanical properties of tendon.

Expression of VEGF and its receptors, VEGFR-1 and VEGFR-2, were recently shown to be present in degenerative Achilles and fetal tendons but not in normal adult tendon (33, 34, 36, 40). Petersen et al. (38) found mRNA and protein expression of the VEGF receptors in injured Achilles tendons at the site of rupture; other sites were not investigated. In our model, the highest cell densities for VEGF and VEGFR-1 were found at the outer region at the enthesis, which may indicate that this region is more susceptible to damage.

Other investigators have reported increased number of capillaries, infiltrates of inflammatory cells, and edema (5, 6, 28) after just 15h of repetitive loading (5 weeks) in the rabbit and rat. These changes were not observed in our model, but the loading pattern used in our experiment differs from that used by other researchers and may not be adequate to cause changes at earlier time points as in the other studies. In our model, the force and loading frequency were selected to be within a range that may be experienced by workers and athletes (30). Backman et al. (5) , for example, used a loading frequency of 2.5Hz, which is considered a fast hopping rate for rabbits. Archambault et al. (3) used a loading frequency which was half that used in Backman's study. The loading frequency was decreased in the Archambault study because it was considered a slow hopping rate for rabbits and within physiological limits. Mechanical stimulation is important for cell survival and growth as well as various tissue-specific functions (22, 44). However, excessive mechanical loading and overuse likely triggers a repair response that may eventually contribute to the degenerative changes observed in tendinopathies. This study demonstrates that prolonged, repetitive tendon loading leads to an increased production of the growth factors VEGF, VEGFR-1 and CTGF by tendon cells. Furthermore, the highest VEGF, VEGFR-1 and CTGF cell densities occurred along the outer regions of the loaded tendon. These locations may be the at-risk regions in the tendon that will ultimately demonstrate the changes typical of tendinosis, such as degenerative changes with new capillary formation.

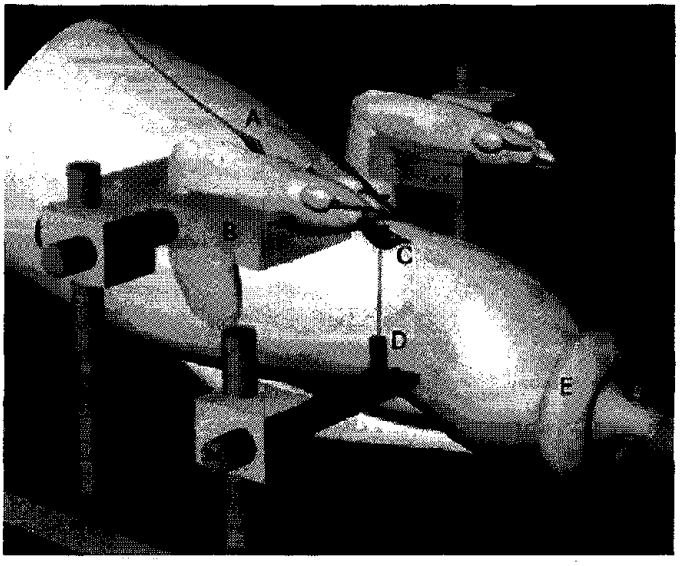

Figure 3.1. Cartoon of loading apparatus with rabbit in a supine position with head to the right and forearms supported. A) stimulation needle, B) forearm support, C) third digit with metal glove, D) load cell, E) anesthesia mask.

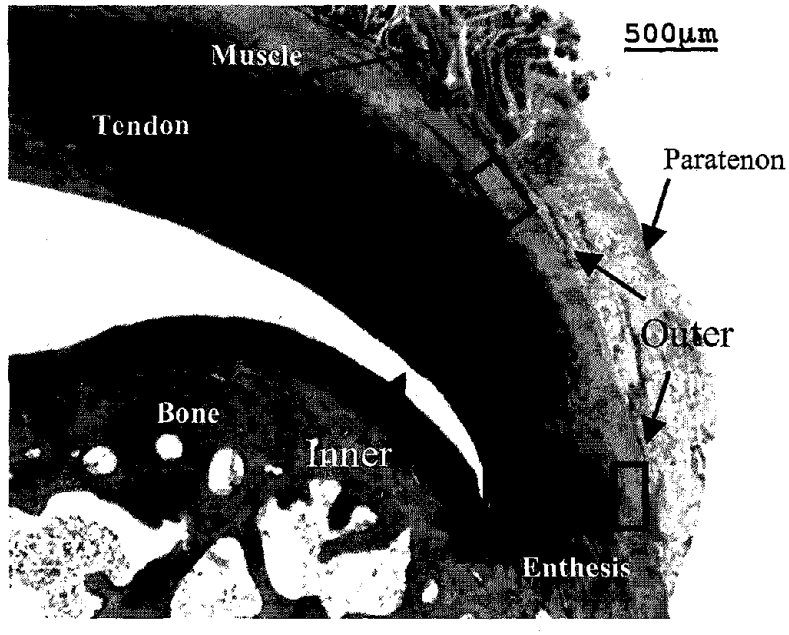

Figure 3.2. Safranin O and Fast Green stained epicondyle with bone, tendon, paratenon, and muscle. Six regions of interest are highlighted, three along the enthesis, and three 1500μm distal to the enthesis. The regions of interest are 200μm by 400μm.

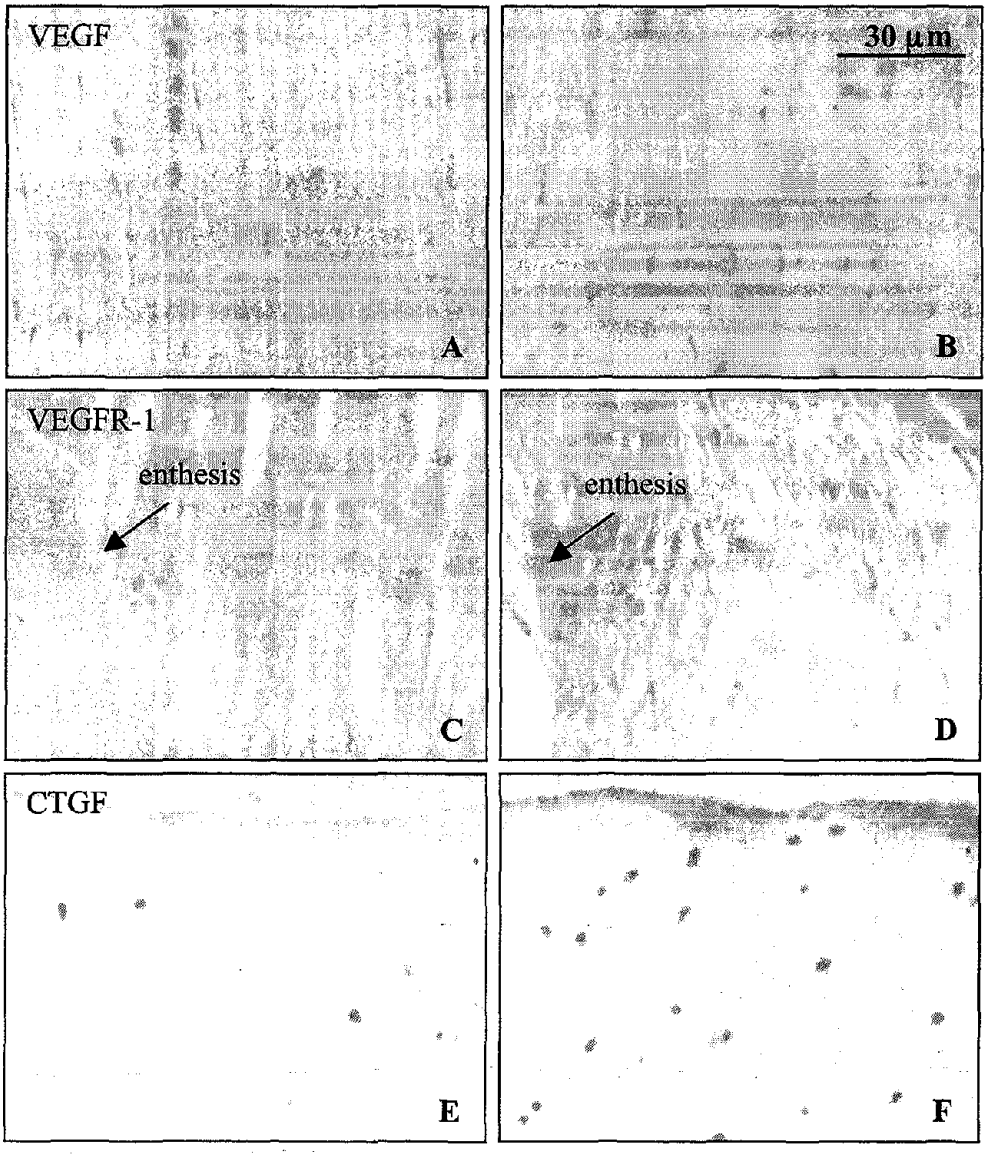

Figure 3.3. VEGF stained cells in the unloaded (A) and loaded (B) center distal region. VEGFR-1 stained cells in the unloaded (C) and loaded (D) outer enthesis region. CTGF stained cells in unloaded (E) and loaded (F) inner distal region of the tendon. 400x magnification.

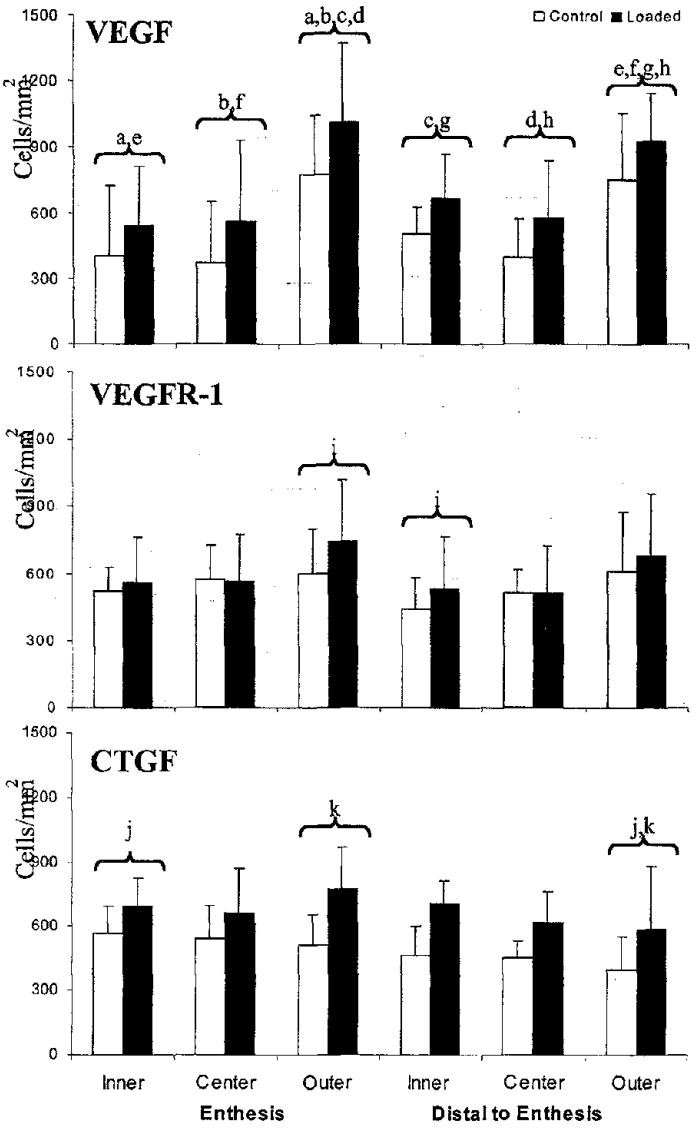

Figure 3.4. VEGF, VEGFR-1 and CTGF cell staining densities (mean ± s.d.) for loaded and unloaded tendon at the epicondyle. Across all regions, cell densities were significantly increased in the loaded tendon compared to unloaded tendon. The limb x region interaction terms were not significant for all three. Regions marked with the same lower case letter are significantly different based on the Tukey follow-up test (N= 9).

REFERENCES

1. Alfredson H, Lorentzon M, Backman S, Backman A, Lerner UH: cDNA-arrays and real-time quantitative PCR techniques in the investigation of chronic Achilles tendinosis. *J Orthop Res* 21:970-5, 2003
2. Allander E: Prevalence, incidence, and remission rates of some common rheumatic diseases or syndromes. *Scand J Rheumatol* 3:145-53, 1974
3. Archambault JM, Hart DA, Herzog W: Response of rabbit Achilles tendon to chronic repetitive loading. *Connect Tissue Res* 42:13-23, 2001
4. Babic AM, Chen CC, Lau LF: Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. *Mol Cell Biol* 19:2958-66, 1999
5. Backman C, Boquist L, Friden J, Lorentzon R, Toolanen G: Chronic achilles paratenonitis with tendinosis: an experimental model in the rabbit. *J Orthop Res* 8:541-7, 1990
6. Barbe MF, Barr AE, Gorzelany I, Amin M, Gaughan JP, Safadi FF: Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *J Orthop Res* 21:167-76, 2003
7. Barr AE, Barbe MF: Inflammation reduces physiological tissue tolerance in the development of work-related musculoskeletal disorders. *J Electromyogr Kinesiol* 14:77-85, 2004
8. Bidder M, Towler DA, Gelberman RH, Boyer MI: Expression of mRNA for vascular endothelial growth factor at the repair site of healing canine flexor tendon. *J Orthop Res* 18:247-52, 2000
9. Blalock TD, Yuan R, Lewin AS, Schultz GS: Hammerhead ribozyme targeting connective tissue growth factor mRNA blocks transforming growth factor-beta mediated cell proliferation. *Exp Eye Res* 78:1127-36, 2004
10. Boyer MI, Watson JT, Lou J, Manske PR, Gelberman RH, Cai SR: Quantitative variation in vascular endothelial growth factor mRNA expression during early flexor tendon healing: an investigation in a canine model. *J Orthop Res* 19:869-72, 2001
11. Bradham DM, Igarashi A, Potter RL, Grotendorst GR: Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 114:1285-94, 1991
12. Brigstock DR: Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 5:153-65, 2002
13. Chard MD, Cawston TE, Riley GP, Gresham GA, Hazleman BL: Rotator cuff degeneration and lateral epicondylitis: a comparative histological study. *Ann Rheum Dis* 53:30-4, 1994

14. Chard MD, Hazleman BL: Tennis elbow--a reappraisal. *Br J Rheumatol* 28:186-90, 1989
15. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989-91, 1992
16. Deroanne CF, Hajitou A, Calberg-Bacq CM, Nusgens BV, Lapiere CM: Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. *Cancer Res* 57:5590-7, 1997
17. Dvorak HF, Brown LF, Detmar M, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146:1029-39, 1995
18. Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR: Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 107:404-11, 1996
19. Gerber HP, Condorelli F, Park J, Ferrara N: Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem* 272:23659-67, 1997
20. Jozsa L, Kannus P: Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 7:113-8, 1997
21. Kannus P: Tendons--a source of major concern in competitive and recreational athletes. *Scand J Med Sci Sports* 7:53-4, 1997
22. Kjaer M: Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 84:649-98, 2004
23. Kraushaar BS, Nirschl RP: Tendinosis of the elbow (tennis elbow). Clinical features and findings of histological, immunohistochemical, and electron microscopy studies. *J Bone Joint Surg Am* 81:259-78, 1999
24. Lau LF, Lam SC: The CCN family of angiogenic regulators: the integrin connection. *Exp Cell Res* 248:44-57, 1999
25. Leask A, Abraham DJ: The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. *Biochem Cell Biol* 81:355-63, 2003
26. Leask A, Abraham DJ: TGF-beta signaling and the fibrotic response. *Faseb J* 18:816-27, 2004
27. Maffulli N, Khan KM, Puddu G: Overuse tendon conditions: time to change a confusing terminology. *Arthroscopy* 14:840-3, 1998
28. Messner K, Wei Y, Andersson B, Gillquist J, Rasanen T: Rat model of Achilles tendon disorder. A pilot study. *Cells Tissues Organs* 165:30-9, 1999
29. Nakama L, King K, Abrahamsson S, Rempel D: Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. *J Orthop Res* In Press, 2005
30. National Research Council (U.S.). Panel on Musculoskeletal Disorders and the Workplace., Institute of Medicine (U.S.): *Musculoskeletal disorders and the workplace : low back and upper extremities,* pp xv, 492. Washington, D.C., National Academy Press, 2001

31. Nishida T, Nakanishi T, Asano M, Shimo T, Takigawa M: Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. *J Cell Physiol* 184:197-206, 2000
32. Perry SM, McIlhenny SE, Hoffman MC, Soslowsky LJ: Inflammatory and angiogenic mRNA levels are altered in a supraspinatus tendon overuse animal model. *J Shoulder Elbow Surg* 14:79S-83S, 2005
33. Petersen W, Pufe T, Kurz B, Mentlein R, Tillmann B: Angiogenesis in fetal tendon development: spatial and temporal expression of the angiogenic peptide vascular endothelial cell growth factor. *Anat Embryol (Berl)* 205:263-70, 2002
34. Petersen W, Pufe T, Unterhauser F, Zantop T, Mentlein R, Weiler A: The splice variants 120 and 164 of the angiogenic peptide vascular endothelial cell growth factor (VEGF) are expressed during Achilles tendon healing. *Arch Orthop Trauma Surg* 123:475-80, 2003
35. Petersen W, Pufe T, Zantop T, Tillmann B, Mentlein R: Hypoxia and PDGF have a synergistic effect that increases the expression of the angiogenetic peptide vascular endothelial growth factor in Achilles tendon fibroblasts. *Arch Orthop Trauma Surg* 123:485-8, 2003
36. Petersen W, Pufe T, Zantop T, Tillmann B, Tsokos M, Mentlein R: Expression of VEGFR-1 and VEGFR-2 in degenerative Achilles tendons. *Clin Orthop*:286-91, 2004
37. Petersen W, Varoga D, Zantop T, Hassenpflug J, Mentlein R, Pufe T: Cyclic strain influences the expression of the vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-1alpha) in tendon fibroblasts. *J Orthop Res* 22:847-53, 2004
38. Pfahler M, Jessel C, Steinborn M, Refior HJ: Magnetic resonance imaging in lateral epicondylitis of the elbow. *Arch Orthop Trauma Surg* 118:121-5, 1998
39. Potter HG, Hannafin JA, Morwessel RM, DiCarlo EF, O'Brien SJ, Altchek DW: Lateral epicondylitis: correlation of MR imaging, surgical, and histopathologic findings. *Radiology* 196:43-6, 1995
40. Pufe T, Petersen W, Tillmann B, Mentlein R: The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. *Virchows Arch* 439:579-85, 2001
41. Regan W, Wold LE, Coonrad R, Morrey BF: Microscopic histopathology of chronic refractory lateral epicondylitis. *Am J Sports Med* 20:746-9, 1992
42. Schild C, Trueb B: Mechanical stress is required for high-level expression of connective tissue growth factor. *Exp Cell Res* 274:83-91, 2002
43. Schild C, Trueb B: Three members of the connective tissue growth factor family CCN are differentially regulated by mechanical stress. *Biochim Biophys Acta* 1691:33-40, 2004
44. See EK, Ng GY, Ng CO, Fung DT: Running exercises improve the strength of a partially ruptured Achilles tendon. *Br J Sports Med* 38:597-600, 2004
45. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC: Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-6, 1995

46. Soslowsky LJ, Thomopoulos S, Esmail A, Flanagan CL, Iannotti JP, Williamson JD, 3rd, Carpenter JE: Rotator cuff tendinosis in an animal model: role of extrinsic and overuse factors. *Ann Biomed Eng* 30:1057-63, 2002
47. Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter JE: Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *J Shoulder Elbow Surg* 9:79-84, 2000
48. Steinborn M, Heuck A, Jessel C, Bonel H, Reiser M: Magnetic resonance imaging of lateral epicondylitis of the elbow with a 0.2-T dedicated system. *Eur Radiol* 9:1376-80, 1999
49. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P: Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun* 187:1579-86, 1992
50. Tsubone T, Moran SL, Amadio PC, Zhao C, An KN: Expression of growth factors in canine flexor tendon after laceration in vivo. *Ann Plast Surg* 53:393-7, 2004
51. Unemori EN, Ferrara N, Bauer EA, Amento EP: Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol* 153:557-62, 1992
52. United States. Bureau of Labor Statistics.: *Occupational injuries and illnesses--counts, rates, and characteristics,* p v. Washington, DC, The Bureau : For sale by the U.S. G.P.O. Supt. of Docs., 1995
53. Wakabayashi I, Itoi E, Sano H, Shibuya Y, Sashi R, Minagawa H, Kobayashi M: Mechanical environment of the supraspinatus tendon: a two-dimensional finite element model analysis. *J Shoulder Elbow Surg* 12:612-7, 2003
54. Wetzel BJ, Nindl G, Swez JA, Johnson MT: Quantitative characterization of rat tendinitis to evaluate the efficacy of therapeutic interventions. *Biomed Sci Instrum* 38:157-62, 2002
55. Yang GP, Lau LF: Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. *Cell Growth Differ* 2:351-7, 1991

CHAPTER IV:

THE EFFECT OF REPETITION RATE ON THE FORMATION OF MICROTEARS IN TENDON IN AN IN VIVO CYCLICAL LOADING MODEL

4.1 ABSTRACT

Previously **(17)**, we reported the formation of microtears in an *in vivo* loaded FDP rabbit tendon with a repetition rate of 60 repetitions per minute and a peak force of 15% of maximum (Po). Tear area as a percent of tendon area, tear density (tears/mm^2) and mean tear size (μm^2) were higher in tendons from the loaded limb compared to the unloaded control limb. The purpose of the present study was to compare those results to results obtained with a repetition rate of 10 while maintaining the same peak force and work (equal force-time integral) (n=8). Due to a strain gradient between the inner and outer sides of the FDP tendon, microtears were quantified in four regions, two regions each along the inner and outer side of the tendon. The tear area as a percent of total tendon area and the mean tear size were significantly greater in the loaded limb compared to the unloaded limb ($p < 0.03$). However, the effects were less than those observed at 60 repetitions/min. The high repetition rate loading pattern resulted in an increase in tear measures in all four regions, while the lower rate produced changes only in the outer regions of the tendon. This finding may establish where the initial sites of damage occur

in tendons that insert into bone in a similar arrangement as the FDP. The results suggest that repetition rate is associated with tendon damage in a dose-response pattern.

4.2 Introduction

Tendon injuries due to overuse are a common problem for both athletes and workers. Overuse injuries account for 30 to 50% of all sports-related injuries (9, 10). Although overuse tendon injuries are related to forceful and repetitive hand activities, little is known about the early mechanisms of injury that ultimately lead to tendinopathy. Elucidating the early structural, cellular, and molecular changes in the tendons exposed to cyclical loading may ultimately improve prevention and treatment options.

Previously we reported the formation of microtears in the tendons of rabbits that were cyclically loaded *in vivo* at 60 repetitions per minute for 80 hours of cumulative loading (17). The mean tear densities ranged from 650 to 1788 tears/mm^2 in the tendon of the loaded limb compared to 358 to 1491 in the unloaded limb. On average, the tears in the loaded limb ranged in size from 13 to 26 μm^2 compared to 9 to 21μm^2 in the unloaded tendon. Larger tears (on the order of cm^2) have been observed in tendons of humans with tendinosis using high-resolution ultrasound (7, 11), MR imaging (5, 24, 28), and 3D volume-rendered images from multi-detector computer tomography (MDCT) (19). The larger tears may occur after prolonged exposure to repetitive load due to accumulation of injuries from microtears.

Several epidemiologic studies in the workplace suggest that rate of repetition may be an important risk factor for tendon injuries (14, 27). Latko et al. (12) categorized repetition as high/medium/low in their investigation of the relationship between repetitive work and the prevalence of upper extremity disorders. They found that people exposed

to high repetition jobs had two to three times higher risk of developing upper extremity disorders. They observed a linear relationship betweeen the three levels of repetition and the risk of tendinitis.

The purpose of this study was to investigate microstructural changes; specifically the formation of microtears in the Flexor Digitorum Profundus (FDP) tendon at the medial epicondyle following cyclical digit loading using a rabbit model with a repetition rate of 10 repetitions/min and compare these results to those obtained with a higher repetition rate (60 repetitions/min) from our previous study (17). The duty cycle (20%) and peak force (0.42N, 15% P_o) were the same between loading groups.

4.3 Methods

4.3a Animal Model

The animal loading model was described previously (17). Eight female, young adult, New Zealand White rabbits weighing 3.58 kg (± 0.84) were used. Under general anesthesia, the FDP muscle of one forelimb was electrically stimulated to contract repetitively for 2 hours per day, 3 days a week, for 80 hours of cumulative loading. The contralateral limb, although supported in the same posture, did not receive a stimulus and therefore served as the control. The stimulation train was adjusted to maintain a mean peak digit flexion force of 0.42N (15% of P_o (peak tetanic force)). The previous study (17) used a repetition rate of 60 repetitions/minute with a train duration of 200ms while this set of animals were exposed to 10 repetitions per minute with a train duration of 1200ms (Figure 1). This study was approved by the University of California, Berkeley's Committee on Animal Research. Weekly examinations of the paw, forearm and elbow

revealed no tenderness, limping, nodules, swelling, limitation in range of motion, reduction in gross claw flexion strength, or skin breaks.

4.3b Tissue and Histological preparation

After 80h of cumulative loading, the animals were weighed (4.00 ±0.55kg), euthanized, and the medial epicondyle block (tendon and bone) were harvested. Tissue and histological preparation along with image acquisition were identical to the previous study (17). Briefly, four regions of interest (ROI) (200 x 400 μm^2) of the FDP tendon were captured and analyzed for tears (tear area as a percent of tendon area, tear density and mean tear size) with a custom image analysis software program.

4.3c Statistical Analysis

A mixed model repeated measures ANOVA was used to analyze differences in tear measures (tear as a percent of tendon area, tear density and mean tear size) by region (inner enthesis, outer enthesis, inner distal or outer distal) and by limb loading status (loaded or unloaded) in the 10 repetitions/min group. Follow-up analysis was performed using the Tukey method for multiple comparisons. The distribution of tears by tear size were transformed into normal distributions using a log transformation preceded by the addition of the smallest value to each data point to avoid taking the logarithm of a zero; then the transformed tear density was compared between loaded and unloaded limbs with the paired t-test using an $\alpha < 0.01$ to adjust for multiple comparisons.

To examine the differences between different repetition groups, a two factor ANOVA was used to analyze differences between loaded and unloaded limbs in the tear parameters by repetition rate (60 versus 10 repetitions/min) and region (4 regions). Follow-up tests were performed using the Tukey method for multiple comparisons.

4.4 Results

4.4a Tear area as a percent of tendon area (10 repetitions/min)

Across the four ROIs, the average tear area as a percent of tendon area ranged from 0.33% to 3.74% in the loaded tendon compared to 0.44% to 2.66% in the unloaded tendon (Figure 2A). The outer enthesis (110% increase) and outer distal (86.3% increase) regions had the greatest percent increase in the tear area as a percent of tendon area when comparing the loaded to unloaded tendon. The inner enthesis (12.3% decrease) and the inner distal (10.7% increase) ROIs exhibited much smaller changes. The limb by region interaction term in the RMANOVA was not significant ($p = 0.054$) while the limb ($p = 0.01$) and region ($p < 0.001$) effects were significant. Using the Tukey follow-up test, significant differences between regions were found. The tear area as a percent of tendon area at the inner enthesis was significantly lower than the outer enthesis ($p < 0.001$) and outer distal ($p < 0.001$) regions. Similarly, the tear area as a percent of tendon area in the inner distal ROI was significantly lower than the outer enthesis ($p < 0.02$) and outer distal ($p < 0.001$) regions. There were no significant differences between the two inner ROIs; however the outer distal ROI had a significant increase in tear area percent when compared to the outer enthesis ($p = 0.016$) ROI.

4.4b Tear Density (10 Repetitions/min)

The tear density (tears/mm^2), on average, ranged from 270.4 to 1672.7 tears/mm^2 in the loaded limb compared to 315.6 to 1442.8 tears/mm^2 in the unloaded limb across the four ROIs. Similar to the tear area as a percent of tendon area, greater changes were observed in the outer regions of the tendon. The outer enthesis (61.3% increase) and outer distal (45.4% increase) regions had a higher percent change than the inner enthesis

(10.7% increase) and the inner distal (7.2% increase) regions when comparing loaded to unloaded tendons. The limb by region interaction term was not significant ($p = 0.57$). There was no significant difference between limbs ($p = 0.25$) but there was a regional effect ($p < 0.0001$). The differences were primarily between the inner regions and the outer regions. The inner region of the enthesis had a significantly lower tear density than the other three ROIs: outer enthesis ($p < 0.001$), inner distal ($p = 0.04$) and outer distal ($p < 0.001$). In a similar manner, the inner distal region had a significantly lower tear density than the outer enthesis ($p = 0.001$) and outer distal ($p < 0.001$) ROIs (Figure 2B).

4.4c Mean tear size (10 Repetitions/min)

The mean tear size (μm^2), on average, ranged from 11.0 to 24.2 μm^2 in the loaded tendon compared to 13.4 to 17.6 μm^2 in the unloaded tendon (Figure 2C) in the four ROIs. Similar to the other two tear measures, the greatest changes occurred along the outer regions of the tendon. The outer enthesis (37.2% increase) and outer distal (38.9% increase) regions had larger changes in mean tear size compared to the inner enthesis (17.1% decrease) and inner distal (7.29% increase). The limb ($p = 0.03$), region ($p < 0.001$) and interaction ($p = 0.03$) terms were all significant in the RMANOVA. The ROIs in the loaded tendon were significantly different from one another. The loaded inner enthesis ROI had a smaller mean tear size than the loaded outer enthesis ($p = 0.0167$) and loaded outer distal ($p < 0.0001$) ROIs (Figure 2C). The loaded inner region distal to the enthesis had a significantly smaller mean tear size than the outer region distal to the enthesis ($p = 0.001$).

4.4d Distribution of tears by size (10 Repetitions/min)

The distribution of tears by size are presented in Figures 4 and 5. The only significant difference found in the 10 repetition/min loading group was for tears 200 μm^2 in size (100-200 μm^2), as noted in the figure.

4.4e Comparing 60 v 10 repetitions/min

Differences in tear parameters between limbs were compared to the data from the 60 repetition/min group study (17). The interaction term in the ANOVA (repetition rate x region) for the differences in tear area as a percent of tendon area was not significant ($p = 0.91$). The 60 repetition/min group had greater differences between limbs in tear area as a percent of tendon area ($p = 0.01$) compared to the 10 repetition/min group (Figure 3A). Regional differences were also present ($p < 0.0001$); there were larger tear differences between unloaded and loaded tendons along the outer regions of the tendon than along the inner regions of the tendon ($p < 0.05$).

The interaction term for the tear density was not significant ($p = 0.82$) in the ANOVA. Differences in tear densities were not significantly different between the two loading groups ($p = 0.07$) nor were there regional effects ($p = 0.18$) (Figure 3B).

The interaction term for the mean tear size was not significant ($p = 0.23$) in the ANOVA. The difference in the mean tear size between the two loading groups was not significantly different ($p = 0.18$). Regionally ($p = 0.005$), there were differences, most notably the outer ROIs had greater changes between loaded and unloaded tendons than the inner enthesis (Figure 3C).

When the tears were grouped by size (Figure 4 and 5), the 60 repetitions/min loading group demonstrated a broader range of differences in tear density **(17)**. Across

nearly all tear sizes, the tear density in the loaded tendon was greater in the 60 repetition/min compared to the 10 repetition/min loading group, especially in the outer enthesis region. These broad range of differences were not observed in the 10 repetition/min group where the only significant difference between loaded and unloaded limbs occurred at the 200 μm tear size (Figure 5).

4.5 Discussion

This is the first study to evaluate the effect of repetition rate on tendon damage, specifically the formation of microtears, in an *in vivo* cyclical loading model. A loading rate of 10 repetitions/min significantly increased tear area as a percent of tendon area and the mean tear size but had negligible effect on tear density. This differs from what we previously found for a repetition rate of 60 repetitions/min (17) where all parameters of tear (tear area percent, tear density, tear size) were significantly greater in the loaded limb compared to the unloaded limb. Comparing the two loading regimens, the higher repetition group had larger changes in the tear area as a percent of tendon area ($p = 0.01$) whereas differences were borderline significant for the tear density ($p = 0.07$) and not significantly different for the mean tear size ($p = 0.18$) (Figure 3). An examination of tear densities by size revealed that the loaded tendons from the higher repetition group consistently had higher tear densities across the majority of tear sizes (Figures 4 and 5), whereas only tears of 100 to 200μm^2 in size were significantly different in tear density for the lower repetition group. These differences are masked when the tear sizes are grouped. These findings provide some insight into the effect of loading rate on tendon microtear formation; lower rates cause relatively large tears but high rates cause tears across a spectrum of tear sizes.

Overall, these findings suggest a dose-response relationship between repetition rate and various measures of microtears. The higher repetition rate caused greater microtear formation. This effect was independent of work load since the differences between the two loading patterns was the repetition rate; peak load and work (area under the force – time curve) were the same. The tendon-muscle-tendon unit has viscoelastic properties that include creep, history-dependence, and loss of energy during cyclic loading. Although the cumulative amount of time that the forces are elevated are equal in both loading conditions, the time allowed for the tendon to recover (0.8s for 60 repetition/min v. 4.8s for 10 repetitions/min) before the next loading cycle begins are not equivalent. In addition, during a loading cycle, energy dissipation may occur and may transfer into internal heat, and effect the physiological state of the tendon. As the tendon is repetitively loaded at a high rate, the tendon has less time to revert toward its original, nonloaded state. The stresses may, therefore, accumulate more during the high repetition rate loading.

A number of animal models have been developed to study the effect of cyclical loading on tendon (1-4, 16, 20, 23). Some studies examined the effects on different outcomes (histological, cellular, biochemical or mechanical) of one loading regimen at different time points (3, 4, 20, 22, 23). Others (1, 2) investigated the effects of loading at one time point. A few studies (1, 2, 16) compared loaded to unloaded tendons of the same animal.

These previous *in vivo* loading models utilized loading rates as low as 4 repetitions per minute (3) to as high as 150 repetitions per minute (2) . Barbe et al. (3) utilized low forces ($F_{peak} < 0.15MVC$) and low repetition rates (4/min) in a rat volitional

study and found several changes including both behavioral and histological changes. These changes started in week 5 and continued until the end of the study in week 8. They reported a decrease in reach rate, task duration and a change in the preferred grasping technique utilized by the rats. Histologically, tendon fraying was present after 5 weeks at the musculo-tendon junction. As early as week 3, the number of resident and infiltrating macrophages were significantly higher from the baseline control group. Soslowsky's overuse rotator cuff model exposing rats to treadmill running used repetition rates of approximately 120 repetitions per minute (4, 22, 23). Peak forces were unknown. If the forces are similar to those involved during gait and jogging, the forces were high relative to our study and other studies (1-3, 16). Soslowsky et al. showed gross mechanical changes at 4 weeks of running (1h/d, 5d/wk) including an increase in tendon cross sectional area, a decrease in maximum tensile stress, and a decrease in the elastic modulus relative to a control group. Perry et al. (2005) , using the same overuse model, showed an increase in mRNA of inflammatory markers (COX-2 and FLAP) and angiogenic factors (VEGF, VWF) after 3 days of exercise. COX-2 and flap expression peaked at 8 weeks. Previously (18), we demonstrated in our model that other angiogenic components (VEGF, VEGFR-1, and CTGF) increased with 60 repetitions per minute of cyclical loading of the FDP muscle.

Although the differences in tear measures between limbs were greater at the higher rate, there were similar regional differences ($p < 0.001$) for both the 60 and 10 repetition rates. Larger differences were present in tear measures between unloaded and loaded tendons between the two loading groups (10 vs. 60 repetitions/min) at the outer regions of the tendon (Figure 3). Along the inner regions of the tendon, there were little

differences between the loaded and unloaded tendons in the lower repetition rate group (Figure3). The inner regions had greater tear measures than their unloaded counterpart for the higher loading rate (60 repetitions/min). This regional variation may be due to the inhomogenous stress distribution that occurs with normal loading (8, 15, 25, 26). As the FDP tendon is loaded, the region adjacent to bone (inner enthesis) experiences tension and compression but the compression results in fibrocartilage formation (8, 15, 25, 26). Fibrocartilage has different mechanical and biological properties that allow it to absorb compressive stresses (26) and as a result is found in tissues bearing compressive loads such as cartilage. These differences in the composition may contribute to the different regional response to loading. The inner and outer enthesis are structurally different and may have different modes of failure and strain differences under repetitive loads. The outer regions of the tendon are composed mainly of type I collagen (30% wet weight), which provides the tensile strength. Although collagen has a high tensile strength, these collagen fibrils may be at risk for failure under cyclical loading patterns as the fibrils continually slide past one another.

Clinical symptoms of overuse injuries to tendon include pain, local edema and/or tenderness, which may be the tissue's response to the formation of microtears. Biopsy specimens of overuse tendon injuries demonstrate degeneration without inflammation and have been termed tendinosis rather than tendinitis due to the absence of inflammatory cells (13, 14). In tendonitis, local pain near the tendon insertion site is usually associated with swelling. Swelling is absent in tendinosis, however, pain may be generated through the development of new nerve fibers or disruption of nerve fibers by tear formation. Messner et al. (16) found groups of multiple nerve fibers in the epitenon and in the

paratendinous fatty tissue of the bursa in their rat model of Achilles tendon disorder. Control tendons showed sparse occurrence of singular nerve fibers. The presence of tears may induce nerve activity causing pain. Eventually, the tears may weaken the tendon structure mechanically resulting in larger tears or even mechanical failure.

Several limitations of this model should be noted. The regions studied were limited and possible changes at the muscle-tendon junction or other regions in the tendon were missed. However, the study has several important strengths. Tissue preparation and tear analysis were blinded to limb-loading status. The loading patterns selected maintained equivalent peak loads during loading so the differences are not due to differences in peak load. The loading patterns involved the same work (force-time integral) therefore the differences were not due to differences in work.

In conclusion, overuse tendon damage is likely associated with several biomechanical loading parameters. Peak force, repetition rate, posture, and duration are associated risk factors based on epidemiologic studies (6, 21, 27) in the upper extremities. Our studies suggest a dose-response relationship between repetition rate and the formation of microtears. The higher repetition rates (60 repetitions/min) led to greater measures of tear damage than the lower repetition rate (10 repetitions/min) across all regions of the tendon. The lower repetition rate had little effect along the inner regions of the tendon; the greater changes occurred along the outer regions. These differences are likely due to differences in tissue properties or the presence of an inhomogenous strain distribution during peak loads. Performing the same 'work' or applying the same peak force at lower repetition rates may prevent microtears in tendon compared to applying the

same load at higher loading rates. These findings may be useful in the management and prevention of tendinopathies.

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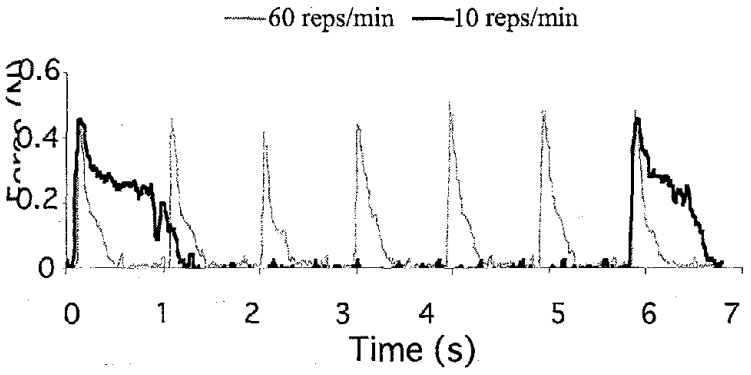

Figure 4.1. Typical tendon loading profiles for the 60 and 10 repetitions/min loading patterns. Peak force is equivalent in the two loading groups. The Force-time integral (work) and duty cycle are also equivalent.

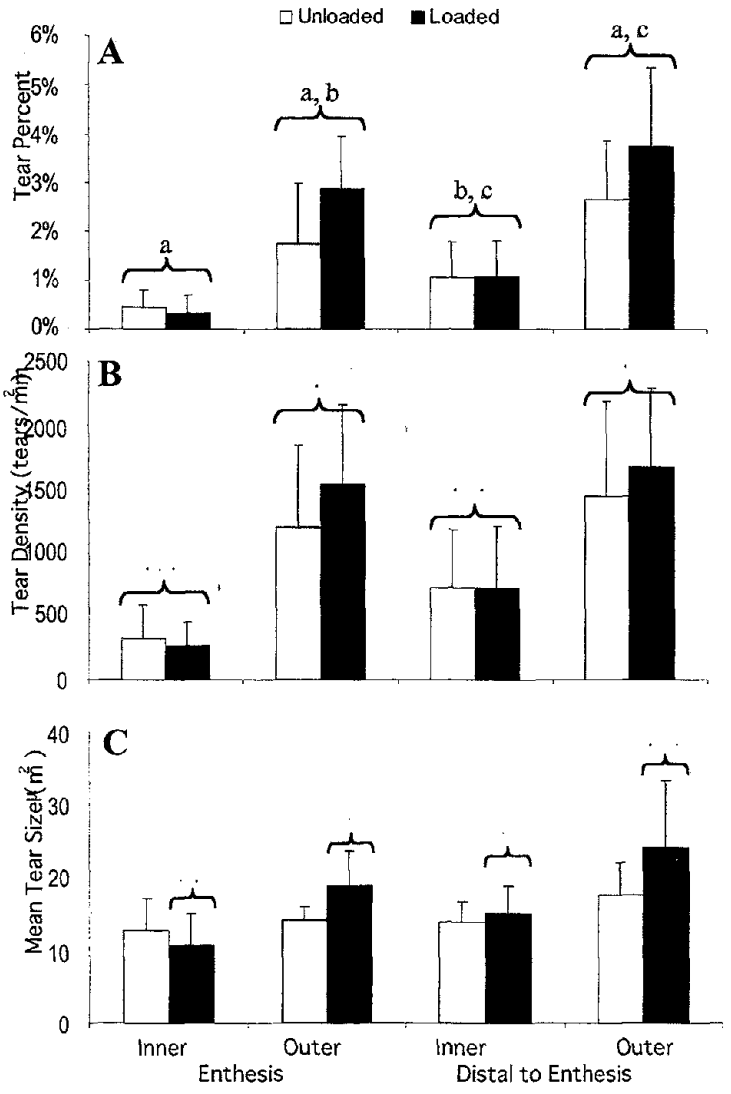

Figure 4.2. The tear area as a percent of tendon area (A), the tear density (B), and mean tear size (C) for the 10 repetition/min group. The interaction terms (loading group x region) for (A) and (B) were not significant ($p > 0.05$) whereas it was significant in (C) ($p = 0.03$). There was a significant ($p = 0.01$) effect of limb in (A) while for (B) it was not significant ($p = 0 .25$). Regions marked with the same lower case letter are significantly different based on the Tukey follow-up test. Columns are marked ± s.d., $n = 8$.

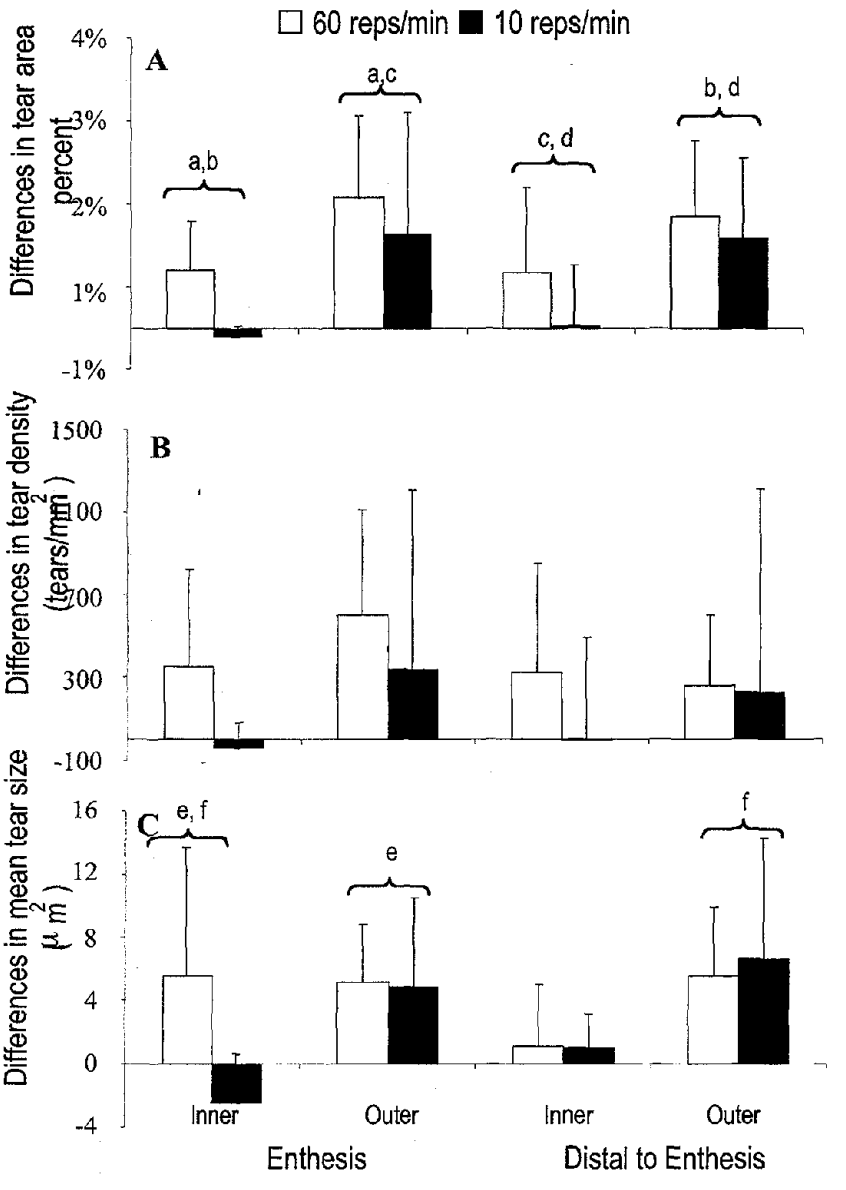

Figure 4.3. Comparison of tear measures for 60 (n=9) and 10 (n=8) repetitions/min loading conditions. Values represent mean (± s.d.) differences between loaded and unloaded tendons of the same animal. Changes in tear area as a percent of tendon area (A), changes in tear density (B), and changes in mean tear size (C). Significant differences between limbs by repetition rate only occur for (A). Regions marked with the same lower case letter are significantly different based on the Tukey follow-up test.

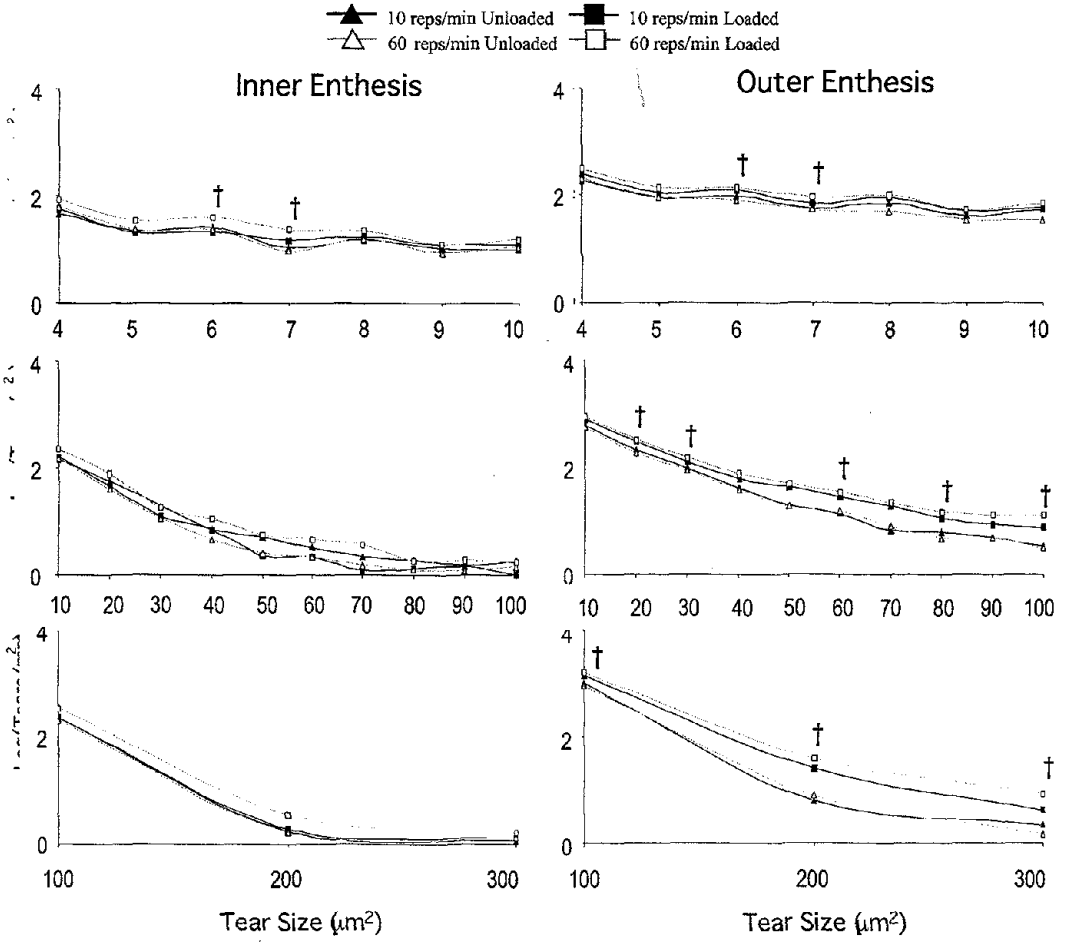

Figure 4.4. The log of the distribution of microtears by tear size is shown for the inner and outer regions of the tendon at the enthesis for both loading groups (10 and 60 repetitions/min). No significant differences were found in the 10 repetition/min group (paired t-test, $p < 0.01$). The symbol † indicates a significant difference between unloaded and loaded limbs (paired t-test, $p < 0.01$) in the 60 repetitions/min group $n = 9$.

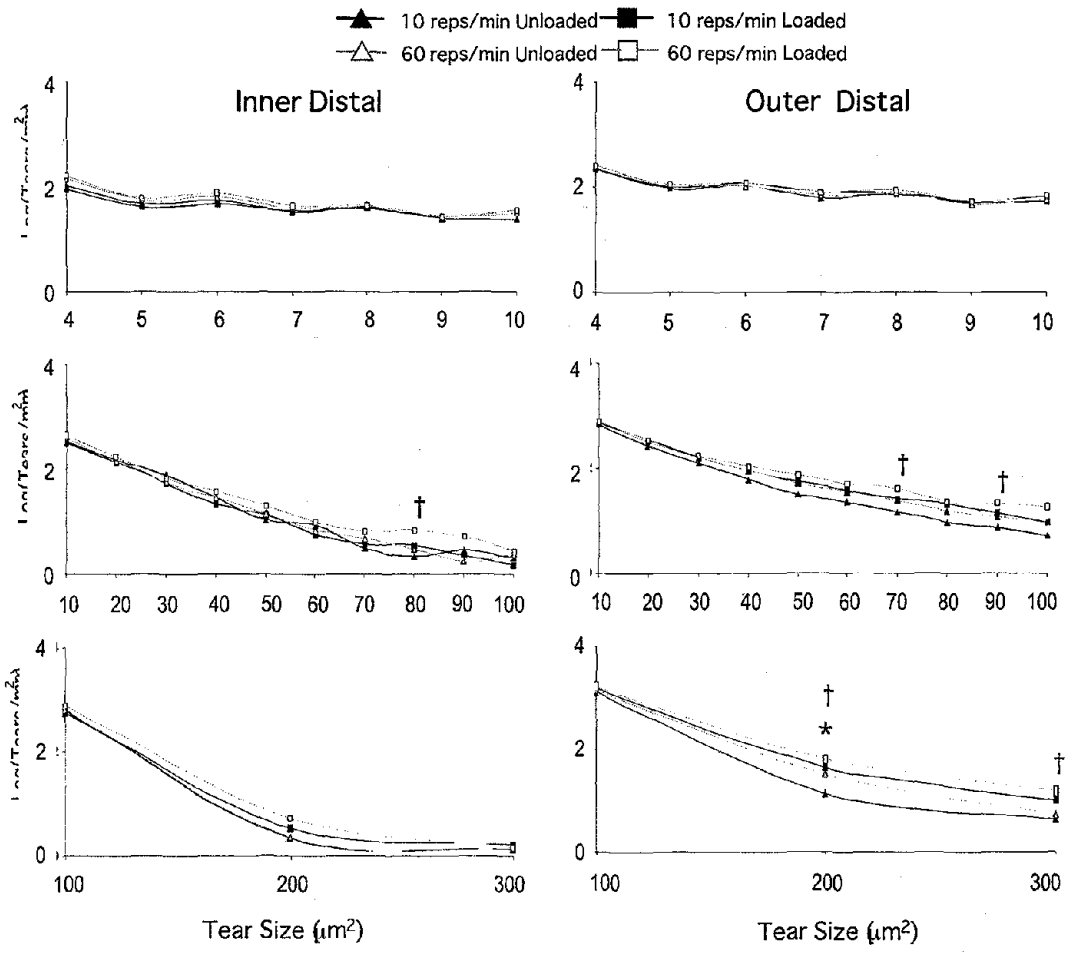

Figure 4.5. The log of the distribution of microtears by size is shown for the inner and outer parts of the tendon distal to the enthesis for both loading groups (10 and 60 repetitions/min). The symbols † and * indicate a significant difference between unloaded and loaded limbs (paired t-test, $p < 0.01$) in the 60 ($n = 9$) and 10 ($n = 8$) repetitions/min group, respectively.

REFERENCES

1. Archambault JM, Hart DA, Herzog W: Response of rabbit Achilles tendon to chronic repetitive loading. *Connect Tissue Res* 42:13-23, 2001
2. Backman C, Boquist L, Friden J, Lorentzon R, Toolanen G: Chronic achilles paratenonitis with tendinosis: an experimental model in the rabbit. *J Orthop Res* 8:541-7, 1990
3. Barbe MF, Barr AE, Gorzelany I, Amin M, Gaughan JP, Safadi FF: Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *J Orthop Res* 21:167-76, 2003
4. Carpenter JE, Flanagan CL, Thomopoulos S, Yian EH, Soslowsky LJ: The effects of overuse combined with intrinsic or extrinsic alterations in an animal model of rotator cuff tendinosis. *Am J Sports Med* 26:801-7, 1998
5. Cvitanic O, Henzie G, Skezas N, Lyons J, Minter J: MRI diagnosis of tears of the hip abductor tendons (gluteus medius and gluteus minimus). *AJR Am J Roentgenol* 182:137-43, 2004
6. Descatha A, Leclerc A, Chastang JF, Roquelaure Y: Medial epicondylitis in occupational settings: prevalence, incidence and associated risk factors. *J Occup Environ Med* 45:993-1001, 2003
7. Gibbon WW, Cooper JR, Radcliffe GS: Sonographic incidence of tendon microtears in athletes with chronic Achilles tendinosis. *Br J Sports Med* 33:129-30, 1999
8. Huang CY, Wang VM, Pawluk RJ, Bucchieri JS, Levine WN, Bigliani LU, Mow VC, Flatow EL: Inhomogeneous mechanical behavior of the human supraspinatus tendon under uniaxial loading. *J Orthop Res* 23:924-30, 2005
9. Jozsa L, Kannus P: Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 7:113-8, 1997
10. Kannus P: Tendons--a source of major concern in competitive and recreational athletes. *Scand J Med Sci Sports* 7:53-4, 1997
11. La S, Fessell DP, Femino JE, Jacobson JA, Jamadar D, Hayes C: Sonography of partial-thickness quadriceps tendon tears with surgical correlation. *J Ultrasound Med* 22:1323-9; quiz 1330-1, 2003
12. Latko WA, Armstrong TJ, Franzblau A, Ulin SS, Werner RA, Albers JW: Cross-sectional study of the relationship between repetitive work and the prevalence of upper limb musculoskeletal disorders. *Am J Ind Med* 36:248-59, 1999
13. Maffulli N, Khan KM, Puddu G: Overuse tendon conditions: time to change a confusing terminology. *Arthroscopy* 14:840-3, 1998
14. Maffulli N, Wong J, Almekinders LC: Types and epidemiology of tendinopathy. *Clin Sports Med* 22:675-92, 2003
15. Malaviya P, Butler DL, Boivin GP, Smith FN, Barry FP, Murphy JM, Vogel KG: An in vivo model for load-modulated remodeling in the rabbit flexor tendon. *J Orthop Res* 18:116-25, 2000
16. Messner K, Wei Y, Andersson B, Gillquist J, Rasanen T: Rat model of Achilles tendon disorder. A pilot study. *Cells Tissues Organs* 165:30-9, 1999

17. Nakama LH, King KB, Abrahamsson S, Rempel DM: Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. *J Orthop Res* 23:1199-205, 2005
18. Nakama LH, King KB, Abrahamsson S, Rempel DM: VEGF, VEGFR-1, and CTGF cell densities in tendon are increased with cyclical loading: An in vivo tendinopathy model. *J Orthop Res* 24:393-400, 2006
19. Ohashi K, El-Khoury GY, Bennett DL: MDCT of tendon abnormalities using volume-rendered images. *AJR Am J Roentgenol* 182:161-5, 2004
20. Perry SM, McIlhenny SE, Hoffman MC, Soslowsky LJ: Inflammatory and angiogenic mRNA levels are altered in a supraspinatus tendon overuse animal model. *J Shoulder Elbow Surg* 14:79S-83S, 2005
21. Roquelaure Y, Mariel J, Fanello S, Boissiere JC, Chiron H, Dano C, Bureau D, Penneau-Fontbonne D: Active epidemiological surveillance of musculoskeletal disorders in a shoe factory. *Occup Environ Med* 59:452-8, 2002
22. Soslowsky LJ, Thomopoulos S, Esmail A, Flanagan CL, Iannotti JP, Williamson JD, 3rd, Carpenter JE: Rotator cuff tendinosis in an animal model: role of extrinsic and overuse factors. *Ann Biomed Eng* 30:1057-63, 2002
23. Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter JE: Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *J Shoulder Elbow Surg* 9:79-84, 2000
24. Steinborn M, Heuck A, Jessel C, Bonel H, Reiser M: Magnetic resonance imaging of lateral epicondylitis of the elbow with a 0.2-T dedicated system. *Eur Radiol* 9:1376-80, 1999
25. Waggett AD, Ralphs JR, Kwan AP, Woodnutt D, Benjamin M: Characterization of collagens and proteoglycans at the insertion of the human Achilles tendon. *Matrix Biol* 16:457-70, 1998
26. Wakabayashi I, Itoi E, Sano H, Shibuya Y, Sashi R, Minagawa H, Kobayashi M: Mechanical environment of the supraspinatus tendon: a two-dimensional finite element model analysis. *J Shoulder Elbow Surg* 12:612-7, 2003
27. Werner RA, Franzblau A, Gell N, Hartigan A, Ebersole M, Armstrong TJ: Predictors of persistent elbow tendonitis among auto assembly workers. *J Occup Rehabil* 15:393-400, 2005
28. Yu JS, Popp JE, Kaeding CC, Lucas J: Correlation of MR imaging and pathologic findings in athletes undergoing surgery for chronic patellar tendinitis. *AJR Am J Roentgenol* 165:115-8, 1995

CHAPTER V:

Peak force has a greater effect than repetition rate on the formation of microtears in tendon in an *in vivo* cyclical loading model.

5.1 Abstract

Previously (17, 18) we reported the formation of microtears in an *in vivo* cyclically loaded FDP rabbit tendon. One loading pattern applied a peak force of 15% of Po (0.42N) and a repetition rate of 60 repetitions per minute (High force, high repetition: HFHR) and another loading pattern had the same peak force but lower repetition rate of 10 repetition/min (HFLR). The higher repetition rate led to greater changes in tear measures between tendons from the loaded limb compared to the unloaded control limb. The purpose of this study was to compare those results to results from low force, high repetition loading pattern (LFHR) ($n = 7$). There were no significant differences observed for the LFHR loading pattern in tear measures of the loaded limb compared to the unloaded limb ($p > 0.05$). The regional differences were similar to the other loading patterns; greater measures of tears were observed in the outer region of the tendon than the inner regions. Larger differences in tear measures were found between loaded and unloaded limbs under loading conditions with high forces rather than high repetition rates. HFHR loading had larger differences in tear area as a percent of tendon area than

both HFLR ($p = 0.03$) and LFHR ($p = 0.002$) loading pattern. Furthermore, the mean tear size was also significantly larger in the HFHR loading pattern than the LFHR pattern ($p = 0.003$). The results suggest that with repetitive loading, tendon microtears are more likely due to peak force levels than repetition rate.

5.2 Introduction

Previously, we reported the formation of microtears in the tendons of rabbits that were cyclically loaded *in vivo* for 80 hours of cumulative loading (16, 17) at either high forces combined with high repetition (HFHR) or high forces combined with low repetition (HFLR). We reported a dose-repsonse relationship with greater changes occurring with the HFHR pattern than the low repetition rate (HRLR). Regionally, the HFLR loading pattern did not generate significant changes at the inner regions of the tendon. Outer regions were effected but not as great as the HFHR pattern. Regardless of region, the HFLR pattern did not lead to significant changes between loaded and unloaded tendons when looking at tear density (tears/mm^2) whereas the HFHR pattern did lead to differences. Additionally, the HFHR loading pattern led to larger differences than the HFLR pattern in the tear area as a percent of tendon area ($p = 0.01$) when comparing loaded to unloaded tendons.

Several epidemiologic studies in the workplace have identified important risk factor for tendon injuries due to overuse (21, 22, 25, 26, 28, 30), including high rate of repetitive hand activity and high peak forces. These risk factors are present in many occupational settings. In meat packing plants, where high grip forces are common, the annual incidence rate for epicondylitis was reported to be as high as 11.3% for female sausage packers. (9). Repetitive activity has been found to be associated with tendinitis

and other soft tissue injuries (5, 12, 14, 28, 29). Latko et al. (10) found a dose-response relationship between three levels of repetition and tendinitis ($p < 0.01$). Prevalence rates for tendinitis were reported as 4.2% for low repetition jobs compared to 14.5% for high repetition jobs.

The purpose of this study was to investigate the effect of LFHR loading on microstructural changes, specifically the formation of microtears in the Flexor Digitorum Profundus (FDP) tendon at the medial epicondyle insertion site following cyclical digit loading using a rabbit model loaded for 80 hours of cumulative loading (14 weeks). HFHR, HFLR and LFHR loading patterns were compared to evaluate the effect of repetition rate and peak force on microtear formation.

5.3 Methods

5.3a Animal Model

The animal loading model was described previously (17, 18). Seven female, young adult, New Zealand White rabbits weighing 3.58 kg (± 0.84) were used. Under general anesthesia, the FDP muscle of one forelimb was electrically stimulated to contract repetitively for 2 hours per day, 3 days a week, for 80 hours of cumulative loading. The contralateral limb, although supported in the same posture, did not receive a stimulus and therefore served as the control. The stimulation train was adjusted to maintain a mean peak digit flexion force of 0.14N. The repetition rate was set at 60 repetitions per minute with a train duration of 600ms (train = 100 pulses/sec and pulse width = 2ms). The previous studies (17, 18) used a repetition rate of 60 repetitions/minute with a train duration = 200ms and a F_{peak} = 0.42N) (HFHR); 10 repetitions/minute with a train duration = 1200 ms and a F_{peak} = 0.42N) (HFLR) (Figure

5.1). The train duration selected for this study maintained the same Force-Time integral as the previous studies (16, 17). This study was approved by the University of California, Berkeley's Committee on Animal Research. Weekly examinations of the paw, forearm and elbow revealed no tenderness, limping, nodules, swelling, limitation in range of motion, reduction in gross claw flexion strength, or skin breaks.

5.3b Tissue and Histological preparation

After 80h of cumulative loading, animals were weighed (4.00 ±0.55kg) and euthanized. Tissue and histological preparation along with image acquisition were identical to a previous studies (17, 18). Briefly four ROIs (200 x 400 μm^2) were captured and analyzed for tears (tear area as a percent of tendon area, tear density and mean tear size) with a custom software program.

5.3c Statistical Analysis

For the Low Force, High Repetition loading pattern a mixed model repeated measures ANOVA was used to analyze differences in tear measures (tear percent of tendon area, tear density and mean tear size) by region (inner enthesis, outer enthesis, inner distal or outer distal) and by limb loading status (loaded or unloaded) in the LFHR pattern. Post hoc analysis was performed using the Tukey method for multiple comparisons. The distribution of tears by tear size were transformed into normal distributions using a log transformation preceded by the addition of the smallest value to each data point to avoid taking the logarithm of a zero, then the transformed tear density was compared between loaded and unloaded limbs with the paired t-test applying an $\alpha < 0.01$ to adjust for multiple comparisons.

To examine the differences between different loading patterns, a two factor ANOVA was used to analyze differences (loaded versus unloaded) in the tear parameters by loading pattern (HFHR, HFLR and LFHR) and by region (4 regions). Follow-up analysis was performed using the Tukey method for multiple comparisons.

5.4 Results

5.4a Tear area as a percent of tendon area (Low Force, High Repetition)

Across the four ROIs, the average tear area as a percent of total tendon area ranged from 0.43% to 3.12% in the loaded tendon compared to 0.43% to 2.48% in the unloaded tendon (Figure 5.2). The limb by region interaction term in the RMANOVA was not significant ($p = 0.55$). The limb ($p = 0.33$) effect was not significant but the region ($p < 0.0001$) effect was significant. Significant differences between regions, based on the Tukey follow-up test, are marked by the same lower case letter in Figure 5.2. Similar to the HFHR and HFLR patterns (17, 18), there were significantly less tear area as a percent of tendon area in the inner regions of the tendon than the outer regions (Figure 5.2).

5.4b Tear Density (Low Force, High Repetition)

The tear density (tears/mm^2), on average, ranged from 267 to 1224 tears/mm^2 in the loaded limb compared to 271 to 1071 tears/mm^2 in the unloaded limb across the four ROIs. The limb by region interaction term was not significant ($p = 0.78$). There was no significant difference between limbs ($p = 0.48$) but there was a regional effect ($p < 0.0001$). Similar to the tear area as a percent of tendon area, the differences were primarily between the inner regions and the outer regions (Figure 5.2).

5.4c Mean tear size (Low Force, High Repetition)

The mean tear size (μm^2), on average, ranged from 15.6 to 26.3 μm^2 in the loaded tendon compared to 15.8 to 25.2 μm^2 in the unloaded tendon (Figure 5.2) in the four ROIs. The limb ($p = 0.89$) and interaction ($p = 0.91$) terms were not significant in the RMANOVA, but the region effect was significant ($p < 0.0001$) (Figure 5.2).

5.4d Distribution of tears by tear size (Low Force, High Repetition)

There were no significant differences between the distribution of tears by tear size (Figure 5.3 and 5.4) in the loaded and unloaded tendon for LFHR loading pattern. The HFHR loading pattern resulted in the greatest number of significant differences between unloaded and loaded tendon (17) (10 significant differences out of 20 tear sizes in the outer region at the enthesis). The HFLR loading pattern resulted in one significant difference between loaded and unloaded tendon (16).

5.4e Comparing HFHR, HFLR and LFHR loading patterns

Data from this study was compared to the data from the previous two loading patterns: HFHR (17) and HFLR (18). The interaction term (loading pattern x region) for the tear area as a percent of tendon area was not significant using (ANOVA, $p = 0.80$). There was a significant effect of loading ($p = 0.0002$) and region ($p < 0.0001$). The HFHR pattern had greater differences in tear area as a percent of tendon area compared to the HFLR pattern (Tukey test, $p = 0.03$) and the LFHR pattern (Tukey test, $p = 0.002$) (Figure 5.5). The inner enthesis region had smaller changes associated with the tear area as a percent of tendon area compared to the outer regions, outer enthesis ($p < 0.01$) and distal to enthesis ($p = 0.001$). Similarly, the inner region of the tendon distal to the

enthesis had smaller changes compared to the outer regions; at the enthesis ($p = 0.008$) and distal to the enthesis ($p = 0.01$) (Figure 5.5).

The interaction term for the tear density was not significant (ANOVA, $p = 0.71$). Differences in tear densities were not significantly different between the three loading patterns ($p = 0.42$) nor were there regional effects ($p = 0.58$) (Figure 5.5).

The interaction term for the mean tear size was not significant (ANOVA, $p = 0.16$). The differences in the mean tear size between the three loading patterns were significantly different ($p = 0.005$) as was the effect of region ($p = 0.01$). Follow-up tests revealed that the HFHR pattern had larger changes between limbs compared to the LFHR pattern. The inner enthesis had smaller changes than the outer region distal to the enthesis ($p = 0.03$) (Figure 5.5).

5.5 Discussion

This study, combined with our prior studies (16, 17), are the first to evaluate the effects of both peak force and repetition rate on tendon degeneration, specifically the formation of microtears, in an *in vivo* repetitive loading model. High repetition rates with low forces (HRLF) led to no significant differences in tear measures between loaded limbs and their contralateral unloaded limbs. Whereas the High Repetition, High Force (HRHF) rate produced significant changes when comparing the loaded to unloaded tendon (17). Reducing the repetition rate but keeping the same peak force (HFLR) produced changes between the HRLF and HFHR loading patterns. The results of the three loading patterns over the range of loading parameters selected suggest that peak force has a larger effect on injury, as measured in microtear formation, than repetition rate.

When microtears were categorized by size (Figure 5.3 and 5.4), the HFHR loading pattern caused tear density to increase across a broader range of tear sizes (10 of 20 categories in the outer region of the tendon at the enthesis) while for HFLR there was only one significant difference (1 of 20 categories in the outer region of the tendon at the enthesis) due to loading **(17)**. LFHR caused no significant difference in tear density at any tear size.

Similar regional differences in microtear density were observed across all three loading patterns. The outer regions of the tendon exhibited higher tear measures regardless of the loading pattern. Thus, regional differences were present, even when there were no effects of loading (LFHR). This inhomogeneity in the microtear distribution has been discussed previously (6, 16-18, 27) and is likely due to the inhomogenous distribution of stress in the tendon. This study provides further evidence that the outer regions of tendons with a similar anatomy as the rabbit FDP tendon are more susceptible to damage as measured by microtear formation.

Several occupational epidemiologic studies have identified biomechanical factors for workplace overuse injuries such as epicondylitis (5, 7-9, 11, 14, 15, 19, 25). These include repetitiveness of work, forceful exertion, mechanical stress and posture. A review by the Centers of Disease Control found there to be a strong evidence for the risk of developing epicondylitis when there is high force and hand repetition in the occupation setting, signifying the importance of evaluating both risk factors. Werner et al. (29) reported the effect of several ergonomic risk factors on the development of tendinitis or elbow pain in industrial and clerical workers. Hand repetition did not have a significant effect ($p = 0.33$) but there was an effect of peak force ($p = 0.04$). Descatha et al. (4)

investigated the associated risk factors of medial epicondylitis in occupational settings and found that forceful work was a risk factor (OR = 1.9; CI = 1.15 – 3.32) whereas exposure to repetitive work (OR = 1.11; CI = 0.59 – 2.10) was not a risk factor. Latko et al. (10) found a strong effect of repetition when investigating predictors of epicondylitis ($p = 0.01$); however, they did not evaluate the effects of force as a risk factor because the force levels were homogeneous across subjects in the study.

Several in vivo animal studies have evaluated the effects of repetitive loading in tendon on structural or biochemical changes (1, 2, 13, 20, 24). However, no in vivo studies evaluated mechanical and cellular effects for different loading patterns. These *in vivo* loading models utilized loading rates as low as 0.07 reps/min (2) and as high as 2.5 reps/min (1) . Peak forces ranged from levels below 15% of strength to high (not unrecorded) forces associated with running. Perry et al. (2005), using an overuse rotator cuff model involving rats running on a treadmill, showed an increase in mRNA of inflammatory markers (COX-2 and FLAP) and angiogenic factors (VEGF, VWF) after only 3 days of exercise. Barbe et al. (2) reported a decrease in reach rate, task duration and a change in the preferred grasping technique utilized by the rats performing repetitive grasping. Changes were noted as early as week 3 involving an infiltration of macrophages in tendons (2). Cellular responses involving inflammatory products may have effects on the structural integrity of the tendon. Macrophages can produce MMP-1, which breaks down type I collagen. Growth factors associated with healing may stimulate new collagen formation; the new collagen may not have established intermolecular collagen cross-links. Under repetitive loading, reduced cross-linking may increase the presence of microtears.

Limitations to this study should be noted. The sample size for LFHR (n = 7) was low but even if it were increased, there is no trend in the data to suggest that the conclusion would change. Another limitation is the loading pattern. The parameters picked for the loading pattern cover a limited range and may not include some loading patterns found in the occupational or sports settings. However, the values picked fit within a physiological range. In the workplace, hand repetition rates can be as high as 1 grip per second (11). Peak force exposures of the hand have been reported to be greater than 100N, close to worker strength (3, 23). Loading duration may be is considered another limitation. When the rate of injury exceeds the rate of repair, disease progression may occur. Therefore, it is important to study different timepoints to fully understand the structural and cellular effects of loading on tendon injury. Another limitation involves the loading schedule. The animals were loaded for 2 hours per day, three times a week; a total of 80 hours of cumulative loading over 14 weeks. In the workplace, employees are frequently exposed to longer periods of hand intensive work. Therefore, the loading duration tested is less than found in many workplaces. Other timepoints may also be useful in revealing other changes in that occur in the disease progression of tendinosis (e.g. angiofibroblastic dysplasia, larger tears, etc.)

Overuse tendon injuries are common in the workplace. These injuries are multifactorial in etiology and the underlying cellular mechanisms are still not well known. Based on this model, peak force has a greater impact than repetition rate in causing microtears in tendon. The results presented here suggest that under certain loading conditions, lowering peak force will have a greater effect on preventing or managing tendon overuse injuries than lowering repetition rate.

Acknowledgement

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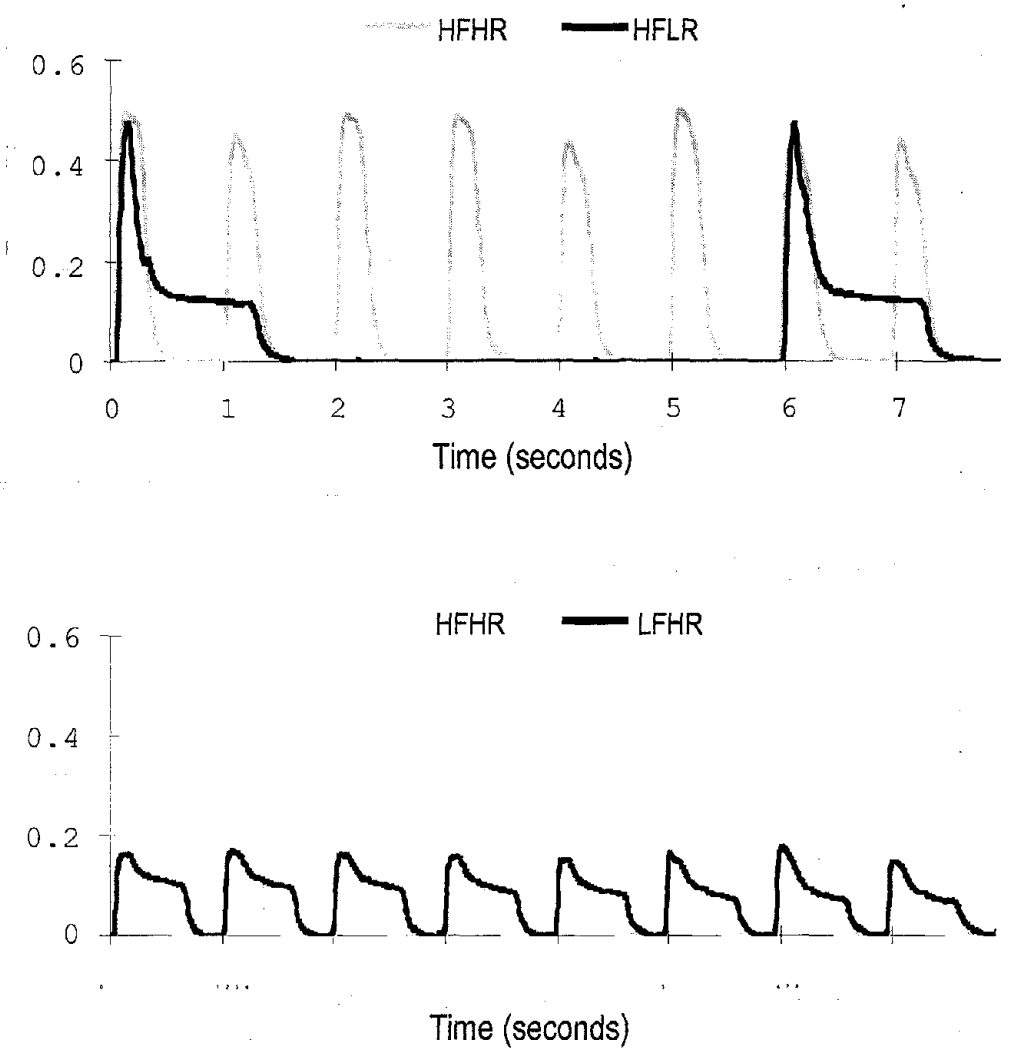

Figure 5.1. Typical loading profiles for High Force, High Repetition (HFHR), High Force, Low Repetition (HFLR) and Low Force High Repetition (LFHR). Peak force is equivalent in HFHR and HFLR while repetition rate varies. Repetition rate is equivalent between HFHR and LFHR while peak force varies.

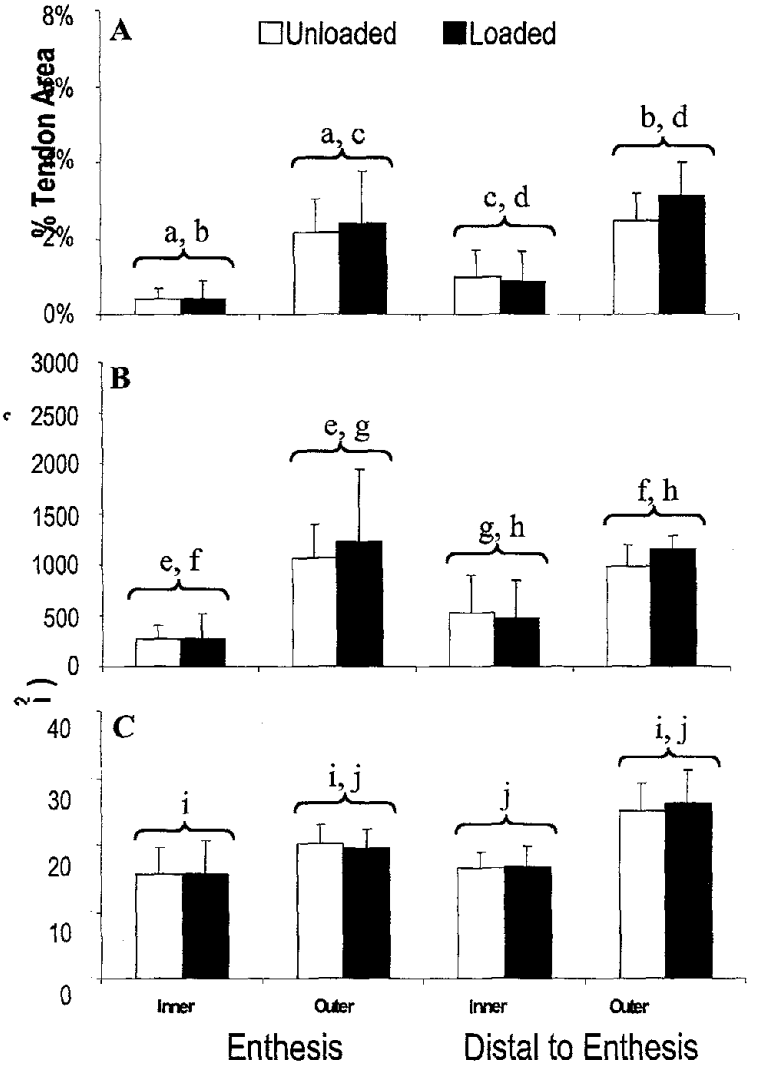

Figure 5.2. Tear area as a percent of tendon area (A), tear density (B), and mean tear size (C) for the LFHR loading pattern. The interaction terms (loading pattern x region) for all tear measures were not significant ($p > 0.05$). Similarly, there was no significant ($p > 0.05$) effect of loading for the tear measures. However, regional effects were seen. Regions marked with the same lower case letter are significantly different, based on the Tukey follow-up test. Error bars are ± SD, n = 8.

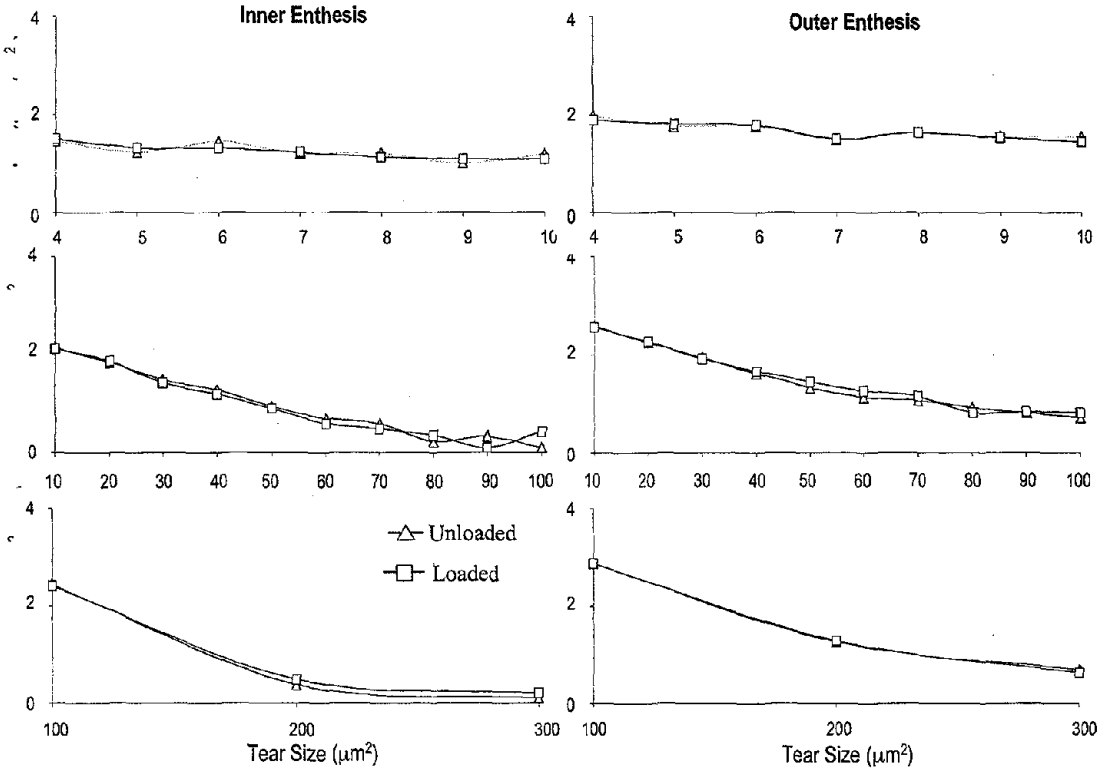

Figure 5.3. The log of the distribution of microtears by tear size is shown for the inner and outer regions of the tendon at the enthesis for the LFHR loading. No significant differences were found (paired t-test, $p > 0.01$) [N = 7]. For significant differences in the HFHR and HFLR patterns see Fig 5 and 6 of (16).

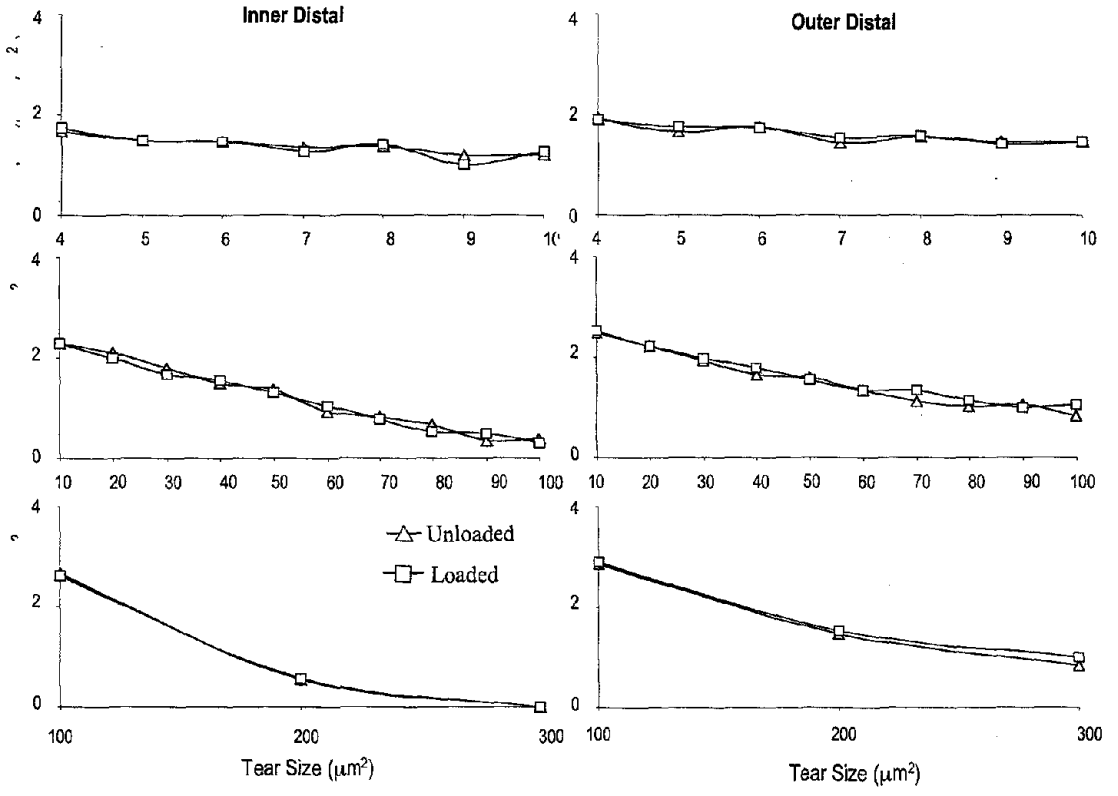

Figure 5.4. The log of the distribution of microtears by size is shown for the inner and outer parts of the tendon distal to the enthesis for the LFHR loading pattern. No significant differences were found (paired t-test, $p > 0.01$) [$N = 7$]. For significant differences in the HFHR and HFLR patterns see Fig 5 and 6 of (16).

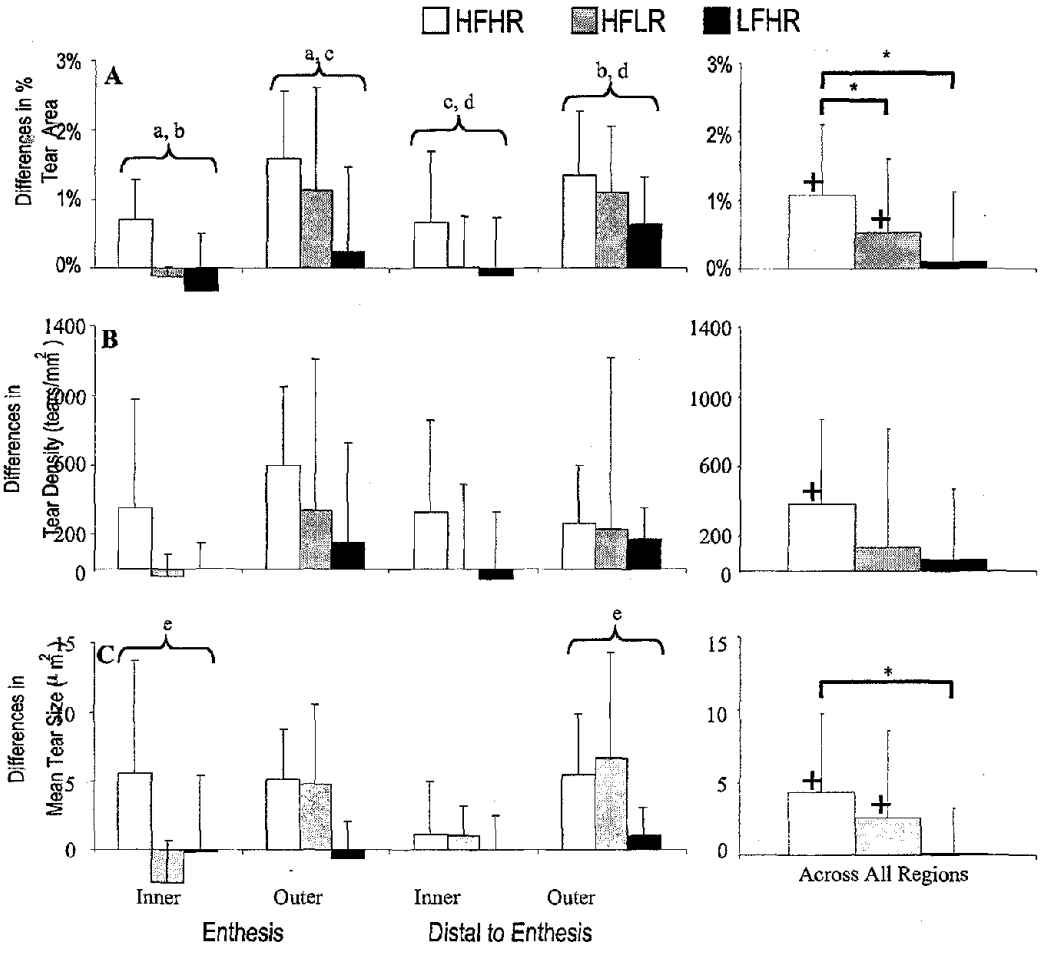

Figure 5.5. Comparison of tear measures for HFHR, HFLR and LFHR loading condition by region and across the four regions. Values represent mean differences (± s.d.) between loaded and unloaded tendons of the same animal. Tear area as a percent of tendon area (A) was significantly affected by loading pattern and region. HFHR demonstrated greater changes than both the HFLR and the LFHR patterns. Tear density (B) was not affected by loading pattern or region ($p > 0.05$). Mean tear size (C) was significantly affected by both loading pattern and region. Regions marked with the same lower case letter are significantly different, based on the Tukey follow-up test ($p < 0.05$). Charts on the right are overall effects of loading pattern averaged across regions. HFHR

demonstrated greater changes than the LFHR loading pattern. Loading patterns marked with a * are significantly different from one another ($p < 0.05$). Loading patterns marked with a † had significant differences between loaded and unloaded tendons within that loading pattern. N = 24

REFERENCES

1. Backman C, Boquist L, Friden J, Lorentzon R, Toolanen G: Chronic achilles paratenonitis with tendinosis: an experimental model in the rabbit. *J Orthop Res* 8:541-7, 1990
2. Barbe MF, Barr AE, Gorzelany I, Amin M, Gaughan JP, Safadi FF: Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *J Orthop Res* 21:167-76, 2003
3. Burdorf A, van Riel M, Brand T: Physical load as risk factor for musculoskeletal complaints among tank terminal workers. *Am Ind Hyg Assoc J* 58:489-97, 1997
4. Descatha A, Leclerc A, Chastang JF, Roquelaure Y: Medial epicondylitis in occupational settings: prevalence, incidence and associated risk factors. *J Occup Environ Med* 45:993-1001, 2003
5. Gerr F, Letz R, Landrigan PJ: Upper-extremity musculoskeletal disorders of occupational origin. *Annu Rev Public Health* 12:543-66, 1991
6. Huang CY, Wang VM, Pawluk RJ, Bucchieri JS, Levine WN, Bigliani LU, Mow VC, Flatow EL: Inhomogeneous mechanical behavior of the human supraspinatus tendon under uniaxial loading. *J Orthop Res* 23:924-30, 2005
7. Hume PA, Reid D, Edwards T: Epicondylar injury in sport: epidemiology, type, mechanisms, assessment, management and prevention. *Sports Med* 36:151-70, 2006
8. Jacobson JA, Miller BS, Morag Y: Golf and racquet sports injuries. *Semin Musculoskelet Radiol* 9:346-59, 2005
9. Kurppa K, Viikari-Juntura E, Kuosma E, Huuskonen M, Kivi P: Incidence of tenosynovitis or peritendinitis and epicondylitis in a meat-processing factory. *Scand J Work Environ Health* 17:32-7, 1991
10. Latko WA, Armstrong TJ, Franzblau A, Ulin SS, Werner RA, Albers JW: Cross-sectional study of the relationship between repetitive work and the prevalence of upper limb musculoskeletal disorders. *Am J Ind Med* 36:248-59, 1999
11. Luopajarvi T, Kuorinka I, Virolainen M, Holmberg M: Prevalence of tenosynovitis and other injuries of the upper extremities in repetitive work. *Scand J Work Environ Health* 5 suppl 3:48-55, 1979
12. McKean ML, Costello K, Scordato R, Ligugnana R: [D.L.vo n. 626/94 - Musculoskeletal diseases caused by use of micropipette in laboratory]. *G Ital Med Lav Ergon* 27:240-3, 2005
13. Messner K, Wei Y, Andersson B, Gillquist J, Rasanen T: Rat model of Achilles tendon disorder. A pilot study. *Cells Tissues Organs* 165:30-9, 1999
14. Moore JS: Biomechanical models for the pathogenesis of specific distal upper extremity disorders. *Am J Ind Med* 41:353-69, 2002
15. Moore JS, Garg A: Upper extremity disorders in a pork processing plant: relationships between job risk factors and morbidity. *Am Ind Hyg Assoc J* 55:703-15, 1994
16. Nakama LH, King KB, Abrahamsson S, Rempel DM: The effect of repetition rate on the formation of microtears in tendon in an in vivo cyclical loading model. *J Orthop Res*, Submitted

17. Nakama LH, King KB, Abrahamsson S, Rempel DM: Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. *J Orthop Res* 23:1199-205, 2005
18. Nakama LH, King KB, Abrahamsson S, Rempel DM: VEGF, VEGFR-1, and CTGF cell densities in tendon are increased with cyclical loading: An in vivo tendinopathy model. *J Orthop Res* 24:393-400, 2006
19. Ono Y, Nakamura R, Shimaoka M, Hiruta S, Hattori Y, Ichihara G, Kamijima M, Takeuchi Y: Epicondylitis among cooks in nursery schools. *Occup Environ Med* 55:172-9, 1998
20. Perry SM, McIlhenny SE, Hoffman MC, Soslowsky LJ: Inflammatory and angiogenic mRNA levels are altered in a supraspinatus tendon overuse animal model. *J Shoulder Elbow Surg* 14:79S-83S, 2005
21. Roquelaure Y, Mariel J, Fanello S, Boissiere JC, Chiron H, Dano C, Bureau D, Penneau-Fontbonne D: Active epidemiological surveillance of musculoskeletal disorders in a shoe factory. *Occup Environ Med* 59:452-8, 2002
22. Roquelaure Y, Mechali S, Dano C, Fanello S, Benetti F, Bureau D, Mariel J, Martin YH, Derriennic F, Penneau-Fontbonne D: Occupational and personal risk factors for carpal tunnel syndrome in industrial workers. *Scand J Work Environ Health* 23:364-9, 1997
23. Silverstein BA, Fine LJ, Armstrong TJ: Hand wrist cumulative trauma disorders in industry. *Br J Ind Med* 43:779-84, 1986
24. Soslowsky LJ, Thomopoulos S, Tun S, Flanagan CL, Keefer CC, Mastaw J, Carpenter JE: Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *J Shoulder Elbow Surg* 9:79-84, 2000
25. United States. Bureau of Labor Statistics.: *Occupational injuries and illnesses--counts, rates, and characteristics,* p v. Washington, DC, The Bureau : For sale by the U.S. G.P.O. Supt. of Docs., 1995
26. Viikari-Juntura E, Kurppa K, Kuosma E, Huuskonen M, Kuorinka I, Ketola R, Konni U: Prevalence of epicondylitis and elbow pain in the meat-processing industry. *Scand J Work Environ Health* 17:38-45, 1991
27. Wakabayashi I, Itoi E, Sano H, Shibuya Y, Sashi R, Minagawa H, Kobayashi M: Mechanical environment of the supraspinatus tendon: a two-dimensional finite element model analysis. *J Shoulder Elbow Surg* 12:612-7, 2003
28. Werner RA, Franzblau A, Gell N, Hartigan A, Ebersole M, Armstrong TJ: Predictors of persistent elbow tendonitis among auto assembly workers. *J Occup Rehabil* 15:393-400, 2005
29. Werner RA, Franzblau A, Gell N, Ulin SS, Armstrong TJ: A longitudinal study of industrial and clerical workers: predictors of upper extremity tendonitis. *J Occup Rehabil* 15:37-46, 2005
30. Werner RA, Franzblau A, Gell N, Ulin SS, Armstrong TJ: Predictors of upper extremity discomfort: a longitudinal study of industrial and clerical workers. *J Occup Rehabil* 15:27-35, 2005

CHAPTER VI:

THE EFFECT OF PEAK FORCE, REPETITION RATE AND MICROTEAR FORMATION ON VEGF, VEGFR-1 AND CTGF CELL DENSITIES IN AN IN VIVO CYCLICAL LOADING MODEL

6.1 Abstract

Different cyclical loading patterns were used in an in vivo loading model of tendinosis to understand the effects of loading on growth factor production (VEGF, VEGFR-1 and CTGF). It was previously shown that cell densities of cells staining with these proteins were increased undergoing High Force High Repetition Loading (HFHR) (18). In the current study, High Force Low Repetition (HFLR) and Low Force High Repeition (LFHR) loading resulted in no significant differences ($p > 0.05$) in cell densities between loaded and unloaded limbs with the exception of VEGFR-1. Regional differences were observed between the outer regions of the tendon and the inner regions of the tendon; these differences were similar to our findings for the distribution of microtears (17). Associations between protein cell densities (VEGF, VEGFR-1 or CTGF) and tear density were examined using linear regression. There is a relationship between tendon microtear density and the growth factors VEGF and VEGFR-1. The slope of the VEGFR-1 cell density vs. tear density was significantly different between

loaded and unloaded limbs (m_{loaded} = 0.2 and $m_{unloaded}$ = 0.04, p = 0. 03) for HFHR loading. VEGF cell density and tear density were moderately associated (m_{loaded} = 0.51, $m_{unloaded}$ = 0.32). Blood vessel density (capillary and arteriole) in the paratenon was not significantly different between loaded and unloaded limbs within a loading pattern, but across the loading patterns, the density was higher with HFLR loading than the LFHR loading pattern. The results from this study demonstrate no effect of HFLR loading pattern on cell density for any of the growth factors, but a possible protective effect (decreased density) for VEGF and VEGFR-1 for the LFHR loading pattern. Long term loading with low repetitive force appears to decrease growth factors and may be protective for tendinopathy.

6.2 Introduction

Studies involving *in vivo* (1, 18, 21, 23-25, 27) and *in vitro* (26, 28) models of tendon overuse injuries have identified a relationship between loading and growth factors, such as VEGF and CTGF. An increase in VEGF, VEGFR-1 and CTGF may indicate the tendon's response to loading by activating pathways leading to neovascularization (6-10, 21, 23-25), cellular proliferation or differentiation (20, 33) or matrix repair (11, 13, 30, 31, 34, 35). The dose-response relationships between external loading and changes in growth factors are unknown. It is likely that the magnitude of load and the repetition rate are important.

Epidemiologic evidence supports a relationship between overuse tendon injuries and forceful and repetitive hand activities (19). Werner et al. (32) reported that people whose jobs had required high levels of hand repetition and force, were at increased risk for having persistent elbow tendinitis. Latko et al. (12) reported that higher repetition

rates led to higher prevalence rates of tendinitis (12). There was a linear trend ($p = 0.004$) relating rates of repetition with prevalence rates of 4.2%, 8.1% and 14.5%, to repetition rates of low, medium and high, respectively.

Previously, our in vivo rabbit model demonstrated that cyclical loading of tendons led to an increase in microtears of the Flexor Digitorum Profundus (FDP). At high forces and a high repetition rate (HFHR), synthesis of VEGF, VEGFR-1 and CTGF were increased under cyclical loading (18). The application of two additional loading patterns High Force, Low Repetition (HFLR) and Low Force, High Repetition (LFHR) demonstrated that microtears were observed to be more sensitive to peak force than repetition rate (17).

The purpose of this study is to determine the effects of cyclical loading under different loading patterns (HFLR and LFHR) on VEGF, VEGFR-1 and CTGF production in tendon, and to determine the relationship, if any, between the synthesis of growth factors and tear density.

6.3 Methods

6.3a Animal Model

The animal loading model was described previously (15-18). This study was approved by the University of California, Berkeley's Committee on Animal Research. Weekly examinations of the paw, forearm and elbow revealed no tenderness, limping, nodules, swelling, limitation in range of motion, reduction in gross claw flexion strength, or skin breaks.

6.3b Tissue and Histological preparation

After 80h of cumulative loading, animals were weighed and euthanized. Tissue and histological preparation along with image acquisition for cell densities were identical to a previous study (18). The paratenon of the tendon (measured from the enthesis to the muscle-tendon junction) was digitally photographed under 200x magnification using an Axioskop MOT 2 microscope with an Axiocam digital camera (Carl Zeiss, Germany). The area was calculated using Axiovision software. Blood vessels were identified in the paratenon of the tendon and classified as capillaries or arterioles depending on size. Vessels with diameters less than 10μm were graded as capillaries and those greater than 10μm were marked as arterioles (Figure 6.3).

6.3c Statistical Analysis

A mixed model repeated measures ANOVA was used to analyze differences in cell density by region (6 regions) and by limb loading status (loaded or unloaded) within each loading pattern. Post hoc analysis was performed using the Tukey method for multiple comparisons. To examine the differences between different loading patterns, a two factor ANOVA was used to analyze differences between loaded and unloaded limbs in the cell densities by loading pattern (HFHR, HFLR, LFHR) and region (6 regions). Post hoc analysis was performed using the Tukey method for multiple comparisons.

The paratenon area was measured along with the capillary density (capillaries/mm^2), arteriole density (arterioles/mm^2) and overall vessel density (capillaries + arterioles per area). A paired t-test was used to evaluate differences between vessel density and paratenon area among the animals loaded. An ANOVA was used to analyze differences in vessel density by limb loading status (loaded or unloaded) and loading pattern (HFHR, HFLR and LFHR).

A standard linear regression and correlation coefficient were obtained for cell density versus tear measures. Comparison of the two regressions was performed to compare slope changes between loaded and unloaded limbs.

6.4 Results

High Force, Low repetition loading

6.4a VEGF

The limb by region interaction term in the RMANOVA was not significant ($p = 0.71$). The limb effect ($p = 0.62$) was not significant but the region effect ($p < 0.0001$) was significant. Using the Tukey follow-up test, significant differences between regions were found and are noted in Figure 6.2. Across the six ROIs, the average VEGF cell density ranged from 121 to 1675 (cells/mm^2) in the loaded tendon compared to 210 to 1973 in the unloaded tendon (Figure 6.2). The inner enthesis (22.3%) and outer distal (23.5%) regions had the greatest percent increase in the VEGF cell density when comparing the loaded to unloaded tendon. The other regions exhibited much smaller changes (1.2 – 8.9% increases).

6.4b VEGFR-1

The limb by region interaction term in the RMANOVA was not significant ($p = 0.89$). The limb effect ($p = 0.76$) was not significant but the region effect ($p = 0.0002$) was significant. Using the Tukey follow-up test, significant differences between regions were found and are noted in Figure 6.2. Across the six ROIs, the average VEGFR-1 cell density ranged from 375 to 1346 cells/mm^2 in the loaded tendon compared to 369 to 1653 cells/mm^2 in the unloaded tendon (Figure 6.2). The inner distal (22.1%) had the greatest percent increase in the VEGFR-1 cell density when comparing the loaded to unloaded tendon. The other regions exhibited negligible changes (-3 – 7.9% changes).

6.4c CTGF

The limb by region interaction term ($p = 0.77$) in the RMANOVA was not significant. The limb effect ($p = 0.89$) was not significant but the region effect ($p < 0.0001$) was significant. Using the Tukey follow-up test, significant differences between regions were found and are noted in Figure 6.2. Across the six ROIs, the average CTGF cell density ranged from 554 to 2402 cells/mm^2 in the loaded tendon compared to 385 to 2522 cells/mm^2 in the unloaded tendon (Figure 6.2). Increased CTGF cell densities were observed in all regions except the outer distal regions (-3.8% decrease). The outer enthesis, inner distal and center distal regions had somewhat large increases (17.4% to 20.7%).

Low Force, High repetition loading

6.4d VEGF

The limb by region interaction term ($p = 0.29$) together with the limb effect ($p = 0.08$) in the RMANOVA was not significant. The region effect ($p < 0.0001$) was significant. Using the Tukey follow-up test, significant differences between regions were found and are noted in Figure 6.3. Across the six ROIs, the average VEGF cell density ranged from 312 to 1994 cells/mm^2 in the loaded tendon compared to 326 to 1754 cells/mm^2 in the unloaded tendon (Figure 6.4). Cell densities for VEGF (-20.6% decrease to 14.2% increase) were observed in all regions except the outer distal regions (-3.8% decrease).

6.4e VEGFR-1

The limb by region interaction term ($p = 0.97$) in the RMANOVA was not significant. However the limb effect ($p = 0.02$) and region effect ($p = 0.0007$) was

significant. Using the Tukey follow-up test, significant differences between regions were found and are noted in Figure 6.3. Across the six ROIs, the average VEGFR-1 cell density ranged from 162 to 1330 cells/mm^2 in the loaded tendon compared to 356 to 1257 cells/mm^2 in the unloaded tendon (Figure 6.3). All regions except for the outer enthesis (5.9%) experienced decreases in VEGFR-1 cell density (-0.3% to –22), where the center distal had the largest decrease.

6.4f CTGF

The limb by region interaction term ($p = 0.08$) together with the limb effect ($p = 0.57$) in the RMANOVA was not significant. The region effect ($p < 0.0001$) was significant. Using the Tukey follow-up test, significant differences between regions were found and are noted in Figure 6.3. Across the six ROIs, the average CTGF cell density ranged from 166 to 2284 cells/mm^2 in the loaded tendon compared to 494 to 1941 cells/mm^2 in the unloaded tendon (Figure 6.3). A large increase (38.8%) in CTGF cell density was observed in the outer distal region. The cell density in the other regions fluctuated between –12.6% to 2.6% changes.

6.4g Differences between HFHR, HFLR and LFHR loading on VEGF, VEGFR-1 and CTGF cell densities.

The effect of loading pattern (HFHR, HFLR, or LFHR) on VEGF, VEGFR-1 and CTGF was evaluated by comparing data from this study to data from the prior study (15-18). Interaction terms (loading pattern x region) for the differences in cell densities (loaded – unloaded) for VEGF ($p = 0.90$) and VEGFR-1 ($p = 0.94$) were not significantly different. Furthermore the differences in cell densities for VEGF ($p = 0.09$) and VEGFR-1 ($p = 0.99$) did not vary significantly by region. However loading pattern had a

significant effect on cell density for VEGF ($p < 0.001$) and VEGFR-1 ($p = 0.01$). The differences in VEGF cell density were greater with HFHR loading than with HFLR ($p = 0.03$) and LFHR ($p < 0.0001$) loading (Figure 6.4). The differences in VEGFR-1 cell density were greater with HFHR loading than with LFHR loading ($p = 0.01$) (Figure 6.4).

The interaction term (loading pattern x region) for the differences in CTGF cell density was significant ($p = 0.02$). Follow-up tests revealed that LFHR loading resulted in greater differences in the outer distal region of the tendon than the LFHR inner distal region ($p = 0.04$) and the HFLR outer distal region ($p = 0.04$).

6.4h Differences between HFHR, HFLR and LFHR loading on vascularization.

The paratenon area was not different between loaded and unloaded tendons for each of the three loading patterns ($p > 0.05$, paired t-test) or between the three loading patterns ($p = 0.10$, ANOVA). The capillary density in the paratenon was not different between loaded and unloaded tendons within each of the three loading patterns ($p > 0.05$, paired t-test) but there were differences found between loading patterns. HFLR loading led to more capillaries per mm^2 than the LFHR group ($p = 0.02$, Tukey test). There were no significant differences between HFHR loading and the HFLR loading. The arteriole density was not different between loaded and unloaded tendons within any of the three loading patterns ($p > 0.05$) or between loading patterns ($p = 0.79$, ANOVA).

6.4i The relationship of microtear density to VEGF, VEGFR-1 and CTGF cell densities.

Growth factor density was plotted against tear density in both the loaded and unloaded limbs for the three loading patterns (HFHR, HFLR and LFHR). The slope, correlation coefficients and p values (slope significantly different from 0) can be found in

Table 1. VEGF is directly related to tear density, regardless of loading. This can be seen in Table 1 where the slope for VEGF is significantly different from 0 (versus tear density) in all three loading patterns. In addition, the VEGF-tear density slopes between loaded and unloaded limbs are not significantly different from one another. Although both VEGF and tear density increase from unloaded to loaded limb under HFHR loading, the relationship between VEGF and tear density does not seem to adjust with loading. Figure 6.5 illustrates the VEGF versus tear density under HFLR loading.

VEGFR-1 in the unloaded tendon under HFHR loading (Figure 6.5) had a weak correlation to the tear density and the slope was not significantly different from zero ($p = 0.48$). However the results were found to be different in the loaded limb, where the correlation was higher ($r^2 = 0.4$) and the slope was 0.20 ($p = 0.0001$). The two slopes (VEGFR-1 versus tear density) were significantly different from one another ($p = 0.03$). This may indicate that VEGFR-1 has no relationship to tear density except when the loading is high (HFHR). Both VEGFR-1 and tear density are increased with HFHR loading and HFHR loading may provide a pathway for the cells to actively produce VEGFR-1 when tear density is high.

CTGF had no relation to tear density ($p > 0.05$, Table 1) under HFHR and HFLR loading. Slopes did not differ between unloaded and loaded limbs ($p = 0.54$ HFHR; $p = 0.14$ HFLR; $p = 0.97$ LFHR) but the slope of CTGF versus tear density was greater than zero in both the loaded ($p = 0.01$) and unloaded limb ($p = 0.02$) under LFHR loading.

6.5 Discussion

The results presented here are the first to evaluate the effect of both peak force and repetition rate on VEGF, VEGFR-1 and CTGF cell density in an *in vivo* model of

cyclically loaded tendon. The greatest increase in growth factors occurred with HFHR loading. The differences between limbs nearly disappears at the lower repetition rate (HFLR) and became inverted (unloaded tendon has higher densities than loaded tendons for VEGFR-1) at the lower peak force pattern(LFHR). Regional differences were present and were similar to the microtear distribution where the outer regions exhibited greater changes.

VEGF, VEGFR-1 and CTGF have been shown to have important functions associated with healing such as angiogenesis and matrix repair (7, 8, 11, 22, 23, 27, 29, 36). VEGF and its receptor are increased in degenerated tendons (24, 27) and not present in normal, healthy tendons. The results of our study suggest that the increase in VEGF, VEGFR-1 and CTGF may be an attempt to initiate pathways to repair the microstructural damage caused by loading.

Previously we reported the formation of microtears in tendons exposed to three loading patterns: HFHR, HFLR and LFHR (17). Microtear formation was increased most with HFHR loading, followed by HFLR, followed by LFHR loading. The formation of microtears may have an effect on the cellular response since the cells are embedded in the tendon's matrix and a disruption in the surrounding substance may trigger a cellular response. Both tear density and VEGFR-1 cell density increased under HFHR loading. VEGFR-1 was moderately related to tear density; the VEGFR-1 cell density increased as the tear density increased in the loaded tendon ($r^2 = 0.4$), whereas in the unloaded limb there was no relationship between VEGFR-1 and tear density ($r^2 = 0.01$). VEGFR-1 is a key receptor for VEGF. Therefore, under HFHR loading conditions VEGFR-1 may provide a pathway for VEGF to initiate angiogenesis and attempt matrix repair. The

relationship between VEGF and loading was different. VEGF cell density was related to tear density in both the loaded and unloaded tendon, independent of loading.

Under LFHR loading, VEGFR-1 cell density was lower in the loaded tendon compared to the unloaded tendon ($p = 0.02$). Similarly, there was a trend for VEGF cell density to be lower in the loaded tendons ($p = 0.08$). Therefore, repetitive loading at a lower peak force may have a protective effect (less VEGF and VEGFR-1) while maintaining the high peak force but lowering the repetition rate had a null effect (no difference between tendons) in terms of cell densities.

The effect of repetitive loading on tendon has been documented previously but the inter-relationships between some these outcome measures have not been explored. The tendon's mechanical and biological response to loading has impacts on one another. It has already been reported that mechanical deformation can trigger a cell to produce VEGF (26) and CTGF (28). Therefore a disruption in the tendon's ECM, as measured by microtears, may up-regulate certain growth factors.

A proposed mechanism is illustrated in Figure 6.6. With continued repetitive loading, the inter-relationships between the tendon's mechanical and biological response to loading are diagramed. Repetitive loading affects both the cellular and structural aspects of the tendon. Structurally, as the tendon fibers continually slide past eachother, tendon cross-links may weaken, reducing the overall structural stability of the tendon. Cellularly, the tendon's cells react to load by producing growth factors and/or cytokines in response to loading. Depending on the magnitude and extent of damage, the tendon may respond at a level that reflects a healthy adaptation. However, if the rate of damage exceeds the rate of repair, the tendon may enter a degenerative pathway. This may

include tear accumulation, neovascularization, increased production of proteoglycans in the matrix, cellular changes including disorganized alignment and hyperplasia.

Other authors (2, 14) have reported hypervascularization in tendons exposed to repetitive loading. Backman et al. (2), reported semiquantitatively that rabbit tendons from the exercised limb exhibited moderate increases of capillaries compared to the control limb (an average of 2.1/3 for 13 animals; 1 = slight change, 2 = moderate change and 3 = marked change). We did not observe a difference in vessel density between loaded and unloaded limbs. There was an increase in capillary density for both limbs of the HFLR group compared to the LFHR group, suggesting a positive systemic effect of loading. There was, however, no corresponding difference in VEGF staining cell density to explain this difference. The changes in vasculature may occur later in our model. Barr (3-5) has proposed several mechanisms that suggest that inflammation and its mediators, both local and systemic, play a large role in the development of a musculoskeletal injury. Our model did no evaluate systemic mediator of vascularization.

Some of the limitations in this study include a low sample size, although the data suggests that increasing the sample size will not change the trend seen in the results. The cumulative period of loading may be considered another limitation. The animals were loaded for 2 hours per day, three times a week for 80 hours of cumulative loading for a 14 week period. In the workplace, employees are frequently exposed to longer periods of work. A longer loading period may have let to microvascularization and other changes that occur further downstream. The growth factors (and receptor) considered in this study were not meant to be conclusive of all markers associated with degeneration. Future studies may evaluate proteases and other inflammatory markers.

Previously (17) we reported that microtears were more sensitive to peak force than to repetition rate. A similar relationship is observed for the growth factors VEGF, VEGFR-1 and CTGF. The results of this study suggest that the growth factors we examined are both equally sensitive to changes in peak force and repetition rate. However, the formation of microtears may affect other degenerative factors in tendons, including the synthesis of these growth factors. Microtears in the tendon are more sensitive to peak force but growth factor synthesis is affected by both a reduction of repetition rate and peak force. After sustained exposure to low levels of force, the damage tolerance of the tendon may not be reached, instead healthy adaptation may occur as seen in the decreased synthesis of VEGFR-1 and VEGF. By looking at the tendon's response to selected loading patterns, this study provides an opportunity to study the interrelationships between the tendon's biological and mechanical responses to loading, which may have been overlooked if they were considered independent. Both structural (microtear formation) and cellular (change in growth factor synthesis) changes were affected by a reduction in repetition rate and peak load, where both may be considered as players in the degenerative pathway of tendinosis. Reducing peak hand forces in the occupational setting may be an important step in preventing tendon degeneration associated with repetitive loading.

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VEGF, VEGFR-1 and CTGF versus Tear Density

	Unloaded			Loaded			Slope Difference*
	slope	r^2	p	slope	r^2	p	p
HFHR							
VEGF	0.26	0.26	**0.0002**	0.22	0.27	**0.001**	0.72
VEGFR-1	0. 04	0.01	0.48	0.20	0.40	**0.0001**	**0.03**
CTGF	-0.015	0.00	0.73	0.02	0.01	0.61	0.54
HFLR							
VEGF	0.3	0.32	**0.0001**	0.35	0.51	**0.0001**	0.55
VEGFR-1	0. 0004	0.00	0.95	0.10	0.12	0.06	0.30
CTGF	-0.102	0.02	0.45	0.14	0.07	0.16	0.14
LFHR							
VEGF	0.24	0.16	**0.04**	0.28	0.26	**0.01**	0.77
VEGFR-1	0.01	0.00	0.92	0.08	0.03	0.34	0.64
CTGF	0. 39	0.2	**0.02**	0.39	0.29	**0.01**	0.97

Table 1. Slope, correlation coefficients and p values between tear density and VEGF, VEGFR-1 and CTGF cell density. The slope difference* column indicates the p value for the difference in slopes between loaded and unloaded tendons.

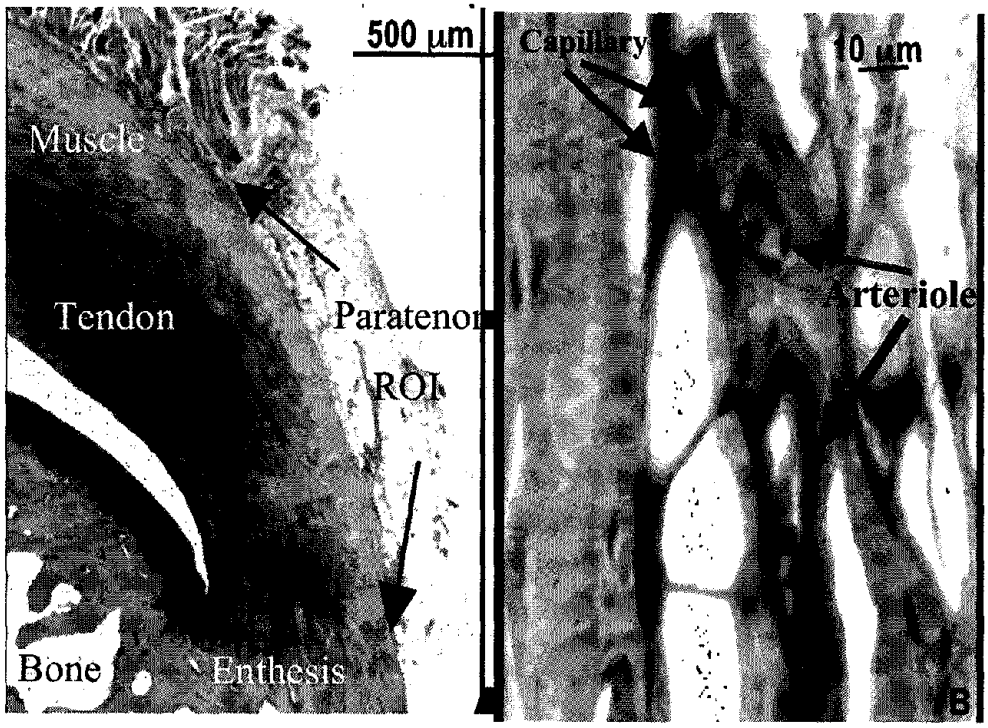

Figure 6.1. **(A)** Paratenon region of interest is adjacent to tendon extending from the enthesis to the muscle. **(B)** Capillaries and arterioles shown within paratenon.

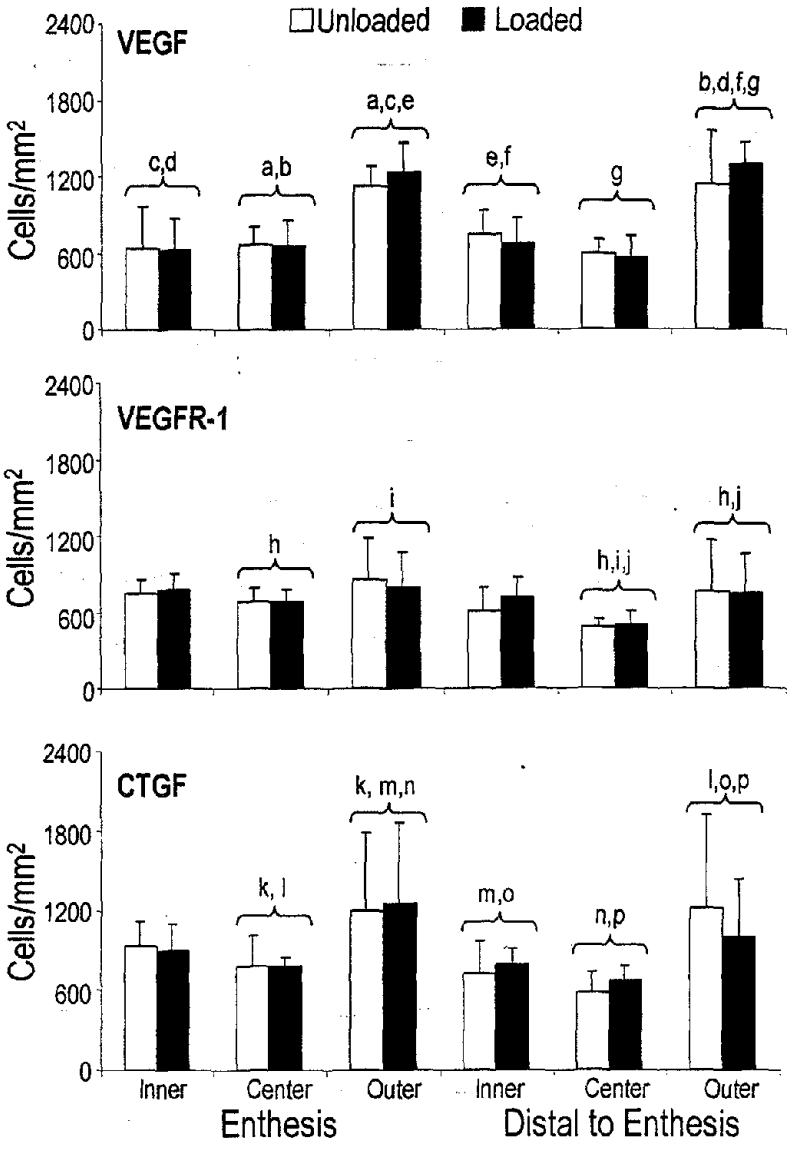

Figure 6.2. VEGF, VEGFR-1 and CTGF cell staining densities (mean ± s.d.) for loaded and unloaded tendon for HFLR loading. Across all regions, cell densities for the three growth factors were not significantly different between loaded and unloaded tendons. The limb x region interaction terms were not significant for all three. Regions marked with the same lower case letter are significantly different based on the Tukey follow-up test (n= 8).

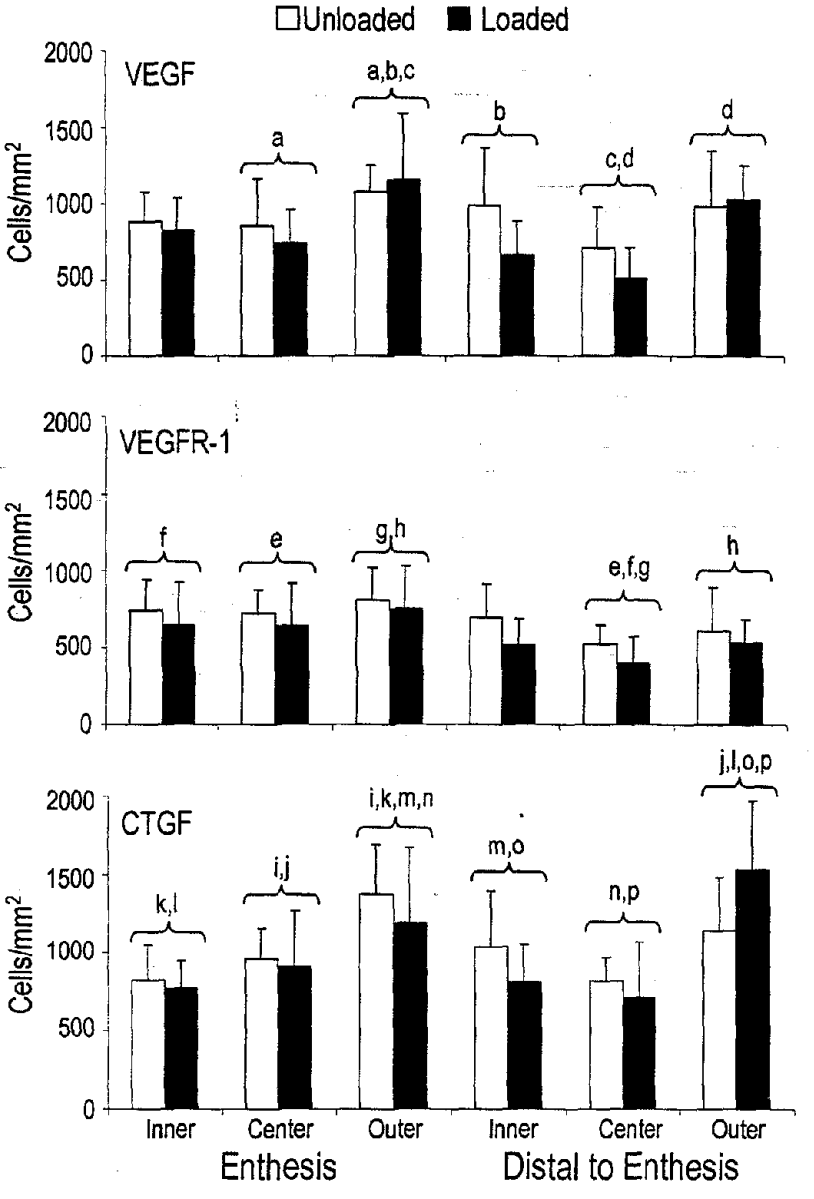

Figure 6.3. VEGF, VEGFR-1 and CTGF cell staining densities (mean ± s.d.) for loaded and unloaded tendon for LFHR loading. VEGFR-1 was the only protein found to be higher in the unloaded versus loaded limbs ($p = 0.02$). The limb x region interaction terms were not significant for all three. Regions marked with the same lower case letter are significantly different based on the Tukey follow-up test (n= 8).

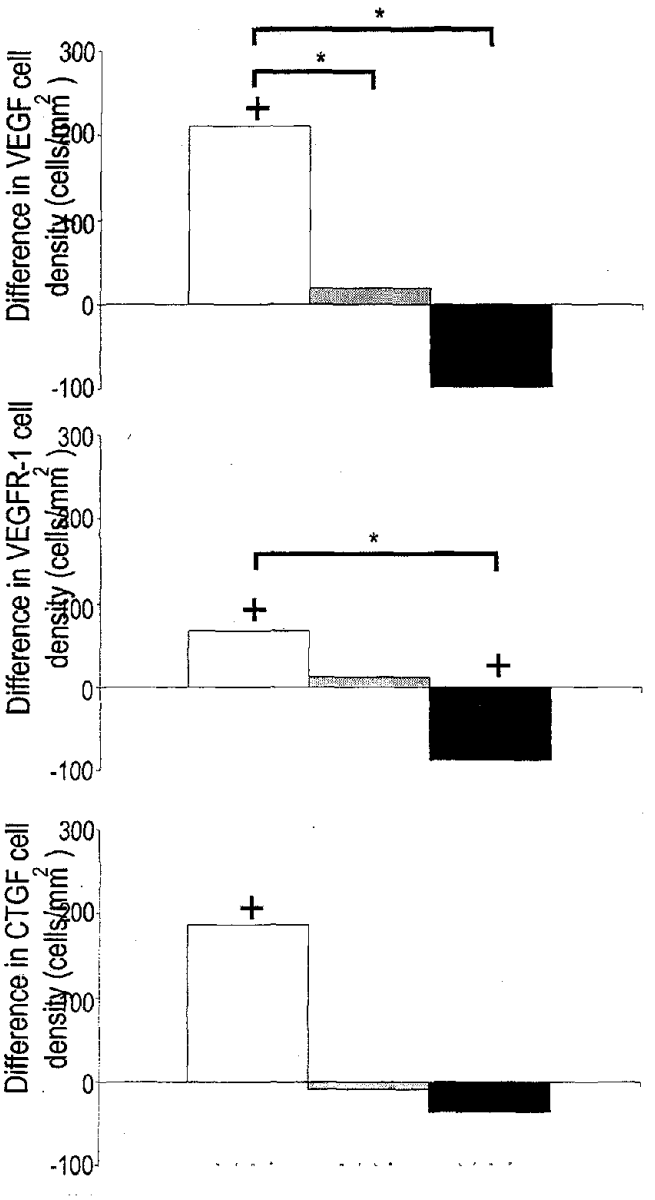

Figure 6.4. Differences (loaded – unloaded) for VEGF, VEGFR-1 and CTGF cell staining densities for HFHR, HFLR and LFHR loading patterns. Differences in VEGF cell density were significantly greater in the HFHR pattern compared to both the HFLR ($p = 0.03$) and LFHR ($p < 0.0001$) patterns. Differences in VEGFR-1 cell density were significantly greater in HFHR than the LFHR ($p = 0.003$) pattern. † indicates significant differences between limbs ($n = 25$).

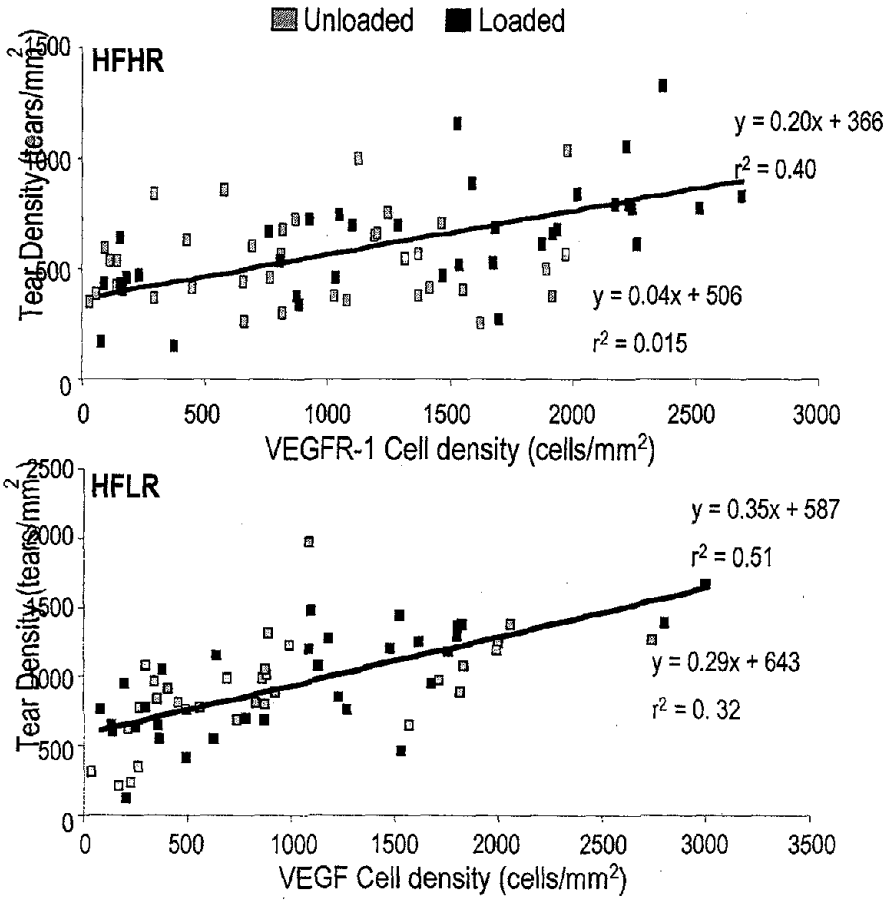

Figure 6.5. A. Plot of VEGFR-1 cell density versus tear density (tears/mm^2) for HFHR loading. The slope for the loaded limb was larger than the unloaded limb ($p = 0.03$, $n = 9$. **B.** Plot of VEGF cell density versus tear density (tears/mm^2) for HFLR loading ($n = 8$).

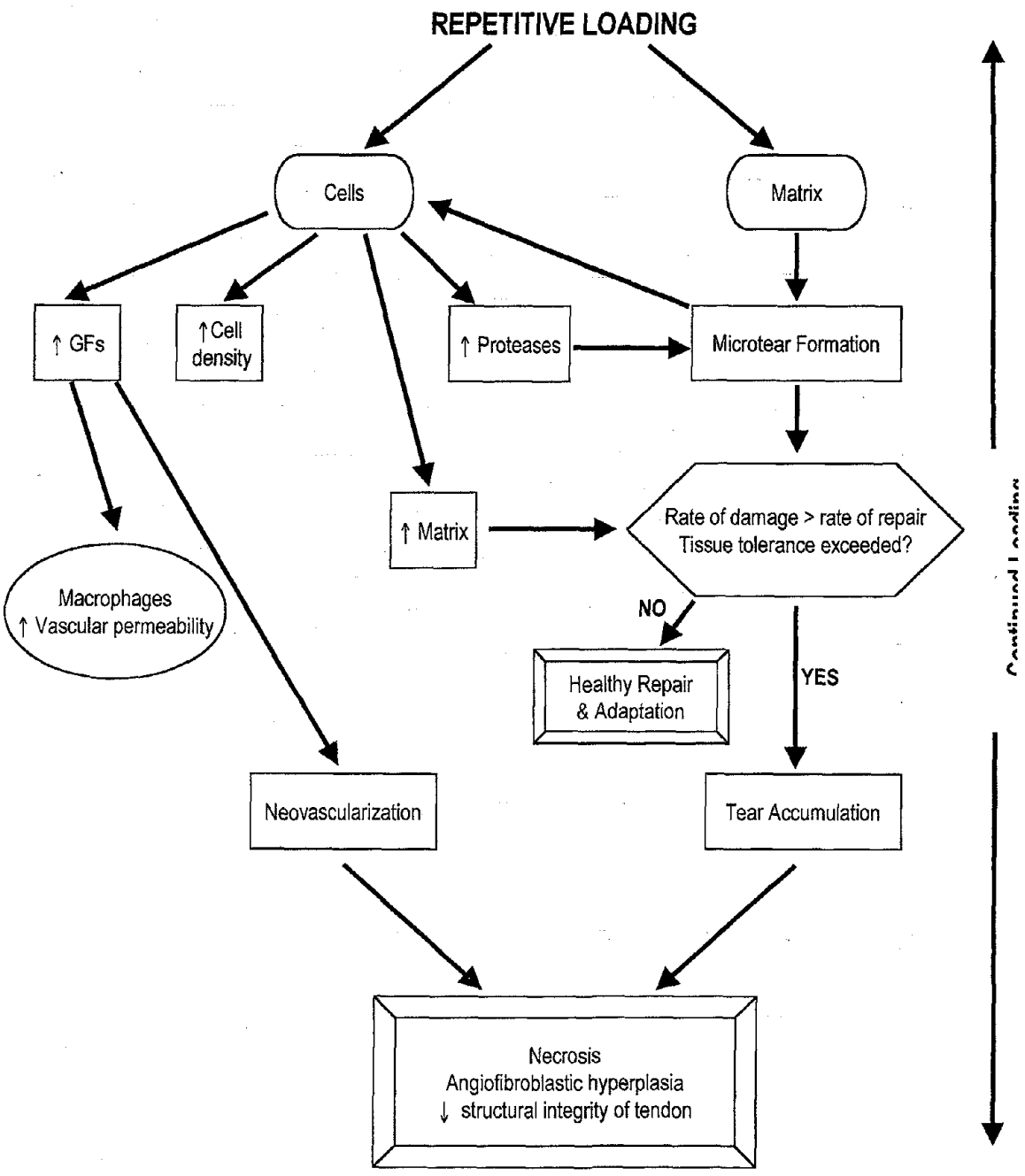

Figure 6.6. Proposed pathway for tendon degeneration due to repetitive loading.

REFERENCES

1. Alfredson H, Lorentzon M, Backman S, Backman A, Lerner UH: cDNA-arrays and real-time quantitative PCR techniques in the investigation of chronic Achilles tendinosis. *J Orthop Res* 21:970-5, 2003
2. Backman C, Boquist L, Friden J, Lorentzon R, Toolanen G: Chronic achilles paratenonitis with tendinosis: an experimental model in the rabbit. *J Orthop Res* 8:541-7, 1990
3. Barbe MF, Barr AE: Inflammation and the pathophysiology of work-related musculoskeletal disorders. *Brain Behav Immun*, 2006
4. Barr AE, Barbe MF: Inflammation reduces physiological tissue tolerance in the development of work-related musculoskeletal disorders. *J Electromyogr Kinesiol* 14:77-85, 2004
5. Barr AE, Barbe MF, Clark BD: Systemic inflammatory mediators contribute to widespread effects in work-related musculoskeletal disorders. *Exerc Sport Sci Rev* 32:135-42, 2004
6. Bidder M, Towler DA, Gelberman RH, Boyer MI: Expression of mRNA for vascular endothelial growth factor at the repair site of healing canine flexor tendon. *J Orthop Res* 18:247-52, 2000
7. Boyer MI, Watson JT, Lou J, Manske PR, Gelberman RH, Cai SR: Quantitative variation in vascular endothelial growth factor mRNA expression during early flexor tendon healing: an investigation in a canine model. *J Orthop Res* 19:869-72, 2001
8. Brigstock DR: Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 5:153-65, 2002
9. Deroanne CF, Hajitou A, Calberg-Bacq CM, Nusgens BV, Lapiere CM: Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. *Cancer Res* 57:5590-7, 1997
10. Dvorak HF, Brown LF, Detmar M, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146:1029-39, 1995
11. Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR: Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* 107:404-11, 1996
12. Latko WA, Armstrong TJ, Franzblau A, Ulin SS, Werner RA, Albers JW: Cross-sectional study of the relationship between repetitive work and the prevalence of upper limb musculoskeletal disorders. *Am J Ind Med* 36:248-59, 1999
13. Leask A, Abraham DJ: TGF-beta signaling and the fibrotic response. *Faseb J* 18:816-27, 2004
14. Messner K, Wei Y, Andersson B, Gillquist J, Rasanen T: Rat model of Achilles tendon disorder. A pilot study. *Cells Tissues Organs* 165:30-9, 1999
15. Nakama LH, King KB, Abrahamsson S, Rempel DM: The effect of repetition rate on the formation of microtears in tendon in an in vivo cyclical loading model. *J Orthop Res*, Submitted

16. Nakama LH, King KB, Abrahamsson S, Rempel DM: Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. *J Orthop Res* 23:1199-205, 2005
17. Nakama LH, King KB, Abrahamsson S, Rempel DM: Peak force has a greater effect than repetition rate on the formation of microtears in tendon in an in vivo cyclical loading model. *J Orthop Res*, Submitted
18. Nakama LH, King KB, Abrahamsson S, Rempel DM: VEGF, VEGFR-1, and CTGF cell densities in tendon are increased with cyclical loading: An in vivo tendinopathy model. *J Orthop Res* 24:393-400, 2006
19. National Research Council (U.S.). Panel on Musculoskeletal Disorders and the Workplace., Institute of Medicine (U.S.): *Musculoskeletal disorders and the workplace : low back and upper extremities,* pp xv, 492. Washington, D.C., National Academy Press, 2001
20. Nishida T, Nakanishi T, Asano M, Shimo T, Takigawa M: Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. *J Cell Physiol* 184:197-206, 2000
21. Perry SM, McIlhenny SE, Hoffman MC, Soslowsky LJ: Inflammatory and angiogenic mRNA levels are altered in a supraspinatus tendon overuse animal model. *J Shoulder Elbow Surg* 14:79S-83S, 2005
22. Petersen W, Pufe T, Kurz B, Mentlein R, Tillmann B: Angiogenesis in fetal tendon development: spatial and temporal expression of the angiogenic peptide vascular endothelial cell growth factor. *Anat Embryol (Berl)* 205:263-70, 2002
23. Petersen W, Pufe T, Unterhauser F, Zantop T, Mentlein R, Weiler A: The splice variants 120 and 164 of the angiogenic peptide vascular endothelial cell growth factor (VEGF) are expressed during Achilles tendon healing. *Arch Orthop Trauma Surg* 123:475-80, 2003
24. Petersen W, Pufe T, Zantop T, Tillmann B, Tsokos M, Mentlein R: Expression of VEGFR-1 and VEGFR-2 in degenerative Achilles tendons. *Clin Orthop*:286-91, 2004
25. Petersen W, Unterhauser F, Pufe T, Zantop T, Sudkamp NP, Weiler A: The angiogenic peptide vascular endothelial growth factor (VEGF) is expressed during the remodeling of free tendon grafts in sheep. *Arch Orthop Trauma Surg* 123:168-74, 2003
26. Petersen W, Varoga D, Zantop T, Hassenpflug J, Mentlein R, Pufe T: Cyclic strain influences the expression of the vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-1alpha) in tendon fibroblasts. *J Orthop Res* 22:847-53, 2004
27. Pufe T, Petersen W, Tillmann B, Mentlein R: The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. *Virchows Arch* 439:579-85, 2001
28. Schild C, Trueb B: Mechanical stress is required for high-level expression of connective tissue growth factor. *Exp Cell Res* 274:83-91, 2002
29. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC: Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376:62-6, 1995

30. Thomopoulos S, Hattersley G, Rosen V, Mertens M, Galatz L, Williams GR, Soslowsky LJ: The localized expression of extracellular matrix components in healing tendon insertion sites: an in situ hybridization study. *J Orthop Res* 20:454-63, 2002
31. Unemori EN, Ferrara N, Bauer EA, Amento EP: Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol* 153:557-62, 1992
32. Werner RA, Franzblau A, Gell N, Hartigan A, Ebersole M, Armstrong TJ: Predictors of persistent elbow tendonitis among auto assembly workers. *J Occup Rehabil* 15:393-400, 2005
33. Yang G, Crawford RC, Wang JH: Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. *J Biomech* 37:1543-50, 2004
34. Yang G, Im HJ, Wang JH: Repetitive mechanical stretching modulates IL-1beta induced COX-2, MMP-1 expression, and PGE2 production in human patellar tendon fibroblasts. *Gene* 363:166-72, 2005
35. Yang GP, Lau LF: Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. *Cell Growth Differ* 2:351-7, 1991
36. Zhang F, Liu H, Stile F, Lei MP, Pang Y, Oswald TM, Beck J, Dorsett-Martin W, Lineaweaver WC: Effect of vascular endothelial growth factor on rat Achilles tendon healing. *Plast Reconstr Surg* 112:1613-9, 2003