ESTABLISHMENT AND VALIDATION OF A RAT ACHILLES TENDON OVERUSE EXERCISE MODEL WITH CHARACTERIZATION OF HISTOLOGY, BIOCHEMISTRY, BIOMECHANICS AND COLLAGEN CROSSLINKING

by

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ABSTRACT

Non-insertional disorders of the Achilles tendon cause pain, disability, may predispose to catastrophic rupture, and are increasing in frequency. Currently, the pathobiology of Achilles tendon disease is poorly understood. This thesis seeks to improve the understanding of Achilles tendon disease biology by establishing an animal model for Achilles tendon disease. The animal model is based on an over-exercise uphill treadmill running regime imposed on rats. It has been validated as providing tendon samples with histological characteristics similar to those commonly cited in the literature for human Achilles tendon disease. These include increased cellularity, and more disorganized, more intensely stained collagen fibers. Immunohistochemical staining of the diseased tendons show that the increased cell numbers are principally fibroblasts and/or endothelial cells-not leukocytes. This is suggestive that the underlying biological response is more reparative than inflammatory. Biochemical testing revealed that the tendons had increased levels of glycosaminoglycans but no increase in collagen content. The collagen thermomechanics of the Achilles tendons from running rats demonstrated decreased intrahelical crosslinking that was similar to the features observed in maturing rat tendons. These biochemical changes did not translate into altered biomechanical properties when the tendons were subjected to mechanical testing. In summary, an animal model for Achilles tendon disease has been established that produces rat Achilles tendons with histological characteristics similar to those observed in human Achilles tendon disease. The underlying histology and biochemistry is suggestive that overuse-exercise running in the rat may induce biological repair or remodeling of the Achilles tendon.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

BPHV	Bioprosthetic Heart Valve
DAB	3,3-Diaminobenzidine
DCS	Differential Scanning Calorimetry
FOV	Field of View
FsOV	Fields of View
GAG	Glycosaminoglycan
H&E	Hematoxylin and Eosin
HIT	Hydrothermal Isometric Tension
HPLC	High Performance Liquid Chromatography
MNNFOV	Mean Number of Nuclei per Field of View
$NaBH_4$	Sodium Borohydride
Rat-A-PED	Rat Achilles Passive Exercise Device
$t_{1/2}$	Half life of stress relaxation associated with peptide bond hydrolysis
T_d	Denaturation Temperature

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"Everything should be done in moderation including moderation"

J.C. Hyndman

STUDENT MANUSCRIPT CONTRIBUTION

The doctor of philosophy thesis below was envisioned through clinical studies during the PhD candidate's orthopedic surgery residency. The experiments on hydrothermal testing were recommended by Dr. Michael Lee, PhD Supervisor.

All personal funding was raised by the candidate through a combination of scholarships and finances earned from a clinical practice in orthopaedic surgery that occupied no more than 20% of the candidate's working time. All funds for the experimental studies were raised by the candidate through peer-reviewed grants, all with the candidate as principal investigator, and most with Dr. Michael Lee as co-applicant. The experimental studies were completed solely by the candidate with the following exceptions:

1. Biochemical assays for collagen content, collagen type, and glycosaminoglycan content were completed with technical and hands-on assistance from lab technician, Maxine Langman.
2. Hydrothermal testing experiments were completed with technical assistance from a fellow graduate student, Ali Jahangir.
3. The mechanical testing apparatus was operated by PhD supervisor, Dr. Michael Lee.
4. Histological techniques of hematoxylin & eosin staining and immunohistochemical staining of tendon specimens were performed by lab technician, Janet Tam.

5. Supervision of portions of the rat running on the rodent treadmill was by students, Cathy MacLeod, Krysten Smith, and Laura MacDonald and by lab technician, Maxine Langman.

6. Manual counting of nuclear numbers present on the cross-sections of tendons was performed by a student, Krysten Smith acting as a second, blinded observer.

7. Semi-quantitative grading of the cross sections of tendons collagen organization and staining intensity was confirmed by blinded observers Dr. Michael Lee and Dr. James Wright.

The writing of the thesis was completed with editorial support of supervisor Dr. Michael Lee and Dr. James Wright.

CHAPTER 1: INTRODUCTION

1.0 Introduction: Tendon Disease

Tendons serve as the attachments of the muscles to the bones. Forces generated by the muscle are transmitted to the bone through the tendon allowing motion across the joint that the muscle tendon unit spans. Overuse activity of a tendon may cause disease resulting in pain, disability or catastrophic rupture. The most common forms of tendon disease have been termed "tendonitis". These include such conditions as rotator cuff tendonitis, patellar tendonitis or Achilles tendonitis. The term tendonitis implies an inflammatory process resulting in the clinical signs of *rubor, tumor, calor, dolor, and functio laesa* (redness, swelling, heat, pain and loss of function).

Achilles tendon disease may involve the paratenon and/or the tendon itself. Involvement of either of these two components may occur in isolation or in combination as classified by Puddu et al. [1]:

1. Pertendonitis consists of edema, fat necrosis and formation of adhesions within the paratenon [2].

2. Tendinosis describes the degenerative process occurring within the tendon itself. Pathological changes include hyaline degeneration with a decrease in the normal cell population, mucoid degeneration with chondroid metaplasia of tenocytes, fatty degeneration of tenocytes, lipomatous infiltration of large areas of

tendon, an increase in matrix mucopolysaccharides, and fibrillation of collagen fibers [3].

3. Pertendonitis with tendinosis is the most common type of process with acute and chronic changes present both in the tendon and the paratenon to varying degrees [3]. The paratenon changes usually occur at the same level as the tendinosis.

These non-insertional Achilles tendon disorders are cited to be increasing in frequency [4, 5] but the literature lacks a clear understanding of the underlying biology that limits effective scientifically based therapeutic intervention strategies. For example, the terminology used to describe Achilles tendon disorders is somewhat confusing and there appears to be a lack of consistency in the literature as to whether the condition is inflammatory [6, 7]. Some use the term Achilles tendonitis with the suffix "itis" denoting inflammation, which is often the case when the paratenon is involved with or without involvement of the tendon itself [1, 8]. Others use the term Achilles tendinosis with "osis" denoting a degenerative process of the tendon with no evidence of an inflammatory infiltrate [3, 9-12].

Arner [9] was one of the earliest to report Achilles tendon disease as a degenerative process rather than an inflammatory process. Arner studied 74 cases of human subcutaneous Achilles tendon ruptures using light microscopy with hematoxylin and eosin (H & E), van Gieson's and Weigart's staining. The histopathological features described included edematous disintegration and irregular collagen fibers with no clear evidence of inflammation. Jozsa et al. [11] used electron microscopy to examine tendon specimens from human subjects within 48 hours of rupture and noted no signs of

inflammation. Subsequent studies [10, 11] noted a predominance of a degenerative process rather than an inflammatory process. The most extensive study of ruptured tendons was by Kannus et al. [3] who studied 1146 ruptured tendons including 397 Achilles tendon specimens taken from patients suffering a spontaneous rupture. It was concluded that there was very little evidence of an inflammatory process based on the histopathology studied using light, polarized light, scanning and transmission electron microscopy. Recently, the predominance of a degenerative process was confirmed by Maffulli et al. [12]. This study used H & E staining with light microscopy to study biopsies from the Achilles tendons subjects who suffered a catastrophic rupture (27 men, 11 women; mean age, 45.3 +/- 13.8 years). The control group consisted of specimens of Achilles tendons from persons with no known tendon ailments (43 men, 3 women; mean age, 64.2 +/- 9.7 years). Semi-quantitative assessment of cross-sections revealed differences in fiber structure and arrangement, rounding of the nuclei, regional variations in cellularity, vascularity, collagen staining, hyalinization, and glycosaminoglycans. It was concluded that there was significantly higher degeneration in the ruptured tendons and that degeneration was not a feature of tendons from healthy, older persons.

In contradiction to the above studies, a recent study by Cetti et al. [13] suggested the presence of widespread bilateral and ipsilateral acute inflammation in the Achilles tendons from 60 patients with a spontaneous rupture of the Achilles. These findings were noted to coexist with the features of tendon degeneration in earlier studies [3, 9-12]. It is interesting to note that these studies were done on specimens harvested at a mean time of 36 hours post rupture (range 7-112 hours). This may suggest that the acute inflammatory response documented is a result of the traumatic event and it is possible that the

degenerative process preexisted and predisposed the tissue to rupture. Further, the incidence of symptoms of Achilles tendonitis prior to tendon rupture has been reported to be in the range 10 to 30 percent [3, 4, 14] leaving one to question the presence of a pre-existing inflammatory process.

The purpose of this thesis is to gain a better understanding of Achilles tendon disease, structure, and function. To this end, an over-exercise animal model for Achilles tendon disease was established and validated on a histologic basis. Experimental tendons from this model were compared to control tendons to determine their structural differences or more specifically the biochemical features of collagen content, glycosaminoglycan (GAG) content, and collagen crosslinking. Biomechanical testing was used to determine if the structural differences have any functional implications. Finally, the tendons derived from this model were also examined using immunohistochemical techniques to gain insight into the tendon's biological response to overuse exercise.

In this thesis there are three chapters each serving as papers submitted for publication in a peer-reviewed journal. The first of these chapters (Paper I) begins with the development and histological validation of an over-exercise animal model for Achilles tendon disease. Paper II advances to the study of the diseased tendon structure by examining the effect of age and over-exercise running on the crosslinking of the collagen in the Achilles tendon. Finally, Paper III continues with further structural characterization of diseased tendons and then advances to biomechanical studies in an attempt to determine if the structural differences have any functional implications with respect to tendon strength.

The findings of these studies suggest that a 12 week overuse exercise uphill running regime causes the rat Achilles tendon to undergo a biological response that is similar to that observed in humans with Achilles tendons afflicted with disease. This biological response was characterized by increased glycosaminoglycans, collagen disorganization, altered collagen crosslinking and an increased cellular response that consisted of fibroblasts or endothelial cells rather than inflammatory cells. These findings suggest a repair or remodeling response to overuse exercise rather than a sustained inflammatory response.

1.1 Animal Models for Achilles Tendon Disease and Histology

An experimental animal model that is validated to produce tendons that are similar to those observed in patients with Achilles tendon disease would be a useful tool to investigate tendon biology and response to therapeutic interventions [15, 16]. Such a model will assist in characterizing the biochemistry, histology and biomechanics of Achilles tendon disease. An ideal animal model for any disease should include certain criteria such as: (1) appropriate tissue type modeled, (2) accurate simulation of the injury conditions, (3) reproduction of pathological animal tissues that are similar to human diseased tissues and finally (4) ease of application [15, 16].

1.1.1 Tissue type to be modeled

In the human, a merging of the insertions of the gastrocneimus, which has its origin at the femoral condyle posteriorly, and the soleus, originating from the posterior superior aspect of the tibia and fibula, forms the Achilles tendon. The soleus component varies from 3 to 11 cm while the gastrocneimus varies from 11 to 26 cm. The confluence

of these two tendons inserts on the calcaneus posterior to the superior calcaneal tuberosity [17]. The Achilles tendon is covered by a paratenon sheath allowing 1.5 cm of glide through a thin layer of fluid.

The rat serves as an appropriate animal to model Achilles tendon disease due to certain similarities with the human. First, the osseous and muscular hind foot gross anatomy is analogous albeit on a smaller scale. Further, the intermetatarsal angle between the first and second rays of the hind limb is more similar to that in humans than in any other mammal or primate. Drawbacks to the rat as an animal model for Achilles tendon disease would include variations in the anatomy, the rat's innate activity patterns, and a rapid rate of maturation, achieving puberty at age 50 to 60 days [18, 19]. However, as we will see, this earlier pubertal development will provide an opportunity to study the maturation process that may be analogous to the biology of tendon disease.

The rat also offers unique features in that it is an animal that is used widely for scientific research. This adds a variety of biological tools such as commercial antibodies and histological techniques that may be used for cell identification pertinent to the current study. Indeed, the rat animal model has been used in an attempt to model tendon disease previously [20-23].

1.1.2 Simulation of the injury conditions

Once the rat was chosen as an appropriate animal model, the next objective was to determine the means by which to induce injury into the experimental Achilles tendon. Others have attempted to induce pathology in animal tendons by partial tendon laceration [24], or injection [25]. Hitchcock et al. [24] used a special designed "tenotome" to create

a uniform tendon laceration in the flexor tendons of White Leghorn chickens while Stone et al. [25] injected New Zealand White rabbit patellar tendons with species-specific preparations of cytokines to model mild, seemingly reversible tendon injury.

In the current study, a more physiological approach to inducing disease into the rat Achilles tendon was utilized in an attempt to parallel the etiological factors for Achilles tendon disease seen in humans. Unfortunately, the etiology of human Achilles tendon disease is not well known, but most of the current literature suggests a number of possible mechanisms. These include age [26], preexisting disease [27, 28], hyperthermia [29] pharmacological agents [30, 31] and overuse running [32] - the latter being most consistently cited [32-36].

In a review of injuries to human subjects participating in running programs, McCroy et al. [32] identified 31 runners symptomatic for Achilles tendonitis and 58 runners who were non-symptomatic. There was a trend linking increased running duration and Achilles tendonitis, with those symptomatic averaging 52 kilometers per week and those not symptomatic averaging 44 kilometers per week.

Previous studies using overuse activity have attempted to develop an animal model for Achilles tendon disease [8, 20]. Backman et al. [8] attempted to simulate overuse running using a complex "kicking" machine. This device was designed to dorsiflex the foot of New Zealand White rabbits with concurrent costimulation of the gastroc-soleus complex. The daily regime included repeated daily anesthesia with fentanyl-fluanisone to allow gastroc-soleus electrical costimulation and 9000 passive dorsiflexion-plantarflexion cycles (~60° of foot motion) three times per week over 6 weeks. This regime did produce degenerative changes in the Achilles tendons that were

concluded to be consistent with chronic Achilles paratendonitis with tendinosis. Specifically, semi-quantitative assessments revealed variation in H & E staining and fibrillar thickness, altered nuclear morphology, increased capillary numbers, edema, fibrosis, and inflammatory cell infiltrate. These findings did not exist in the Achilles tendons taken from the contralateral non-exercised limbs.

Messner et al. [20] also attempted to simulate overuse running using a complex "kicking" machine applied to male Sprague Dawley rats. The daily regime was identical to that of Backman et al. with the exceptions of Ketamine anesthesia, 35° of foot motion and a 3-5 week protocol. This regime produced tendon fibrillation and hypervascularity in 2 of the 5 Achilles tendons taken from exercised limbs.

Neither the regime of Backman et al. [8] nor that of Messner et al. [20] were utilized in this study due to a number of factors. First, the animal models were not completely physiological due to the non-weight bearing position of the hind limb and the immobilization of the foot, leg and thigh. Second, the regimes were both cumbersome and dangerous to the systemic health of the animals due to the requirement of repeated daily anesthesia. Further, personal communication with Messner et al. [20] indicated that further study on their part with this regime was abandoned due to inconsistent histology results and the rats inability to tolerate the regime.

Most of the problems with the animal models discussed above were solved by Soslowsky et al. [22] in their development of a rat model for shoulder tendonitis. In that study, the rat model was chosen to study the effect of overuse exercise on the rat rotator cuff tendon, which again has very similar anatomy to that of the human. The overuse exercise protocol consisted of 10° downhill treadmill running at a speed of 17 m/min for

one hour, 5 days per week. This regime was equivalent to about 7500 strides per day, which was similar to the number of strokes an elite swimmer may take during a typical training protocol. After 16 weeks of this over-exercise regime, the rotator cuff tendons were harvested and stained with H & E to allow a semi-quantitative assessment of tendon cellularity, cell shape, and collagen fiber organization. The results showed that these tendons demonstrated increased cellularity, disorganized collagen fibers and changes in cell shape to more rounded cells. These histological changes were deemed consistent with those observed for human tendinopathy and the authors suggested that the animal model may be used to further study rotator cuff tendinopathy.

In the current study, methods were used that parallel those of Soslowsky et al. [22] to develop an over-exercise running rat animal model for Achilles tendon disease. This model utilizes an overuse running regime, the most commonly cited injury condition for Achilles tendon disease that is both physiological and practical. This regime confines rats to a 10^{o} uphill treadmill running regime at distances (once scaled down to the rat stride length) equivalent to a human 52 km/week protocol that has been previously suggested to increase the incidence of Achilles tendon disease in humans [32]. Further, uphill running at a 10^{o} incline was chosen to simulate an activity requiring eccentric muscle contractions that result in a greater risk for tendon injury [37-40].

1.1.3 Reproduction of pathological animal tissues similar to human diseased tissues

The single most important feature of the rat animal model for Achilles tendon disease would be that it must produce Achilles tendons that have biological features similar to those observed in human diseased Achilles tendons. Other studies have used

commonly cited histological features of human tendon disease to validate animal models [8, 20-22]. The normal Achilles tendon has well-described histological features [41], including highly organized, tightly packed parallel collagen bundles [41, 42]that stain pink-red (eosinophilic) under H & E staining [42]. The cellular component shows sparse nuclei that are elongate and found interspersed between the collagen bundles [41, 42].

A review of the histopathology of diseased Achilles tendons from the human and animal literature can be found in Appendices 1 and 2 respectively. The diseased Achilles tendon specimens have been derived from human patients with subcutaneous rupture [3, 9-13] and human patients with chronic localized Achilles tendon symptoms [10, 43, 44]. The histopathological features most commonly cited in the human specimens include: disorganized collagen fiber arrangement with more intensely eosinophilic staining, and increased numbers of cells that exhibit rounding of cell nuclei (Appendix 1). These features have been cited to result from repetitive damaged that is sustained by the Achilles tendon with overuse-running [32-34, 45-48].

The animal model developed in this study was intended to produce Achilles tendons that bear histological features that are similar to the histopathological findings of biopsies from ruptured human Achilles tendons [3, 9-13], diseased human Achilles tendons [10, 43, 44], and tendons from animals subjected to over use exercise models [8, 20-22, 49].

1.1.4 Application of the animal model

Previous authors that have attempted to model Achilles tendon disease have had success with simulation of the injury condition and have been able to induce histological

changes (see section 1.1.3) in the Achilles tendon that are similar to those observed in human Achilles tendons afflicted with disease [3, 9, 43, 44, 50]. However, the problem with these models stem from the complexity of a "kicking" exercise regime demanding repeated general anesthesia and electrode-induced co-stimulation of the lower leg muscles (discussed previously in section 1.1.2). These features made the models less practical and more costly, thus limiting the application of the animal model.

The current study uses a more practical animal model where the rat is encouraged to perform 10^{o} uphill treadmill running by confinement on a rolling belt treadmill (Letica Scientific Instruments, Barcelona, Spain). This regime is technically simple to administer, more physiologic and ethical. This ease of application will thus facilitate the model's future utility in this field.

1.2 Achilles Tendon Biochemistry

The fundamental function of all tendons is to transmit forces generated by muscles to allow movement of the musculoskeletal elements. The Achilles tendon serves to transmit the contractile force of the gastrocnemius and soleus muscles to the calcaneus to facilitate plantarflexion of the foot and ankle. The underlying biochemical structure of the Achilles is suited for these tensile forces. This includes a predominance of large diameter, fibrillar collagen type I that is aligned in parallel bundles and heavily crosslinked to resist the tensile forces [51, 52]. These collagen bundles are embedded in a matrix of proteoglycans and glycosaminoglycans (GAGs) that has been shown to associate with collagen [53]. The GAGs may associate with collagen through electrostatic interactions and with the proteoglycans by binding covalently to their protein cores. It is suggested that the proteoglycan-GAG matrix binding is important to resisting

deformation within a single collagen fiber bundle [54]. The viscous like properties of the glycosaminoglycans and proteoglycans may also facilitate reorientation of the collagen fibers along the direction of stretch to enhance resistance to tension [55].

1.2.1 Collagen

The biosynthesis of collagen has recently been reviewed by Patino et al. [56]. Collagen biosynthesis includes intracellular and extracellular events. Within the fibroblast cytoplasm, polypeptide chains are synthesized by translation of specific mRNA on membrane-bound polyribosomes on the rough endoplasmic reticulum. The collagen polypeptides are then hydroxylated and glycosylated yielding a triple-helix conformation where cysteine residues are juxtaposed for the formation of disulfide bridges that will link individual pro-α chains at their C-terminal ends. The amino acid sequence of these polypeptide chains consists of about 33% glycine and 25% proline. The large proline content dictates the left-handed helical conformation of the polypeptide. Three polypeptides (alpha chains) then aggregate into a triple helix referred to as procollagen. The triple helix is characteristic of all collagens, which are named according to their alpha chains that are a distinct gene product. The steric conformation is due to the repeating motif of Gly-Xaa-Yaa (where Xaa and Yaa are usually proline and hydroxyproline, respectively) with glycine, the smallest amino acid, positioned within the interior of the triple helix.

After intracellular synthesis the procollagen bundles are then secreted by the fibroblasts where they undergo extracellular modifications including hydrolysis of peptide bonds to release telopeptides (the globular non-helical end of the procollagen due

to a lack of the repeating motif of Gly-Xaa-Yaa) at terminal peptide ends to form the collagen molecule (tropocollagen in the older literature). It is these collagen molecules, which align in a head to tail fashion forming the quarter-staggered array, that ultimately yield a collagen fibril. The fibrillar collagens will polymerize side-by-side and end-to-end into a long, fibrillar aggregate [57].

During exercise, one might expect an increase in tendon collagen content to facilitate a tendon's ability to resist tensile forces. A number of studies have failed to demonstrated an increase in the collagen content of tendons in response to exercise [58-61]. These include a study by Woo et al. [59] where it was shown that a 12 month running exercise regime (40 km/wk) in a swine animal model did not cause an increase in collagen content of the swine digital flexor tendons. This study was in contradiction to an earlier study [62] that demonstrated increased collagen concentrations in the same animal model, resulting in greater strength and stiffness of swine extensor tendons. The differences in these two studies were attributed to differences in the biochemical composition and function of flexor and extensor tendons. In another study by Eriksen et al. [63] it was shown that the collagen content of ruptured human Achilles tendons was decreased but there was an overall increase in Type III collagen at the rupture site. It was hypothesized that this relative change in collagen subtypes, with no overall increase in collagen concentration, was due to previous micro trauma and subsequent healing that caused the human Achilles tendon to be more susceptible to rupture.

The discrepancy in these and other studies may be reconciled by the fact that there exists at least indirect evidence and some direct evidence that many determinants of strength exist in a collagenous tissue such as a tendon. These determinants of strength

include, but are not limited to, collagen crosslinking [64, 65], tendon size [62], collagen subtypes [63, 66], architecture and density of collagen fibers present [3, 9, 12, 67], and extracellular matrix components (see review by Silver [54]) such as elastin and glycosaminoglycan (GAG) content [3, 12, 68, 69]

The type of collagen crosslinks was shown to act as a determinant of strength in rat tail tendons by Davison [64]. This study demonstrated increased tensile strength of all tendons that were subjected to a sodium borohydride reduction technique over tendons that were not reduced. This effect was more pronounced in the more immature rats. The density of collagen crosslinks may also act as a determinant of the strength of a collagenous tissue. Frank [65] showed that rabbit medial collateral ligament scar weakness was associated with decreased collagen pyridinoline crosslink density.

Tendon size has also been shown to serve as a determinant of strength in the study of Woo et al. [62], where swine extensor tendons taken from animals subjected to exercise were larger and stronger than those from sedentary animals.

While there exists no direct evidence that the types of collagen present in a tendon serve as determinants of strength, there is some evidence for this dependence in other collagenous tissues such as the mouse aorta. Vouyouka et al. [66] studied the biomechanical properties of aortas from a unique genetic variant mouse containing a collagen type I defect. Mechanical testing results showed decreased strengths and stiffness of the aortas from the mice with the mutant form of collagen type I compared to the wild type.

There is also some indirect evidence that collagen subtypes may act as determinants of strength in human Achilles tendons [63]. As discussed above, Eriksen et

al. [63] showed a localized increase in collagen type III at the rupture site of Achilles tendons compared to controls and it was suggested that pre-existing increases in collagen type III content at the rupture site predisposed the tendon to rupture due to a decrease in tensile strength.

The possibility that the architecture and density of collagen fibers present in a tendon may also serve as determinants of strength was evidenced by Vilarta et al. [67]. In this study, Wistar rats were subjected to an exercise regime of 5° uphill treadmill running at a velocity of 11 m/min daily for 1 hour over an unspecified time period. The Achilles tendons from exercised rats were subjected to mechanical testing and birefringence. Results demonstrated increased strength that was attributed to a reorganization of the collagen architecture including increased collagen fibril alignment and more intense molecular packing. Other indirect evidence that collagen architecture acts as a determinant of strength exists in studies of human Achilles tendon ruptures that consistently demonstrate a more disorganized collagen architecture [3, 9, 12].

Although collagen is the principal component of collagenous tissues that provides strength and stiffness, there are other components that play roles as well. Elastin fibers have been shown to provide the resistance to tensile forces in arteries at low strains [68]. When arteries were differentially digested to remove the collagenous components it was shown that the elastin component provided stiffness and strength at low strains and to a lesser extent at physiological strains. On the other hand, when the elastin was removed, the collagenous components were shown to be most important at physiological strains and even more important at high strains.

Glycosaminoglycans are another component of collagenous tissues that may be important for imparting strength and stiffness on collagenous tissues [69]. In cyclic fatigue studies on bioprosthetic heart valves (BPHVs) it has been shown that GAG content significantly decreases compared to non-fatigued controls [69]. In the same study these fatigued BPHVs also displayed decreased cuspal bending strengths. While it is possible that the decreased strength resulted from changes in the collagen I at the molecular level it is also likely that the loss of GAGs played a role. For example, in BPHVs the GAGs play an important role in maintaining a biomechanical relationship between the major cuspal planes.

The above study yielded evidence that GAG content of collagenous tissues may be a determinant of strength. In histological analysis of tendons that have ruptured, there is a similarly consistent finding of decreased GAGs content when compared to control, non-ruptured tendons [3, 12]. The evidence for GAGs playing a role in determining strength and stiffness in the tendon is indirect since mechanical testing was not utilized.

The current study is limited in its ability to precisely examine the determinants of strength in the Achilles tendons from rats subjected to over exercise since it does not consider all determinants of strength. It does however; take into consideration the GAG content, collagen content and collagen crosslinking in the tendon to help explain the tendons response to over-exercise.

1.2.2 Glycosaminoglycans

Glycosaminoglycans are disaccharide polymers which are an important component of the tendon extracellular matrix. The three major GAGs that are found

within tendons include hyaluronate, chondroitin sulfate and dermatan sulfate. Hyaluronate and chondroitin sulfate are predominantly interfibrillar with respect to collagen while dermatan sulfate is entirely fibril-surface associated [53].

The binding of these GAGs and their associated proteoglycans to collagen have been shown to play roles in the development of collagen fibril diameter [70] and have been shown to be important in determining the mechanical properties of a tendon [71, 72]. In a study using isolated fascicles from mouse tail tendons [73], it was shown that during maturation there was a decrease in the content of GAGs. Specifically, 8-week-old mice had lower chondroitin and dermatan sulfate contents and a higher collagen content than 3-week-old mice resulting in subsequent changes in ultra structural and mechanical properties of the tendon [71].

Since the process of collagenous tissue maturation is similar to repair and remodeling after injury, one would expect that changes in GAG concentration and mechanical properties may result from overuse injuries [74]. With respect to the effect of exercise, studies are limited and conflicting on changes in glycosaminoglycan content. Vailas et al. [60] showed increased GAG content in tendons of Long-Evans rats that were allowed increased voluntary running on an exercise wheel for 6 months. This increase in GAGs was documented by comparing the amount of galactosamine in the tendons of 9-month-old running and non-running rats, a figure reflective of the tendon's total and dermatan sulfate concentrations. This study was consistent with the results of Heikkinen and Vuori [75] who documented that running caused an increase in GAG as measured by increased hexosamine concentration of Achilles tendons of mice after a 4-week exercise regime. Another study by Curwin et al. [61] showed no change in the GAGs as

determined by measurement of hexuronate content of Achilles tendon from Leghorn chickens subjected to an exercise regime. While the former two studies have concluded that exercise increases the GAG content of tendons, the latter study did not confirm this. These conflicting and limited results prohibit any definitive conclusions on the effect that exercise will have on GAG content of tendons and suggest that further studies are necessary.

1.2.3 Collagen crosslinking

Collagen molecules are stabilized by a variety of different chemical bonds including disulfide bonds forming between adjacent cysteine amino acids [76], non-covalent bonds such as hydrogen and electrostatic bonds, and collagen crosslinks [51]. It is generally accepted that the collagen crosslinking plays the largest role in determining the biomechanical properties of a collagenous tissue such as the tendon [64, 65]. Crosslinks are formed when lysyl oxidase converts the ε-amino side group of the amino acid lysine to a semi-aldehyde that in turn interacts with another ε-amino group of an adjacent amino acid to form an immature, reducible, or unstable aldimine type of crosslink. These unstable crosslinks are thought to reduce with time by a condensation reaction rendering the collagens more hydrolytically stable, a process often referred to as maturation of collagen. This may also be done artificially using reducing agents such as sodium borohydride [77].

Crosslinks that form between amino acids of the alpha-chains within the same collagen molecule are referred to as intramolecular linkages. Those found between adjacent collagen molecules are referred to as being intermolecular. The role of

intermolecular crosslinks is that of resisting tensile forces by preventing the "slippage" of collagen molecules past one another [78]. Correlations between intermolecular crosslinking and tensile strength have been demonstrated in skin, tendon and bone [79, 80].

The role of intramolecular crosslinking appears to be that of minimizing the distances between neighboring polypeptide chains within the same collagen triple helix and therefore preventing the incorporation of excess water that would otherwise act to break down hydrogen and electrostatic bonds [81]. Indeed, covalent intrahelical crosslinks that exist within a collagen molecule's triple helix structure have been suggested to further stabilize collagen from denaturation [82]. This denaturation process entails the transformation of a collagen molecule from a highly organized hydrogen-bonded triple helical structure to a random coil as a result of increased thermal gyration sufficient to disrupt various types of reversible bonds (e.g. hydrogen, ionic, and polar), within the collagen triple helix [83-85]. Thus, intramolecular crosslinking acts to make collagen less susceptible to denaturation.

1.2.4 Methods for assessing crosslinking in collagenous tissues

A variety of methods exist to assess the type and extent of crosslinking of collagenous tissues. These include biochemical tests such as High-Performance Liquid Chromatography (HPLC) of hydrolyzed samples and thermal stability tests such as differential scanning calorimetry (DSC), shrinkage temperature tests and hydrothermal isometric tension (HIT) tests.

HPLC is a sensitive means for quantifying the 3-hydroxypyridinium, pentosidine and pyridoxine crosslinks of collagen in tendon and other tissues [54, 55]. For example, Eyre et al. [86] established methods to use HPLC to quantify the 3-hydroxypyridinium crosslinks in crude lysates of all types of connective tissues. Curwin et al. [61] used HPLC techniques to show that tendon collagen from Leghorn chickens subjected to over use running contained fewer (50%) pyridinoline crosslinks.

Thermal stability tests measure the temperature at which collagen denatures changing from its hydrogen-bonded triple-helical structure to a random coil [87]. The denaturation temperature will vary with the extent of intra- and intermolecular crosslinking. DSC is an example of a thermal stability test used to determine the thermal behavior of a polymer as it undergoes chemical and physical denaturation. During a DSC test, the amount of heat required to raise the temperature of a sample at a constant temperature rate is measured. The denaturation temperature is the temperature recorded at the peak in heat flow on the heat flow/temperature curve recorded.

Shrinkage temperature tests are another means of measuring denaturation. In a shrinkage temperature test, a collagenous material is held under isometric constraint with the length of the sample being monitored. The sample is then heated and the temperature at which the sample shrinks in length is referred to as the 'shrinkage temperature', which is analogous to the collagen denaturation temperature.

Hydrothermal Isometric Tension (HIT) tests are another means of assessing the crosslinking in a collagenous tissue and is the method utilized in this study. HIT is used to investigate the crosslinking of collagenous tissues by observing changes in the denaturation temperature (T_d) during that heating phase and the half-life of stress

relaxation associated with peptide bond hydrolysis ($t_{1/2}$) during the isometric relaxation phase [88-91]. During HIT, both ends of a collagenous tissue are held by two grips and the sample is maintained at a constant length (isometric constraint). One grip is attached to a force transducer and the other to a fixed rod. The samples are immersed in a water bath while the temperature is increased. The water bath temperature, load (resultant tensions at the grips), and time are recorded concurrently and plotted against time (Figure 4.1 in Chapter 4 below). When the collagen denatures there is a sudden increase in load recorded. Tendons that do not fail and surpass the denaturation temperature provide further information after a constant elevated temperature of 90°C (isotherm) is maintained. This portion of the HIT testing is referred to as the isometric relaxation phase (Figure 4.1). During this segment, the relaxation of rubbery collagen (due to breaking of peptide bonds) is inhibited by any hydrolytically stable crosslinks that survived the increase in temperature to 90 °C. Thus, the isometric relaxation phase provides an estimate of the density of thermally stable, mature, non-reducible crosslinks, a value reflected in changes in the half-time of the relaxation, $t_{1/2}$.

Without artificial chemical stabilization of the unstable immature crosslinks, the relaxation during the isometric relaxation phase is inhibited by mature crosslinking only. If there is a lack of mature crosslinks, then the tissues may fail prior to achieving a sustained isometric relaxation phase or display a nearly vertical slope and decreased $t_{1/2}$ value during the isometric relaxation phase. This indicates a prohibitively faster rate of stress decay due to the lack of thermally stable mature crosslinks.

If tissue samples are treated with sodium borohydride ($NaBH_4$) reduction prior to HIT, there is a conversion of reducible immature crosslinks to non-reducible mature

crosslinks that are thermally stable and inhibit relaxation during the isometric segment [77]. Thus, reduction treatment of collagenous tissues with sodium borohydride provides information on the extent of total crosslinking while a comparisons of HIT profiles from reduced and non-reduced tissues provide an estimate of the proportion of reducible versus non-reducible crosslinks [77].

1.2.5 Collagen crosslinking and tissue remodeling

Past studies using hydrothermal isometric testing on ovine aorta [92] and skin from humans and rats [88] suggest that during growth and maturation there exists changes in the extent of collagen crosslinking in developing collagenous tissues. Further, it has been suggested that this increase in crosslinking is largely due to a conversion of immature crosslinks into their mature form [92]. It may be hypothesized that changes in collagen crosslinking with maturation may be a remodeling response to ageing of tissues. A similar remodeling response may occur in tendons if subjected to excess activity as with over-exercise running. For example, Curwin et al. [61] have shown that when Leghorn roosters were subjected to treadmill over-exercise running their Achilles tendon collagen contained fewer (mature) pyridinoline crosslinks than did non-runners. The conclusion from this study suggested that high-intensity exercise causes greater matrix-collagen turnover in growing chickens, resulting in reduced maturation of tendon collagen. In the current study, this potential remodeling process will be further studied using hydrothermal isometric testing on rat Achilles tendons under the two experimental conditions of maturation and overuse-activity.

1.3 Achilles Tendon Biomechanics

There is very limited information in the literature to indicate the magnitude of strain that causes the human tendon to begin to fail in vivo. Literature does exist on activities that will cause catastrophic rupture. Studies have shown that Achilles ruptures most often occur in subjects who have been participating in sports [93, 94]. Arner and Lindholm [95] outlined mechanisms accounting for the rupture of the Achilles. Pushing off with the weight bearing foot with concurrent extension of the ipsilateral knee joint (i.e. classic eccentric gastrocnemius contraction) accounted for 53.3 % of the ruptures. Sudden, unexpected and violent dorsiflexion of a plantar flexed foot accounts for 17.4 % and 9.8%, respectively. None of these studies, however, provide the exact strain or stress level experienced by the tendon during rupture. Further, there are no studies that demonstrate what activities cause permanent deformation to the human Achilles tendon in vivo.

The biomechanical response of the human (and horse) Achilles tendons to tensile forces was investigated in vitro by Abrahams [96]. The stress strain-curve showed three distinct regions when a strains of 100 % /min was applied to 60 different human and horse tendons. The primary region (or the toe region) was described as the region where there are considerable increases in extension of the tendon with small increases in tension. This occurred at strain rates of 0-1.5 % and was attributed to straightening of the wavy crimp pattern of the collagen fibers that was observed when the histology of strained and unstrained tendons was examined using H & E staining. As strain increased, a second region was characterized where the collagen fibers were fully oriented to the tensile force and an increasing modulus of elasticity was reflected by a steeper portion of

the tendon stress-strain curve. This occurred at strains between 1.5 and 3 %. At strains of 3-5 %, a third region of the stress-strain curve was identified where there was a completely linear relationship between stress and strain. This region was presumed to be a result of the behavior of the collagen fibers. Physical rupture of the fibers commenced at the 5-6 % strain level when the tendons could no longer tolerate further increases in load causing the curve to become convex as sections of the tendon began to rupture. The maximum strain reached for most human tendons was 8% corresponding to a stress of 345 kg/cm^2. This would suggest that the ultimate tensile failure strain of the human Achilles tendon was about 8%.

The next set of experiments in this study [96] was designed to determine the tendon's elastic limit through cycling experiments where horse tendons were strained 10 times to 2, 3 and 4%. The results showed that if a tendon was strained beyond the 2-3 % level, permanent deformation resulted as evidenced by histological observations of collagen fiber disruption and change in the stress-strain curve. This change in the stress-strain curve was described as an increasing residual strain whereupon with the removal of the stress, the tendon did not return to it's original length. Histological observations revealed areas of the tendon that were no longer wavy or crimped. As long as the strain remained less than 4%, the process was reversible and hence the elastic limit was defined. Others have more recently provided further evidence of permanent deformation with strains beyond the 2-3 % level as a result of alterations in the collagen structure [97, 98].

Although the results of Abraham's [96] biomechanical studies on the human Achilles tendon were performed in vitro, they still provide some guidelines for how the Achilles tendon may behave in vivo. Fukashiro et al. [99] measured peak forces of 2233,

1895, and 3786 N when human subjects were jumping, squat jumping and hopping respectively, using a buckle-type transducer implanted under local anesthesia. Presumably these stresses did not exceed ultimate failure stress of the human Achilles since none of the subjects experienced a rupture of the Achilles tendon. It is equally unlikely that the ultimate failure strain (8% in vitro) was experienced either. However, one cannot be sure that the strain experienced by these tendons was greater than 3 % which may result in tendon microscopic tendon failure according to the in vitro study of Abrahams [96].

Other in vitro studies on Wallaby tendons [100] have shown that tendons may also fail by fatigue and creep [100, 101]. In the fatigue studies [100], tendons failed after repeated applications of stresses that were much lower than the ultimate tensile stress. However, these in vitro studies do not easily translate to the in vivo human scenario for it is extremely uncommon for tendons to completely fail with repeated applications of stress that are lower than the ultimate tensile stress. For example, the human Achilles tendon experiences a peak stress of about 50 megaPascals (MPa) in each running stride [102] at a stride frequency of about 1.4 Hz [103]. When this stress and frequency are compared to the fatigue tests for the Wallaby tendon [100], one would expect the Achilles tendon to fail after one hour of running. The reason for the perceived lack of failure of the Achilles with fatigue is a subject of intense study. Some authors [104, 105] would suggest that cyclic stress on the Achilles tendon during running does indeed cause failure at the microscopic level. However, no concrete evidence exists to support this.

It is generally hypothesized that during the mechanical failure of a collagenous structure the underlying collagen molecules fail by sliding of adjacent molecules past one

another rather then by breakage of the actual collagen molecules. Wang et al. [97] provides the best evidence for this collagen molecule sliding theory by using Raman microscopy to show that when rat tail tendons were strained beyond 4 % there was a shift in the relative positions of the collagen molecules. Kastelic and Baer [106] also showed evidence of this collagen molecule damage by using mechanical testing, histological methods, electron microscopy and small angle x-ray diffraction to show that as the rat tail tendon is strained, damage occurs between collagen fibrils. Others have suggested that tendons ruptured by tensile forces undergo local denaturation at the rupture site [107, 108]. This presumed denaturation of tendons from tensile forces would suggest that micro-trauma or molecular damage may results from tensile forces. Further, it is likely that even in the absence of complete rupture, activities such as overuse running may cause localized damage to the tendon in the form of localized denaturation. Thus, an analysis of the types of collagen crosslinks in tendons that were subjected to overuse exercise should demonstrate decreases in the intrahelical crosslinks that play an important role in resisting denaturation.

Given that it is possible that overuse running causes localized damage or denaturation of the tendon's collagen, then there probably exists a biological mechanism to repair the damage. Stevens and Minns [107, 108] have postulated that local denatured collagen must be enzymatically removed by phagocytic cells to allow an organized repair of the damaged collagenous tissues. These cells are most likely to be predominately fibroblasts that are capable of both phagocytosis and collagen synthetic functions essential to repair and remodeling [109]. It has been suggested that degradation of collagen by fibroblasts occurs by phagocytosis under normal remodeling conditions and

by proteinase activity using matrix metalloproteinases under rapid remodeling conditions [74, 109]. Further, phagocytosis is usually a receptor binding-dependent mechanism, and it has been shown that certain otherwise hidden recognition sequences in collagen such as arginine-glycine-aspartic acid become exposed when collagen is thermally denatured and enable binding by integrin receptors [110].

CHAPTER 2: THESIS HYPOTHESIS

The broad hypothesis of this thesis is that with overuse-running (10^{o} uphill treadmill running at a speed of 17 meters per minute for one hour, five times per week over a 12 week treatment period) there will be repeated injury to the microstructure of the Achilles tendon causing excessive damage or denaturation that outstrips the animal's ability to restore normal tissue. This should result in a repair response that is characterized by a mixture of denatured and disorganized newly synthesized collagen, increased glycosaminoglycans and a cellular infiltrate that is more characteristic of a repair response rather than an inflammatory response.

The hypothesis will be investigated with the proposed animal model that has all the necessary criteria to serve as a tool to facilitate investigation of the biology of diseased Achilles tendons and response to therapeutic interventions [15, 16]. This model will be used to provide Achilles tendon tissue samples that can be compared to control tissue samples to characterize differences in their biology, biochemistry and biomechanics.

Thus, the objective of this thesis is to gain a better understanding of Achilles tendon disease, structure and function through the study of the following specific hypotheses:

1. An over-exercise uphill treadmill running regime imposed on rats will serve as an animal model that will produce Achilles tendons exhibiting pathological changes consistent with those most commonly observed

in the literature for human and animal diseased Achilles tendons (Appendix 1 and 2). Specifically:

a. Achilles tendons harvested from rats subjected to overuse uphill treadmill running will have increased cell numbers when compared to Achilles tendons harvested from control rats subjected to normal cage activity.
b. Achilles tendons harvested from rats subjected to overuse uphill treadmill running will exhibit more disorganized collagen architecture when compared to Achilles tendons harvested from control rats subjected to normal cage activity.
c. Achilles tendons harvested from rats subjected to overuse uphill treadmill running will stain more intensely with H & E staining methods when compared to Achilles tendons harvested from control rats subjected to normal cage activity.

2. An over-exercise uphill treadmill running regime imposed on rats or changes in age will cause changes in collagen crosslinking. Specifically:

a. Achilles tendons harvested from rats subjected to overuse uphill treadmill running or from rats of younger ages will contain less intramolecular and intermolecular collagen crosslinking in their Achilles tendons.

b. Achilles tendons harvested from rats subjected to overuse uphill treadmill running or from rats of younger ages will contain less stable non-reducible collagen crosslinking in their Achilles Tendons.

3. An over-exercise uphill treadmill-running regime imposed on rats will induce biochemical changes in the Achilles tendon. Specifically:
   a. Achilles tendons harvested from rats subjected to overuse uphill treadmill running will contain more collagen than Achilles tendons harvested from control rats subjected to normal cage activity.
   b. Achilles tendons harvested from rats subjected to overuse uphill treadmill running will contain more glycosaminoglycans than Achilles tendons harvested from control rats subjected to normal cage activity.
4. An over-exercise uphill treadmill-running regime imposed on rats will induce biomechanical changes in the Achilles tendon. Specifically:
   a. Achilles tendons harvested from rats subjected to overuse uphill treadmill running will have decreased mechanical strength.

CHAPTER 3: PAPER 1: HISTOLGICAL ANALYSIS OF ACHILLES TENDONS IN AN OVERUSE ANIMAL MODEL

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3.1 ABSTRACT

Non-insertional Achilles tendon disease has a variable clinical presentation that ranges from intermittent pain and stiffness to catastrophic rupture. The timing and extent of inflammatory response within the tendon are poorly understood, as are the etiological factors. Overuse activities such as excessive running have been implicated. In this study, an overuse uphill running animal model has been established using a custom designed rodent treadmill. Subsequent histology revealed histopathological alterations in the rat Achilles tendon that are consistent with those described in the literature from animal and human tendon tissues. These features include: decreased collagen fiber organization, more intense collagen staining and increased cell nuclei numbers. Further, subsequent immunohistochemical cell typing suggests that the increased cellularity does not include a significant inflammatory component but is secondary to increased numbers of endothelial cells (i.e. vascularization) and fibroblasts. These histological features likely represent a biological response to repair or remodel the damaged or denatured collagen that resulted from overuse running.

3.2 INTRODUCTION

Disorders of the Achilles tendon cause pain, disability and may predispose to catastrophic rupture. These disorders represent a small portion of all tendon disorders but are cited to be increasing in frequency [4]. The etiology of Achilles tendon disease is multifactorial and may involve overuse running [32], biomechanical factors [33, 35], age-

related changes in the tendon [26], pre-existing collagen disorders [27], exercise-induced hyperthermia [29, 111], or side-effects of pharmacological agents [31, 112].

It is important to have a clear understanding of the location and biology of the underlying pathology when considering treatment recommendations for Achilles tendon disease. Puddu et al. [1] have provided a clinical classification system for Achilles tendon disease which suggests that the underlying pathology may represent a spectrum of diseases that may or may not involve an inflammatory component

t. This is consistent with the animal work of Backman et al. [8] who documented a degenerative process occurring in the Achilles tendon of rabbits subjected to overuse activity with a co-existing inflammation of the paratenon. A recent study by Cetti et al. [13] has suggested that Achilles tendon disease precedes catastrophic rupture of the human Achilles tendon. The pre-existing disease was characterized after biopsy of 60 human subjects who suffered a ruptured Achilles tendon. The disease was cited to be consistent with a widespread ipsilateral acute inflammation and was suggested to result from an underlying inflammatory process evidenced by infiltrate of neutrophils. Prior to this study Achilles tendon disease has been more consistently cited to involve tendon degeneration [3, 9-12]. Thus it is not entirely clear as to whether Achilles tendon disease is inflammatory and/or degenerative based.

The normal Achilles tendon has been well described with respect to it's histological features [41, 42]. These features include organized, tightly packed parallel collagen bundles that stain pink-red (eosinophilic) with H & E staining (e.g. Figure 3.1a and 3.2a). Sparse elongated cell nuclei are seen interspersed between the collagen bundles.

The histopathology of the diseased human Achilles tendon has been previously described on the basis of biopsies from the Achilles tendons of patients with subcutaneous rupture [3, 9-13] and chronic, localized Achilles tendon symptoms [10, 43, 44]. These studies demonstrate many consistent histopathological features suggestive of a common pathological process that may represent a continuum, with Achilles rupture as the end-stage. The histopathological features include: disorganized collagen arrangement, increased number of cells, rounding of cell nuclei, variable collagen staining and increased glycosaminoglycan content.

An animal model for the study of tendon pathology is a useful tool to investigate tendon biology and response to therapeutic interventions [15]. Experimental tendonopathies have been induced by partial tendon laceration [24], needle puncture [113] or injection [25]. Others have attempted more physiological approaches based upon suspected overuse etiologies [8, 20, 21].

The animal model for tendon disease in the rat shoulder developed by Soslowsky et al. [22] has demonstrated validity when compared to the human literature and supports overuse activity as an etiologic factor in the development of tendinopathy. This running model is more physiological and practical than the more complex rabbit and rat models for Achilles tendon disease developed by Backman et al. [8] and Messner et al. [20] respectively. The problems with the models of Backman et al. [8] and Messner et al. [20] stem from the complexity of the "kicking" exercise regime. These models demand that animals sustain a general anesthetic daily with electrode-induced co-stimulation of the lower leg muscles to simulate overuse activity. This makes the model impractical and more costly.

The purpose of the present study was to establish a Rat Achilles Pathology Exercise Device (Rat-A-PED) model that can be used to gain a better understanding of Achilles tendon disease. This animal model is similar to the animal model for shoulder disease by Soslowsky [21, 22]. Achilles tendon samples taken from the rats subjected to the Rat-A-PED model have been compared to Achilles tendons taken from rats that were subjected only cage activity using H & E staining and immunohistochemical techniques. The goal was to validate the Rat-A-PED model's ability to produce Achilles tendons with pathology similar to that observed in biopsies of Achilles tendons from: (1) humans who ruptured their Achilles tendons [3, 9-13], (2) humans with chronic, localized Achilles tendon symptoms [10, 43, 44] and (3) animals subjected to overuse exercise models [8, 20, 22, 49].

3.3 METHODS

3.3.1 Rat tendinosis regime

The Rat-A-PED model was developed, using methods that parallel those of Soslowsky [21] by scaling down to a rat-size version of a marathon runner's training regime previously shown to increase the incidence of Achilles tendon disease [32]. All experiments were performed with the approval of the Dalhousie University Committee on Laboratory Animal Care and in accordance with the Canadian Council on Animal Care guidelines.

Ten Sprague Dawley rats (61-63 days old and 300-325 grams at the beginning of the running regime) were subjected to 10^{o} uphill treadmill running by confinement on a rolling belt treadmill (Letica Scientific Instruments, Barcelona, Spain) (Figure 3.3).

Incline running was chosen since it requires eccentric muscle contractions that result in a greater risk for tendon injury [39, 40]. A grid at the trailing end of the treadmill administered a shock that encouraged the animals to run in avoidance. The frequency of shocking was monitored to assure humane treatment. The running regime consisted of a 12 week treatment period at a speed of 17 meters per minute for one hour, five times per week. Rats were acclimated to treadmill running over a four-day period prior to the start of the regime, increasing the running time by 15 minutes per day. When not running, the rats were allowed to freely weight-bear with normal cage activity and were fed rat chow (LAB DIET, Brentwood, MO, USA) with additional pieces of sweet cereal (Fruit Loops, Kelloggs, London, Ontario) after each running period as a treat. During the exercise period, the animals were monitored for limping, problems weight-bearing, or other signs of pain under advice of a veterinarian. Weight gain was monitored to assure good systemic health.

3.3.2 Tendon harvest and cross-sectional area calculation

After the completion of the 12-week overuse running protocol, the rats were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. The gastrocnemius and soleus muscles were dissected free of all soft tissues including the plantaris muscle tendon unit if it existed. The two merging tendons were isolated by amputation: proximally at the distal end of the gastrocnemius soleus muscle belly, and distally at the calcaneus insertion. Tendon cross-sectional area was determined by using Mitutoyo Digimatic calipers (Model#CD-6"BS, Mitutoyo Corp. Tokyo, Japan) to take two measurements for each of (i) the coronal medial to lateral width and (ii) the sagital

anterior to posterior thickness. To calculate a value for the cross-sectional area, an assumption of elliptical shape was made and ½ of the mean coronal width was then multiplied by ½ of the sagital thickness and then by π (3.141). The tendon samples taken from the right hind limb were then fixed in 10 % formalin and used for histology. The samples taken from the left hind limb were used for mechanical testing (See Chapter 5).

3.3.3 Histology

The tendon samples (10 tendons harvested from rats subjected to the RAT-A-Ped model and 10 tendons from control, non-running rats) previously fixed in 10 % formalin were processed, embedded in paraffin and then sectioned at 4 μm for hematoxylin-eosin staining. In order to archive the entire tendon specimen, the shape and size of all tendon sections were viewed under a microscope (Nikon Eclipse E600) using the 1X objective and a digital image was captured using NIH Image-J software (Scion Corp. Frederick, MD). Digital images of three distinct Fields of View (FsOV) were then captured using the 10 X objective. The 3 FsOV were captured from the central portion of the width and length of the tendon. The positions of the FsOV were determined in a consistent fashion by first positioning the center of FOV2 at the center of the tendon specimen. Next, FOV 1 and 3 were placed directly proximal and distal to FOV 2 along the longitudinal axis (Figure 3.4).

In total, 60 FOV images were captured from the tendon sections of the right lower limbs of 20 rats. Three FsOV were captured from each of the 20 tendons harvested. The images were then printed on Kodak Premium Picture Paper ($8^1/_2$ x 11 inch) using a Hewlett-Packard color photographic quality inkjet printer (Model # 970cse). These FOV

photographs were then assessed for three of the most consistent and characteristic histological features of human and animal tendon: nuclear numbers, collagen fiber organization and collagen staining intensity.

3.3.4 Nuclear numbers

Two observers (MG and KS) quantified nuclear numbers per FOV in a blinded randomized fashion by manually numbering each individual nucleus that stained intense purple on all FsOV. The Mean Number of Nuclei per FOV (MNNFOV) was calculated for each tendon from the mean number of nuclei in each of the 3 FsOV. Inter- and intra-observer comparisons were then made between the two observers. Finally the observers' 10 MNNFsOV were averaged to provide an overall MNNFOV for the 30 FsOV. The overall MNNFOVs were compared between the tendons harvested from rats subjected to the Rat-A-Ped model and non-running rats. Statistical comparisons were made using the paired Student's t-Test for two samples for means (Microsoft Excel Software). Data are presented as the mean ± one standard deviation.

3.3.5 Collagen organization

A semi-quantitative grading scale was designed to allow a comparison of the collagen organization of Achilles tendons harvested from either rats subjected to the Rat-A-Ped treatment or non-running rats. Each FOV was graded in a blinded and randomized fashion by two observers (JML and JRW). Each FOV was again graded by assigning a value from grade 1 to 5 for collagen organization. Grade 1 was used to designate poorly organized collagen (Figure 3.1b) and Grade 5 to designate highly organized collagen

(Figure 3.1a). Reference images (Figure 3.1) that demonstrated poorly and highly organized collagen were provided to the observers for reference during grading.

3.3.6 Collagen staining intensity

A similar semi-quantitative grading scale was designed to allow comparison of the collagen staining intensity of Achilles tendons harvested from either rats subjected to the Rat-A-Ped treatment or non-running rats. Each FOV was graded in a blinded and randomized fashion by two observers (JML and JRW). Each FOV was graded by assigning a value from grade 1 to 5 for collagen staining. Grade 1 was used to designate poorly stained collagen (Figure 3.2a) and Grade 5 to designate highly stained collagen (Figure 3.2b). Reference images (Figure 3.2) that demonstrated poorly and highly stained collagen were provided to the observers for reference during grading.

3.3.7 Immunohistochemistry

Paraffin sections were cut at 4-5 μm and mounted on in-house-prepared glass slides, double coated with poly-l-lysine and 4% 3-aminopropyltriethoxysilane (both from Sigma) and dried at 60°C for at least 1 hr in an attempt to promote specimen adherence [114]. Sections were then deparaffinized and rehydrated, cooked for 30 min in 90°C 0.01 M citrate buffer, washed in water, treated for 30 minutes with 3% hydrogen peroxide in methanol to inactivate endogenous peroxidase, rinsed in PBS, incubated with normal horse serum (diluted 1:20 in PBS) and then incubated overnight with either mouse anti-rat CD45 (Leukocyte common antigen: BD Biosciences Pharmingen # 550566) or mouse anti-vimentin monoclonal antibody (DAKO, Clone V9 # M0725, Lot 057) at 4 °C. Next,

sections were washed with PBS, incubated with biotinylated horse anti-mouse IgG (1:200 in PBS) for 30 minutes at room temperature, washed with PBS, incubated with ABC elite working solution for 30 minutes, washed with PBS, developed for 2 minutes in 3,3-diaminobenzidine (DAB, Sigma), washed in water, and then counterstained with Mayers hematoxylin. Rat t lymph node tissue was used as a positive control.

Anti-CD45 (purified mouse anti-rat CD45 Leukocyte common antibody: BD Biosciences Pharmingen # 550566) reacts with CD45 (leukocyte common antigen) on all mature hematopoietic cells except erythrocytes. This will allow identification of leukocytes to assess the presence of inflammation. Anti-vimentin, which reacts with both endothelial cells and fibroblasts, by binding to vimentin (a 57 kDa intermediate filament found on mesenchymal cells) was used to assess neovascularization and fibroblast proliferation: i.e., evidence of wound healing. Attempts to stain sections with a stain specific for rat endothelial cells (anti-rat endothelium monoclonal antibody: CL043A, Cedarlane Laboratories) proved unsuccessful as the staining in positive controls was unconvincing.

3.3.8 Statistics

Statistical comparisons of various parameters on the Achilles tendons from non-running and running rats were made using the paired Student's t-Test for two sample means (Microsoft Excel Software). Data are presented as the mean ± one standard deviation.

3.4 RESULTS

3.4.1 Rat tendinosis regime

The Rat-A-PED model proved to be practical, safe and humane. Animal facility staff noted that these rats exhibited neither hostility nor aggression. More objectively, all rats consistently gained weight on a weekly basis during the 12-week exercise period (Figure 3.5). Rats that were subjected to normal cage activity and unlimited diet (mean ±S.D. 280 ± 52g) gained more weight (p= 0.00021) than those that were subjected to the RAT-A-PED model (190 ± 36g) over the 12-week period.

The shock record (Figure 3.6) indicates that the rats experienced a mean shock rate of 1-4 shocks per minute. Most of these shocks were delivered in the first 5-10 minutes for each run with the latter portion of the run resulting in fewer shocks being delivered.

3.4.2 Gross appearance

The mean cross-sectional area of the Achilles tendons harvested from running rats (5.6 ± 1.2 mm^2, n=18) was not significantly different (p= 0.4) from those of non-running rats (6.7 ± 1.6 mm^2, n=19). Further, the Achilles tendons had a similar gross appearance.

3.4.3 Histology

Achilles tendons sections stained with hematoxylin-eosin after harvest from runner rats displayed histological features that were different from Achilles tendons harvested from non-runner (Figures 3.1b & 3.2b and Figures 3.1a & 3.2a respectively).

As shown in figures 3.1a and 3.2a, the Achilles tendons from non-runners were characterized by very highly organized and lightly eosinophilic stained collagen fibers arranged in parallel with occasional elongated basophilic nuclei interspersed between the collagen fibers. Figures 3.1b and 3.2b are examples of Achilles tendons from runners that display a good contrast to those histological features observed in the non-runners. Specifically, the histological features included more disorganized and more intensely stained eosinophilic collagen fibers with numerous, more rounded basophilic nuclei aligned in rows interspersed between the collagen fibers. A more objective analysis of the differences in the nuclear numbers, collagen organization and staining between the runners and non-runners follows.

3.4.4 Nuclear numbers

The mean number of nuclei per FOV in the 30 FsOV calculated by Observer One for running rats (520 ± 79) was greater ($p= 0.01$) than that for non-running rats (391.9 ± 30.5). The mean number of nuclei in the 30 FsOV calculated by Observer Two for running rats (700 ± 120) was also greater ($p= 0.01$) then that for non-running rats (450 ± 79). Inter-observer analysis using a one variable T-test showed a difference ($p< 0.05$) in the absolute values (33 %) of the number of nuclei counted for each FOV by observers however; each observer reached the same conclusion. Finally, each observer's MNNFOVs were averaged to provide comparisons of overall MNNFOV (Table 3.1). The overall MNNFOV for running rats (610 ± 97) was also greater ($p= 0.01$) then that for non-running rats (420 ± 52).

3.4.5 Collagen organization

The mean semi-quantitative grading score assigned by Observer One for Achilles tendons collagen organization of runners (2.9 ± 0.9) was less (p= 0.02) then that for non-runners (3.7 ± 0.6). The mean semi-quantitative grading score assigned by Observer Two for runners (2.8 ± 0.9) was less (p= 0.01) than that for non-runners (3.7 ± 0.7). Inter observer analysis using a one variable T-test showed no difference (p> 0.05) in the semi-quantitative grading score for collagen organization in Achilles tendons (3.5% and no difference for running and non running rats respectively). Finally, each observer's semi-quantitative grading score for collagen organization was averaged to provide comparisons of overall mean semi-quantitative grading score for collagen organization (Table 3.1). The overall mean semi-quantitative grading score for collagen organization for runners (2.83 ± 0.92) was significantly less (p= 0.01) than that for non-runners (3.7 ± .6).

3.4.6 Collagen staining intensity

The semi-quantitative grading score assigned by Observer One for Achilles tendons collagen staining intensity of runners (3.5 ± 0.7) was greater (p= 0.01) than that for non-runners (2.0 ± 0.4). The semi-quantitative grading score assigned by Observer Two for the collagen staining intensity for runners (3.9 ± 0.7) was also greater (p= 0.01) than that for non-runners (2.0 ± 0.4). Inter observer analysis using a one variable T-test showed no difference (p> 0.05) in the semi-quantitative grading score for collagen organization in Achilles tendons (11 % and no difference for running and non running rats respectively).

Finally, each observer's semi-quantitative grading score for collagen staining intensity was averaged to provide comparisons of overall mean semi-quantitative grading score for collagen staining intensity (Table 3.1). The overall mean semi-quantitative grading score for collagen staining intensity for runners (3.7 ± 0.7) was less ($p= 0.01$) than that for non-runners (2.0 ± 0.4).

3.4.7 Immunohistochemistry

Immunohistochemical staining of Achilles tendon sections from running and non-running rats proved difficult as there was a strong tendency for the sections to dislodge from the slides during the heating (antigen-retrieval) process. Double coating the slides improved the results but sections usually had tears and folds. Nevertheless, slides were easily interpretable, but very difficult to photograph. There were only very rare leukocytes present with the cytoplasm appearing brown from anti-CD45 labeling (similar to brown-stained leukocytes in rat lymph node positive control not shown) however the hematoxylin counter-stain turned the nuclei blue-purple in Achilles tendons sections from both the running (Figure 3.7a) and non-running rats (Figure 3.7b). This means the increased numbers of nuclei are not evidence of an inflammatory response.

Anti-vimentin monoclonal antibody staining of the same Achilles tendons sections from non-running rats revealed heavy staining of the cells (Figure 3.8b). When attempting to stain running rat tendons with anti-vimentin monoclonal antibody there was generalized disorganized structural architecture of the tendons (Figure 3.8a), presumably secondary to technical difficulties with the staining techniques due to the highly disorganized collagen matrix and difficulty with specimen adherence to glass slides. Higher magnification of intact areas of these runner tendons revealed a heavy stain of the

cells membrane (Figure 3.8a) similar to the non runners (Figure 3.8b). This suggests that the cells present in these tendon tissues are endothelial cells or fibroblasts. Based upon H & E staining, there appears to be both vascularization and increased numbers of fibroblasts. These changes are suggestive of a healing response.

3.5 DISCUSSION

In the current study we have established an animal model for Achilles tendon disease using methods modified from these of Soslowsky et al. [22] for shoulder tendon disease. Our new model meets the key criteria for a useful animal model including ease of application, appropriate tissue type modeled, and accurate simulation of the injury conditions [15, 16].

It is important to note that the current and related studies (Paper II and III) utilized different methodologies than those of Soslowsky [22]. Specifically, we used uphill rather than downhill running. The principal reason for choosing uphill running is that the gastrocnemius-soleus musculotendinous complex is in a lengthened state. Thus, with concurrent contraction (plantarflexion) during push-off, the appropriate biomechanical conditions for an eccentric contraction exist that have been shown to exert greater forces [115]. Therefore, there is an increased likelihood of damage to the musculotendinous structure [116, 117].

Very recently, Huang et al.[1] [118] attempted to develop an animal model of Achilles tendon overuse injury using Soslowsky's shoulder injury protocol [22], but their

[1] The current study was completed prior to publication of this paper and we were unaware of this parallel but somewhat similar work.

results suggested that the cross-sectional area and biomechanical properties of Achilles tendons from running and non-running rats were not significantly different. These findings are consistent with the current and another related study that showed no differences in the cross-sectional area and biomechanical properties respectively. However, the current study has demonstrated histological differences in the Achilles tendons of running rats while the related studies demonstrated distinct differences in biochemical properties of Achilles tendons from running rats as well.

In this study, we report on an animal model that produces rat Achilles tendons with histopathological features that are consistent with those described in the literature from three relevant sources: 1. ruptured human Achilles tendons [3, 42, 50], 2. diseased human Achilles tendons [43, 44], and 3. tendons from animals subjected to over-use exercise models [8, 20, 22, 49]. Even though these histopathological changes indicate that the Achilles tendons are different when compared to controls it must be stressed that the only etiological factor that is simulated in this animal model is overuse-running. Thus, all results should be discussed in terms of the effects of overuse-running rather than Achilles tendon disease per se.

The key histopathological features that have been reproduced include Achilles tendons with increased nuclear numbers, more intensely eosinophilic staining of collagen fibers, and collagen fibers that are disorganized. These features are most likely a result of repetitive damage that is sustained by the Achilles tendon with overuse-running [32-34, 45-48].

In order to determine why these histopathological features exist one must first consider the effects of over-use running on the Achilles tendon. During overuse-running the collagen fibers of the Achilles tendon experience stress as a result of the tensile forces placed on it. This stress may cause strain that may be in excess of ~ 4 % and there exists a possibility of irreversible damage to the tendon [38, 119] that may include local denaturation at the site of damage [107, 108]. Thus, it may be possible that in the absence of complete ruptures overuse running may still cause localized damage to the tendon in the form of localized denaturation.

This is consistent with the results of the current study showing that Achilles tendons from running rats are characterized by intensely eosinophilic collagen fibers. In this context it is interesting to note that denatured collagen exhibits increased binding of eosin [120]. This is most likely a result of the nature of eosin stain that binds more readily to acidophilic substrates and thus the denatured collagen may expose more binding sites for eosin. This theory of localized denaturation as a result of overuse running is further supported by a study of the collagen crosslinking in the Achilles tendons of rats subjected to overuse running (Paper II, Chapter 4). In this study, hydrothermal isometric testing was used to determine that overuse running causes a decrease in the denaturation temperature of the rat Achilles tendon. This suggests that overuse running will produce tendons with decreased collagen stability and therefore a decreased resistance to denaturation.

Given that it is likely that overuse running causes localized damage or denaturation of the tendon collagen then there is likely a biological mechanism that exists to repair the damage. Steven and Minns [107, 108] have postulated that local

denaturation must be enzymatically removed by phagocytic cells to allow an organized repair of the damaged collagenous tissues. The current study demonstrates an increase in cell numbers in the tendons of running rats. However, staining for leukocyte common antigen does not show evidence of an inflammatory response. These cells are vimintin-positive and thus likely to be a combination of: (i) fibroblasts that are capable of both phagocytosis and collagen synthetic functions and (ii) endothelial cells (neovascularization) essential to repair and remodeling [109].

Finally, it is suggested that with overuse running there will be repeated injury causing excessive damage or denaturation that outstrips the animal's ability to restore normal tissue. This will result in a repair response that is characterized by a mixture of denatured and disorganized newly synthesized collagen, which is characteristic of the current studies findings demonstrating increased collagen disorganization in the Achilles tendons from running rats.

3.6 CONCLUSION

In conclusion, we have established an animal model for overuse uphill running activity that produces Achilles tendons with histological features similar to those observed in human Achilles tendon disease. These histological features appear to reflect a biological response where fibroblasts and endothelial cells exist in increased numbers to repair or remodel the damaged or denatured collagen that resulted from the tensile forces of overuse-running. Further, there is no significant inflammatory cell infiltrate noted in the Achilles tendons after the 12 week overuse running regime suggesting that inflammation is not a predominate process for this regime.

Thus, it maybe best to reserve the terms such as tendonitis, paratendonitis and tendinosis for use when inflammation has been confirmed and use the more general term tendinopathy otherwise [6, 7].

	Runners (n=10)	Non-runners (n=10)	p-value
Mean Number of Nuclei per Field of View (MNNPFOV)	614 ± 97[a]	421 ± 52	0.000016
Mean Semi-quantitative Grading Score for Collagen Organization	2.8 ± 0.9[a]	3.7 ± 0.6	0.01
Mean Semi-quantitative Grading Score for Collagen Staining intensity	3.7 ± 0.7[a]	2.0 ± 0.4	0.0000094

[a] Indicates a statistical difference from non-runners with p-Value <0.05

Table 3.1 Analysis of the differences (mean from two observers) in the nuclear numbers, collagen organization and collagen staining intensity between the Achilles tendons of running and non-running rats following the overuse running regime.

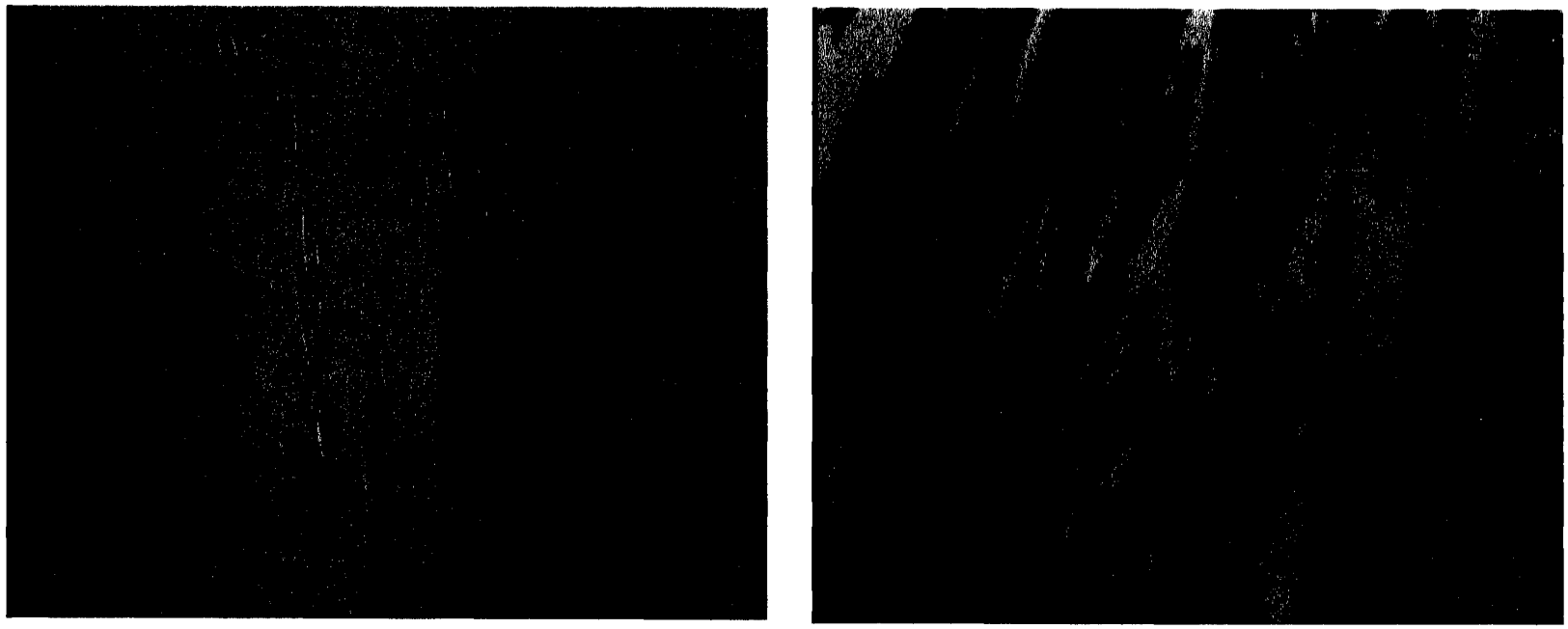

Figure 3.1. Field of view (FOV) coronal section of : (a) an Achilles tendon from a non-runner rat showing very highly organized collagen fibers arranged in parallel, and (b) an Achilles tendon from a running rat showing more disorganized collagen fibers (100x original magnification).

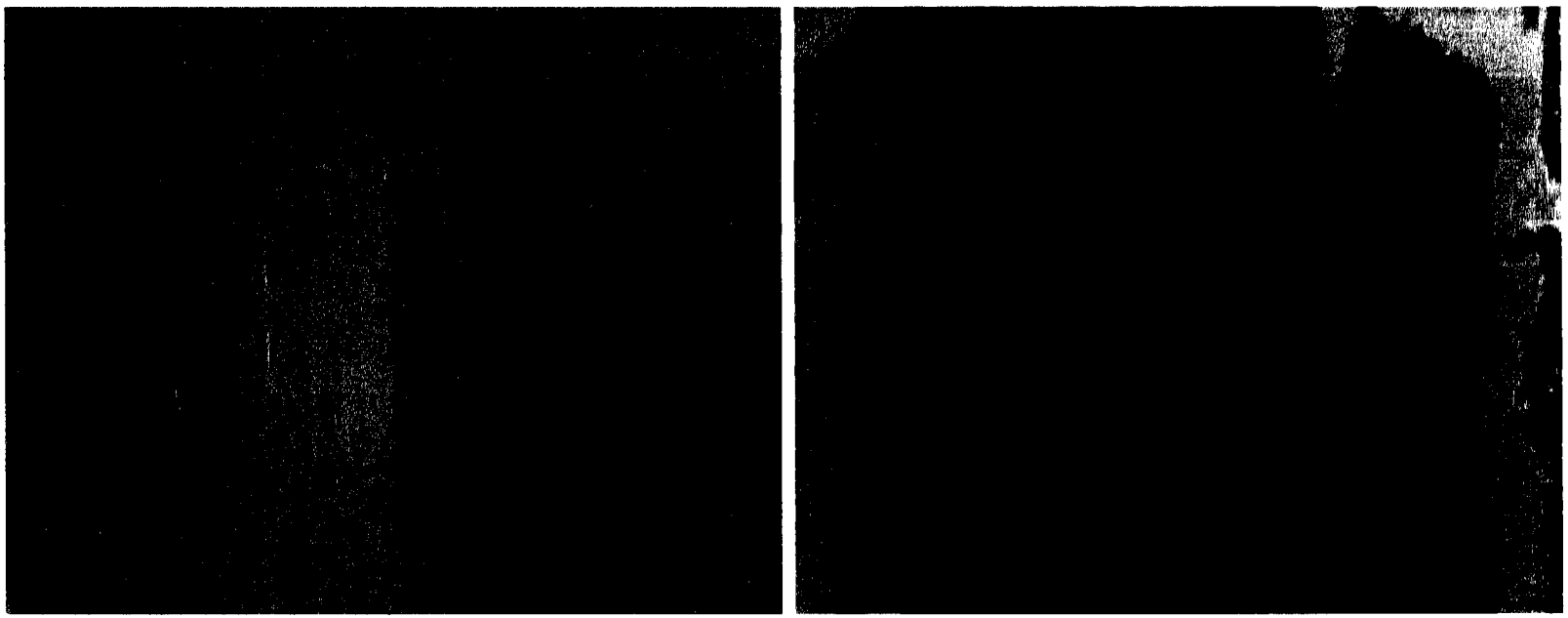

Figure 3.2. Field of view (FOV) coronal section of : (a) an Achilles tendons from a non-runner rat showing lightly eosinophilic stained collagen fibers (b) an Achilles tendon from a running rat showing more intensely eosinophilic stained collagen fibers (100x original magnification).

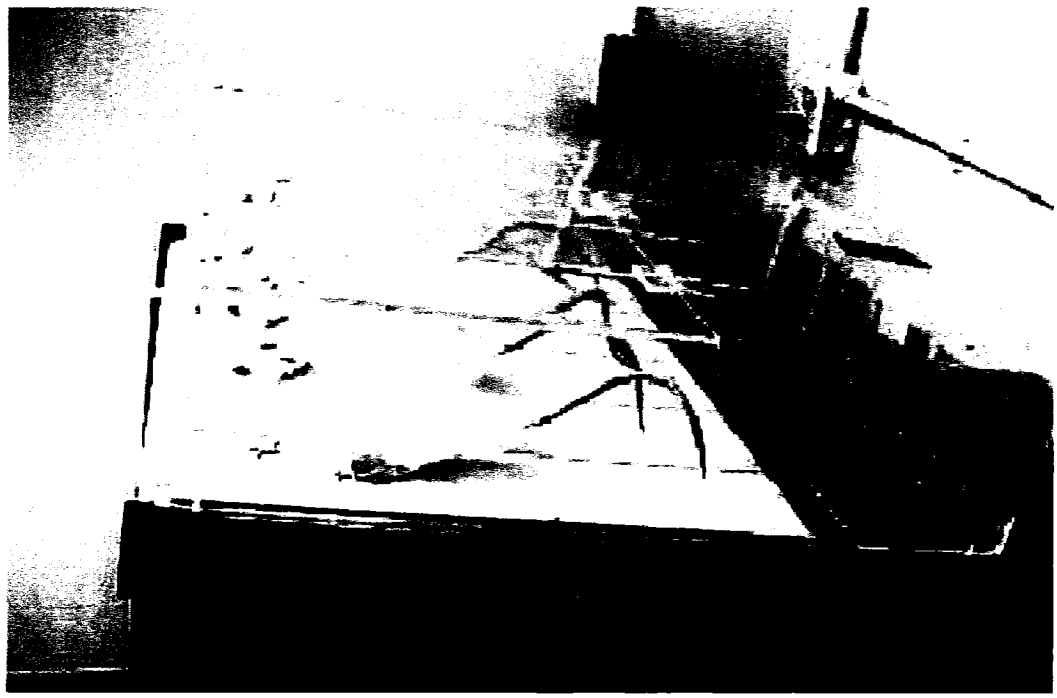

Figure.3.3. Sprague Dawley rats running on treadmill built for rodent over-exercise running. Note plexiglass barriers separating the rats running lanes and the shock grid behind the rats.

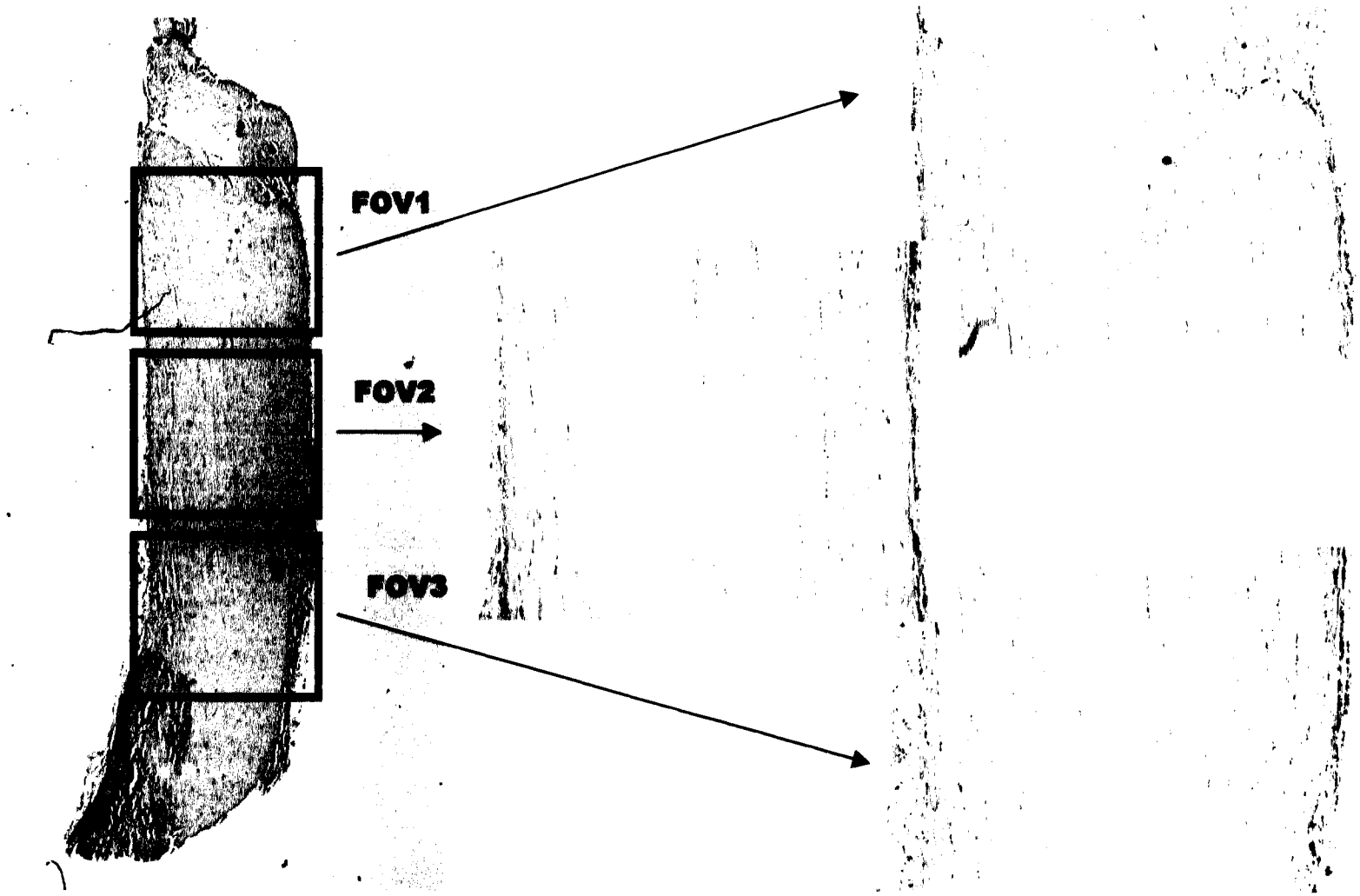

Figure 3.4. Coronal section of a whole rat Achilles tendon sample (left 40X original magnification) and three individual Field of Views 1-3 (right 100X original magnification).

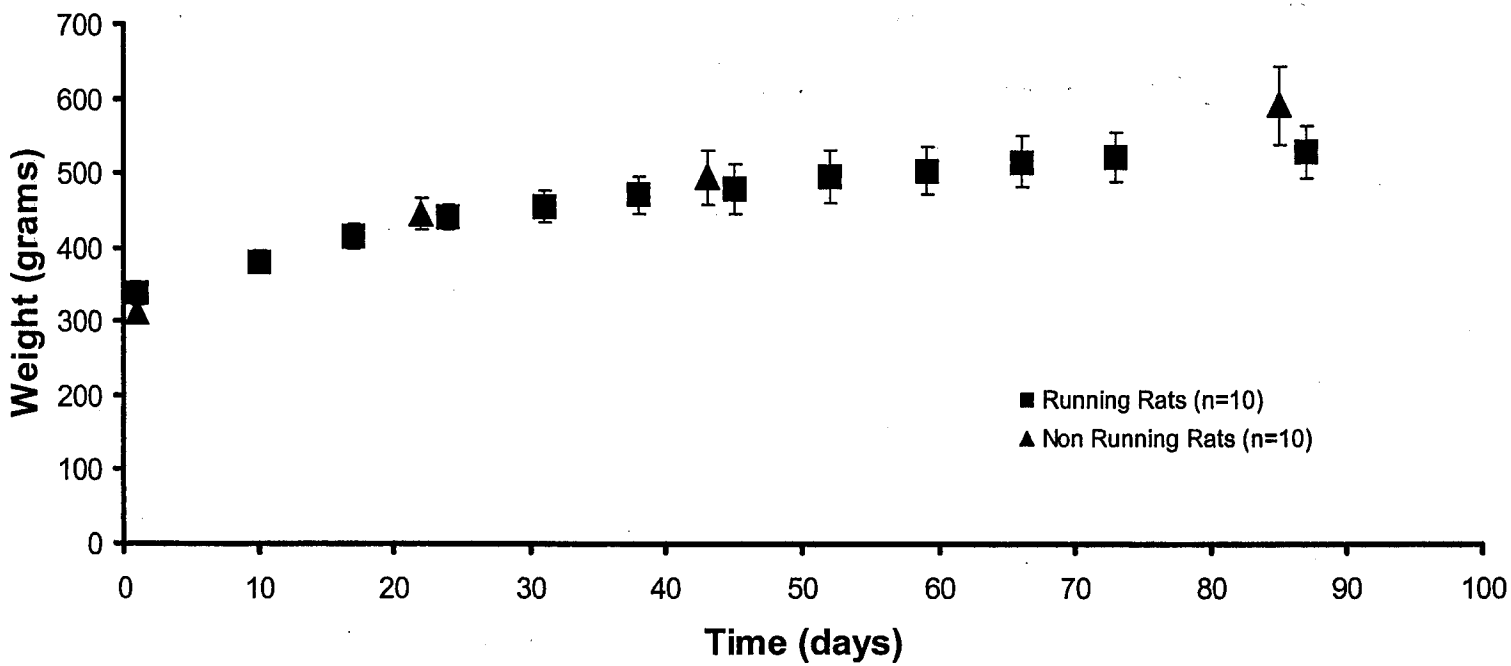

Figure 3.5. Weight gain (grams) of rats over a 12 week period.

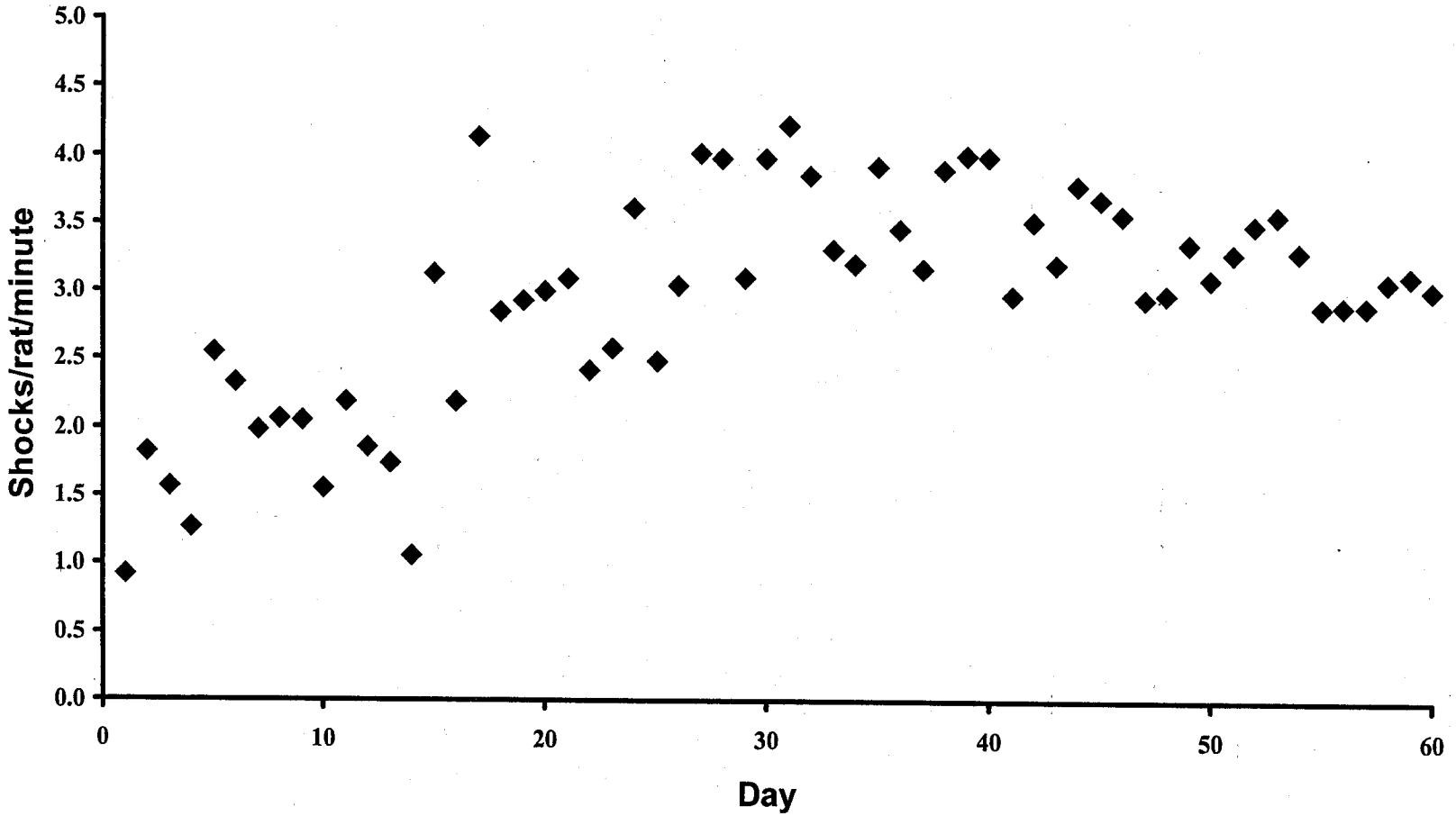

Figure 3.6. Shock record of rats subjected to 60 days of an overuse running regime.

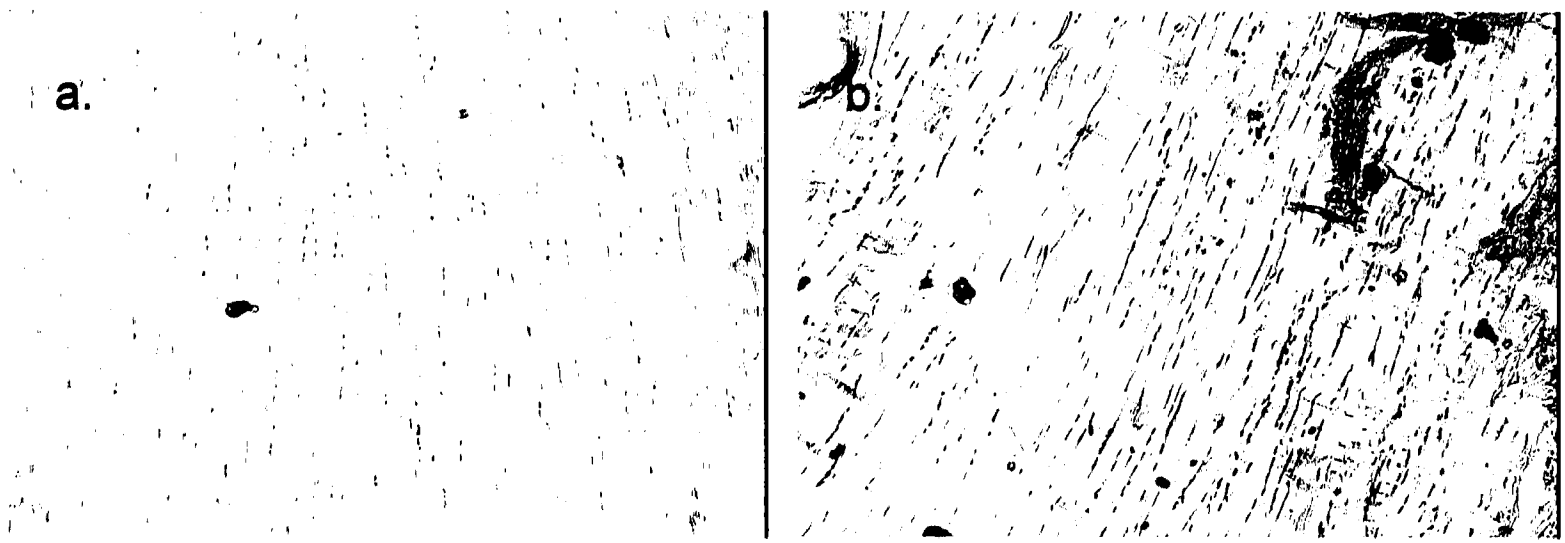

Figure 3.7. Achilles tendon sections incubated with CD45 Leukocyte common antigen showing little or no staining of Leukocytes taken from: (a) non-running and (b) running rats (100 X original magnification). The counter-staining was hematoxylin (blue nuclei) and antibody labeled cells should appear brown.

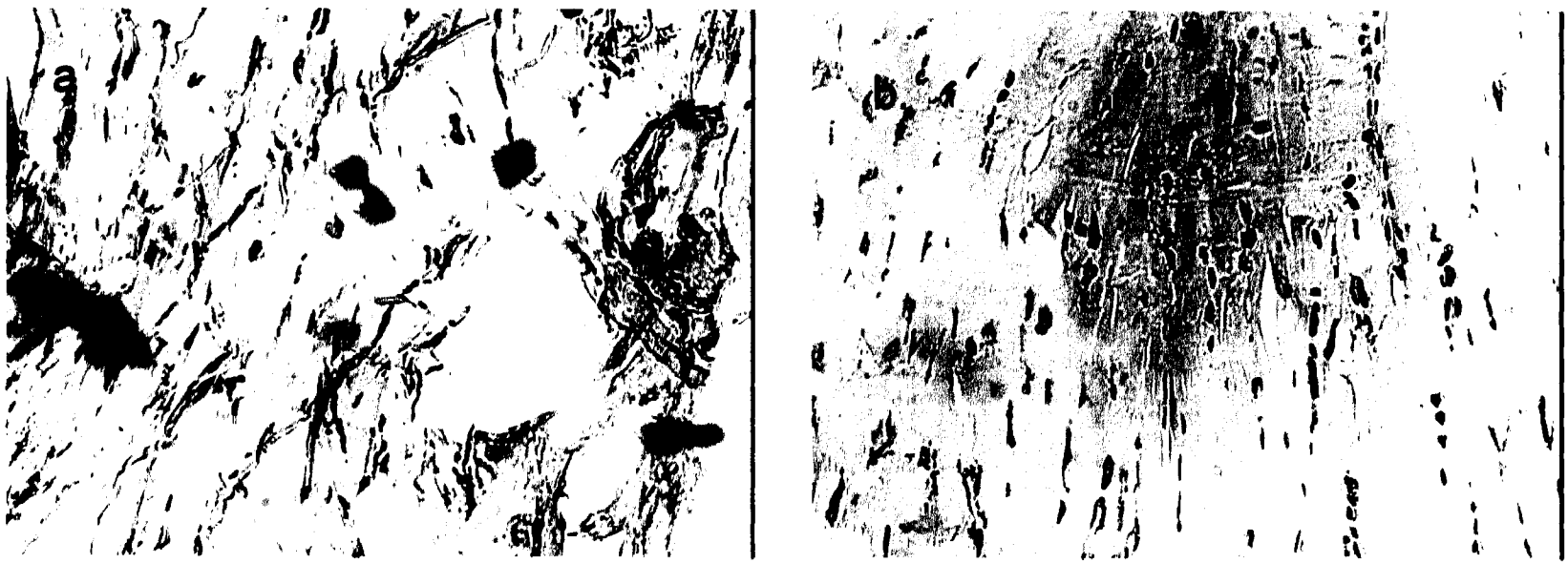

Figure 3.8. (a) Achilles tendon sections taken from running rats and incubated with anti-vimentin monoclonal antibody showing generalized disorganized collagen and heavy staining of cells. (b) Achilles tendons sections taken from non-running rats and incubated with anti-vimentin monoclonal showing less disorganized collagen and heavy staining of cells. (200x original magnification). The counter-staining was hematoxylin (blue nuclei) and antibody labeled cells should appear brown.

CHAPTER 4: PAPER II: AGE AND OVERUSE RUNNING EFFECTS ON COLLAGEN CROSSLINKING PATTERNS IN ACHILLES TENDONS USING HYDROTHERMAL TESTING

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4.1 Abstract

Hydrothermal Isometric Tension (HIT) testing was used to determine semi-quantitative differences in the collagen crosslinking of Achilles tendons from rats of varying age and activity levels. The HIT testing allows determination of the denaturation temperature (T_d) and the half-life of stress relaxation associated with peptide bond hydrolysis ($t_{1/2}$). These parameters are reflective of the total intramolecular (intrahelical) and the mature intermolecular collagen crosslinks, respectively. Achilles tendons from rats of various stages of maturity (3, 8, and 20 weeks) living under normal cage activity (non-runners) were compared. A second group of 20-week-old rats that were subjected to a 12-week overuse running regime (runners) were also compared to the non-running 20-week-old rats. An identical series of HIT experiments was then conducted after matched tendons harvested from the contralateral limb of the rat were subjected to reduction with sodium borohydride to determine the extent of reducible versus non-reducible crosslinks. The findings of this study suggest that the rat Achilles tendon undergoes alterations in its collagen crosslinking during growth and maturation that are similar to changes observed when an over-exercise running regime is imposed. It is suggested that the changes in the crosslinking observed after over-exercise may be the result of a remodeling process.

4.2 Introduction

Disorders of tendons cause pain, disability and may predispose to catastrophic rupture. Achilles tendon disorders represent a small portion of all tendon disorders and are cited to be increasing in frequency [4, 5, 121]. The etiology of Achilles tendon disease is multifactorial and may involve overuse running [32-34, 45-48]. An overuse exercise animal model for the study of tendon pathology is a useful tool to investigate tendon biology and response to therapeutic interventions [8, 15, 25, 49, 122, 123]. Past studies [8, 20, 22, 49] have used such models to document tendon histopathological findings similar to biopsies from ruptured human Achilles [1, 3, 41, 42, 50, 95, 124] and diseased human Achilles tendons [8, 36, 43, 44, 125]. These histopathological features include: disorganized collagen arrangement, rounding and increases number of cells, varied collagen staining and increased glycosaminoglycan content [1, 3, 41-44, 50, 95, 124].

Many hypothesize that the disorganized collagen and increased cell numbers represent a biological response to structural damage or micro trauma that must be repaired to maintain function [61, 79, 88, 92, 126-129]. This most likely occurs by a process similar to tissue remodeling [3, 21, 22, 42-44, 108]. Remodeling of a collagenous tissue, such as a tendon, is a common biological phenomenon and evidence exists that suggests that remodeling occurs with growth and maturation [88, 92] and in response to over-exercise [61]. These suggest that the remodeling that occurs with growth, maturation and repair involves changes in the extent of collagen crosslinking. Changing collagen crosslinking may also affect the mechanical properties of a collagenous tissue [64, 65, 82, 130] and thus may prove clinically important when one considers

pathological conditions where the mechanical strength and stiffness of collagenous tissues is necessary for function.

HIT testing is a useful tool to investigate the crosslinking of collagenous tissues by looking at changes in the denaturation temperature (T_d) and the half-time of stress relaxation ($t_{1/2}$) associated with peptide bond hydrolysis during the isometric relaxation phase [88-91]. During HIT, a collagenous tissue is suspended in a water bath and held at a constant length (isometric constraint). The water bath temperature, load (resultant tension at the grips), and time are recorded concurrently and plotted against time (Figure 4.1.). As the water bath temperature is increased there is an initial decrease in the load recorded on the sample due to stress relaxation. Further increases in temperature will lead to a sudden increase in load when the collagen denatures. Tendons that do not fail as a result of this sudden load will provide further information after a constant elevated temperature of 90°C (isotherm) is maintained. During this phase of HIT testing, there is a relaxation of rubbery collagen (due to hydrolysis of peptide bonds) resulting in a decrease in the load recorded. This relaxation is inhibited by hydrolytically stable crosslinks. Therefore, the isometric relaxation phase will provide a semi-quantitative estimate of thermally stable mature non-reducible crosslinks reflected by changes in the $t_{1/2}$.

Further information on the extent of unstable immature crosslinks in collagenous tissue can be provided if HIT experiments are repeated with samples that are artificially stabilized with sodium borohydride reduction. This chemical reduction causes a conversion of reducible immature crosslinks to non-reducible mature crosslinks that are thermally stable and capable of inhibiting the relaxation during the isometric segment [77]. During the isometric relaxation phase, there will be increased resistance to

relaxation (decreased slope) that will reflect the extent of pre-existing unstable immature crosslinks. Thus, reduction of a collagenous tissues with sodium borohydride provides information on the extent of total crosslinking while comparison of HIT profiles from reduced and non-reduced tissues provides an estimate of the proportion of reducible versus non-reducible crosslinks [77].

In this study, HIT was used to study the collagen crosslinking in rat Achilles tendons under two experimental conditions. First, the collagen T_d and $t_{1/2}$ values were compared in the Achilles tendons of rats at various ages to determine the effect of growth and maturation on the collagen crosslinking in the tendon. Second, 20-week-old rats subjected to an over-exercise running regime (Chapter 3) were compared to a non-running control group to determine the effect of overuse running on collagen crosslinking of the rat Achilles tendon.

4.3 Methods

The effects of growth and maturation on collagen crosslinking were determined by comparing the HIT profiles of six rat Achilles tendons in each of three age groups (3 , 8 and 20 weeks old). Next, the effect of overuse running was determined by comparing the HIT features of the rat Achilles tendons from 20 -week-old non-running control rats (n= 6) and experimental running rats (n=8) that were subjected to over-exercise. Bilateral Achilles tendon harvest from all rats provided tissue for HIT experiments, with and without sodium borohydride reduction, to further characterize the type of crosslinks present. (See below.).

The subjects were Sprague-Dawley rats initially aged 3 or 8 weeks, and initially weighing <50 or 300-325 grams respectively. All rats were allowed normal cage activity and fed a diet of unlimited rat chow and water. The running rats were maintained under identical conditions but were also subjected to the overuse running exercise regime (Chapter 3).

4.3.1 Rat overuse exercise running regime

To determine the effect of overuse running 8 -week-old rats (n=8) were subjected to a 12-week over-exercise running regime that was described in detail in Chapter 3. During this time period control rats (n=6) were allowed normal cage activity under otherwise identical conditions.

4.3.2 Tendon harvest

Upon the completion of the 12-week overuse running protocol, the rats were sacrificed in a humane manner by carbon dioxide asphyxiation followed by cervical dislocation to assure death. The gastrocnemius and soleus muscle was dissected free of all soft tissues including the plantaris muscle tendon unit if it was present. The two merging tendons of the gastrocnemius and soleus muscle were isolated by amputation at the mid-portion of the gastrocnemius soleus muscle belly proximally and the posterior tuberosity of the calcaneus insertion distally.

4.3.3 Hydrothermal isometric testing

HIT was performed using a custom-built multi-sample denaturation temperature testing apparatus for collagenous biomaterials [91]. During the HIT tests, both ends of rat Achilles tendons were held between two custom-designed grips (design by MAG) and maintained at a constant length (isometric constraint). Each grip/sample/grip assembly was hung from an upper hook attached to a force transducer, and the lower grip to a fixed rod. The tension on the grips was adjusted to a preload of 30 grams. The tendons and grips were suspended in a distilled, deionized water bath while the temperature was increased to 90°C. During the heating of the water bath temperature, load (resultant tension at the grips) and time were recorded concurrently, with load being subsequently plotted against water bath temperature (Figure 4.1.). When the collagen denatured, a sudden increase in load was recorded. The temperature at which this rise began was designated as the denaturation temperature T_d. Some tendons failed to resist the force developed after denaturation and during heating to the 90°C isotherm. These samples experienced an intra-substance failure, and yielded T_d values only (and no $t_{1/2}$ values).

Tendons that did not fail at the denaturation temperature provided further information after a constant elevated temperature of 90°C was achieved and maintained. This portion of the HIT testing is referred to as the isometric relaxation phase (Figure 4.1). During this segment, the relaxation of rubbery collagen (associated with breaking of peptide bonds) is inhibited by hydrolytically stable crosslinks that survived the increase in temperature to 90°C. Thus, the isometric relaxation phase provides an estimate of the density of thermally stable (mature, non-reducible) crosslinks. The value of $t_{1/2}$ increases with increasing crosslink density [92, 131].

Without artificial chemical stabilization of the unstable immature crosslinks, the relaxation during the isometric relaxation phase is inhibited by the existing mature crosslinking only. If there is a lack of mature crosslinks, then the tissues may fail prior to achieving a sustained isometric relaxation phase or display a very steep vertical slope and artificially low $t_{1/2}$ value during the isometric relaxation segment phase. This indicates precipitus stress decay due to the lack of thermally stable mature crosslinks.

All HIT experiments included a matched group of rat Achilles tendons that were treated with sodium borohydride reduction prior to HIT [77]. This resulted in the conversion of reducible, immature crosslinks to non-reducible, mature crosslinks that were thermally stable and inhibited relaxation during the isometric segment. Thus, reduction of collagenous tissues with sodium borohydride provided information on the extent of total crosslinking. Further comparisons of HIT data from reduced and non-reduced tissues provided an estimate of the proportion of reducible versus non-reducible crosslinks [77].

It has also been shown that reduction of a collagenous tissue with sodium borohydride results in an increased T_d [132]. Since it has been suggested that intrahelical crosslinks primarily contribute to the collagen molecule's ability to resist denaturation [77], then it is likely that increases in T_d with sodium borohydride reduction may reflect increases in the density of mature intrahelical crosslinks.

4.3.4 HIT data analysis

The denaturation temperature (T_d) was defined as the temperature at which the load began increasing without further decrease after the stress relaxation phase and prior

to the isotherm of 90°C. The half-time of stress relaxation associated with peptide bond hydrolysis ($t_{1/2}$) represents the time required for a force reduction of 50% of the peak force.

The calculation of $t_{1/2}$ has been published elsewhere [77]. A summary follows. The relaxation behavior of a denatured collagen network undergoing peptide bond hydrolysis follows a Maxwell-type relationship [84]:

$$\frac{\sigma(t)}{\sigma(0)} = e^{-kt} \qquad \text{Equation 1}$$

where k is an empirically derived constant. To obtain k, the natural logarithms of stress-time ratio data were plotted against time as:

$$\log\left(\frac{\sigma(t)}{\sigma(0)}\right) = -kt \qquad \text{Equation 2}$$

and the parameter $-k$ was obtained as the slope of the linear regression line through the data points. The half-time of the stress decay, $t_{1/2}$ was defined as the time at which:

$$\frac{\sigma(t_{1/2})}{\sigma(0)} = \frac{1}{2} \qquad \text{Equation 3}$$

Inserting Equation 3 into Equation 1 yields:

$$\frac{1}{2} = e^{-kt_{1/2}} \qquad \text{Equation 4}$$

which, after taking natural logarithms, shows that $t_{1/2}$ can be obtained as:

$$t_{1/2} = \frac{0.693}{k} \qquad \text{Equation 5}$$

Here again, $-k$ is the slope of the plot of $\sigma(t)/\sigma(0)$ versus time.

4.3.5 Statistical analysis

A 2-way ANOVA analysis (Microsoft Excel Software) was used when comparing T_d and $t_{1/2}$ of Achilles tendons between the rats of three age groups and between running and non-running rats with and without sodium borohydride reduction.

If the rat Achilles tendon failed prior to achieving a sustained isometric relaxation phase no $t_{1/2}$ calculation was possible. Note was made of the proportion of tendons that failed in each group.

4.4 Results

During HIT testing of the Achilles tendons there were differences noted in the load versus time plots for Achilles tendons from the rats of various ages and from rats that were subjected to the over exercise running regime. Figure 4.2 shows a typical plot of load in grams versus time in seconds from an HIT experiment with isometric relaxation for two Achilles tendons that were not reduced with sodium borohydride and obtained from 20-week-old rats subjected to an overuse running regime. In general there was an initial load relaxation with increasing temperature, followed by a sharp increase in load at the T_d, which reached a maximum near 90°C. Next, there was a sudden drop in load during the 90°C isotherm depicted as a near vertical slope due to complete tendon failure (Figure 4.2, Tendon 1) or a very steep slop (Figure 4.2, Tendon 2) due to a more gradual tendon failure. These patterns are suggestive of no or insufficient mature intermolecular crosslinks to resist slippage of collagen chains due to peptide bond hydrolysis and resultant intra-substance tendon failure.

Figure 4.3 shows how the typical load versus time plot differed if the tendons were reduced with sodium borohydride prior to HIT. In these plots, the main difference exists during the 90°C isotherm where the load versus time plot demonstrated a shallow slope of exponential decay (Figure 4.3, Tendons 1 & 2). This suggests that a combination of immature crosslinks reduced with sodium borohydride and preexisting mature crosslinks were present in the tendons.

4.4.1 Tendon failure during HIT

It was demonstrated that if rat Achilles tendons from all age and running groups were not reduced with sodium borohydride, then they exhibited high rates of early failure (Tables 4.1 and 4.2). This failure always occurred after the denaturation point was achieved but prior to achieving a sustained isometric relaxation phase. This allowed calculation of the T_d for all tendons tested but prohibited calculations of $t_{1/2}$ in non-reduced tendons that failed. There was no significant difference demonstrated in the $t_{1/2}$ for all groups tested (Table 4.1 and 4.2) but there were differences noted in the T_d (Table 4.1 and 4.2). These are reported below.

4.4.2 Age effects in tendons for non-running rats (Table 4.1)

The untreated tendons from 8-week-old rats tendons had the lowest T_d value and this value was statistically different from the 3-week-old age group with an intermediate denaturation temperature. The 20-week-old age group had the highest T_d however this was only statistically different from the 8-week-old age group and not different from the

3-week-old age groups. Interestingly, this effect of differences in T_d with age was eliminated by reduction of the tendons with sodium borohydride prior to HIT.

A high rate of tendon failure was noted in all age groups during HIT prior to achieving a sustained isometric relaxation phase. It is interesting to note that the 8-week-old age group demonstrated the highest failure rate as well as the lowest denaturation temperature. When the tendons were treated with sodium borohydride reduction prior to HIT the failure rate was reduced to zero for all groups and there was no difference in the T_d between all three age groups. However, it was also interesting to note that sodium borohydride reduction caused a significant increase in the T_d only in tendons harvested from the 8 -week-old age group.

4.4.3 Activity (running vs. non-running) effects (Table 2)

Achilles tendons of 20 -week-old rats that were subjected to the over-exercise running regime had a lower mean denaturation temperature than did Achilles tendons from control, non-running rats. It is interesting to note that reduction of the tendons with sodium borohydride prior to HIT eliminated the effect of overuse running on denaturation temperature just as was the effect of age (above). This effect was not observed with a group of rats that were not subjected to the over-exercise running regime.

A high rate of tendon failure was noted in running and non-running rats during HIT prior to achieving a sustained isometric relaxation phase. Treatment with sodium borohydride reduction prior to HIT reduced the failure rate to zero in the running group just as it did in all age groups.

4.5 Discussion

4.5.1 Age effects

Thermal denaturation of collagen is a transformation from a highly organized, hydrogen-bonded triple helical structure to a random coil as a result of increased thermal gyration sufficient to disrupt various types of reversible bonds (e.g. hydrogen, ionic, and polar) within the collagen triple helix [83-85]. Covalent intrahelical crosslinks that exist within a collagen molecules triple helix structure have been suggested to further stabilize collagen from denaturation [82]. Thus, increases in the amount of intrahelical crosslinks present in the collagenous tissue should increase T_d [83, 85, 133]. The results of the current study show a decrease in the T_d in Achilles tendons harvested from 8-week-old rats compared to 3-week-old rats. However, when the more mature rats (20-week-old) Achilles tendons were compared to the 8–week-old tendons there was an increase in T_d noted (Table 4.1). This would suggest that during the period from 3 to 8 weeks there is a decrease in the number of intrahelical crosslinks and then a subsequent increase with further ageing.

The initial 3 to 8 week decrease in intrahelical crosslinking of the rat Achilles tendon is somewhat perplexing but a consideration of the pattern of growth and development of rats may offer an explanation. The male rat breeding onset is 9-16 weeks with puberty occurring from 6 to 8 weeks [18, 19]. During puberty, mammals experience a rapid period of growth and development that includes widespread tissue remodeling. This remodeling stage has been previously shown to cause differences in the crosslinking of collagenous tissues. Le Lous et al. [88] used HIT testing to demonstrate a striking decrease in $t_{1/2}$ values reflecting a decrease in the rate of formation of mature stable

crosslinks in the rat skin and rat tail tendons during the weeks of puberty. It was suggested that the changes observed in the crosslinking could be reflective of a remodeling process where the destruction of a previously established collagen network is remodeled with the incorporation of newly synthesized, but as yet poorly organized collagen.

In the current study there were parallel changes in the collagen crosslinking of rat Achilles tendons noted during puberty. It has been shown that the initial decrease in intrahelical crosslinks (as reflected by decreased T_d values) from 3 to 8 weeks of age (Table 4.1) corresponds to puberty and thus may be explained in part by a remodeling process. It should be noted that the pattern, but not the types of changes in crosslinks, were similar during puberty when comparing the results of the current and LeLous's [88] study. Specifically, LeLous [88] demonstrated changes in $T_{1/2}$ reflecting the extent of stable interhelical crosslinks while the current reports changes in T_d values reflecting changes in intrahelical crosslinks.

In another study, Naimark et al. [77] showed similar changes in both the pattern and types of crosslinks by demonstrating an increase in T_d of ovine pericardia from the fetal and newborn period to adulthood. The increased T_d likely results from maturation of existing intrahelical crosslinks which can provide increased resistance to uncoiling or denaturation of the triple helical collagen. This process may be similar to the maturation of intermolecular crosslinks described by others [79, 88, 92, 129, 134, 135].

Further, it should be noted that the changes in T_d in this study and the study of Naimark et al. [77] were small and may not be functionally significant even though they are statistically significant. This may suggest that although there may be changes in the

types and quantity of crosslinks in the rat Achilles tendon with maturation this may not translate into changes in the mechanical properties of the tendons.

Lastly, when you compare the absolute values of T_d in this study to other studies such as those for lamb thoracic aorta [92] and pericardial [77] tissues it is noted that the rat Achilles tendons have lower T_d values [mean 61.2-64.3^{0}C (current study and consistent with $T_d \sim 62^0$C from LeLous et al. [88]) and compared to the mean of aorta 65.1-70^{0}C [92] or the mean of pericardia 68.4 -70.2^{0}C] [77]. While these differences in absolute T_d values may be reflective of differences in the chemical composition and structure in the rat and ovine tissues, it is suggestive that the rat Achilles tendon have less intrahelical cross linking to resist denaturation at least when compared to the ovine tissues of the other studies [77, 92].

4.5.2 Running effect

Achilles tendons of 20 -week-old rats that were subjected to the over-exercise running regime had a lower mean denaturation temperature than did Achilles tendons from control non-running rats (Table 4.2). This suggests that there may be a decrease in intrahelical crosslinking of the Achilles tendons of rats subjected to an overuse running regime. This finding may be accounted for by the widespread belief that that overuse of tendons causes continuous micro-trauma, resulting in biochemical alterations that may predispose to tendon rupture [3, 13, 42, 63, 104, 136-139]. It is possible that the tensile stresses from running may result in strains large enough to disrupt collagen crosslinks, thereby leading to denaturation [107, 108] or deformation of the collagen [64, 97, 106]. This damage must be repaired to maintain function and it may occur by a process similar

to tissue remodeling where damaged or denatured collagenous tissue may be enzymatically removed [107, 108] and then replaced with newly synthesized collagen. The current studies (Chapters 3 and 5) lend further support to the remodeling theory by demonstrating an increased concentration of glycosaminoglycans and disorganized collagen fibers in the Achilles tendons of rats subjected to overuse-running.

Thus, the above findings of decreased intrahelical crosslinks in the rat Achilles tendons subjected to the overuse running (Table 4.1) may be reflective of damaged or denatured collagen that will be remodeled. Interestingly this remodeling process may bear some resemblance to that suggested occurring during puberty since the T_d of rat Achilles tendons decreased similarly with overuse exercise and with growth and development from 3 to 8 weeks of age. However, it should be noted that the exact mechanism accounting for the decreased T_d in the Achilles tendons of running and pubertal rats may be different. For example, it is unknown if the decreased T_d was a result of changes in hydrogen bonding involving hydroxyproline amino acids in the alpha-chains since we did not measure the fraction of the collagen amino acids that was hydroxyproline. Further, the hydroxyproline content was not measured in the Achilles tendons from rats of various ages.

4.5.3 Sodium borohydride reduction effects

Sodium borohydride reduction has previously been shown to cause increases in T_d reflective of increased non-reducible intrahelical crosslinks [132] and provides

information on the extent of total crosslinking and an estimate of the proportion of reducible versus non-reducible crosslinks [77]. Thus, in an attempt to further characterize the effect of ageing and overuse running on the collagen crosslinking of the rat Achilles tendon all HIT experiments were conducted with and without prior sodium borohydride reduction.

In the current study there were no differences in the T_d of rat Achilles tendons that were treated with sodium borohydride between all 3 age groups (Table 4.1) suggesting that the extent of total intrahelical crosslinking is similar for all three age groups. It was also noted that there were no differences in the T_d between running and non-running rats treated with sodium borohydride (Table 2.) again suggesting a similar extent of total intrahelical crosslinking in runners and non-runners. However, it is important to note that when the Achilles tendons of running rats were treated with sodium borohydride reduction there was an increase in T_d noted that was similar to the increase in T_d noted when tendons from pubertal (8 -week-old) rats were treated with sodium borohydride. Thus, it may be concluded that Achilles tendons from: (i) pubertal and (ii) older, running rats both contained a greater proportion of reducible intrahelical crosslinks. These findings may lend further support to the proposed remodeling theory since the reducible crosslinks are more characteristic of newly synthesized collagen seen with remodeling.

The most striking finding in this study was the high rate of failure of Achilles tendons in all groups during HIT without prior sodium borohydride reduction (Table 4.1). This suggests that rats in all age and running groups had insufficient non-reducible mature stable crosslinks to resist stress decay during the isothermal relaxation phase.

Further, the fact that sodium borohydride reduction of all tendons reduced the failure rate to zero suggests that there were considerable immature reducible crosslinks present. These findings are consistent with the work of Naimark et al. [77] who demonstrated failure of fetal ovine pericardial samples during HIT if the samples were not reduced with sodium borohydride. The reduction with sodium borohydride rendered the fetal samples capable of resisting failure and resulted in HIT profiles that were similar to adult tissue groups. The authors concluded that the fetal samples primarily contained reducible immature crosslinks that stabilized with ageing or reduction with sodium borohydride. It should be noted that when discussing the rate of failure it is most probable that the type of crosslink of primary importance is the intermolecular crosslinks that resist slippage of collagen molecules under tensile forces [78].

Although the current study showed a similar failure pattern for all rat Achilles tendons it is interesting to note that tendons from 8 -week-old rats demonstrated 100 % incidence of failure (Table 4.1) This failure rate was reduced to 0 (Table 4.1) after reduction with sodium borohydride and again suggesting a high level of immature crosslinks at 8 weeks of age possibly reflective of remodeling.

It should also be noted at this point that when you compare the absolute values of stress decay half life $t_{1/2}$ for the Achilles tendons treated with sodium borohydride reduction to other studies such as those for lamb thoracic aorta [92] and pericardial [77] tissues, it is noted that the rat Achilles tendons have much lower $t_{1/2}$ values [mean 3.3-4.2 hr. (current study) compared to mean 11-20 hr [92] or mean 8.06-44.4 hr [77]]. As with differences in T_d values, these differences in absolute $t_{1/2}$ values may be reflective of differences in the chemical composition and structure in the rat and ovine tissues. Still, it

is suggestive that the rat Achilles tendons may have less total crosslinking than do the ovine tissues of the other studies [77, 92].

Finally, it should be emphasized that the dramatic change in mechanical and thermal properties of all tendons tested after sodium borohydride reduction suggests that the rat Achilles tendon has crosslinking that is principally supported by reducible crosslinks indicative of immature tissues. This may suggest that rats less than 20 weeks of age may not serve as an ideal animal to model tendon disease that commonly occurs in the human is in the adult population.

4.6 CONCLUSION

The findings of this study show that the rat Achilles tendon undergoes alterations in its collagen crosslinking when an over-exercise running regime is imposed that are similar to changes observed during growth and maturation. Specifically, a greater proportion of reducible intrahelical crosslinking was documented in the Achilles tendons of running and pubertal rats. This may suggest that the changes in the crosslinking observed are the result of a remodeling process.

Age	$NaBH_4$ Reduction	Subject Numbers	Mean T_d +/- SD (°C)	Mean $t_{1/2}$ +/-SD (hours)	% Tendon Failure Rate[1] (Ratio Failed)
20 weeks	No	6	64.3 ± 1.5 [b]	N/A	83 (5/6)
	Yes	6	63.5 ± 1.5 [c]	3.7 ± 2.1	0 (0/6)
8 weeks	No	6	61.2 ± 1.0 [abc]	N/A	100 (6/6)
	Yes	6	63.0 ± 0.6	3.3 ± 1.0	0 (0/6)
3 weeks	No	6	63.3 ± 0.5 [a]	N/A	66.7 (4/6)
	Yes	6	63.5 ± 1.0	4.2 ± 1.5	0 (0/6)

[1] Prior to achieving a sustained isometric relaxation phase
[abc] Indicates a statistical difference with p-Value <0.05

Table 4.1. Summary of results of HIT testing with isometric relaxation showing the effect of age and $NaBH_4$ reduction on the denaturation temperature (T_d), stress relaxation half-time ($t_{1/2}$), and failure rate of Achilles tendons harvested from 20, 8, and 3-week-old-rat.

Activity	$NaBH_4$ Reduction	Subject Numbers	Mean T_d +/- SD (°C)	Mean $t_{1/2}$ +/-SD (hours)	% Failure Rate[1] (Ratio Failed)
Runner	No	8	62.7 ± 0.7 [ab]	N/A	62.5 (5/8)
	Yes	9	64.1 ± 0.9 [b]	5.1 ± 2.7	0 (0/6)
Non-runner	No	6	64.3 ± 1.5 [a]	N/A	83 (5/6)
	Yes	6	63.5 ± 1.5	3.8 ± 2.1	0 (0/6)

[1] Prior to achieving a sustained isometric relaxation phase
[ab] Denotes values that are statistically different with p-Value <0.05

Table 4.2. Summary of results of HIT testing with isometric relaxation showing the effect of activity and $NaBH_4$ reduction on the denaturation temperature (T_d), stress relaxation half-time ($t_{1/2}$), and failure rate of Achilles tendons harvested from 20-week-old-rats.

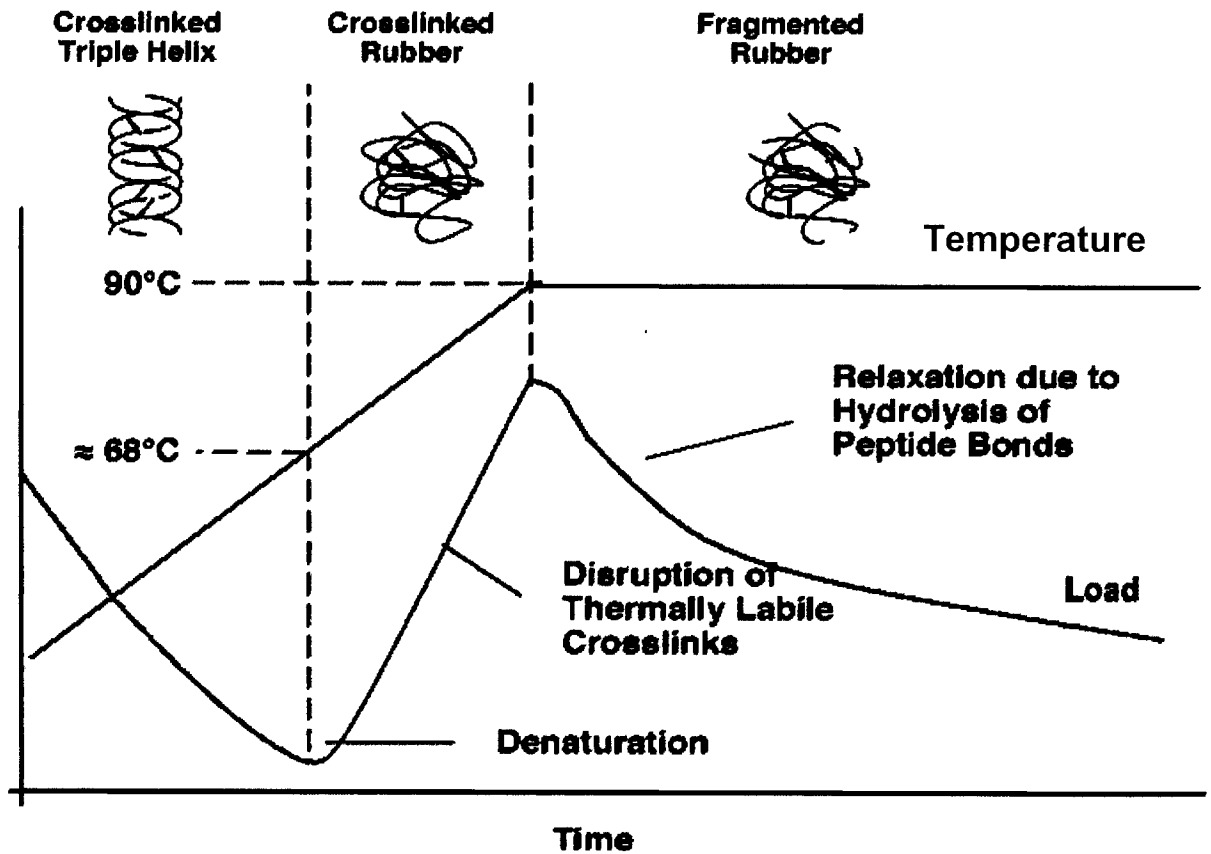

Figure 4.1. Cartoon of load and temperature versus time (seconds) depicting a typical Hydrothermal Isometric Tension (HIT) experiment with isometric relaxation of a collagenous tissue. The plot illustrates an initial stress relation phase with subsequent sudden rise in slope reflective of denaturation and finally a gradual decrease in load due to hydrolysis of peptide bonds. Figures at top show the collagen triple helix with immature and mature crosslinks (left) before and denaturing losing immature crosslinks (center), and mature crosslinks only (right). [Courtesy of JML).

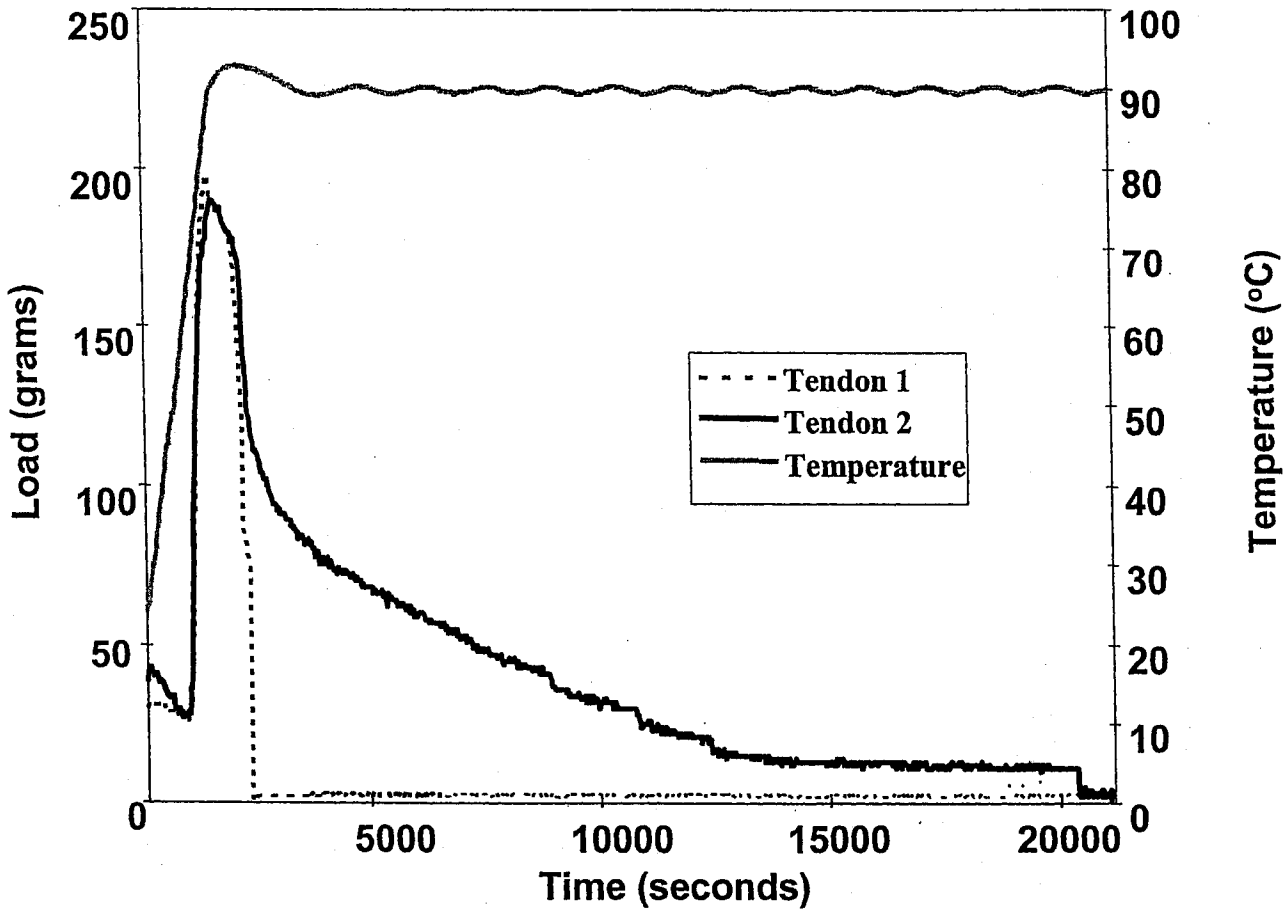

Figure 4.2 Typical plots of load in grams versus time in seconds from HIT experiments with isometric relaxation. Test samples were Achilles tendons obtained from 20-week-old rats subjected to an overuse running regime. Tendon 1 failed during the isometric relaxation phase and thus the plot has a vertical slope indicting the presence of insufficient mature crosslinking to resist slippage resulting from the breaking of peptide bonds. Tendon 2 did not fail but the plot does have a very steep slope suggesting minimal mature crosslinking capable of resisting slippage from breakage of peptide bonds.

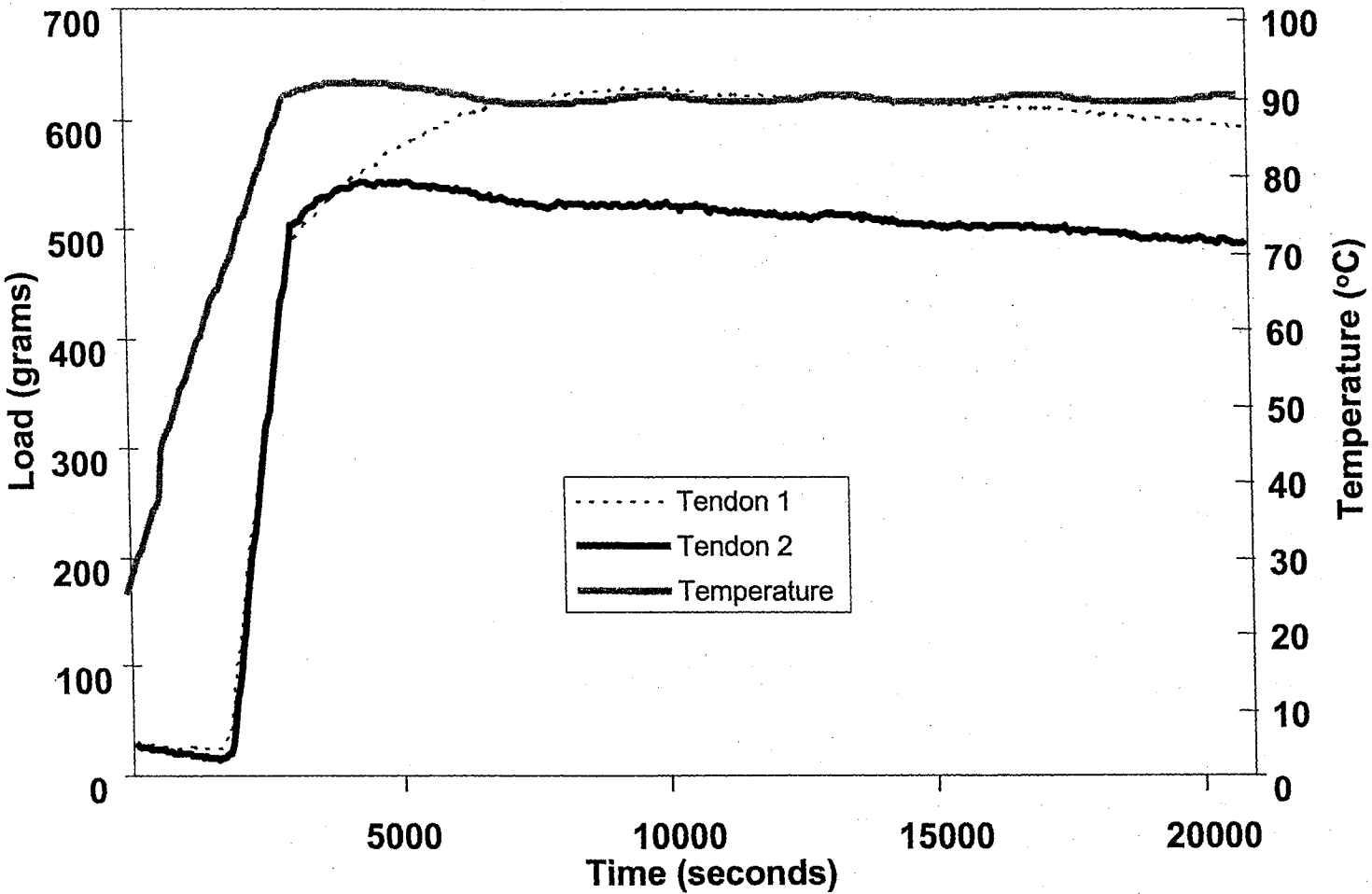

Figure 4.3. Plot of load in grams versus time in seconds from an HIT experiment with isometric relaxation. Test samples were Achilles tendons obtained from 20 week old rats subjected to an overuse running regime. The samples were reduced with sodium borohydride prior to HIT. The Tendons 1 and 2 did not fail during the isometric relaxation phase due to the artificial reduction of unstable immature mature crosslinks with sodium borohydride to more stable cross-links capable of resisting slippage resulting from the breaking of peptide bonds.

CHAPTER 5: PAPER III: BIOCHEMICAL AND BIOMECHNICAL ANALYIS OF ACHLLES TENDONS IN AN OVERUSE ANIMAL MODEL

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5.1 INTRODUCTION

A merging of the insertions of the gastrocnemius and soleus muscles forms the Achilles tendon. Its primary function is to facilitate planter flexion of the foot by transmitted muscle generated forces to the hindfoot. The biomechanical response of a tendon to a tensile force was reviewed by Stanish et al. [39]. At low tensile forces (strains ~ 1 %) tendons undergo reversible deformation as a result in straightening of the crimped collagen. When larger tensile forces are applied tendons (strain levels of 2-3%) the collagen fibrils slide past one another and the crosslinks begin to break [64, 106]. This crosslink failure may be responsible for localized denaturation which has been shown to occur at the rupture ends of tendons experiencing tensile failure [107, 108]. These tensile failures may then lead to micro-tears an event causing clinical symptoms [104]. Finally, at larger tensile forces (strain levels > 8%) there is complete failure of the tendon that is a catastrophic event causing pain, suffering and loss of the muscle tendon units function.

To date, it is generally accepted that tendons derive their tensile strength from the underlying biology of the tendon. This would include the collagen crosslinking [51, 64, 65, 77] [79, 81, 82, 130, 140], tendon size [62, 141], collagen content [54, 142], collagen subtypes [66, 143], density of collagen fibers present [67], diameter of collagen fibers [11, 144], architecture or organization of the collagen [5, 12, 95, 145], ECM components [53-55] and GAG content [3, 12, 68, 69]. Although all determinants of stiffness and strength should be considered together to determine a tendons ability to resist failure, each individual determinant will play some role.

There is increasing evidence in the literature to suggest that exercise will alter the biomechanical properties of the tendon, altering its biochemical composition [146] and thus altering the determinants of strength discussed above. In this paper, biochemical changes that occur in the Achilles tendon from rats subjected to uphill overuse running have been studied in an attempt to determine their effect on biomechanical properties. Specifically, the biochemical analysis will include quantification of the total sulphated glycosaminoglycan and collagen contents with a subsequent review of changes in collagen crosslinking analysis presented previously (Chapter 4).

The mechanical properties were also investigated by mechanical testing to determine if the biochemical changes caused by the over-exercise model translated into changes in the biomechanical properties.

5.2 METHODS

5.2.1 Rat overuse exercise running regime

The Sprague Dawley rats used were initially aged 8 weeks, weighing 300-325 g. Non-running rats were allowed normal cage activity and fed a diet of unlimited rat chow and water. The running rats were maintained under identical conditions but were also subjected to the overuse running exercise regime (Chapter 3). The effect of overuse running on Achilles tendon biochemistry was determined by comparing the Achilles tendons from a group of running and non-running rats for collagen crosslinking, collagen and glycosaminoglycan content. The effect of overuse running on Achilles tendon biomechanics was determined by comparing the failure force, energy and ultimate tensile stress for Achilles tendons from a group of running and non-running rats.

5.2.2 Tendon harvest and allocation

Upon the completion of the 12 week overuse running protocol, the rats were sacrificed in a humane manner by carbon dioxide asphyxiation followed by cervical dislocation to assure death. The gastrocnemius and soleus muscle-tendon unit was dissected free of all soft tissues including the plantaris muscle tendon unit if it was present. The two merging tendons of the gastrocnemius and soleus muscle were isolated by amputation at the mid-portion of the gastrocnemius soleus muscle belly proximally and the posterior tuberosity of the calcaneus insertion distally. Whole tendons were used for HIT (n=8 runners and n=6 non-runners), HIT with $NaBH_4$ (n=9 runners and n=6 non-runners), and mechanical testing (n= 18 runners and n=19 non-runners) while the tendons allocated for biochemical analysis (n=10 runners and n=10 non-runners) were cut though the sagital plane and two equal portions each representing 50 % of the tendon harvested were isolated. One portion was used for collagen typing and the other portion was divided though the sagital plane in two more equal portions allocated for determination of the collagen (25 %) and glycosaminoglycan (25 %) content. The wet weight of all portions of the tendon samples for determination of collagen and glycosaminoglycan content were recorded and then freeze dried for 24 hours to allow a determination of percent dry weight.

5.2.3 Hydroxyproline assay for collagen content

A colorimetric assay for hydroxyproline [147] was used to quantify collagen in the rat Achilles tendons. The assumption of hydroxyproline accounting for 10% of the

amino acid composition of collagen was made as per Woessner's methods [147]. This method utilizes Chloramine-T (SIGMA) to oxidize hydroxyproline to a pyrrole derivative, which is then reacted with Ehrlich's reagent (SIGMA) to give a colored product. After recording the dry weight, each tendon sample allocated to the hydroxyproline assay was hydrolyzed to liberate the hydroxyproline from the peptide linkage by incubation with 1 ml of 6 N HCl for each 25 mg of tissue at 110° C for 18 hours. The hydrolyzate was then neutralized by adding 2.4 ml of 2.5 N NaOH and two drops of 0.02% of methyl red indicator was added directly to 1 ml of hydrolyzate. The pH was finely adjusted to show a faint straw color using 1 N and 0.1 N NaOH and HCl respectively. This mixture was added to a 15 ml plastic Falcon screw cap test tube and it was filled to the 15 ml mark with ddH_2O. A duplicate set for each sample was prepared by adding 150 µl of the neutralized hydrolyzate sample to a separate test tube and then the volume was adjusted to 2 ml using dH_2O. Concurrent with preparation of the samples a set of standards was prepared in duplicate using using 0, 5, 10, 20, 30, and 50 µg of hydroxyproline (SIGMA) with volume adjusted to 2 ml using dH_2O. Next, 1ml of chloramine-T reagent was added to samples and standards and these were vortexed prior to allowing a 20 min oxidation of the hydroxyproline to a pyrrole at room temperature. Then, 1 ml of a 3.15 M solution of Perchloric Acid was then added to the samples and standards and they were vortexed to destroy unreacted chloramine-T over a 5 min period at room temperature. One milliliter of dimethylaminobenzaldehyde (Ehrlich's regent) was then added with vigorous vortexing. This mixture was placed in a hot water bath at 60° C for 20 minutes. The samples and standards were then read at room temperature

using a spectrophotometer (Thermo Spectronic Helios alpha model # 101015) at a 561 nm wavelength.

5.2.4 Collagen type analysis

Freeze dried Achilles tendon tissues were minced with a mortar/pestle tissue grinder (Kontes Glass Co. Model #885451-0023, New Jersey USA) attached to a drill press (Mastercraft model # 55-5915-4, Toronto, Canada) in 0.5 M acetic acid solution. The minced tissue was then allowed to soak for 48 hours at 4 degrees with constant agitation to facilitate solubilization. Pepsin (Sigma P-6887) was added to each sample at a concentration of 1 mg pepsin / 10 mg of initial freeze-dried sample. In an attempt to maximize collagen type III extraction for subsequent gel experiments, the remainder of the extraction process was attempted by three different methods. In the first method the samples were left to digest for an additional 48 hours at 4 degrees with constant agitation and then spun for 90 minutes at 4 degrees at 15,000 rpm. The second method differed from the first by digesting samples at 20 degrees instead of 4 degrees; however, this succeeded only in breaking the collagen down totally. In the third method another attempt at removing the collagen incorporated a second and third 48 hour pepsin digest ($4^{o}C$) each with a subsequent 15,000 rpm spin and collection of extraction supernatant. The three supernatants were combined their pHs adjusted to neutral, and then freeze-dried. The rationale for this approach was that if all of the collagen was not extracted with the first extraction, the process would be repeated it twice more to ensure maximal extraction. The adjustment of pH ensured that acetic acid did not have a negative effect on the electrophoresis gels used in subsequent experiments.

SDS polyacrylamide gel electrophoresis was used to determine the relative contents of collagen types III and I. The method was based on the work of Cheung [34] where the gel was interrupted to allow dithiothreitol to disrupt disulphide bonds thus separating individual collagen α-chains in type III collagen only.

After the collagen was extracted from the tendons, approximately 0.8 mg (range 0.7-1.0 mg) of the freeze-dried supernatant samples was suspended in 200 µl diluted Lammeli buffer (2:1 water:Lammeli buffer) (Bio-Rad Laboratories, Mississauga Ont. #161-0737) to achieve a final concentration of approximately 0.08 mg/200 µl (range 0.07- 0.10 mg/200 µl) and heated in a boiling water bath for 10 minutes. Diluted collagen samples (40 µl) were then loaded on a Criterion precast SDS polyacrylamide gel (Bio-Rad Laboratories, Mississauga Ont. #345-0005). 10 µl of Precision Protein standards (Bio-Rad Laboratories, Mississauga Ont. #161-0372) and 40 µl of type I and III collagen standards (SIGMA) at a final concentration of 0.08mg/200 µl diluted Lammeli Buffer was also loaded onto the same gels. The gels were run on a Criterion cell (Bio-Rad Laboratories, Mississauga Ont. #420-6272) at 200 V in a Tris/Glycine/SDS Buffer (Bio-Rad Laboratories, Mississauga Ont. #161-0732). After the sample front reached a position of 2.5 cm beyond the origin, the gel was interrupted, dithiotreitol (50 µl of 130 mMol solution) was added, and the gel allowed to sit for one hour. The gel was then reloaded on the criterion cell and run at 200V until the sample front reached the bottom of the gel.

Gels were placed on a gently rotating platform and stained in 50 ml Bio-Safe Coomassie stain for 1 hour (Bio-Rad Laboratories, Mississauga Ont. #161-0786) after

washing in 200 ml dH_20 3 times for 5 minutes each. After staining the gels were again rinsed in 200 ml of dH_20 for 30 min and stored in water prior to digital scanning.

Since collagen type III was not detectible using three variations of the extraction process and Coomassie staining (above) a Western blot technique with streptavidin-HRP amplification was utilized. Prior to staining the gels with Coomassie, the gels were equilibrated for 15 min in 50 ml Tris/Glycine transfer buffer (Bio-Rad Laboratories, Mississauga Ont. #161-0734). Transfer of the collagen from gels to nitrocellulose paper (0.045 um Bio-Rad Laboratories, Mississauga Ont. #162-0115) was performed in Tris/Glycine Buffer with 20 % methanol (Bio-Rad Laboratories, Mississauga Ont. #161-0734) using the Criterion Blotter system (Bio-Rad Laboratories, Mississauga Ont. #170-4070 and 170-4071) run at 100 V for 30 min. After transfer, collagen type III was detected using the Opti-4CN Substrate Kit (Bio-Rad Laboratories, Mississauga Ont. #170-8235) with a primary monoclonal anti mouse anti-Collagen Type III antibody (Clone FH-7A, mouse ascites fluid, SIGMA C-7805), secondary goat anti-mouse Horse Radish Protein (GAM-HRP, Bio-Rad Laboratories, Mississauga Ont. #170-8242) and amplified using the Western Blot Amplification Module (Bio-Rad Laboratories, Mississauga Ont. #170-8230).

Detection began with washing of the nitrocellulose membrane in phosphate-buffered saline/0.1%Tween-20 (PBST) 3x for 5 min each. Blocking of the remaining protein binding sites on the nitrocellulose membrane surface was performed using 3% blocker in PBST to minimize non-specific interactions. Nitrocellulose paper was then washed in PBST 3x 5min. each prior to incubation with the primary monoclonal anti-Collagen Type III antibody 1:1000 in a solution of 1% Bovine Serum Albumin (BSA,

SIGMA A-8022) in PBST for 1.5 hours. The nitrocellulose was then washed in PBST 4x for 5 min each prior to incubation with secondary antibody GAM-HRP 1:5000 in 1% BSA in PBST for 1 hour. The nitrocellulose was then washed in PBST 4x for 5 min each prior to an additional amplification process to enhance collagen type III detection by 4-8 fold.

The amplification process began with incubation of the nitrocellulose in Bio-Rad Amplification Reagent for 10 minutes. Washing was then performed 4x 5min. each in a 20 % dimethysulfoxide (DMSO)/ PBST solution followed by an additional 2x wash 5 min. each in PBST. The washed nitrocellulose was then incubated for 30 min in streptavidin-HRP diluted 1:1000 in 1% BSA in PBST. Section Colorimetric detection was accomplished by incubating the nitrocellulose in the diluted Opti-4CN substrate as per module instructions. Colour developed western blots were washed in 50 ml of dH_2O and scanned digitally for qualitative analysis.

5.2.5 Dimethylmethylene blue assay for glycosaminoglycans

A dimethylmethylene blue assay for sulphated glycosaminoglycans [148] was used to estimate the glycosaminoglycan content in the rat Achilles tendons since it detects two (chondroitin and dermatan sulphate) of the three major GAGs that are found within tendons [53]. Hyaluronate was not measured.

Each tendon sample allocated to the dimethylmethylene blue assay was digested in 500 µl of working papain digestion solution for 48 hrs at 65° C. The papain digestion solution was made by diluting stock papain (Sigma P-3125:25 mg/ml) to 80 µl/ml with

papain digestion buffer (50 ml of digestion buffer was prepared by combining 0.130 g ammonium acetate, 0.019 g Na_2.EDTA.$2H_2O$ and 0.015 g. DL-dithiothreitol (DTT) in distilled water with pH adjusted to 6.2 using either hydrochloric acid or sodium hydroxide). After papain digestion, the samples were diluted in 1% BSA in PBS at a 1 to 5 dilution.

To make standards, chondroitin sulphate (Chondroitin Sulfate sodium salt, Sigma cat# C-8529 from bovine trachea) was added to phosphate-buffered saline to achieve a specified concentration: 0, 10, 20, 40 and 60 μg/ml. The chondroitin sulphate standards and 10 μl of the tendon-papain digestion solution were added in quadruplicate to separate wells on a 96-well ELISA plate. Then, 200 μl of dimethylmethylene blue dye solution was added to all the wells and the absorbance read at 525 nm on a plate reader (Bio-Rad model 3550-UV microplate reader).

The dimethylmethylene blue dye solution was stored in a light-protected bottle at room temperature and was prepared by mixing 8 mg of dimethylmethylene blue dissolved in 5ml 100% ethanol (light opaque tube) with 495 ml of 0.2 % formic acid (pH 5.3).

The mean absorbance reading at 575 nm was calculated from the 4 readings of the standards and tendon sample. The standard curve was then plotted and used to determine the quantity of chondroitin sulphate present in each sample. Chondroitin sulphate was then expressed as a percentage of the tendon sample dry weight to yield an estimate of the sulphated GAG concentration in the tendon.

5.2.6 Mechanical testing

Achilles tendons were harvested with a portion of the gastrocneimus muscle and the calcaneus on the proximal and distal end respectively from (i) rats that were subjected to the over-exercise running regime (n=18) and (ii) non-running rats (n=19). The tendons were mounted on custom grips that consisted of a screw-tightened clamp lined with # 180 grit waterproof sandpaper secured to the grips with epoxy to minimize tendon slippage. Mechanical testing was performed using a MTS 486-series servohydraulic mechanical planar biaxial testing apparatus in uniaxial mode. All tendons were extended at a rate of 1.25 mm/sec to tendon failure. The force in Newtons (N), time (sec) and distance (mm) of grip excursion were sampled simultaneously using custom-written LabVIEW software (JML). Prior to tendon testing, the tendon cross-sectional area was determined to allow an estimation of the ultimate tensile stress. Electronic calipers were used to take two measurements for each of the coronal medial to lateral width and the sagital anterior posterior thickness. The rat lower extremity was held at a 90^{o} flexion angle with tendon dissected free of surrounding soft tissues while the coronal and sagital measurements were made. To calculate a value for the cross sectional area, the assumption of an axial elliptical cross-section was made and ½ of the mean coronal width was then multiplied by ½ of the mean sagital thickness and then by π (3.141). The peak failure force was recorded as the maximum force prior to tendon failure while the energy required for failure was calculated as the area under the graph of force vs. extension for each tendon trial (LabVIEW software, National Instruments Version 7 by JML). Statistical comparisons were made for peak failure, ultimate tensile stress and energy to failure between the tendons from the runners and non-runners.

It is important to note that measurements of changes in tendon length were not made due to the extremely small size of tendons and therefore calculations of strain or modulus were not possible.

5.2.7 Statistics

Statistical comparisons of various biochemical and biomechanical parameters on the Achilles tendons from non-running and running rats were made using the Student's t-Test for two sample means (Microsoft Excel Software).

5.3 RESULTS

5.3.1 Hydroxyproline assay for collagen content

When the Achilles tendons of rats subjected to the overuse exercise running regime were compared to those of control non-running rats, there was no difference in mean hydroxyproline content based on dry weight of the tendon tissue (Table 1). This would suggest that the mean collagen content of Achilles tendons was not changed when rats were subjected to the overuse running regime.

5.3.2 Dimethylmethylene blue assay for glycosaminoglycans estimation

The Achilles tendons of rats subjected to the overuse exercise running regime demonstrated significantly greater mean sulphated glycosaminoglycan content based on dry weight of the tendon than those Achilles tendons of non-running rats. This suggests that the sulphated glycosaminoglycan content of the Achilles tendons is increased when rats were subjected to the overuse running regime (Table 5.1).

5.3.3 Collagen typing

Collagen type I but, no collagen type III, could be identified from running and non-running rats using gel electrophoresis (Figures 5.1a. and 5.11b. respectively). Western blotting techniques were then used assuming that collagen type III may be present in such small quantities that gel electrophoresis was not sensitive enough to detect it. Collagen type III standards were detected indicating the western blotting techniques were working properly but there was still no collagen type III detected from the Achilles tendons harvested from the running and non running rats (Figures 5.2a, and 5.2b, respectively).

Concerns regarding the processing of the Achilles tendons were then addressed by altering the temperature and length of time of the Pepsin digest. Collagen type III remained undetected. The reason for not detecting collagen III may be one of two possibilities. First, there may be no collagen type III in the Achilles tendons of running and non-running rats or second, the processing of collagen type III does not allow isolation of intact separated collagen type III α-chains.

5.3.4 Hydrothermal testing with isometric relaxation (From Chapter 4)

During HIT experiments [149], a decrease in mean denaturation temperature was noted after the Achilles tendons from 20 -week-old rats were subjected to the over-exercise running regime and compared to tendons from rats that did not run (Table 1.). This decreased denaturation temperature noted in the running rats may be reflective of a decrease in the quantity of intrahelical crosslinks [83, 85, 133]. It is interesting to note that reduction of the tendons with sodium borohydride prior to HIT eliminated this effect

indicating that the running rat Achilles tendons had a greater proportion of immature or reducible crosslinks [77].

Another interesting finding arising from the HIT experiments [149] was a high rate of tendon failure prior to achieving a sustained isometric relaxation phase noted in the Achilles tendons of both running and non-running rats during HIT (Table 5.1). Chi square analysis showed no significant differences between groups. This indicates a lack of mature non-reducible crosslinks in the collagen of those failing tendons. Treatment of these tendons with sodium borohydride reduction prior to HIT reduced the failure rate to 0 in both running and non-running groups (Table 5.1). This again suggested the presence of significant amounts of immature reducible crosslinks.

5.3.5 Mechanical testing

A typical force versus extension curves for an Achilles tendon from running and non-running rats are shown in Figure 5.3. The Achilles tendons of rats subjected to the overuse exercise running regime did not demonstrate any differences in mean peak failure force, energy required for complete failure or ultimate tensile failure stress when compared to the Achilles tendons of non-running rats (Table 5.1).

5.4 DISCUSSION

The etiology of Achilles tendon disease is multifactorial and has been cited to include overuse running [32-34, 45-48]. However, the underlying biology of Achilles tendon disease is unclear and whether Achilles tendon disease compromises the mechanical strength of the tendon is also unclear [146]. A study by Sommer et al. [150]

demonstrated decreased ultimate tensile strength of Achilles tendons harvested from rats subjected to 12 or 16 weeks of overuse running. In this study rats were subjected to three different over exercise regimes that included endurance, endurance and speed, and speed only running for a training period lasting 2, 4, 8, 12 and 16 weeks. The tendons taken from the rats subjected to the 12 or 16 weeks of speed running showed the largest decrease in tensile strength.

A recent study by Huang et al.[2] [118] closely parallels the methods of the current study with the exception of downhill running. That study did not document any changes in the mechanical properties of rat Achilles tendon subjected to over-exercise. There, 30 rats were subjected to 10^{o} decline treadmill running at 17 m/min for 1h per day, 5 days per week. The control group was subjected to 4 weeks of cage activity and the ages of those latter rats did not match those of the experimental group. Ten of the rats subjected to treadmill running were sacrificed after 2, 8 or16 weeks of treadmill running. Mechanical testing was performed using an Instron electromechanical testing system and tendon morphometric measurements were made using a high-resolution linear variable differential transformer and an optically based image processing system used in prior studies [151]. The results suggest that there was no difference in maximum load, stress or tissue modulus between the control group and the exercise group.

These results are in agreement with our results which showed no difference in tendon failure force, energy and ultimate tensile stress. We did not assess any mechanical parameters of the tendons that involve measurements of the tendon length during extension, such as tissue modulus, due to the small size of the rat Achilles tendon

[2] The current study was completed prior to publication of this paper and we were unaware of this parallel but somewhat similar work.

that did not permit accurate measurements of change in length. Further, our study was limited to a 12-week uphill over-exercise running regime that may be too long or too short to enhance or compromise the biomechanical strength of a tendon.

Finally, a review by Buchanan [146] suggested that most studies demonstrate an increase in mechanical strength of tendons subjected to exercise which is in contrast to the current study and the studies of Sommer et al. [150] and Huang et al. [118]. This contradiction may be explained by the importance of the type of exercise as a factor in determining the mechanical properties of the tendon. For example, some studies have shown that when a tendon is subjected to increased loads during exercise it is less likely to demonstrate increases in mechanical strength [23, 59, 118, 152]. These studies are consistent with the current study where the Achilles tendons of rats were targeted to be loaded eccentrically during the uphill overuse exercise regime. These eccentric contractions have been shown to exert greater forces [115] and therefore increase the likelihood of damage to the musculotendinous structure [116, 117]. Thus one must carefully consider the overuse running protocol used when interpreting the mechanical testing data.

In summary, our study both confirms and contradicts previous published studies published due to confounding variable's of type, duration, and intensity of exercise. Clearly, such variables need to be studied in a controlled manner to determine each variables affect on the mechanical properties of tendon. Further, in order to better understand the effect of overuse activity on the mechanical properties of a tendon, one must gain an understanding of the effect of overuse activity on the determinants of the mechanical properties of the tendon. These determinants have been listed above (see

introduction) and should be considered together to determine a tendons ability to resist failure however each individual determinant will play a role.

Since it is not possible to examine all the determinants of strength for a collagenous tissue, it was decided that a focus should be placed on collagen since it comprises ~ 87 % of an Achilles tendon's dry weight [153]. Further, collagen crosslinking [64, 65] and the concentration of collagen [62] has also been shown to be important in determining the strength and stiffness of a tendon. GAGs were also studied here since their importance arises from their interaction with collagen to enhance the biomechanical properties of a collagenous tissue [71, 72]. The binding of GAGs and their associated proteoglycans to collagen have also been shown to play a role in the determination of collagen fibril diameter [70].

Currently, there exists a lack of integrative studies on tendon mechanical properties with biochemical studies. This makes it difficult to definitively associate changes in mechanical properties with chemical composition [146]. In the current study, an attempt has been made to provide data that integrates mechanical strength with changes observed in the collagen crosslinking, sulphated glycosaminoglycan and collagen content, and histology of Achilles tendon from running and non-running rats. Our results indicate that when an over-exercise running regime is imposed for 12 weeks on the rat, there is a distinct change in the biochemistry and histology of the rat Achilles tendon (chapter 3, 4 and 5), yet this does not translate into changes in mechanical strength.

It should be noted that a power analysis on the mechanical testing data (University of California Los Angeles Website) suggests that, given the variability in our

data, observed changes of 20 N, 4.5 N mm and 52 Nmm^2 would be required to indicate significant differences for force, energy or ultimate tensile stress respectively. These differences were not achieved and thus, it is possible that either (i) there is no statistically significant difference in these variables for running and non running rat Achilles tendons, or (ii) if lesser differences existed in the sample populations, then more tendons would have to be tested to detect this. Thus, it is likely that these findings reflect the human clinical condition since most human subjects who rupture their Achilles tendon experience trauma that does not involve huge increases in force over regular physical activity (e.g. running off a curb) and are therefore probably consistent with a force increase of 21% as detectable in this study demonstrated by the power analysis.

This study also reports the quantity of collagen remained unchanged (Table 5.1.) in Achilles tendons taken from rats subjected to overuse running. This is consistent with other studies reporting no significant change in the concentration of collagen in a tendon that has been subjected to physical activity [58-61]. These findings would suggest that if there is on going active synthesis of collagen, then there must be similar removal of collagen to maintain the same concentration as previously suggested [61]. This may suggest a remodeling process where there may be differences in the organization [22, 43, 44] of the collagen fibers and/or the collagen subtypes [42, 154].

An attempt to investigate collagen subtypes was also undertaken in this study in an attempt to determine if there were any changes in the amount of collagen types I and III in rat Achilles tendon with over-exercise running. This aspect of the study demonstrated many difficulties with the quantification of collagen type I and III in rat collagenous tissues. Our hypothesis for the observations made would be that Type III

collagen is either present in very low concentrations or is extremely resistant to biochemical extraction. While Naimark et al. [82] found a similar problem in mature dogs tissues due to dense crosslinking, the present HIT studies suggest that this mechanism is unlikely and that type III collagen may not be present in rat Achilles tendon tissuesused in this study.

Other authors who used HPLC techniques [63] have shown that the collagen content of human ruptured Achilles tendons was decreased but there was an overall increase in Type III collagen at the rupture site.

In the current study, we also report increased GAG content in the Achilles tendons from running rats. It is interesting to note that other studies have demonstrated decreases in GAG content in ruptured tendons when compared to non-ruptured tendons [3, 12, 155] but these studies used a semi-quantitative method for GAG analysis using acid Schiff histochemical staining techniques.

Other studies that used biochemical assays for GAGs are consistent with the results of this study. Vailas et al. [60] found increased GAG content in tendons of rats subjected to overuse running using the sulphated GAG detection method identical to ours while Cartmell et al. [156] used total GAG detection methods to arrive at the same conclusion.

These findings may suggest that an increased GAG content may play a role in stabilizing the mechanical properties of tendons since the tendons from our study with increased GAG content did not demonstrate a decrease in ultimate tensile failure stress despite decreases in collagen fiber organization (Chapter 3) and decreased intramolecular collagen crosslinking [149]. The findings of increased GAGs in the Achilles tendons of

the running rats may also be suggestive of a remodeling process characteristic of an early phase of tendon repair. It has been shown that proteoglycans containing a variable amount of glycosaminoglycans covalently bound to the protein core have the capacity to regulate collagen fiber formation [53, 157, 158]. Further, increased levels of proteoglycans have been observed in granulation tissues [159] and hypertrophic scars [157]. Thus, the increased GAG content demonstrated in tendons subjected to overuse running may represent a biological response to repair or remodel [160] the damage caused by the overuse running [32, 48].

This repair or remodeling theory gains further support when one considers the collagen crosslinking of Achilles tendons from rats subjected to overuse exercise (Chapter 5). In that study, hydrothermal isometric testing was used to determine that the rat Achilles tendon undergoes alterations in its collagen crosslinking during growth and maturation that are similar to changes observed when an over-exercise running regime is imposed. Specifically, a greater proportion of reducible immature intrahelical crosslinking was documented in the Achilles tendons of pubertal and running rats. This suggests that the changes in the crosslinking observed may result from a remodeling process.

5.5 CONCLUSION

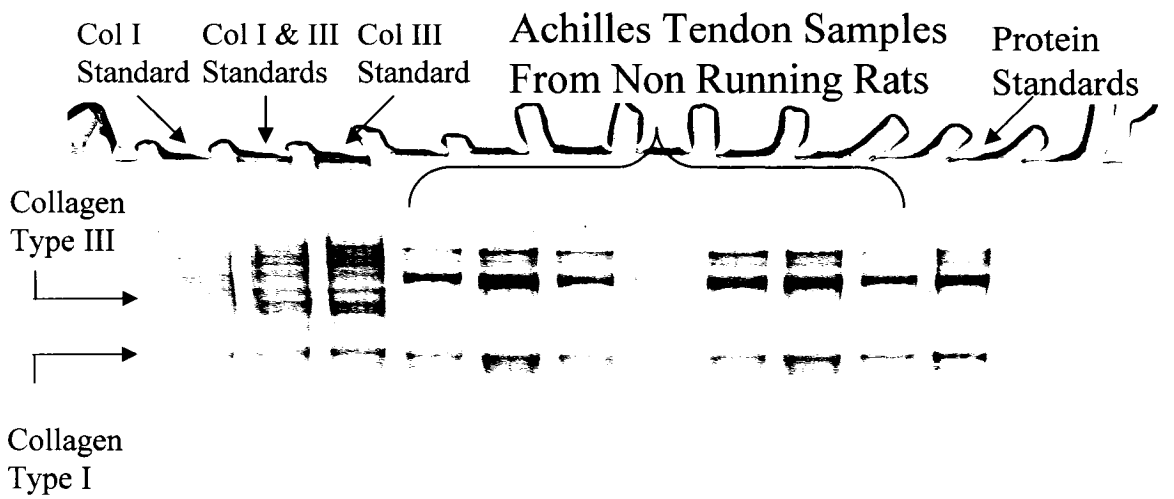
In conclusion, this study shows that when rats are subjected to uphill overuse running activity, their Achilles tendons demonstrate biochemical and biomechanical changes that may be similar to a repair or remodeling response to damage caused by the overuse running.

Activity	$NaBH_4$ Reduction	Collagen Content % Dry Weight +/- SD	Chondroitin Sulphate Content ug/ml +/- SD	Mean T_d °C +/- SD	% Tendon Failure Rate[1] (Ratio Failed)	Mechanical Testing: Force (N) Energy (Nmm) UTS (Nmm^2) +/- SD
Runner	No	86.0 ± 11.0 (n=10)	17.5 ± 7.4[a] (n=10)	62.7 ± 0.7 [bc] (n=8)	62.5 (5/8)	86.8 ± 20.5 15.7 ± 4.2 173.5 ± 54.3 (n=18)
	Yes			64.1 ± 0.9 [c] (n=9)	0 (0/6)	
Non Runner	No	89.1 ± 10.6 (n=10)	9.9 ± 4.9[a] (n=10)	64.3 ± 1.5 [b] (n=6)	83 (5/6)	95.4 ± 21.4 15.3 ± 5.2 183.5 ± 55.6 (n=19)
	Yes			63.5 ± 1.5 (n=6)	0 (0/6)	

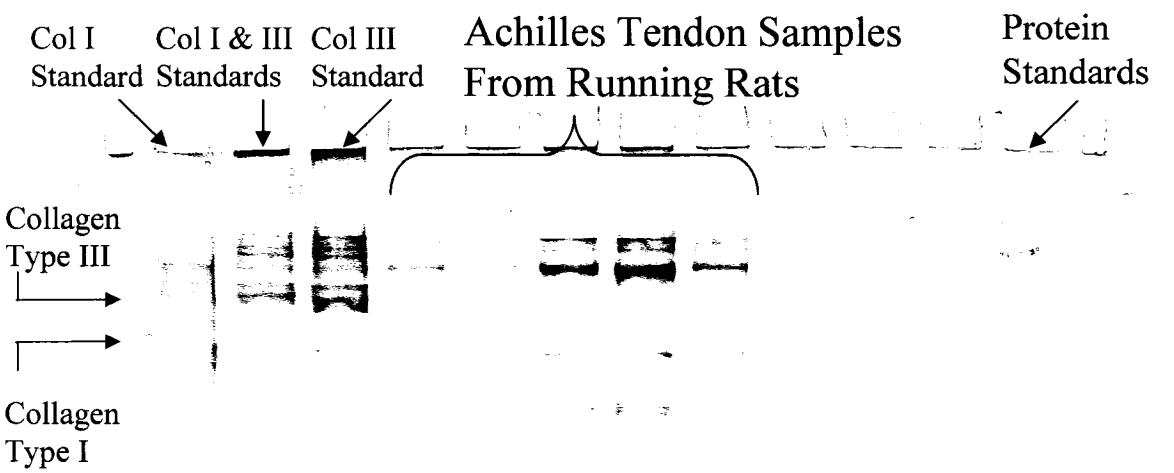
[1] Prior to achieving a sustained isometric relaxation phase

[abc] Denotes values that are statistically different with p-Value <0.05

Table 5.1. Mean collagen content, chondroitin sulphate content, denaturation temperature, tendon failure rate and mechanical testing data for Achilles tendons with or without sodium borohydride reduction. Tendons were harvested from 20 week old rats subjected to normal cage activity (non runners) or a 12 week overuse exercise regime (runners).



(a)



(b)

Figure 5.1. Digital scan of polycrylamide gel electrophoresis of protein standards, collagen type I & III standards and rat Achilles tendons harvested from (a.) non running and (b.) running rats. Note is made of collagen type III present in standard lanes but not in the lanes of Achilles tendons.

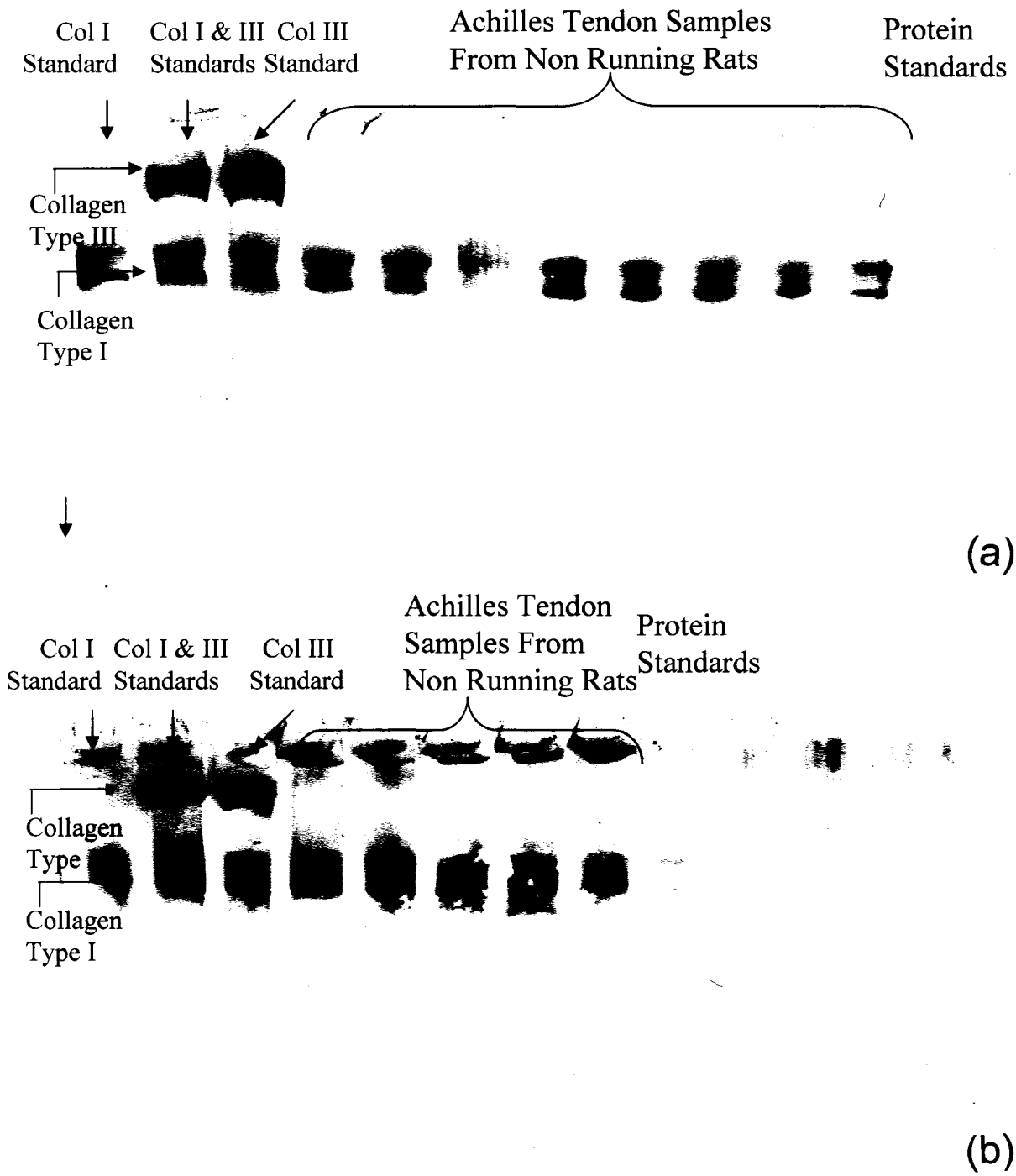


Figure 5.2. Digital scan of Western blots of protein standards, collagen type I & III standards and rat Achilles tendons harvested from (a) non running and (b) running rats. Note is made of collagen type III present in standard lanes but not in the lanes of Achilles tendons.

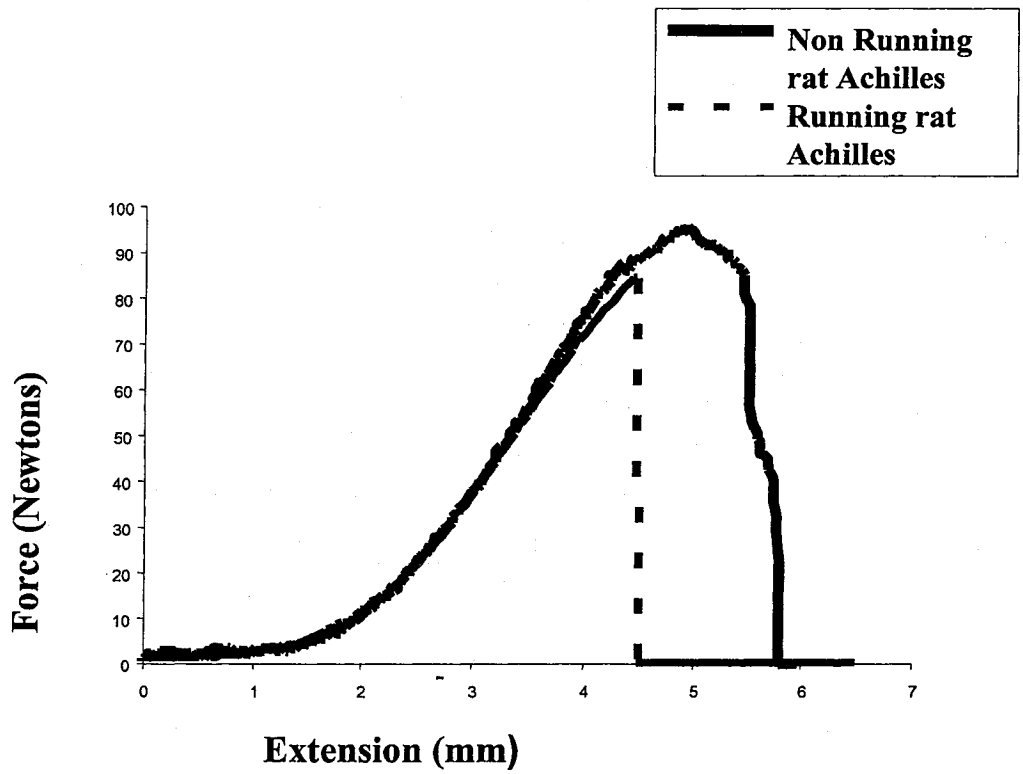


Figure 5.3. Force versus extension plot of Achilles tendons harvested from running and non running rats during mechanical testing of extension to failure.

CHAPTER 6: THESIS DISCUSSION

Non-insertional Achilles tendon disease is a clinical problem that causes pain and disability that is similar to that seen in other tendons of the body such as the knee, shoulder and elbow. The classical treatment of these disorders consist of non-operative and operative modalities that have very little scientific basis for their limited success. Numerous clinical outcome studies have been published to guide clinical care (examples [7, 34, 105]) but little information is available to explain how treatments restore normal biology to the tendon to alleviate pain and suffering. For example, patients with chronic pain and swelling at the Achilles tendon will often be treated with surgical debridement [161, 162] if they fail non-operative treatment modalities. Surgical debridement consists of removal of tendon tissues that look "grossly abnormal" and incision of remaining tissues to "stimulate a normal healing response". Results have been reported as "good to excellent" in 30-80% of patients treated in this manner [161, 162]. These studies are more anecdotal than scientifically based. Thus, current clinical care of Achilles tendon disease is more of an art than science.

In order to better understand these clinical problems one must have a clear understanding of both the non-diseased tendon and the diseased tendon. The purpose of this thesis was to develop an animal model that fulfills the necessary criteria [15, 16] to serve as a tool to facilitate investigation of the biology of the diseased Achilles tendon. The necessary criteria that this model should include are: 1) appropriate tissue type modeled, 2) accurate simulation of the injury conditions, 3) reproduction of pathological

animal tissues that are similar to human diseased tissues and finally, 4) ease of application.

Others have attempted to develop animal models for investigation of diseased tendons [8, 20-25, 49] but have fallen short in at least one of the above listed criteria. Stone et al. [25] attempted to model tendon disease by injecting rabbit patellar tendons with species-specific preparation of cytokines. This strategy fails to model the most commonly cited etiological factor of tendon disease which is that of overuse [32]. Backman and Messener [8, 20] have attempted to model overuse exercise in the rabbit and rat Achilles. Unfortunately their models were impractical due to a complex "kicking" apparatus and especially the need for repeated anesthesia. Further, the simulation of kicking with co-stimulation of leg muscles did not accurately reproduce overuse running.

Sommer et al. [150] used a model similar to the one in this study but did not attempt any validation or histological analysis to compare the Achilles from their rats to human diseased Achilles tendons. Their study was limited to assessing the rat Achilles tendons gross appearance, oxygen uptake and mechanical testing discussed earlier.

The study of Soslowsky et al. [22] set a new standard for animal models of tendon overuse due to the running regime and the detailed examination of the tendons. This study utilized a downhill treadmill-running regime imposed on the rat to induce disease in the tendons of the shoulder that was similar to shoulder tendon disease in humans. Recently, this model was used in an attempt to examine the biomechanical properties of Achilles tendon after overuse running [22]. The results suggested that the biomechanical properties of Achilles tendons from running and non-running rats were not significantly

different. These findings are consistent with the current study that shows no differences in the biomechanical properties.

In the current study, methods were chosen that were distinctly different from those of Soslowsky [22] with some modifications to maximize stress in the Achilles tendon of the rat. These modifications included uphill running to cause a different type of contraction by the gastrocnemius and soleus muscle complex. Further, it has been previously shown that uphill running causes a significant increase in cross-sectional area [163] likely as a result of increased eccentric contractions.

The rat model is currently the gold standard for animal models for tendon disease [21], however it is important to emphasize that this is merely a model and, as with all animal models, caution should be exercised when translating any information to humans. With respect to our rat model for Achilles tendon disease, it is important to highlight and reemphasize some of the differences in the human and rat that were discussed earlier. This would include an anatomy that is analogous but not identical to the human, physiology that supports a nocturnal and likely more anaerobic activity pattern (Chapter 1), a young and rapid rate of maturation (Chapters 3 and 4) that may result in significant variations in the maturation state of tissues at various ages (Table 4.1, Chapter 4).

Regardless of the uncertainties with translation of information from animal models to the human clinical condition, our rat model has caused the Achilles tendons of running rats to demonstrate distinct differences in histology and biochemistry when compared to non-running rats. Specifically, this animal model has been validated to produce Achilles tendons that have histological features (Chapter 3) similar to the histopathological findings of biopsies from ruptured human Achilles tendons [1, 3, 41,

42, 50, 95, 124], diseased human Achilles tendons [8, 36, 43, 44, 125], and tendons from animals subjected to over use exercise models [8, 20, 22, 49]. These histological features included increased cell numbers, more intensely eosinophilic staining and disorganized collagen fibers: all of which are features that most likely result from repetitive damage that is sustained by the Achilles tendon with overuse-running [32-34, 45-48].

In order to determine why these histopathological features exist one must first consider the effects of over-use running on the Achilles tendon. During overuse-running the collagen fibers of the Achilles tendon experience stress as a result of the tensile forces placed on it. This stress may cause strain that may be in excess of ~ 4 %, which has been cited to cause irreversible damage to the tendon [39, 119]. Further, it has also been shown that tendons ruptured by tensile forces undergo local denaturation at the rupture site [107, 108]. Thus, it is likely that even in the absence of complete rupture; overuse running may still cause localized damage to the tendon in the form of localized denaturation.

The suggestion of local denaturation being caused by tensile forces [107, 108] gains indirect support from the current study. Here it has been shown that Achilles tendons from running rats are characterized by intensely eosinophilic collagen fibers, a feature that has been previously suggested to occur with denatured collagen [120]. Further, it is commonly known that denaturation of a collagen molecule causes the molecule to transform to a disorganized state and the molecular level which may have effects on the histological appearance as well.

The theory of localized denaturation as a result of overuse running is further supported in the current study of collagen crosslinking in the Achilles tendons of rats subjected to overuse running (Chapter 4). Here, hydrothermal isometric tension testing

was used to determine that overuse running caused a decrease in the denaturation temperature of the rat Achilles tendon. This suggests that overuse running will cause tendons to have decreased intrahelical collagen crosslinking, perhaps rendering the collagen molecule more likely to denature and subsequently stain more intensely with eosin.

Given that it is likely that overuse running causes localized damage or denaturation of the collagen in tendons, then there is likely a biological mechanism that exists to repair the damage. Stevens and Minns [107, 108] have postulated that local denaturation must be enzymatically removed by phagocytic cells to allow an organized repair of the damaged collagenous tissues. In the current study, immunohistochemical staining of Achilles tendons taken from overuse running rats demonstrates that the cellular proliferation does not include a significant inflammatory component but a combination of fibroblasts and endothelial cells were present. This likely represents a biological response where a proliferation of increased blood vessels supply nutrients to fibroblasts capable of both phagocytosis and collagen synthetic functions that are essential to repair and remodeling [109].

The possibility of overuse exercise causing damage to the Achilles tendon that is repaired or remodeled allows us to return to our opening argument centered on the uncertainties of the nature of the underlying biology of Achilles tendon disease (Chapter 1). In short, the literature does not clearly define the pathobiology of Achilles tendon disease with argument for [1, 8, 13] and against [3, 9-12] inflammation. This study strongly supports a lack of inflammation and more likely a reparative response to repetitive injury evidenced by our findings of: i) cellular proliferation that is non-

inflammatory in nature (Chapter 3 and 4), ii) increased glycosaminoglycan concentrations, iii) no net increase in collagen concentration (Chapter 5) and (iv) tendon collagen crosslinking that bears similarities to remodeling collagenous tissues (Chapter 4). These findings support an original contribution from this thesis to the knowledge of Achilles tendon disease and provides a validated animal model that will be useful for further investigation.

CHAPTER 7: THESIS CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

In conclusion, the objective of this thesis, to gain a better understanding of Achilles tendon disease, was realized. The findings of this study suggest that an animal model for Achilles tendon disease has been established where over-exercise running in the rat causes changes in rat Achilles tendons that are similar to the histology and biochemistry observed in human tendon disease.

The studies on tendon histology demonstrate that tendons harvested from rats subjected to overuse uphill treadmill running have increased cell numbers, more disorganized collagen architecture and stain more intensely with H & E staining methods when compared to Achilles tendons harvested from control rats subjected to normal cage activity. Further immunohistochemical studies have demonstrated increased fibroblasts and endothelial cells with no significant inflammatory cell infiltrate noted in the Achilles tendons from running rats, possibly suggesting that inflammation is not a predominate process for this running regime.

Biochemical testing demonstrated a greater collagen and glycosaminoglycan content in running rat Achilles tendons while HIT studies revealed alterations in collagen crosslinking. Specifically, a greater proportion of reducible intrahelical crosslinking was documented in the Achilles tendons of running rats. These changes were similar to changes observed during growth and maturation of pubertal rats

which may suggest that the changes in the crosslinking observed are the result of a remodeling process.

Finally, the changes in histology and biochemistry observed in Achilles tendons from running rats did not translate into changes in the mechanical strength.

Future studies will be focused on two fronts: 1) further validation of our animal model for Achilles tendon disease in the human and 2) further studies to strengthen the theory that Achilles tendon disease is a reparative response to repetitive damage. The continuation of validation of our rat model will be based on characterizing the biology of the diseased human Achilles tendon. This will include ethical consented harvest of portion of ruptured and diseased human Achilles tendons in my clinical practice of subspecialty Foot and Ankle Orthopedics and the Queen Elizabeth II Health Sciences center. This research will involve experiments that parallel those of the current PhD project and will utilize the established methodologies. Further studies to strengthen the reparative theory of Achilles tendon diseased will be focused on an investigation of the presence of collagen type III that will involve further collaborative studies using immunohistochemistry techniques and High Performance Liquid Chromatography to determine if collagen type III is present in increased quantities in humans with Achilles tendon disease and in Achilles tendons harvested from rats subjected to our overuse running model.

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Appendix 1: Tendinopathy Histopathology Literature Search Summary (Human)

Author	Histological Stain(s) Used	Histopathology Features
<u>Tendon Source: Human Subcutaneous Rupture (Achilles)</u>		
Arner et al. (1959)	H & E	edematous disintegration
	van Gieson (collagen)	Fiber Disorganization
	Weigarts elastin	
Kannus et al. (1991)	H & E	Increased cell #'s
	picrosirius (collagen)	more rounded cells
	orcein (elastin)	Fiber Disorganization
	Masson trichrom(denatured & Non denat collagen)	variations in fiber diameter
	Alcian Blue periodic acid-Schiff	mucoid degeneration
	Sudan Black	Increased GAG
Maffulli (2000)	H & E	Increased cell #'s
	Alcian Blue periodic acid-Schiff	more rounded cells
		Fiber Disorganization
		Varied Staining affinity
		Increased Vascularity
		Increased GAG
		Increased Hyalinization

Tendon Source: Human Tendinosis (Achilles)

Astrom & Rausing (1995)	H & E	Fiber Disorganization
	van Gieson (collagen)	Varied Staining affinity
	Prussian Blue (iron)	Decreased Birefringence
	Birefringence	Increased ground substance
		Increased cell #s in rows
		more rounded cells
		regional decreased cell #s
		hypervascular (irregular)
		sparse iron deposits
		fibrinogen
		no inflammation
		70% normal paratenon
Movin et al. (1997)	H & E	Increased cell #'s
	Alcian Blue periodic acid-Schiff	more rounded cells
		Fiber Disorganization
		Increased Vascularity
		Decreased Collagen Stainability
		Hylalinization
		Increased GAG Content

Appendix 2: Tendon Histopathology Literature Search Summary (Animal)

Author	Histological Stain(s) Used	Histopathology Features
Tendon Source: Animal Tendinosis (Achilles)		
Backman et al. (1990) Kickicking Machine Rabbit	H & E	Varied Staining affinity Fiber Disorganization Varied nuclear appearance Paratenon thickness increased Paratenon fibrosis Paratenon Edema Paratenon hypervascularity Paratenon inflammation
Messner et al. (1999) Kickicking Machine Rat	H & E Alcian Blue periodic acid-Schiff Immunohistochemistry (collagen I & II) Immunohistochemistry (neurofilament) Immunohistochemistry (calcitonin gene related peptide) Immunohistochemistry (substance P)	Fiber Disorganization Hypervascular Decreased proteoglycans Paratenon increased nerves Paratenon increased CGRP Paratenon increased SP
Tendon Source: Animal Tendinosis (Supraspinatus)		
Soslowsky et al. (1996) Collagenase Acromial impingement Rat		Increased cell #'s more rounded cells Fiber Disorganization No tendon inflammation Paratenon inflammation (some)

Carpenter et al.1 (1998) Collagenase Acromial impingement overuse running Rat	H&E	Increased cell #'s more rounded cells Fiber Disorganization
Soslowsky et al. (1999) overuse running Rat	H & E	Increased cell #'s more rounded cells Fiber Disorganization