A HISTOLOGICAL OBSERVATION OF INFLAMMATORY CELLS IN THE RABBIT ACHILLES

TENDON DUE TO OVERUSE INJURY

by

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Abstract

The presence or absence of inflammatory cells in chronic Achilles tendinopathy has been a controversial subject in previous studies. Macrophages, T lymphocytes, and neutrophils have previously been detected in injured human Achilles tendons, whereas other authors have reported that there is no evidence for their occurrence. This controversy may stem from the fact that human Achilles tendon overuse injuries usually develop gradually over time, and the time course of inflammation in response to overuse has been difficult to establish in clinical populations. The aim of my study was to examine the presence of inflammatory cells in the Achilles tendon of rabbits that were subjected to repetitive mechanical loading of defined durations.

Twenty-Four New Zealand male rabbits were subjected to repetitive mechanical loading of the Achilles tendon and grouped into four groups in this study, according to the exercise time period for each group: 0, 1, 3, and 6 weeks. Achilles tendons were harvested at the end of each time period. Achilles tendons sections were stained with Hematoxylin and Eosin to examine the histological changes. Both Neutrophils and T-lymphocytes were detected by Immunohistochemistry. Macrophages were detected using the Prussian blue staining.

A very small number of inflammatory cells were detected in some tissue sections in the control group. Tissue sections from exercised groups 1, 3, and 6 weeks respectively, showed some qualitative changes in tendon morphology. Collagen bundles were disorganized, and hyalinized patches and spaces between collagen fibers were observed. Tenocyte nuclei were rounder and basophilic, and there was an increase in their numbers with loss of parallel alignment. Macrophages, T-lymphocytes, and neutrophils were detected in tendon sections, specifically in the paratenon. Statistically both lymphocytes and macrophages were significantly higher than control at 6 weeks. While the number of macrophages in the control was lower than the 6 weeks group, there was no

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significant difference between 1 week and 3 weeks. However, no lymphocytes were found at week 3. Neutrophils in all groups showed no significant difference. The evidence of inflammation was not evenly distributed, as some tissue sections from the same groups showed no evidence of inflammatory cells.

Preface

All animal procedures were performed according to the guidelines for animal experimentation. The certificate number is A12-0265.

This thesis is unpublished, independent work by the author, B. Almohimeed.

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List of Abbreviations

AT: Achilles tendon

MP: Macrophages

T cells: Thymus cells

N: Neutrophils

NK: Natural killer cells

AB: Antibody

IHC: Immunohistochemistry

H&E: Hematoxylin and eosin

MPS: Mononuclear phagocyte system

MPO: Myeloperoxidase

OCT: Optimum cutting temperature

IL-1B: Interlukin 1 Beta

US: Ultrasound

DAB: 3,3'-Diaminobenzidine

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Dedication

This dissertation is dedicated in the first place to ALLAH, without whom this work would not be possible.

Next, I dedicate this thesis to my parents for their encouragement and prayers.

This work is dedicated to my beloved wife, Kholod Aladwan and our two amazing children, Ghassan and Elias for their continuous support and patience.

Chapter 1: Introduction

Overview

Sports participation is increasing among people of all ages, particularly in adults due to changes in their life style and an increase in their leisure time compared to previous generations,[77] thus increasing the prevalence of sports injuries, resulting in tendon injuries in many cases.[58, 92, 96] The growth of sports-related injuries among people is high; according to Sandelin J. (1988), there are more than 200,000 sports-related injuries per year among 5 million people in Finland and this number is greater than the number of traffic injuries.[96] It was reported that the number of people with overuse injuries is not known, however the estimated number of sports-related injuries among people in the United States is about 30% to 50%.[93] Interestingly, in some countries downhill skiing was reported as the most common cause of Achilles tendon rupture.[40] To reduce the risk of sports-related injuries, epidemiological studies must be done in each individual country to assess the risk factors which could be addressed by preventative measures.[47] Interestingly, many epidemiological reports that were published have identified the main problems in sports medicine and have helped to understand the background of sports-related injuries.[77] Anatomically, the Achilles and patellar tendons are the most commonly affected tendons among sports practitioners, while the upper extremity tendons are more affected among workers.[47, 61, 110, 111] However, gender and age are two important factors that must be considered in studying tendons problems in sports.[47] While men experience a greater number of sports-related and tendon injuries than women, an increasing rate of injuries among women has been identified.[77] Reportedly, the male-to-female ratio of incidence of total Achilles tendon ruptures is between 7 to 1 and 4 to 1.[25, 40, 77, 94, 112] Achilles tendon overuse injuries are mostly reported among middle and long distance runners, tennis players, and in those who participate in ball games.[92, 67]

Achilles Tendon Anatomy and Biomechanics

The Achilles tendon (AT) is considered the strongest and thickest tendon in the human body. [83] The human leg consists of four muscle compartments; anterior, lateral, superficial posterior, and deep posterior. [29, 95] The gastroc-soleus complex and the plantaris muscle are in the superficial posterior compartment. [29, 97] AT is the conjoined tendon of the two muscles, gastro cnemius and soleus. The AT begins near the middle of the gastrocnemius. [71, 95] It connects the gastro cnemius (calf), and soleus muscles to the calcaneus (heel) bone. [59, 97] In humans, the tendon crosses posterior to the ankle. It is approximately 15 centimeters long. [71] The part of the tendon which experiences mechanical loading is inserted into the middle part of the posterior surface of the calcaneus bone, and the calcaneal bursa is located between the tendon and the upper surface of the calcaneus bone. [94] The narrowest part of the tendon is at its mid-portion, about 2-4 cm above its insertion. [74] The tendon is covered by a membrane (the paratenon) and the fascia, and sticks out conspicuously behind the calcaneus bone. [74] The paratenon is "[a]thin gliding membrane of loose areolar tissue that permits free movement of the tendon within the surrounding tissues" [56] surrounding the tendon throughout its length. [78] The AT obtains its innervation from surrounding muscles and cutaneous nerves, especially the sural and the tibial nerves. [84, 108] Previous studies confirmed that there are three vascular supplies to the Achilles tendon. [1] The posterior tibial artery and peroneal artery supply the peritendinous tissues, [29] while the mid-substance of the tendon is supplied mostly through its anterior surface. [29] Blood vessels of muscle bellies supply the proximal third of the tendon and the distal one-third of the tendon is supplied by small vessels. [29] According to some previous studies, the area of mid-substance of the tendon is where most problems occur due to hypovascularity [15, 23] but other studies have not replicated this finding. [108] Tendons transmit high levels of force from muscle to bone. [106] Tendons have robust physical properties, they are flexible and tough with high tensile

strength and they can be strained up to 4% of their initial length before micro-damage begins to occur. [85] Interestingly, Wren *et al* (2000) reported in their study on human Achilles tendon the mean failure strains of 16.1%, (SD, 3.6) and 12.8% (SD, 1.7) for faster and slower rates (10 mm s^{-1}, 1 mm s^{-1}) respectively. [85] Actin and myosin are found in tenocytes,[43] and a previous study reported that the tendon itself may have its own ability to regulate the force transmission by an active contraction–relaxation mechanism. [83] Loading in the AT is almost 9 kN during running as determined by *in vivo* measurements, and this equals 12.5 times body weight, or 11.1 kN/cm^2 per cross-sectional area of the tendon.[60] Interestingly, some studies showed that the AT force during running approaches 6-8 times body weight. [21, 97] Because the Achilles tendon is inserted into the calcaneus, it can also be subjected to forces secondary to subtalar motion; this was noted in both the hypopronated foot (*pes cavus*) and in runners who pronate excessively. [48] As noted in previous studies, either a hyper-pronated (*pes planus)* or hypopronated (*pes cavus*) foot may predispose to Achilles tendon injury because of the reduced shock absorption and altered subtalar motion associated with these malalignments. [97] Accordingly, biomechanical factors have been shown to be involved in AT injuries. [21,33] Schepsis (2002) reported that "the hindfoot movement going from a supinated to a pronated position and then back during running gait cycle may create a 'whipping' action in the Achilles tendon, which creates shear forces across the Achilles tendon, placing particularly high stresses on the medial side of the tendon. Malalignment factors above the ankle, such as *genu varum*, can also contribute to increased stress on the Achilles tendon." [97]

Histological Features of the Achilles Tendon

Normal Achilles tendon cells are well organized into longitudinal arrays. The main cells in the Achilles tendon are fibroblasts, which can be categorized morphologically as tenocytes or

tenoblasts, however there is no validated marker for these two proposed cell types.[52] Ippolito (1980) reported that tenoblasts can be in different shapes and sizes but are generally more rounded in appearance than tenocytes, which are highly elongated.[43] Tendon consists of collagen fibers which are tightly packed in parallel, longitudinally arranged bundles.[54] Tendon collagen contains mainly Type I collagen, and small proteoglycan molecules that hold the collagen to form parallel bundles.[69] Collagen is arranged in hierarchical levels; tropocollagenmicrofibril, which unites into fibrils, fibers, fascicles, tertiary bundles, and finally the tendon itself.[52] Additionally, tendon contains elastin fibers that contribute to the flexibility of tendon.[52] Collagen fibers are surrounded by ground substance which is a mixture of small and large proteoglycans and glycoproteins.[43] Ground substance has a viscosity property which provides structural support, lubrication, and spacing of the fibers. Ground substance contains between 60% and 80% water, whereas proteoglycans and glycoproteinare less than 1% of the dry tendon weight.[52]

Chronic Tendinopathy

Chronic tendinopathy is a clinical syndrome, which has also been known as chronic tendinitis, chronic tendon injury, and chronic tendinosis, although each of these terms has different meanings. Tendinopathy is generally defined as a clinical syndrome of chronic pain and tendon swelling/thickening resulting from overuse; the underlying histological appearance is known as tendinosis.[109] Previous studies reported that tendinosis may be caused by microtears in the connective tissue in and around the tendon, leading to an increase in tendon repair cells and reduced tensile strength.[54, 81] Accordingly, the histological characteristics of tendinosis are hypercellularity, degenerative changes[49] in the collagenous matrix hypervascularity, and a lack of inflammatory cells.[102] The term "tendinosis" is used by many sources to explain the underlying histological, or

sometimes radiological, appearance of degenerative changes in the tendon due to chronic overuse. [33] The term "tendinitis" has been used to refer to the inflammation of a tendon when the musculotendinous unit is acutely overloaded or when a tensile force is too heavy and sudden, resulting in a more acute-onset injury with more prominent signs and symptoms of inflammation. [36, 39, 54, 98]

Interestingly, there has historically been confusion about the difference between tendinitis and tendinosis. In 1998, Khan *et al*. argued that "most currently practicing general practitioners were taught, and many still believe, that patients who present with overuse tendinitis have a largely inflammatory condition and will benefit from anti-inflammatory medication. Unfortunately this dogma is deeply entrenched" and "instead of adhering to this myth, physicians should acknowledge that painful overuse tendon conditions have a non-inflammatory pathology." [56] Microscopic investigation of tendons from patients who underwent surgery due to longstanding (chronic) tendon pain demonstrated thin tendon fibrils, collagen separation, increased vascularity, and an increase in tenocytes with myofibroblastic differentiation. [36, 80] However, no inflammatory cells were observed in these early studies. In contrast, other studies in animals and humans have reported the presence of inflammatory cells in chronic Achilles tendinopathy. [98, 102]

Etiology of Achilles Tendinopathy

Although the etiology of Achilles tendinopathy is still under investigation, some previous studies have reported that risk factors include prior injury, poor vascularity, genetic makeup, gender, endocrine and metabolic factors. [65, 70] However, repetitive loading of the Achilles tendon is considered one of the main pathological stimuli that predisposes to tendinopathy. [10] To understand

the etiology of Achilles tendinopathy, some studies have suggested that there are intrinsic and extrinsic factors that lead to Achilles tendinopathy. Tendon vascularity, triceps muscle dysfunction, age, body weight and height, gender, and lateral ankle instability are intrinsic factors, while previous injuries, sudden changes in training pattern, poor technique, inadequate warm-up and stretching prior to training, footwear, and environmental factors like training on hard, slippery, or slanting surfaces are extrinsic factors. [23, 78, 84, 86]

Clinical Features

The main symptom of Achilles tendinopathy is pain which occurs during or after an exercise session. However, after a period of time and as pathological process progresses, pain also occurs during activities of daily living, and may also occur at rest or at night in severe cases. [28] In the acute case, the tendon may appear swollen and edematous. [107] Notably, the tendon on palpation shows tenderness, typically with a location between 2 and 6 cm proximal to the tendon insertion. In the chronic phase, the main symptom is still pain, while crepitations and effusions diminish. [52, 86] A nodular, mobile thickening in the substance of the Achilles tendon can be seen in chronic cases which is often taken to signify tendinosis.[70]

Diagnosis

Case history and clinical examination of the tendon are applied in the clinics for diagnosis purposes. However, diagnostic imaging is also commonly used to examine tendon problems and also to gauge the extent of tendinopathic change. Ultrasound (US) is often used because of its availability, quick results, safety, and low cost. However, US is operator-dependent, offers limited soft-tissue contrast and may be less sensitive than Magnetic Resonance Imaging (MRI).[100]

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According to Gibbon WW. (1996), another important advantage of US is the interactive facility, which helps reproduce symptoms by transducer compression and concentration on the pathologic area.[37] Recently, power or colour Doppler imaging has become a more accurate method for the vascularity assessment in the tendon, and this feature is important when assessing inflammation. The US can be used to observe the hyperemia of inflammation within the tendon, the mesotendon and synovium. Furthermore, Doppler can also be used to show the hyperemia within the tendon, and it also can help to differentiate hypoechoic hyperemic pannus from hypoechoic fluid; this can be useful to evaluate disease progression and therapy.[99]

Treatment and Prevention

Many sports clinics follow conservative treatments in the early phase of Achilles tendinopathy.[117] Early treatment of tendon disorder often leads to positive outcomes, while treatment of the chronic condition is more complicated.[25] Since tendinosis is a chronic condition, deep friction massage has been recommended as part of the treatment plan but there is insufficient evidence to establish this as an effective treatment for AT.[20] Stretching is also frequently recommended, to restore tissue elasticity and reduce the muscle–tendon tension.[82] Surgery is often recommended to patients who have experienced non-operative management without improvement for at least 6 months. A number of patients with Achilles tendon disorders (from 24% to 45.5%) require surgery because they failed to respond to conservative treatment.[61,67,87] According to some authors, preventing Achilles tendon injuries can be achieved by using a footwear that provides good cushion for heel strike, walking and stretching to warm up the leg muscles before performing any exercise, cooling down after exercise, and avoiding any unaccustomed movement.[41] A previous literature by Scott *et al* (2011) discussed the evidence base for conservative management of chronic mid-portion

Achilles tendinopathy in randomized controlled trials. A number of suggested conservative treatments were reviewed in the study including non-steroidal anti-inflammatory drugs (NSAIDS), laser therapy and ultrasound, dry needling, friction massage, and nutritional supplements. Heavy-load exercise in randomized controlled trials was applied to provide a strong, controlled, mechanical force to the Achilles tendon, and demonstrated a consistent clinical benefit in several randomized controlled trials. Furthermore, orthotics may be useful in the treatment of Achilles tendinopathy, whereas braces and splints did not show positive outcome for the treatment of Achilles tendinopathy in the clinical trials that were reviewed. [99] Ice, transverse friction massage, therapeutic ultrasound and exercise, as part of physiotherapy which lead to short-term effects, have been applied in a trial group compared with control (no treatment); the physiotherapy group experienced improvements in activity related pain compared with controls. NSAIDS have been used in the treatment of Achilles tendionpathy pain, but demonstrated little long-term effect or ability to resolve the pathology. The evidence, either for or against, other options such as extracorporeal shock wave therapy and glyceryl trinitrate are still inconclusive. [99]

Understanding the Role of Inflammation in Tendinopathy

Inflammation is a biological reaction of vascular tissues to a damaging exciter, such as pathogens, irritants, and damaged cells. [33] Inflammation can be classified into acute and chronic. [74] While acute inflammation is the first response of the body to any damaging exciter and causes movement of plasma and leukocytes from the blood stream into the injured tissues, chronic inflammation is characterized by an increasing shift in the type of cells present at the site of inflammation. Moreover, chronic inflammation is distinguished by simultaneous destruction and healing of the tissue during the inflammatory process. [74] In addition, chronic inflammation can

be distinguished by infiltration with mononuclear cells, such as macrophages and lymphocytes. [113] Macrophages are the most dominant cells during chronic inflammation and are one component of the mononuclear phagocyte system (MPS). MPS is comprised of blood monocytes and tissue macrophages which arise from a precursor in the bone marrow. [113] Later, monocytes migrate from the blood stream into tissues and differentiate into macrophages. The whole process is regulated by differentiation factors, adhesion molecules, and cytokines. It was reported that Transforming growth factor beta (TGF beta) that plays a role in the wound healing may also has a role in damaged tendon healing process but this is still not fully understood. [32] As discussed earlier, monocytes are transformed into macrophages when they reach extra-vascular tissue, [114] and macrophages can be activated by a variety of irritants and harmful stimuli and thus eliminate injurious agents such as microbes and initiate repair process. Importantly, macrophages contribute to the tissue injury in chronic inflammation. [113] However, if the irritant is removed in acute inflammation, macrophages disappear by normal body processes. But, in cases of chronic inflammation, "macrophage accumulation persists." [113] In addition to the macrophages, lymphocytes are involved in chronic inflammation. Lymphocytes and macrophages act in a two-way partnership, and this kind of acting plays an important role in chronic inflammation. [113] T cells are a type of lymphocyte that play an important role in cell mediated immunity, and the presence of T-cell receptors (TCR) on the cell surface distinguish them from other lymphocytes, such as B cells and natural killer cells (NK). [45, 46] Previous studies reported that macrophages display antigens to T cells and produce membrane molecules and cytokines, such as IL-12, that lead T-cells to respond; however, cytokines are also produced by activated T lymphocytes. These interactions between T cells and macrophages can cause the inflammation to become chronic due to the immune system's involvement in the inflammatory reaction. [45, 46, 113] Previous studies mentioned that neutrophils, a type of white blood

cell, are generally only found in acute inflammation, but even so, many cases of chronic inflammation show large numbers of neutrophils, induced by different factors, such as microbes and mediators produced by activated macrophages and T lymphocytes. [46]

There is evidence both for and against a role of inflammation in Achilles tendinopathy in humans. In 1976, Puddu et al [91] published a classic histological description of human Achilles tendon pathology. The methods used (e.g. types of histological preparations) and the number of patients or samples and symptom duration were not specified, and apparently no immunohistochemistry or specific cell labeling or any quantification was performed. The authors classified Achilles tendon pathology into three categories: pure peritendinitis, peritendinitis with tendinosis, and tendinosis. The authors reported qualitative evidence of "production of inflammatory cells within the peritendinous tissue [and] the adherence between the tendon and the peritendinous tissue was confirmed." The authors also distinguished between (1) cases of spontaneous Achilles tendon rupture which were asymptomatic, and demonstrated no evidence of inflammatory cell infiltration, with (2) ruptures that were preceded by symptoms and which did demonstrate inflammatory cell infiltration. In 2003, Cetti et al [24] reported on 60 consecutive patients with spontaneous Achilles tendon ruptures treated surgically. As controls, 50 patients provided contralateral Achilles tendon biopsies, and in addition 3 cadaver Achilles tendons were examined. In contrast to Puddu et al, immunohistochemistry was employed to label neutrophils (antibody to calgranulin). Unlike Puddu et al, Cetti et al reported acute inflammation at the rupture site in all cases, as well as proximal (49/56) and distal (35/55) to the rupture site. Interestingly, the authors concluded that widespread inflammation **preceded** the rupture, but it is not clear how this can be concluded. In 1991, Kvist conducted histopathology on the Achilles paratendon of 14 athletes undergoing surgical management for chronic Achilles tendinopathy, and compared them to 4

cadaver controls using immunofluorescence against fibronectin and fibrinogen. The average symptom duration was 20.4 months. The major findings were that "increased vascular permeability and fibrin formation still persist in chronic Achilles paratendonitis" and that there was a mild inflammatory reaction in all cases which was absent from controls. The results were interpreted as evidence of inflammation and immature scar tissue in chronic paratendonitis, however no labeling of specific inflammatory cell types was conducted. Astrom[9] examined histopathology of 163 patients undergoing surgical management (82% male, 75% athletes). Routine histopathology including H&E and Prussian blue were conducted (no immunohistochemistry). Paratendinous edema or scarring was noted in 40% of the cases, and tendinosis (non-inflammatory degenerative changes) was present in 67% and partial rupture in 19% of the cases. However, inflammatory cells were noted to be absent, presumably based on absence of Prussian blue staining, although this was not mentioned or quantitated. Most recently, Schubert et al (2005)[98] examined 10 Achilles tendon biopsies from patients with painful Achilles tendinosis (symptom duration 6 – 120 months) and 10 biopsies from patients with an Achilles tendon rupture. Immunohistochemistry for CD3 (T lymphocytes), CD20 (B lymphocytes) and CD68 (macrophages) was conducted, and iron positive cells (haemosiderophages) were also identified. Granulocytes were counted on H&E sections. T lymphocytes, B lymphocytes and macrophages were all increased in the tendinosis samples compared to the ruptured samples. Similar to Cetti et al, the number of granulocytes was increased in the ruptured Achilles tendons. This paper would have been stronger had they been able to include samples from controls (e.g. cadaver).

In addition to histological evidence for or against a role of inflammation in Achilles tendinopathy, some authors have used other biological assays including gene expression and microdialysis. Legerlotz et al (2012)[66] collected Achilles tendon biopsies from controls (9 male

cadavers) or patients undergoing surgery for chronic Achilles tendinopathy (13 males and 7 women) or acute rupture (14 men and 14 women, on average all patients were middle-aged. Gene expression analysis was conducted for several inflammatory substances (e.g. VEGF, COX2, IL6). COX2 and IL6 were significantly upregulated in the painful AT compared to controls (2-fold and 6.3 fold, respectively). In contrast, Ireland et al [44] conducted a gene array study on a small number of patients (n=4) compared to cadaver controls (n=3) and found no significant difference in the expression level of CD70, CD27, CD30 or CD33. Surprisingly, CD4 was downregulated in Achilles patients. This study is limited primarily by its small sample size, and by the fact that of the 4 patients, 2 had chronic Achilles tendinopathy and 2 had a spontaneous rupture. More recently, Pingel et al [89] randomized 30 patients (recreational athletes or workers with > 6 months Achilles tendon pain) to either to a gene expression study (n=16) or a structural study (n=14) in which collagen fibril density and size, volume fraction of cells and nucleus: cytoplasm ratio of cells were quantitated. Unlike the previous studies, the samples from these patients were taken by biopsy (from two areas – most symptomatic, or 4 cm proximal to this), rather than surgically. In contrast to Legerlotz *et al*, IL6 expression could not be detected in any sample. Surprisingly, Ki67 could also not be detected (neither could COX2 or IL-1R), and these substances have been previously shown to be expressed in both normal and painful tendons. Finally, there is another line of evidence about the role of inflammation in Achilles tendinopathy, which comes from biochemical measurement of inflammatory substances. Langberg et al [64] used microdialysis on six young healthy adults to determine interstitial levels of inflammatory substances including prostaglandin E_2 (PGE_2) and thromboxane B2 (TXB_2). After 30 minutes of repeated plantar-flexion exercise, PGE_2 and TXB_2 levels increased by 100%. The authors concluded that "inflammatory activity is accelerated in the peritendinous region of the human Achilles with dynamic loading." In contrast, Alfredson et al [3]

examined baseline levels of PGE_2 using microdyalsis in a small number of patients (n =4) with chronic Achilles tendinopathy and found no elevation of PGE_2. To my knowledge, no study has examined the levels of PGE_2 in acute Achilles tendinopathy in humans. [96]

The effectiveness of various anti-inflammatory strategies has been examined for Achilles tendinopathy, and this may be taken as another line of evidence for a role of inflammation in this condition. Mazieres et al, [73] in a double-blind clinical study, randomized 172 patients with various tendinopathies to receive topical ketoprofen (n=87) or placebo (n=85). Of this number, only 7 patients had Achilles tendinopathy. Overall, pain was significantly lower after 1 week of treatment in the ketoprofen group compared to placebo (56% reduction vs. 37% reduction). This response was statistically and clinically significant. Most of the patients had rotator cuff tendinopathy, which has a more established inflammatory component than Achilles tendinopathy. Dreiser et al [30] tested the efficacy of niflumic acid gel (an inhibitor of COX2) in a double-blind placebo controlled trial with similar findings to those of Mazieres et al, but again only a small minority of these patients had Achilles tendinopathy. Sundqvist et al [105] conducted a double blind study of 60 patients (recreational athletes) with chronic Achilles peritendinitis. 29 patients received GAGPS (glycosaminoglycan polysulphate) and 30 received indomethacin (IM). In the IM group, a statistically significant reduction in pain was seen after 2 weeks but not in the GAGPS group. The GAGPS group achieved a significant reduction in pain after 4 weeks. A strength of this study is that it included patients with both acute/subacute symptom duration (<3 months, n=13 in the IM group) and patients with chronic symptoms (n=16 in the IM group). 85% of those with acute/subacute symptoms experienced significant pain relief after 2 weeks with IM compared to only 11% of those with chronic symptoms. Interestingly, in the long term (52 weeks), 66% of the GAGPS treated patients had a good clinical

result compared to only 33% in the IM treated patients. A limitation of this study is that the determination of "good clinical result" was made by a global physician evaluation, and the success of blinding after 1 year is not reported. In 1992, Astrom and Westlin randomized 70 adults with Achilles tendinopathy of various durations to NSAID (piroxicam) or placebo. 52/70 cases were engaged in sports. Evaluations up to 28 days revealed no differences between the groups in clinical effect (pain, tenderness, swelling, strength or range of motion). [26] In 2009, Fredberg and Ostgaard conducted a small pilot study of adalimumab (TNF inhibitor) or anakinra (IL1 receptor inhibitor) in 20 athletes with Achilles tendinopathy. [35] There were small reductions in resting and walking pain in the adalimumab group but not the anakinra group, but these were not statistically or clinically significant and there was no change in tendon thickness. However, after a single peritendinous corticosteroid injection, both groups of patients experienced substantial and statistically significant reductions in tendon thickness, resting pain and walking pain after 4 weeks. [35] It should be noted though that a 2009 systematic review [76] included 72 articles examining the effect of glucocorticoids on Achilles tendinopathy, but identified only 1 RCT that met their inclusion criteria, and concluded "there is no consensus as to whether local glucocorticoid injections have a therapeutic role" and that "they may incur a risk or tendon damage" and should be avoided until further research becomes available. However, the fact that the majority of patients in the subsequent trial by Fredberg and Ostgaard responded favourably to corticosteroids indicates that there may indeed be an inflammatory component to this disorder.

Animal Models of Tendinopathy

Due to the difficulties in studying the early stages of tendon overuse injury in humans, using animal models is significantly important. One of the advantages of using animal models is that an

injury can be induced in an animal model for the study of tendinopathy and evaluated and treated over time. [116] An animal model should ideally have the ability to result in pathological processes that are known to be involved in the human conditions. For many years, a wide range of animal species were used in tendinopathy studies (including horses, dogs, goats, rabbits, primates, mice and rats)but there is still no single species reflecting a standard or universally accepted model. [116]

Non-human primates were reported to be the most ideal species to use in tendon research due to their anatomy and physiology which are very similar to human's body, but using them in research poses many complicated ethical issues, and also their availability is not guaranteed.

Large animals such as horses, goats, and dogs have been considered good models in tendinopathy but their size makes using them hard because a minimum of two investigators should work on each animal.[116] In addition, large animals create higher costs for housing and purchasing. In contrast, small animals such as rabbits, mice, and rats are the most convenient and widely used models for tendinopathy research.

Rabbits are used as models for tendinopathy due to their popularity since their anatomy and tissue pathology is close to humans. In addition, rabbits are easy to handle. However, one disadvantage of using rabbit in research is the higher cost of their housing and purchasing compared to rodents; also investigators should have experience in dealing with rabbits due to the high possibility for them to be injured.

Rats and mice have good advantages as models for several types of research due to their short life span and gestation period, and they also can give multiple offspring as well as their rapid growth rates. [116] Interestingly, rat and mouse anatomy and physiology are analogous to humans. And as models for tendinopathy, they possess a similar limb anatomy and tendon histology as humans. [116] A disadvantage of rodents is the small tendon size which makes it difficult to perform

biomechanical testing or to extract proteins for quantification, or to apply electrical stimulation to create a controlled tendon loading stimulus. Previous studies have been conducted using different animal models to induce tendinopathy as well as to study inflammatory processes at the site of injured tendons.

Several studies have used different animal models to study the changes in the Achilles tendon due to inflammation induced by overuse or by a variety of other pro-inflammatory stimuli. Backman et al [12] subjected rabbits to a combination of passive ankle movement and calf muscle stimulation (as described in the section "Backman model" below) 3 times per week over a 5.5 week duration. Basic histological methods were used (H&E staining). The authors reported degenerative changes in the tendon accompanied by chronic paratendonitis (edema, lymphocytes) and fibrosis (fibroblast proliferation) and concluded that repetitive overuse of the Achilles tendon causes paratendonitis with tendinosis. One strength of this study is the presentation of a model which allows the location of inflammatory cells to be identified (e.g. predominantly paratendon, with adjacent areas of tendinosis). A limitation is that the type of inflammatory cell populations was not conclusively shown. A follow-up study [13] using the same model confirmed the basic histological changes and measured increased substance P levels with immunoassay, immunohistochemistry and in situ hybridization, but again inflammatory cell populations were not detailed. Archambault et al [6] used a similar model except a lower frequency (1.25 Hz), however there was no evidence of tendon damage or inflammation using this less aggressive loading protocol and no evidence of injury. Messner et al [75] used a rat model to induce Achilles paratendonitis with tendinosis. Ten adult male rats were exposed to 1 hour of electrical stimulation of the calf muscles 3 x /week for 1 hr, 30 contractions per minute. Trained rats were euthanized either after 7-11 weeks of overuse (21-23 sessions) or when evidence of limping was demonstrated. Histology and immunohistochemistry (not

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for inflammatory cells, but for type I collagen, substance P and CGRP) were conducted. Unlike the Backman model, there were no macroscopic changes (e.g. tendon thickening) evident. [75] The authors did not comment on the presence or absence of inflammatory cells, but noted chronic changes in the epitendon including thickening, hyperplasia, hypervascularity and increased substance P expression associated with vascular structures (presumably nerves), whereas tendons were unchanged. The authors contrast their results to those of Backman et al and argue that the lack of tendon injury may have been due to the lower stimulation frequency. Zhang and Wang [119] subjected adult mice to treadmill running to exhaustion, daily for 1 week, following which the patellar and Achilles tendons were removed and the level of PGE_2 was determined by immunoabsorbent assay. PGE_2 levels were increased compared to control (sedentary) animals in both the Achilles and patellar tendons. The authors conclude that PGE_2 is increased in tendons by mechanical loading and may play a role in the injury process. Inflammatory cell populations were not identified in this study. Sullo et al [104] subjected Sprague Dawley rat tendons to repeated (weekly) peritendinous injections (800ng, which is consistent with levels measured by Zhang and Wang) for 1, 3 or 5 weeks; there were several controls (saline only, needle only, no treatment). Basic histology (H&E) was performed, however the authors specifically assessed for extent of inflammation (blood extravasation, swelling, neutrophils, etc). In the PGE_1 treated tendons, inflammation peaked after 1 week, including presence of neutrophils, macrophages, and lymphocytes and hypervascularity. Despite repeated PGE_1 injection, by 5 weeks there was minimal evidence of acute inflammatory change but extensive degenerative changes in the tendon (variable tendon cell density, collagen fibre degeneration). Marsolais et al [27] administered a single carrageenan injection into the adult rat Achilles tendons and euthanized 1 or 3 days later. Although tendon inflammation was observed (neutrophils and macrophages, identified via immunohistochemistry, mainly in the paratendon), there was no

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decrease of biomechanical properties (load to failure). A limitation of this study is that only a single injection and acute time points were evaluated, but a strength is that immunohistochemistry was used to identify inflammatory cell types. The same group [72] also examined the impact of a more powerful pro-inflammatory stimulus (collagenase) on the rat Achilles tendon, and again used immunohistochemistry to label neutrophils and macrophages. Both cell types peaked 1 day after injury; neutrophil levels declined to baseline by 14 days, but macrophage numbers remained increased even 28 days after injury. Biomechanical properties were significantly reduced and blood vessels density was increased, especially in the paratendon. Diclofenac inhibited inflammation and inflammatory cell invasion, although it did not improve the recovery of tendon biomechanics (stiffness, rupture point), which were approximately 50% recovered after 28 days. A limitation of this study is that the extent of injury created by collagenase injection may be more than what is seen in an overuse injury.

Previous studies that have been reviewed demonstrated that animals may be useful as models for Achilles tendinopathy if the loading regime or injury stimulus is of sufficient intensity, and can provide some evidence as to whether inflammatory processes may contribute to tendinopathy or not.

Significance of Research

      Chronic Achilles tendinosis is a serious problem among people in our society, causing substantial pain, time lost from work, and reduced participation in healthy physical activity. The presence or absence of inflammatory cells in chronic Achilles tendinopathy has been a controversial subject in previous studies. Macrophages, T lymphocytes, and neutrophils, have been detected in injured tendon by some authors, whereas other authors have reported that there is no evidence of inflammatory cells in or around the tendon. If our hypotheses are verified (stated immediately below), this study may lend support to the suggestion that inflammatory cells contribute to the development of chronic tendon overuse injuries. This may allow us to conduct further research to test new treatments in order to prevent the inflammation at the site of injury and to examine the effects of anti-inflammatory treatments on the development of chronic Achilles tendinopathy.

Hypotheses

The overall hypothesis is that there will be an increased number of inflammatory cells in the Achilles tendons of rabbits who have been subjected to an Achilles overuse injury, compared to control rabbits (uninjured). Specifically, we hypothesized that rabbits subjected to 1, 3, and 6 weeks of Achilles overuse will demonstrate a higher density of inflammatory cells compared to control tendons (not subjected to loading, i.e. 0 weeks), defined as:

1) Macrophages, identified by Prussian blue stain.

2) T cells and neutrophils, identified by immunohistochemistry.

Twenty-four New Zealand male rabbits were used in this study. Animals were divided into four groups according to Achilles overuse time periods of 0, 1, 3, and 6 weeks.

Chapter 2: Materials and Methods

Rabbits

In our study we used twenty-four male New Zealand rabbits purchased from Charles River Canada with an age ranging from 6 to 9 months to obtain a weight of approximately 4 kg. All animal procedures were performed according to the guidelines for animal experimentation approved by Animal Care Committee of University of British Columbia. Some extra rabbit Achilles tendon tissues were brought from Sweden, Umea University. These tissues had been previously obtained from rabbits that had undergone the identical Achilles tendon overuse protocol and tissue fixation procedure outlined here, and those experiments were also conducted according to the guidelines for animal experimentation approved by Umea University.

Backman model

The animals were divided into four groups of six rabbits. One group was a control group and three groups were subjected to an overuse protocol in the right leg of the animal. The Backman model [12] was used to exercise each rabbit's right ankle joint for a total period of 1, 3, and 6 weeks to induce Achilles tendinosis. [5] In the Backman model, rabbits were anaesthetized and positioned supine, and each animal was injected intramuscularly with Xylazine hydrochloride (2.5-5mg/kg) and ketamine hydrocholoride (20-22mg/ml) followed by inhalation of isoflurane (1.5-2%), during the exercise. The duration of anesthesia during the exercise only was 2.5h, but when rabbits were subjected to exercise and other experimental interventions (e.g. ultrasound) the duration of anesthesia was approximately 5h, and this was only once a week for every rabbit. To prevent excessive body movement we used a band around the rabbit's pelvis, to keep the rabbit in position while the ankle is exercised. [12, 13] The right leg of each rabbit was attached to a pneumatic piston, which moves 9.5cm,

between 20-25 degree dorsi flexion and 35-40 degree plantar flexion. [12] Electrical stimulation using

surface electrodes (pediatric electrodes 40- 426A; Hewlett Packard, Andover, Massachussetts,

USA)were used to trigger a pulse of 0.2ms duration, 35-50V in the triceps surae muscle. The right

ankle was subjected to 150 flexions per minute. In a subset of the animals, an ultrasound image of

the tendon was taken at the end of every week of exercise. For analgesic purposes, buprenorphine

(0.02-0.05mg/kg) was given subcutaneously to each rabbit after every exercise session. After the last

week of each exercise time period the rabbits were euthanized under anaesthesia using Euthanyl

administered intravenously in the marginal ear vein using a 22 gauge catheter. Achilles tendons

from each rabbit were removed for further studies.

Figure 1. The Backman model

Tissue collection and processing

Achilles tendon specimens were taken from both right and left legs of each animal. Samples were then divided into two groups. Tissue from the first group was frozen with the Optimum Cutting Temperature (OCT) Compound (TissueTek, Miles Laboratories, Naperville, IL, USA), and stored at -20° C. Tissue from the second group was fixed in 10% neutral buffered formalin and kept at room temperature until further processing.

Histology

Frozen and formalin fixed tendon tissues were taken to a certified histology laboratory for embedding in paraffin, sectioning, and hematoxylin & eosin (H&E) staining (Centre for Translational and Applied Genomics, BC Cancer Agency, Vancouver, B.C.). Slides stained with H&E were viewed under a Zeiss Axiophot Upright Light Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

Ultrasound assessment

A General Electric (GE)Venue 40 ultrasound unit with an L8-18i probe was used to qualitatively examine the tendons pre- and post-exercise. The optimal probe frequency that is used for musculoskeletal examination is 7.5 – 20 MHz. [11] Furthermore, linear probes are highly preferred for clear tendon structure demonstration.[11] US was performed on animals Achilles tendons of right legs of groups 0, 1, 3, 6 weeks pre- and post-exercise and with and without muscle stimulation at 0cm, 1cm, and 2cm respectively proximally from the calcaneal insertion. Animals were examined by US once a week under general anesthesia.

Immunohistochemistry

Immunohistochemistry (IHC) was used to detect and quantify the presence of T cells in rabbit

tendon tissue and in control (spleen) tissue. Formalin-fixed tissues were dehydrated and embedded

in paraffin. Tissues in paraffin were sectioned 5μm thick using a microtome and then mounted on

glass slides (Fisher Scientific). Slides were incubated at 45° overnight. Tissues were deparaffinized

before proceeding with staining protocol of IHC. Tissues were washed twice for 3 minutes in

xylene, followed by 100% ethanol twice for 3 minutes, washed once for 3 minutes each in 95%,

70%, 50% ethanol, respectively. All tissue slides were then rinsed in cold tap water for one minute.

CSA II, Biotin-Free Catalyzed Amplification System (Dako, Burlington, ON, Canada) was used for

immunohistochemistry. The CSA II system is being used widely due to its high sensitivity according

to many studies. [17, 18, 19, 112, 115] After preparing tissue slides for staining as described above, tissue

slides were incubated inside a humidified chamber with Peroxidase Block for five minutes to quench

endogenous peroxidase activity. Tissues were then incubated with a protein block for five minutes to

suppress nonspecific binding of subsequent reagents. A mouse monoclonal anti-rabbit primary

antibody against CD3 for the detection of T lymphocytes (MCA805GA, AbDSerotec, CA, USA)

was added to each tissue slide and incubated for 15 minutes. The primary antibody was diluted

1:100 in Tris-buffer saline 1X (TBS) (Sigma Aldrich, Oakville, ON, Canada) mixed with 1%

Bovine Serum Albumin (BSA). Slides were then incubated for 15 minutes with an anti-mouse

immunoglobulin-HRP, followed by a 15 minute incubation with Fluorescyl-tyramide hydrogen

peroxide (amplification reagent) and during this step, slides were protected from light by covering

the humidified chamber with aluminum foil. An anti-fluorescein-HRP was then added to tissue

slides and incubated for 15 minutes. Slides were then incubated for five minutes with

DAB/hydrogen peroxide. Finally, slides were rinsed in distilled water, counterstained with

hematoxylin for one minute, and rinsed again with tap water until the tap water runs clear. Slides were then cover slipped with Aqua-Mount™ aqueous mounting medium. A wash with phosphate buffered saline 1X (PBS) up to 3 times for approximately 5 minutes was done after each step according to the protocol.

Prussian Blue Staining

Prussian Blue Staining detects the presence of iron plaques in macrophages. [118] In human and some animals, about 10 to 20% of absorbed iron is transferred to cells of the mononuclear phagocyte system, such as macrophages. [4,79] In Prussian blue reaction, sections are treated with acid solutions of ferrocyanides and any ferric ion (+3) present in the tissue will react with the ferrocyanide and results in the formation of a bright blue pigment called Prussian blue. [101] Paraffin embedded slides were deparaffinized and washed in a distilled water. A 20% aqueous solution of hydrochloric acid was prepared by adding 20ml of concentrated hydrochloric acid to 80mldistilled water. A 10% aqueous solution of potassium ferrocyanide was also prepared by adding 10g of potassium ferrocyanide trihydrate (Sigma Aldrich, Oakville, ON, Canada) to 100ml distilled water. A working solution was then prepared by mixing equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide solution, for further usage. Slides were immersed in this mixture for 20 minutes then washed three times in distilled water. Slides were then counterstained for five minutes with nuclear fast red solution (Sigma Aldrich, Oakville, ON, Canada), then rinsed twice in distilled water. Slides were then dehydrated through 95% alcohol and 2 changes of 100% alcohol followed by clearing 2 times in xylene, 3 minutes each. Finally, slides were cover slipped with Aqua-Mount ™ aqueous mounting medium.

Immunoperoxidase Method

Neutrophils were detected using an immunoperoxidase method (VECTASTAIN® ABC KIT from

Vector Laboratories). An anti-Neutrophil Defensin 5 (NP5) monoclonal antibody against rabbit

(MBS603525, MybioSource, Inc. San Diego, CA, USA) was used in this method after dilution

1:100 inTBS+1% BSA.VECTASTAIN® working solutions were first prepared according to the

company's instructions. For blocking serum (normal serum),three (3) drops (150 µl) of stock (yellow

labeled)added to 10 ml of PBS buffer in a mixing bottle (yellow labeled). For biotinylated antibody,

one (1) drop (50 µl) of stock (blue labeled) was added to 10 ml of PBS buffer in a mixing bottle

(blue label).VECTASTAIN® ABC Reagent was also prepared by adding 2 drops (100 µl) of

Reagent A (orange labeled) to 10 ml of PBS buffer in the ABC Reagent mixing bottle. Two (2) (100

µl) drops of Reagent B (brown labeled) were added to the same mixing bottle. Reagent A and

Reagent B were mixed immediately and left for around 30 minutes before using. Paraffin embedded

tissue slides were deparaffinized and washed in distilled water. Tissue slides were incubated inside a

humidified chamber with Peroxidase Block (Dako, Burlington, ON, Canada) for five minutes for

quenching endogenous peroxidase activity followed by washing in PBS buffer for 5 minutes. Slides

were incubated for 20 minutes with diluted normal serum and excess serum in sections was blotted.

Sections were incubated for 30 minutes with primary antibody diluted in buffer then washed in

buffer for 5 minutes followed by incubating with diluted biotinylated secondary antibody solution

for 30 minutes and washed in buffer for 5 minutes. Sections were then incubated for 30 minutes with

VECTASTAIN® ABC Reagent that was prepared and washed with buffer for 5 minutes followed

by incubating the sections in 3,3' DAB/hydrogen peroxide (Dako, Burlington, ON, Canada) until

desired stain developed. Sections were rinsed in tap water for a few seconds and counterstained with

Hematoxylin (Sigma Aldrich, Oakville, ON, Canada) for one minute then washed in tap water until excess stain is removed. Finally, slides were cover slipped with Aqua-Mount ™ aqueous mounting medium and viewed under a Zeiss Axiophot Upright Light Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

Quantification of inflammatory cell numbers

After staining the slides for inflammatory cells, one researcher (AS) blinded and randomized the slides using a random number generator. A single tissue section demonstrating maximum inflammatory cell labeling tendon was selected for quantification. One tissue section per rabbit per time point was counted (by BM). Using a 20x objective lens attached to a digital camera (Carl Zeiss, lcc1), the region of interest was pre-defined as the area demonstrating the greatest number of inflammatory cells, and a digital micrograph was taken. All positively labeled cells within the region of interest were counted, and expressed as cells/field.

Chapter 3: Results

General observations

Any changes in animals during and after each procedure were noted. Animals showed individual variations in terms of accepting the anesthesia and in demonstrating post-exercise side effects. But there were no serious issues requiring us to stop the procedure for any animal, and all animals survived the experimental protocols to their pre-defined time point. In cases where an animal was temporarily experiencing difficulty breathing or developed tachycardia (presumably due to stress), we paused the procedure temporarily until normal breathing and heart rate were restored. After each exercise, temporary lameness was noticed in some animals that were subjected to exercise. Macroscopic (qualitative) findings at the end of each experiment were observed significantly such as thickening of tendon of the right leg comparing to the left leg in animals subjected to 1, 3, and 6 weeks of Achilles overuse. However, no such changes were observed in the control group.

Ultrasound findings

US imaging revealed no qualitative changes in the control group and zero week tendons, however, qualitative changes at 3 and 6 weeks were observed. In the mid-portion of the tendon where the most prominent changes typically occurred, there was an impression of thickening along the whole length of the Achilles tendon in the longitudinal view, while in the transversal view the tendons subjected to overuse appeared more rounded (the normal tendons having a more flattened appearance). In the distal portion of the tendon, there also appeared to be areas of thickening and structural irregularity(Fig. 2 and 3), but these impressions were not quantitated.

We found that using US imaging technique to examine the changes in tendons pre and post exercise was very helpful and easy to handle. Importantly, US helped us to examine the changes on tendons every week and compare it to control, although we did not attempt to quantify the observed changes due to the difficulties with reproducing the exact probe placement from session to session. Therefore, the qualitative impressions reported cannot be taken to be definitive. We also found that using this technique on rabbits was easy to handle because the size of the probe was small enough to conform to the contour of the rabbit Achilles tendon.

Figure 2. Ultrasound image of distal Achilles tendon. Rabbit no. 9, 0 cm, exercised group, baseline. No changes in the tendon noted.

Figure 3. Ultrasound image of distal Achilles tendon. Rabbit no. 9, 0 cm, exercised group, after 6 weeks of exercise. Note the qualitative impression of tendon thickening.

Histology

Rabbit Achilles tendon tissues were examined under the light microscope for qualitative, morphological changes due to excessive exercise. Notably, collagen bundles were disorganized, and large hyalinized patches and spaces between fibers were observed significantly after 3 and 6 weeks of overuse. In these groups, tenocyte nuclei were round and demonstrated darker (more basophilic) staining, and there was an increase of their numbers with loss of parallel alignment. Moreover, there was an increase of vascularity. Inflammatory cells were absent in tendon tissues, except some regions of the paratenon. (Fig. 4).
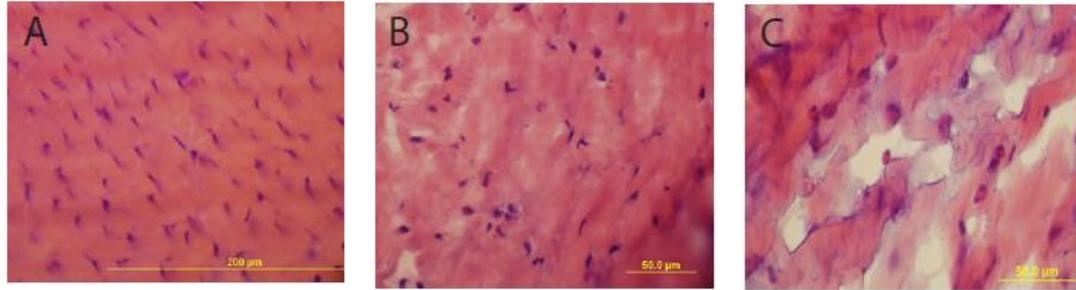
Figure 4. H&E, qualitative (morphological) impressions. 40x objective lens, equal magnifications. A. Unexercised control rabbit, tendon proper – tendon fibroblasts (dark blue) and collagen (pink) are densely packed and well organized (normal). This morphology is also seen in unstimulated (contralateral) tendon at all time points. B. 6 weeks of overuse, tendon. Note disorganization of tendon fibroblasts and collagen (tendinosis). C. Qualitative impression of inflammatory cells after 3 weeks of overuse, paratendon. Similar morphology is seen after 3 and 6 weeks overuse, but with considerable individual variation among rabbits.

T-lymphocytes

Immunohistochemistry staining for T lymphocytes in rabbits tendons that were subjected to exercise showed the presence of cells in the tendons of animals from groups 0, 1, and 6 weeks, however, no cells were observed in three weeks group. The increasing number of T-cells in those groups was statistically significant (Fig. 8). However, the localization of the cells was consistently in the tissue around the tendon, the paratenon, but no cells were found in the tendon itself. (Fig. 5) It should be noted that this antibody stained very few cells both in the control tissue (rabbit spleen), and in the paratendon tissue indicating that the method may suffer from a lack of sensitivity. The positively labeled cells in the paratendon tissue were more elongated in appearance than the cells identified in the spleen tissue.
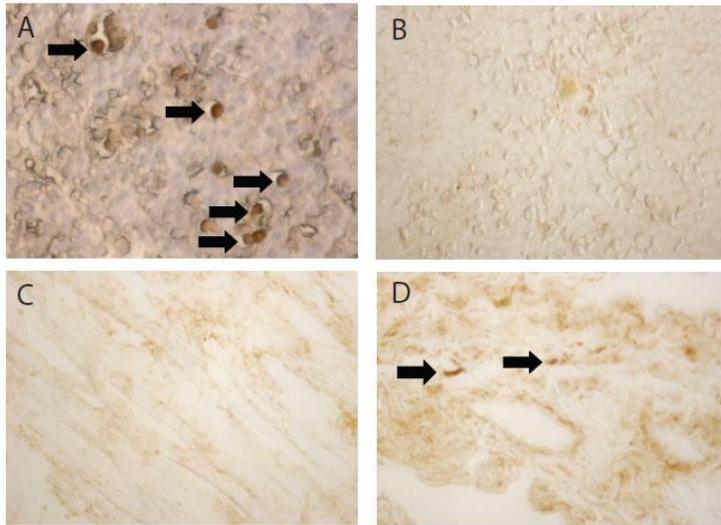
Figure 5. T lymphocytes, 40x objective, 14ms exposure, 6.5/10 illumination.

A. A small number of cells were labeled in the rabbit spleen (a light Haematoxylin counterstain was applied to assist with visualization of the negatively stained cells). B. Rabbit spleen (- control, no counterstain). C. Paratendon, 6 weeks, negative control (no primary antibody). D. Paratendon, 6 weeks, faintly labeled perivascular cells.

Macrophages

Tendons tissues from groups 0, 1, 3, and 6 weeks were stained with Prussian blue to examine the presence of macrophages. Interestingly, macrophages were increasingly observed in the tissues of each exercised group (1, 3, 6 weeks). The presence of these cells was significantly increased in the paratenon of every sample from each animal of these groups. Cells stained with Prussian blue were colored blue and round in their shape. (Fig. 6)
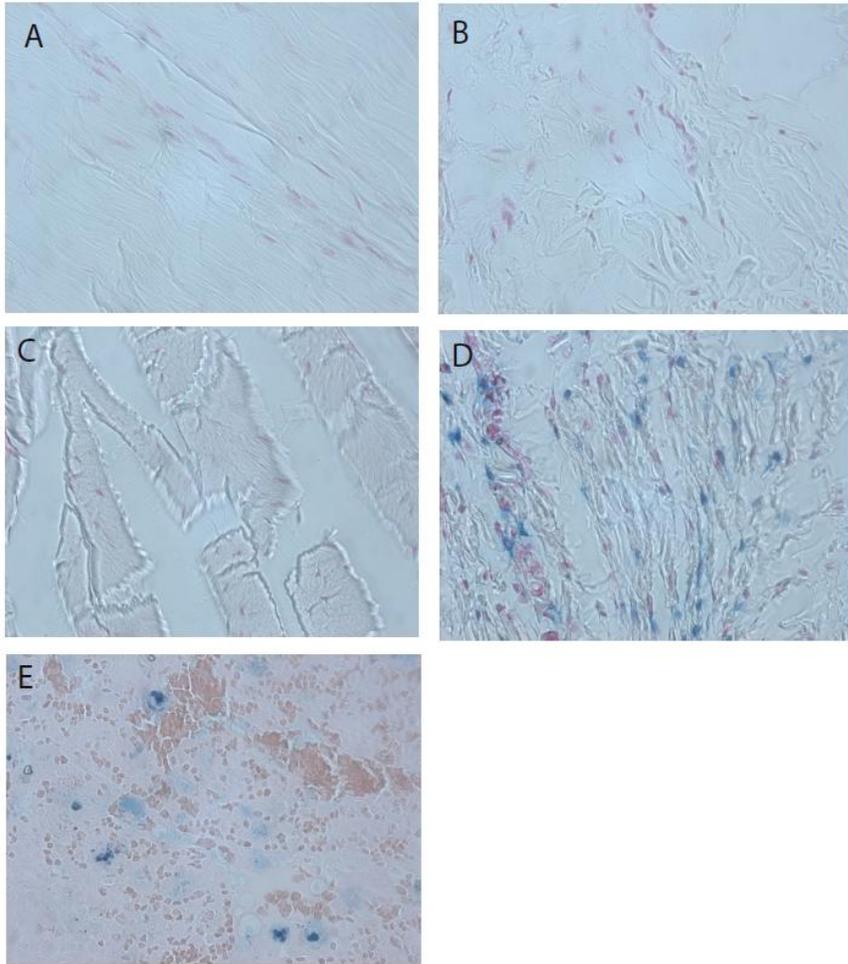
Figure 6. Macrophages (Prussian blue stain, with fast nuclear red counterstain), 40x objective, 12ms exposure, 6.5/10 illumination. There are no, or very few cells in the paratendon (A) of unexercised tendons compared to paratendon after exercise (D, 6 weeks exercise). No detectable macrophages in tendon of control (B) or exercised (C, 6 weeks) animals. E. Macrophages are scattered throughout the spleen. There is no negative control for this staining technique.

Neutrophils

Achilles tendon tissues that were examined under the light microscope demonstrated the presence of

neutrophils in some samples from each group. The localization of these cells was noticed in the

paratendon region of the injured site (Fig. 7). However, tissues were stained with H&E showed

localization of neutrophils in the same groups, and this technique was performed to confirm their

presence in tissues. As discussed earlier, neutrophils may arise in both acute inflammation and

healing stages or chronic inflammation. Their number was not significantly different among groups

1, 3, and 6 weeks as shown statistically (Fig. 8).

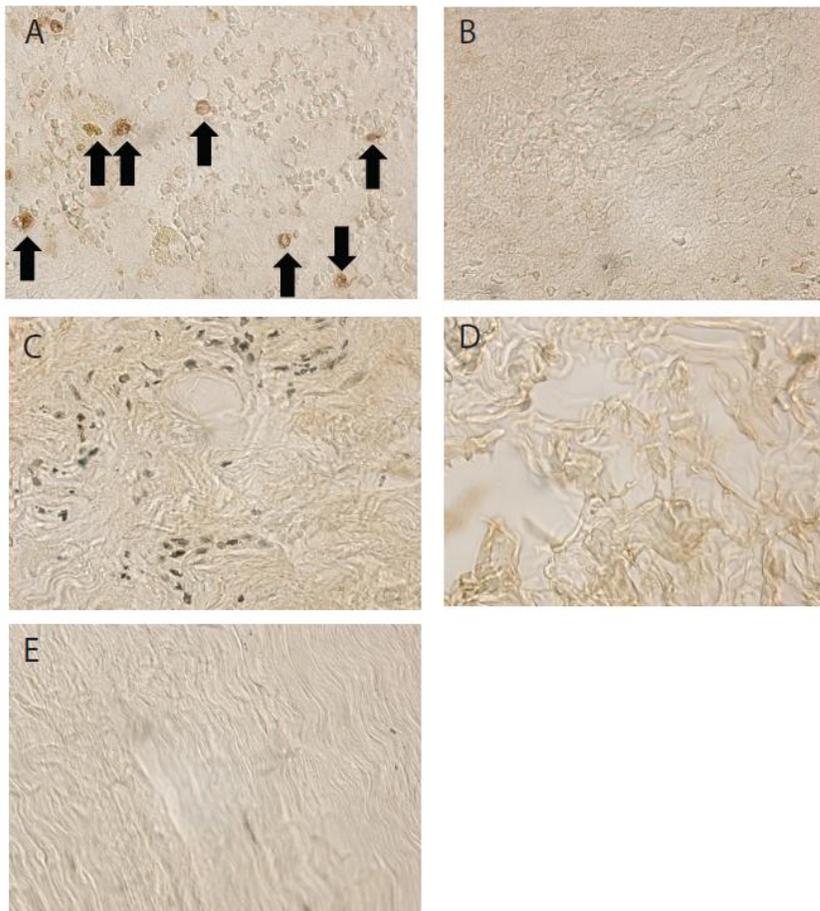**Figure 7**. Neutrophils, 40x objective, 16 ms exposure, 5.5/10 illumination. A. Spleen (+ control). B.

Spleen (- control). C. Paratendon, 6 weeks. Note that neutrophils in the paratendon appear slightly

more dense than in the spleen, similar to data reported by Cetti et al 2003. D. Paratendon, 6 weeks, - control (primary antibody omitted). E. Tendon, 6 weeks – no neutrophils are identified within the tendon proper.

Statistical Analysis

Statistical analysis showed that the densities of both lymphocytes and macrophages were significantly higher than control at 6 weeks (Fig.8). While the number of macrophages in the control is lower than 6 weeks group, there was no significant difference between 1 week and 3 weeks. However, no lymphocytes were found at week 3. Neutrophils in all groups showed no significant difference.
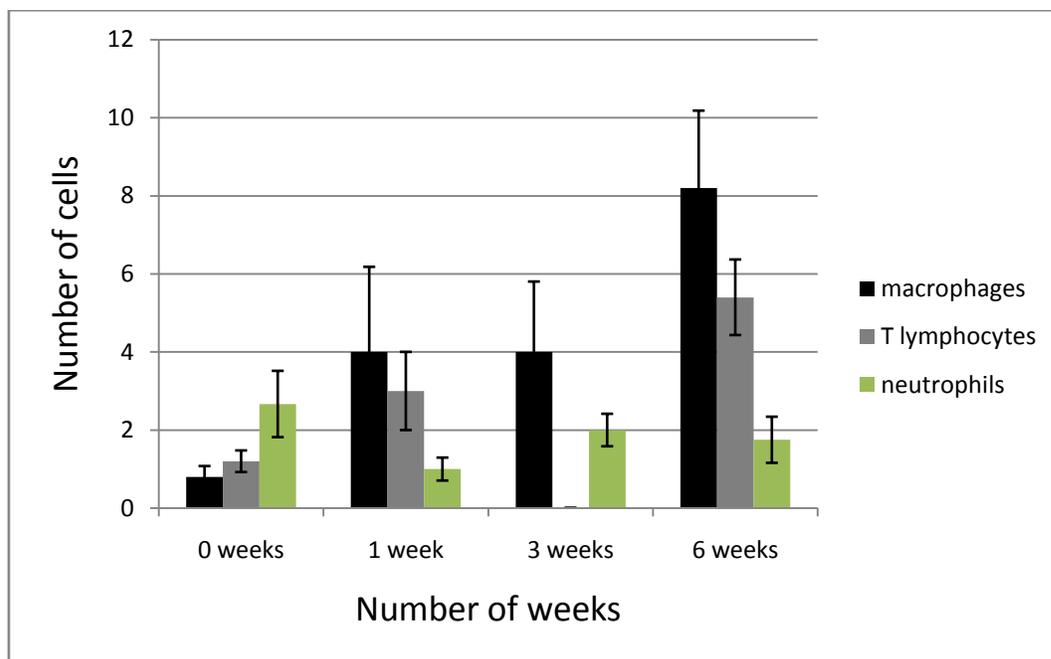

Figure 8. Number of inflammatory cells from 0 to 6 weeks. T lymphocytes and macrophages are both significantly higher than control at 6 weeks (p=0.048, Mann Whitney U, directional)

Chapter 4: Discussion

The main objective of this study was to assess the presence of inflammatory cells in a rabbit model of Achilles tendinopathy, and to determine their possible role in chronic tendon tissue damage. The hypothesis of this study was that animals which had been subjected to an Achilles tendon overuse model for 1, 3, and six weeks would demonstrate a higher density of inflammatory cells in and around the Achilles tendon compared with tendons from the control group. We identified macrophages by the Prussian blue strain, and T lymphocytes and neutrophils by immunohistochemistry in order to determine the numbers of inflammatory cells in and around the Achilles tendon. The prospect of this research for future treatments and study of tendon problems was the primary motivation for reconsidering the role of inflammation in this condition, since current research has not adequately addressed the subject.

This is particularly valuable research because of the confusion that has been persistent in the literature regarding the difference between tendinitis and tendinosis, as highlighted by Khan *et al* (1998)[56]. The concept of "overuse tendinitis "was predominant in the thought behind this current study. The overall question was whether overuse tendon conditions have an inflammatory component. The lack of observation of inflammatory cells in many earlier studies remains a glaring gap that needs to be addressed by more research with appropriate animal models. This study does not attempt to discount the importance of biomechanical factors, which could still be the main provocation of injury; for example, Schepsis' (2002)[97] work examining hindfoot movement and the "whipping" action in the Achilles tendon as a main source of high stress on the medial side of the tendon may be an important starting point for understanding stressors. However, the focus of this

research moves somewhat beyond the typical biomechanical concerns to examine changing cell populations in overused tendon tissue. Therefore, a newer approach and different models were selected.

Though there certainly are major challenges in the study of early stages of tendon overuse in humans, selecting appropriate animal models appeared to be a sound pathway for this specific research. The hope was that the chosen model could provide similar results to those found in the human condition. There is currently no standard model accepted by all researchers, but rabbits were chosen as an appropriate analogue for study. Rabbits have a close proximity to human anatomy and tissue pathology, which is clearly advantageous for this work. [42] Rodents were not preferable due to their small tendon and muscle size and the difficulties involved in extracting proteins and applying electrical stimulation, therefore, they were not performed in this thesis, but could be a planned future direction.

The focus of the proposed experiments with rabbits was on the development of tendinopathy (over a 6 week period), also referred to as chronic tendinitis, because there was a special need for more research to be done on the role of inflammatory cells in the early stages of this condition. The concepts that tendinopathy should be understood as a problem of overuse was retained, and the histological characteristics of importance for more consideration in this case were tied to the degenerative changes in the collagenous matrix, and the presence or absence of inflammatory cells. This seemed to be a productive area of research concentration because the overloading of the musculotendinous unit allowed for the study to consider new approaches to conditions where the tensile force becomes too heavy.

As such, the study design used four groups of six rabbits, where one was a control group, and three groups were subjected to tendon overuse for varying durations. The Backman model was chosen as the exercise technique in order to induce the Achilles tendinosis. The goal here was to achieve the best means for inducement of overuse injury, while keeping ethical concerns at the fore with respect to the rabbit subjects. The duration of the anesthesia given was kept at 2.5 hours when the rabbits were subject to exercise. One concern was to prevent any excessive body movement, and to achieve this, a band was placed around the rabbit's pelvis, while the right leg of each was affixed to a pneumatic piston. That is, thoughtful care went into not only controlling the groups, but also making certain that the rabbits were treated considerately. An ultrasound image of the tendon was then carefully taken at the end of every week of exercise to examine qualitatively whether the presence of injury could be detected (not a primary objective of the thesis).

One of the major results that began to take shape quickly in this research was that macrophages and T lymphocytes were prominently detected in injured tendons. This finding was in contrast to the results of other authors, who reported that there was no evidence of inflammatory cells in or around the tendon. [9] Therefore, there was good reason to believe that this study would serve as a complement to other work on chronic tendinopathy. The gaps in the scientific literature on the topic makes this current approach especially relevant and exciting insofar as it can expand our knowledge about the possible complications due to this cellular presence. This was one of the main hopes of the study, since there is potentially wide application of the results, not the least of which is in sports medicine research.

That is, if the current hypotheses could indeed be verified here and elsewhere, such study may help give support to the idea that inflammatory cells may, in fact, contribute to the development

of chronic tendon overuse injuries. More so, the findings might go some way to suggest other investigations of inflammatory cells not directed at tendon problems. Thus, the conclusions found here may come to prompt further research using different models, or they may become helpful for testing out new treatments centered on inflammatory cells. New studies along these lines could significantly aid in preventing the inflammation at the site of injury and in examining the effects of anti-inflammatory treatments on the development of chronic Achilles tendinopathy.

This is timely work because it has been relatively unclear whether or not the presence or absence of inflammatory cells in Achilles tendinopathy is crucial in evaluating the exact nature of the chronic problems. Though, in general, this has been an addressed subject in previous studies, it has never been elucidated to this degree and through this specific mode of research. It was important then that this study follow from work on macrophages, T lymphocytes, and neutrophils as they have previously been detected in injured human Achilles tendons, but it was likewise key to rigourously examine the assumptions that there would be no evidence for their occurrence. This analytic controversy may stem from the fact that the human Achilles tendon, in terms of overuse injuries, usually develops signs gradually over time. Therefore, the time course of inflammation in response to overuse has been difficult to establish in clinical populations and through sustained study. This was certainly a worry of the current study, and the lack of clinical population studies presents certain challenges and potential limitations for these results. Nevertheless, there were very encouraging, empirical signs suggesting this was the right research to pursue.

For all of these reasons, the study aimed specifically to examine the significant presence of inflammatory cells in the Achilles tendon of rabbits when they were subjected to repetitive mechanical loading of defined durations. It was crucial to make determinations based upon varying durations due to the nature of the stressors and the condition. The larger goal was to demonstrate

some potential paths for medical treatment or rehabilitation along these lines, though this was an assumed implication for this study. The main result was to show that macrophages, T lymphocytes, and neutrophils, have, indeed, been shown to be prominent in injured tendons. Following such a line of inquiry further, there may be more breakthroughs in the treatment of chronic tendon overuse injuries. This was considered to be a potentially important discovery, which validated the selected inquiry.

The study proceeded in the following ways and had significant results. Male rabbits, subjected to repetitive mechanical loading, were grouped into four groups in the study, according to the exercise time period for each group: 0, 1, 3, and 6 weeks. Achilles tendons were harvested at the end of each time period, which ensured the proper time frame for evaluation. The results for the control group showed no changes in tendon morphology, i.e., the tendons conformed to classic descriptions of normal healthy tendon. However, a small number of inflammatory cells were detected in some sections in the exercised groups. For example, the sections from groups 1, 3, and 6 weeks respectively, showed some changes in tendon morphology and cell populations. It is important to remember that collagen bundles were increasingly disorganized. There were also large spaces between fibers, which were likewise observed. Here, it is also key to note that tenocyte nuclei were round and more basophilic, and there was an increase in their numbers with loss of parallel alignment, with increasing durations of exercise.

Because macrophages, T lymphocytes, and neutrophils were detected in tendon sections from groups: 0, 1, 3, 6 weeks, there was a trend displayed, which was specifically in the paratenon. Along these lines, the statistical analysis showed that the density of both lymphocytes and macrophages was significantly higher than control at 6 weeks. One must recognize that while the

number of macrophages in the control is lower than 6 weeks group, there was no significant difference between 1 week and 3 weeks. However, it is also true that no lymphocytes were found at week 3.Neutrophils in all groups showed no significant difference, and this remains a crucial observation which highlighted the differences in leukocyte populations. However, some sections from the same groups showed no evidence for inflammatory cells, which throws in an odd variable for analysis as such. This is a potentially significant variance for the study, and while it does not discount the results, it does present a limitation for assuming a complete case.

What is most important to recognize is that Achilles tendon sections from the control group did in fact show a trace number of inflammatory cells in the study, whereas there was an increasing number of Macrophages, and T-lymphocytes detected in tendon sections from the groups subjected to repetitive mechanical loading. This statistically significant pattern was observed despite the absence of the same types of cells in some sections from the same groups that occurred for unknown reasons.

Questions persisted such as: how well would the animals handle the procedure? Would there be any lameness or any macroscopic change observed (e.g. thickening of the tendon, etc.)? These questions, if answered, tentatively or firmly, could allow for the re-examination of the effects of anti-inflammatory treatments on the development of chronic Achilles tendinopathy. This, again, remained the most important mode of inquiry concerning the initial problematic behind the study. The question of the ultrasound findings hinge upon whether any qualitative impression can be derived finally based on these readings. One key question in this context relates to how the potential technical difficulties relate to the interpretive model at hand. Beyond this, the main query remained tied to the rabbit Achilles tendon tissues as they were examined under light microscope for

morphological changes due to the excessive exercise, and this line of inquiry produced qualitative results.

The immunohistochemistry staining showed the presence of T lymphocytes in rabbit tendons of groups 0, 1, and 6 weeks. What is curious in this respect is that no cells were observed in the weeks 3 group. The increasing number of T-cells in those groups remained statistically significant, which leant support to the main architecture of this study. However, the localization of the cells was totally observed in the tissue around the tendon, the paratenon, and yet no cells were found in the tendon itself. The tendon tissues from groups 0, 1, 3, and 6 weeks were stained with Prussian blue in order to examine the presence of macrophages in the tissues. The macrophages were observed in some tissues of every group increasingly. The presence of these cells was significant in the paratenon of each sample, which has strong implications.

Along these lines, it was found that Achilles tendon tissues that were examined under light microscope showed the presence of neutrophils in some samples from each group. The localization of these cells was noticed in the paratenon region of the injured site. However, it is key to recognize that tissue stained with H&E showed localization of neutrophils in the same groups. The main point to relate here is that this technique was performed to confirm their presence in tissues. Neutrophils may arise in both acute inflammation and healing stages or chronic inflammation. Their number, however, was not significantly different among groups 1, 3, and 6 weeks as shown statistically. It is possible that neutrophils peaked earlier (after 24 or 48 hours) and had returned to baseline before the earliest time point (1 week). Conversely, perhaps neutrophils are not involved in tendon overuse pathology.

In sum, the sections from the control group demonstrated a trace level of inflammatory cells. Those groups subjected to the repetitions of mechanical loading expressed an increasing number of macrophages and T lymphocytes. There was an absence of the same type of cells within some same group sections, and this remains an unexplained result in the study, but is not necessarily a definitive absence. The results gathered do appear to support well the major hypothesis here, which is that inflammatory cells might contribute to the development of chronic tendon issues related to overuse.

Building upon this work, one area of future research in this case will need to speak to the question of how to inhibit inflammatory cells in Achilles tendinopathy and prevent chronic processes at the site of injury. It will be interesting to see to what degree the problem of Achilles tendinopathy can be solved by using anti-inflammatory treatments after confirming the inflammatory cells presence at the site of injury. This study may resolve the question of inflammation as it relates to Achilles tendinopathy development in the paratenon; however, it may not go far enough in exploring the presence of cells in the tendon itself.

Chapter 5: Conclusion

We used New Zealand white rabbits to induce Achilles tendinopathy due to repetitive exercise. In our study we demonstrated that the tendons were thicker and enlarged with cellular changes and collagen fiber disorganization comparing to control. However, inflammatory cells such as macrophages, T-cells, and neutrophils were detected in the exercised tendons using histological and immunohistochemical tests. Further experiments on tissues from exercised animals by using ELISA to examine important pro-inflammatory cytokines such as interleukin 1 beta (IL-1B) can provide more information about inflammation role on Achilles tendinopathy. Moreover, previous and future studies results could be used to prevent the inflammation in its early and late stages at the site of injured tendon.

5.1 Future Directions

More studies are planned to be conducted using new testing methods**.** The localization of inflammatory cells in the exercised tendons tissues that have been demonstrated in our previous study will allow us to examine the early stages of inflammation by measuring specific cytokines that promote the introduction of inflammatory cells such as IL-1B. However, this future study will also allow us to test new or existing anti-inflammatory drugs and as a result prevent or limit the effects of macrophages and other inflammatory cells.

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