THE INDIVIDUAL AND COMBINED EFFECTS OF EXERCISE AND COLLAGENASE ON THE RODENT ACHILLES TENDON

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ABSTRACT
Rachel Candace Dirks

THE INDIVIDUAL AND COMBINED EFFECTS OF EXERCISE AND COLLAGENASE ON THE RODENT ACHILLES TENDON

Tendinopathy is a common degenerative pathology that is characterized by activity related pain, focal tendon tenderness, intratendinous imaging changes, and typically results in changes in the histological, mechanical, and molecular properties of the tendon. Tendinopathy is difficult to study in humans, which has contributed to limited knowledge of the pathology, and thus a lack of appropriate treatment options. However, most believe that the pathology is degenerative as a result of a combination of both extrinsic and intrinsic factors.

In order to gain understanding of this pathology, animal models are required. Because each tendon is naturally exposed to different conditions, a universal model is not feasible; therefore, an appropriate animal model must be established for each tendon susceptible to degenerative changes. While acceptable models have been developed for several tendons, a reliable model for the Achilles tendon remains elusive. The purpose of this dissertation was to develop an animal model of Achilles tendinopathy by investigating the individual and combined effects of an intrinsic and extrinsic factor on the rodent Achilles tendon.

Rats selectively bred for high capacity running and Sprague Dawley rats underwent uphill treadmill running (an extrinsic factor) to mechanically overload the Achilles tendon or served as cage controls. Collagenase (intrinsic factor) was injected into one Achilles tendon in each animal to intrinsically break down the tendon. There were no interactions between uphill running and collagenase injection, indicating that the

influence of the two factors was independent. Uphill treadmill running alone failed to produce any pathological changes in the histological or mechanical characteristics of the Achilles tendon, but did modify molecular activity. Intratendinous collagenase injection had negative effects on the histological, mechanical, and molecular properties of the tendon.

The results of this dissertation demonstrated that the combined introduction of uphill treadmill running and collagenase injection did not lead to degenerative changes consistent with human Achilles tendinopathy. Intratendiouns collagenase injection negatively influenced the tendon; however, these changes were generally transient and not influenced by mechanical overload. Future studies should consider combinations of other intrinsic and extrinsic factors in an effort to develop an animal model that replicates human Achilles tendinopathy.

Stuart J. Warden, Ph.D., PT, Chair

TABLE OF CONTENTS

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xii

GLOSSARY OF TERMS xiv

CHAPTER ONE: INTRODUCTION

1.1 Introduction 1

1.2 Tendon anatomy and function 2

1.2.1 Macroscopic tendon structure 2

1.2.2 Microscopic tendon structure 3

1.2.3 Tendon metabolism 10

1.2.4 Tendon function and biomechanics 11

1.3 Human tendon pathology 17

1.3.1 Pathology 17

1.3.2 Etiology 26

1.3.3 Pathogenesis 31

1.3.4 Prevalence 33

1.3.5 Treatment 34

1.4 Models of tendinopathy 40
1.4.1 *In vitro* models 41
1.4.2 *Ex vivo* models 42
1.4.3 *In vivo* models 43
1.5 The Achilles tendon 48
1.5.1 Anatomy and function 48
1.5.2 Etiology and pathology 50
1.5.3 Symptoms and diagnosis 52
1.5.4 Treatment 53
1.6 Summary and aims 56
1.6.1 Tendinopathy summary 56
1.6.2 Dissertation overview 56
1.6.3 Aims 60

CHAPTER TWO: THE EFFECT OF UPHILL RUNNING ON THE HISTOLOGICAL APPEARANCE OF THE ACHILLES TENDON IN HIGH CAPACITY RUNNING RATS

2.1 Introduction 62
2.2 Methods 64
2.2.1 Animals 64
2.2.2 Treadmill running 64
2.2.3 Histology 66
2.2.4 Statistics 67

2.3 Results....68
2.4 Discussion....70

CHAPTER THREE: THE EFFECTS OF UPHILL RUNNING AND COLLAGENASE INJECTION ON THE ACHILLES TENDON IN HIGH CAPACITY RUNNING RATS

3.1 Introduction....73
3.2 Methods....75
3.2.1 Animals....75
3.2.2 Intrinsic factor....76
3.2.3 Extrinsic factor....77
3.2.4 Histopathological analysis....77
3.2.5 Mechanical testing....79
3.2.6 Gene expression....81
3.2.7 Statistics....82
3.3 Results....82
3.3.1 Histopathological appearance....86
3.3.2 Mechanical properties....86
3.3.3 Gene expression....89
3.4 Discussion....89

CHAPTER FOUR: THE EFFECTS OF UPHILL RUNNING AND COLLAGENASE INJECTION ON THE ACHILLES TENDON IN

SPRAGUE DAWLEY RATS

4.1 Introduction....96

4.2 Methods....97

4.2.1 Animals....97

4.2.2 Treadmill acclimation....98

4.2.3 Intrinsic factor....98

4.2.4 Extrinsic factor....99

4.2.5 Histopathological analysis....99

4.3 Results....102

4.4 Discussion....106

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

5.1 Dissertation summary....108

5.2 Strengths and limitations....109

5.3 Future directions....111

APPENDIX

Representative photomicrographs of the graded histological characteristics....114

REFERENCES....117

CURRICULUM VITAE

LIST OF TABLES

2.1	Running protocol used for the running group of rats	65
2.2	Semiquantitative scale used to grade tendons	68
2.3	Weekly running distances by rats in the run group	69
2.4	Differences in individual histopathological categories and total histopathological score in Achilles tendons	70
3.1	Running protocol used for the running group of rats	78
3.2	Weekly running distances by rats in the 4 week run group	84
3.3	Weekly running distances by rats in the 10 week run group	85
3.4	Collagenase and treadmill running effects on histolopathological characteristics	88
4.1	Running protocol used for the running group of rats	100
4.2	Semiquantitative scale used to grade tendon ground substance	101
4.3	Weekly running distances by rats in the run group	103
4.3	Collagenase and treadmill running effects on histopathological characteristics	105

LIST OF FIGURES

1.1	Structural hierarchy of collagen	7
1.2	The organization of tendon structure	8
1.3	Tendon stress-strain curve	16
1.4	Synthesis pathway of inflammatory mediators	24
1.5	Failed healing theory for the pathogenesis of tendinopathy	32
1.6	Progression of difference between high and low capacity runner rats in running distance during untrained phenotype endurance testing	58
2.1	Cumulative distance ran on the treadmill by rats in the run group	69
2.2	Representative photomicrographs of the Achilles tendon	70
3.1	Collagenase injection into the Achilles tendon	76
3.2	Set-up for the mechanical testing of rat Achilles tendons	80
3.3	Cumulative distance ran on the treadmill by rats in the run group	83
3.4	Collagenase and treadmill running effects on the histological presentation of the Achilles tendon	87
3.5	Collagenase and treadmill running effects on the mechanical properties of the Achilles tendon	90
3.6	Collagenase and treadmill running effects on the gene expression of the Achilles tendon	91
4.1	Cumulative distance ran on the treadmill by rats in the run group	102
4.2	Collagenase and treadmill running effects on the Achilles tendon	104

LIST OF ABBREVIATIONS

5-HPETE	arachidonic acid 5-hydroperoxide
5-LO	5-lipoxygenase
COX	cyclooxygenase
CSA	cross-sectional area
CTGF	connective tissue growth factor
ECM	extra-cellular matrix
ER	endoplasmic reticulum
FLAP	5-lipoxygenase-activating protein
GAG	glycosaminoglycan
HCR	high capacity runner
IL	interleukin
LTB_4	leukotriene B_4
MMP	matrix metalloprotease
MRI	magnetic resonance imaging
MTJ	myotendinous junction
NK-1R	neurokinin-1 receptor
NSAIDS	non-steroidal anti-inflammatory drugs
OTJ	osteotendinous junction
PDGF	platelet-derived growth factor
PLA_2	phospholipase A_2

PRP	platelet-rich plasma
SP	substance P
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of metalloprotease
TNF	tumor necrosis factor
TSCs	tendon stem cells
VEGF	vascular endothelial growth factor

GLOSSARY OF TERMS

Term	Definition
Aggrecan	A protein within the extracellular matrix that withstands compression
Anaerobic glycolysis	The transformation of glucose to pyruvate which serves as a means of energy production when limited amounts of oxygen are available
Angiogenesis	The formation of new blood vessels from pre-existing vessels
Autocrine	A form of cell signaling in which a cell binds and responds to a chemical messenger or hormone that was produced and released by itself
Compression	A force resulting in the shortening of an object
Concentric loading	Shortening of the muscle-tendon unit
Creep loading	The application of a continuous force on an object
Cyclical loading	The application of a fluctuating force upon an object
Cytokine	Small, secreted proteins that function as autocrine or paracrine signaling molecules in cell communication for immunoresponse, growth and development, and injury repair
Decorin	A small leucine-rich proteoglycan that binds to type I collagen and plays a role in matrix assembly
Dorsiflexion	Bending toward the back (e.g. bringing the top surface of the foot toward the front of the leg)
Dynamometry	The measurement of energy used during work
Ehlers-Danlos syndrome	Connective tissue disorders caused by a defect in the synthesis of collagen
Elasticity	A property of materials that opposes deformation when exposed to external forces and allows the material to return to its original form once the force is no longer applied

Ex vivo	"Out of the living", refers to the study of processes on Intact tissue outside of the living organism
Fibrosis	The formation or development of excess fibrous connective tissue
Flexion	Bending or decreasing the angle of a joint
Glycoprotein	A group of proteins with a carbohydrate component which are able to bind macromolecules or cell surfaces together. Examples include fibronectin, thrombospondin, tenascin-C, and undulin, which interact with collagen fibrils to increase mechanical stability of the extra cellular matrix
Ground reaction forces	The force exerted by the ground on a body in contact with it. The force is equal in magnitude and opposite in direction To the force that the body exerts on the surface
Hyperpronation	In the foot, excessive inward roll of the foot at the subtalar talocalcaneonavicular joints resulting in the sole of the foot facing laterally
In vitro	"Within glass", refers to the study of components of an organism that have been isolated from their natural surroundings (i.e. in culture)
In vivo	"Within the living", refers to the study of processes occurring within the living organism
In vivo microdialysis	A technique for measuring extracellular fluid and responses to exogenous agents by inserting a small probe with a semipermeable membrane into living tissue
Krebs cycle	A series of chemical reactions which generate energy through the oxidation of acetate derived from carbohydrates, fats, and proteins into carbon dioxide
Marfan syndrome	A genetic disorder of the connective tissue which affects the connective protein fibrillin-1, a glycoprotein essential for the formation of elastic fibers
Menke kinky hair syndrome	A disorder that affects copper levels in the boy, leading to copper deficiency that may result in diminished tendon reflexes

Paracrine	A form of cell signaling in which a target cell binds and responds to a chemical messenger or hormone that was produced and released by a nearby cell
Pentose phosphate pathway	A pathway for the metabolism of glucose in which five-carbon sugars are synthesized and nicotinamide adenine dinucleotide phosphate (NADPH) is produced
Phagocytosis	The process by which phagocytes ingest or engulf other cells or particles
Pinocytosis	The ingestion of fluid into a cell by turning a portion of the cell membrane inward to form a sheath that is pinched off to form an internal vesicle
Reactive oxygen species	Chemically reactive molecules containing oxygen which are a natural byproduct of normal metabolism and have a role in cell signaling and homeostasis; however, increased levels may damage cell structures, resulting in oxidative stress
Sclerosing agents	Chemical irritants that destroy vasculature in the localized region
Shear	A force applied parallel to the cross-section of an object, as opposed to normal stresses applied perpendicularly
Strain	The deformation caused by the action of stress on an object defined as the change in length per original length. Strain is (+) in tension and (-) in compression
Stiffness	The rigidity of an object determined by the extent to which it resists deformation in response to an applied force
Stress	(σ) An internal distribution of force (load) per unit area that balances external loads applied to a body. $\sigma = F/A$, where F = force applied and A = cross-sectional area
Young's modulus	The measure of the stiffness of an elastic material that may be calculated from the slope of the linear region of a stress-strain curve. May also be referred to as the tensile or elastic modulus
Tendinitis	Inflammation of the tendon

Tendinopathy	A broad term encompassing disease conditions occurring in and around tendons
Tendinosis	Degeneration of the tendon
Tension	A force resulting in the stretching of an object
Ultimate force	The maximum load a material can withstand before failure
Undulin	A glycoprotein in the extracellular matrix found between densely packed, mature collagen fibrils.

CHAPTER ONE: INTRODUCTION

1.1 Introduction

The musculoskeletal system, consisting of bones, muscles, ligaments, and tendons, is a dynamic system that serves several functions in the body, including mechanical support, mineral homeostasis, hematopoiesis, and motion. For motion to occur, the connective tissue components (ligaments and tendons) are essential. To carry out this function, ligaments connect bone to bone and tendons connect muscle to bone. Tendons are dense bands of fibrous connective tissue that were originally thought to simply transmit forces. However, more recent research has revealed complex elastic functions of the tendon. Tendons are viscoelastic structures that undergo growth and remodeling in response to mechanical loading, but may be susceptible to pathology and injury as a result of a variety of factors. Although the understanding of tendons has advanced greatly, major voids in the knowledge of tendons remain. This lack of knowledge extends into the pathology, which has prevented the development of appropriate treatment options, and thus tendon pathology, once present, often persists throughout an individual's life. Because of the limited number of studies pertaining to tendons and tendinopathy, discrepancies in findings are prevalent and there is a need for research on both the normal and pathological characteristics and functions of tendons.

1.2 Tendon anatomy and function

1.2.1 Macroscopic tendon structure

Structurally, tendons have two points of attachment: the myotendionus junction (MTJ) between the tendon and muscle, and the osteotendionous junction (OTJ) between the tendon and bone. These attachments are referred to as the origin at the MTJ and insertion at the OTJ. At the origin, the collagen fibrils of the tendon protrude deep into the myofibroblasts, allowing forces generated by the contractile proteins of the muscle to be transmitted to the tendon collagen fibers [1-3]. This junction is the weakest point of the muscle-tendon unit [4,5]. There are two types of insertions, referred to as the fibrous enthesis and fibrocartilaginous enthesis, with the former describing the tendon attaching directly to the periosteum and the latter consisting of a transitional zone [6,7]. The transitional zone is an area where chondrogenesis has occurred and is composed of four different regions: dense, fibrous connective tissue; uncalcified fibrocartilage; calcified fibrocartilage; and bone. Although these four regions exist within the transitional zone, there are no distinct boundaries between regions, which instead appear as a gradual transition from tendon to calcified bone [8].

Gross observation reveals a white color of tendons with variation in their shape, depending on the muscles and bones to which they attach. Typically, the tendons of more powerful muscles are short and broad, while tendons of muscles performing more delicate movements are long and slender. Tendons may also be surrounded by a combination of other structures, depending upon their location and function. Many structures exist to reduce friction as the tendon moves through its course, including

retinacula, reflection pulleys, synovial sheaths, peritendinous sheaths, and tendon bursae. Retinacula are the canals through which tendons move, and reflection pulleys reinforce the retinacula along curves in order to keep the tendon on its proper course. Synovial sheaths consist of multiple layers and contain peritendinous fluid to prevent friction. These sheets are quite rare, and instead, most tendons have peritendinous sheets (paratenon). The paratenon is made of loose fibrillar tissue and prevents the tendon from moving against surrounding tissues. Finally, the tendon bursae exist to prevent bony prominences from compressing the tendon.

1.2.2 Microscopic tendon structure

Tendons, much like muscle, are organized into a hierarchical structure of fibrils, fibers, and fiber bundles which ultimately form the gross tendon structure. Most tendons are surrounded by the paratenon, which consists of type I collagen, type III collagen, and elastic fibrils, along with a lining of synovial cells on its inner surface [9-11]. Under the paratenon lies the epitenon, which is a thin connective tissue sheath surrounding the entire tendon. The epitenon is made of longitudinal, oblique, and transverse collagen fibrils, which exhibit uniform density and organization and may also fuse to superficial tendon fibrils [12]. The endotenon lies under the epitenon and surrounds each tendon fiber and fiber bundle [12,13]. This layer is made of collagen fibrils in a crisscross pattern [13-15] and carries vessels, nerves, and lymphatics into the tendon [13,16].

a) Tendon cells

Tenoblasts and tenocytes, which are homologous to fibroblasts and fibrocytes, comprise the majority of tendon cells. These cells lie between the collagen fibers and are responsible for production of the extra-cellular matrix (ECM) [16]. In addition, chondrocytes may be found near the insertion, synovial cells are located in the tendon sheath, and cells associated with vascularity (endothelial and smooth muscle cells) are located in the endo- and epitenon. More recently, tendon stem cells (TSCs) have been identified in mice, rats, rabbits, and humans [17-20]. TSCs are able to self-renew, as well as differentiate into adipocytes, chondrocytes, osteocytes, and tenocytes [19]; therefore TSCs are important for tendon maintenance and repair [21].

While adult tendons have minimal cellularity, immature tendons have a much greater number of cells. The tenoblasts found in newborn tendons are arranged in long, parallel chains and vary in shape and size, including long, round, and polygonal [22]. These cells are responsible for the formation of the ECM and their composition enables the high metabolic activity responsible for the synthesis of the matrix. This includes well developed rough endoplasmic reticulum (ER), Golgi apparatus, and long, slender cytoplasmic processes forming desmosomal, tight, and gap junctions between cells [22,23]. The rough ER and Golgi apparatus are vital in the production of proteoglycans and glycoproteins found in the ECM, with the protein being formed in the rough ER and the glycidic portion in the Golgi apparatus [24]. The intercellular junctions are important as they are likely involved in coordinating the response to loading of the tendon [25].

With aging, the number of cells decreases as the amount of matrix increases. During this process, the majority of tenoblasts become tenocytes, as they elongate and undergo changes in composition, leaving them with a nucleus and very little cytoplasm. The elongation is believed to allow for continued contact between the cells and matrix as the number of cells decreases [23]. Because these cells are still metabolically active, the rough ER and Golgi apparatus remain well-developed [26].

b) Extracellular matrix

The main function of the extracellular matrix (ECM) is structural support of the tendon during the transmission of force in the muscle-tendon complex. This strength is dependent on the intra- and intermolecular cross-links and the orientation, density, and length of the collagen fibers. Turnover of the ECM is influenced by physical activity with more turnover existing in tendons that undergo regular physical activity and inactivity leading to decreased ECM turnover [27]. Collagen makes up the majority of the ECM, but the strength is dependent upon the coordination of collagen with many other molecules, including elastic fibers, ground substance, and inorganic components.

Collagen- Tendons are composed of 65-80% collagen and 1-2% elastin within a proteoglycan-water matrix [28-31]. Of the collagen, approximately 90% is type I, less than 10% is type III, and the remainder is any combination of the other forms of collagen [32]. The basic unit of collagen is the α chain, which is a repeating triplet of amino acids (Glycine-X-Y), where X and Y are often proline and hydroxyproline, which contribute to

the rigidity of collagen, as areas lacking proline and hydroxyproline have more flexibility [33,34]. Three α chains then combine to form a triple helix called procollagen, which is roughly 1000 amino acid residues with a length of about 300 nm [35]. The procollagen molecule contains non-helical extensions at each end [36,37], named the amino- (N) and carboxylic- (C) propeptides. The N- and C- propeptides are removed by proteinases prior to the final collagen fibril assembly [38]. Collagen molecules assemble into five row microfibrils, which range from 20 to 280 nm in diameter [39], with larger diameters typically in mature or large tendons. In this configuration, collagen molecules align end-to-end in a staggered pattern leaving a 67 nm gap zone between ends [35] (Figure 1.1). This arrangement leads to the banding pattern that is visible under magnification. Additionally, a crimping pattern is visible under magnification in several tendons [40,41]; however, the geometry of the pattern differs between tendons.

Once in this arrangement, cross-linking can occur between collagen molecules, which affect the overall strength of the collagen. Enzymatic cross-links include lysylpyridinoline, which exist in small quantities near the bone insertion [42,43] and hydroxylysylpyridinoline, which are increased in older tendons [44] and tendons undergoing greater mechanical stresses [45]. Non-enzymatic cross-linking may also occur, resulting in irreversible binding of sugars to matrix proteins [42].

The collagen fibrils form the base of the hierarchical structure of the tendon and aggregate to form collagen fibers. The collagen fibers combine to form a primary fiber bundle (subfascicle), which in turn, combine to form a secondary fiber bundle (fascicle). Finally, the fascicles congregate to form a tertiary bundle, and the tertiary bundles make up the tendon (Figure 1.2).

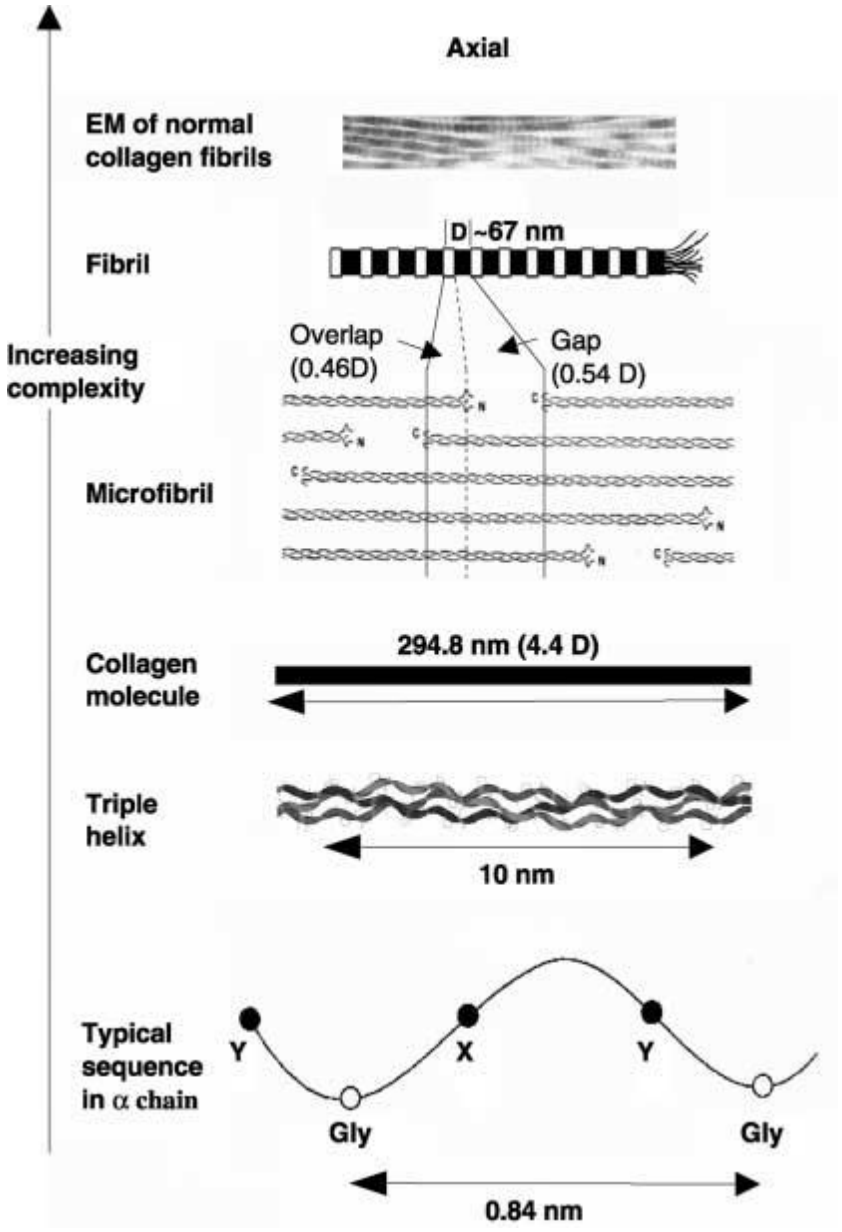


Figure 1.1. Structural hierarchy of collagen (Reproduced from Orgel, et al. [46] with permission from Elsevier).

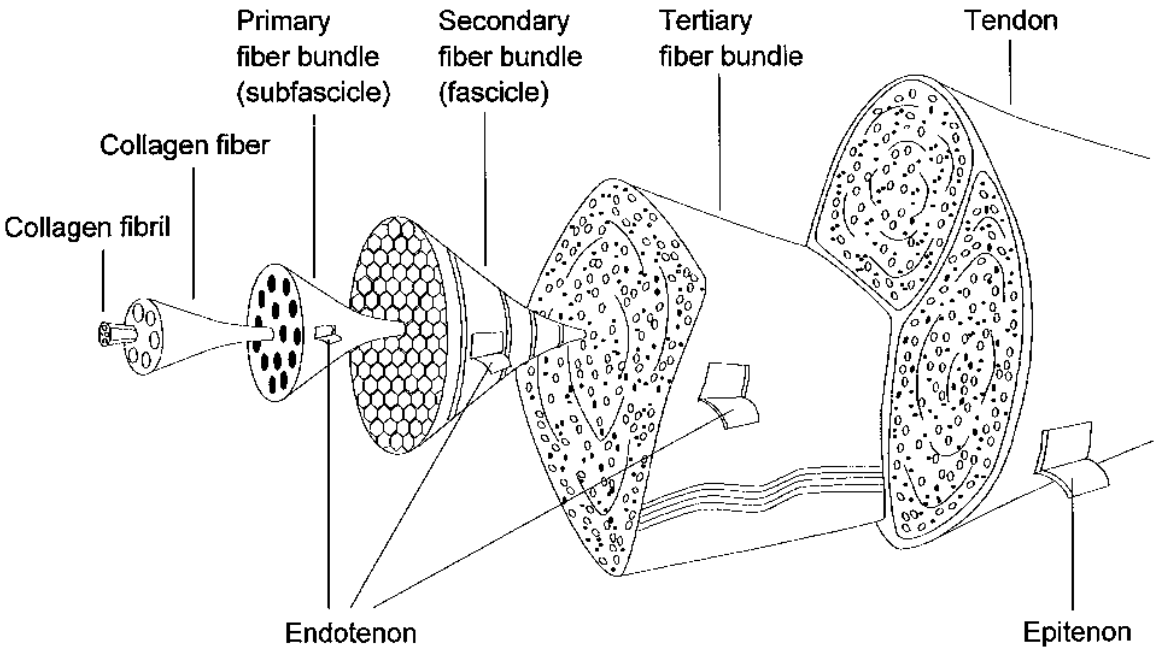


Figure 1.2. The organization of tendon structure (Reproduced from Kannus [26] with permission from John Wiley and Sons).

Ground substance- In tendons, the ground substance surrounds the collagen and consists of proteoglycans, glycosaminoglycans (GAGs), structural glycoproteins, and a variety of other molecules. Proteoglycans are defined by a protein core covalently bonded to one or more GAGs. They are negatively charged, hydrophilic molecules and are typically located within and between collagen fibrils and fibers [26]. Because of their charge, proteoglycans are stiff and resistant to compressive and tensile forces. The amount of proteoglycan within tendon is dependent upon mechanical loading conditions of the tendon, with tendons undergoing greater loads having greater amounts of proteoglycans [47-50]. Examples of proteoglycans within tendons are aggrecan, which holds water and resists compression [50] and decorin, which enables sliding of the fibrils during mechanical deformation [51]. Additionally, proteoglycans may inhibit calcification of the tendon by filling the gap zones between collagen fibrils where minerals are able to deposit [52].

Glyocoproteins are groups of proteins with a carbohydrate component and are able to bind macromolecules or cell surfaces together. Fibronectin, thrombospondin, tenascin-C, and undulin are examples of glycoproteins that have been identified in tendons [53-55], with fibronectin facilitating wound healing [56,57] and all glycoproteins interacting with collagen fibrils to increase mechanical stability of the ECM [58].

Elastic fibers- Elastic fibers are sparse within tendons and are estimated to only account for 1-2% of the dry weight [29]. This percentage may be even less in humans, as Jozsa and Balint [59] found measurable amounts of elastic fibrs in only 10% of healthy human tendons. However, the number and volume of elastic fibers in tendons are increased in patients with certain diseases, such as Ehlers-Danlos syndrome and chronic uremia [59,60].

Elastic fibers consist of two distinct components: elastin and microfibrils. Elastin forms the central core, which comprises about 90% of the elastic fiber. The microfibrils have a diameter of 10-12 nm and form a sheath that surrounds the elastin central core [61]. Once formed, the elastic fibers are oriented parallel to the basic tissue organization of the tendon [62]. These fibers are found in many tissues, including arteries, lungs, cartilage, and connective tissue and have a primary function of recoil, allowing the elastic tissues to undergo repeated stretch and relaxation cycles [62]. Aside from this main function, the specific function of elastic fibers in tendons is unclear, but they are thought to contribute to the recovery of the wavy configuration of the collagen fibers after the tendon is released from a stretch [63].

Inorganic elements- Inorganic components are very scarce in tendons, but a variety have been detected [64,65], with calcium being the most common. These elements are usually found in limited quantities but may function in growth, development, and normal metabolism of musculoskeletal structures [66,67]; however, the actual role of many of these elements is unknown.

1.2.3 Tendon metabolism

Tenocytes are capable of the aerobic Krebs cycle, pentose phosphate pathway, and anaerobic glycolysis, all of which are pathways of energy metabolism necessary for energy production and biosynthesis of the matrix [30,31,60,68]. The ratio of the utilization of these pathways changes during growth of the tendon, as the three pathways are highly active in immature tendons, but only anaerobic glycolysis remains active in adult tenocytes. Therefore, the metabolic pathways of tendons become more anaerobic with age [16,69]. Also changing with age is the synthetic activity, as growing tendons are actively synthesizing collagen, elastic fibers, proteoglycans, and glycoproteins, but this activity decreases with age [70].

Little is known about the metabolism of the tendon matrix with the exception of collagen. Similar to the other tendon characteristics, collagen metabolism changes with age. The rate of collagen production is very high in infancy and drastically reduces as the tendon ages. The rate of collagen turnover remains low throughout adulthood with only areas of newly synthesized collagen being metabolically active [71].

Even less is known about matrix catabolism, but using knowledge of other connective tissue degradation, it may occur in two ways: 1) lysosomes or cytoplasmic

degradative enzymes are produced by tenocytes and secreted into the ECM, and 2) degradation through phagocytosis and pinocytosis [26].

The combination of the low metabolic rate and anaerobic metabolism is crucial for the tendon to effectively carry out its function of carrying loads and remaining in tension for long periods, as they can continue to function with little or no oxygen. However, this combination also has a negative impact of lengthening recovery and healing times after activity or injury [11,72]. This consequence is twofold, because oxygen is required for the synthesis of collagen [73,74] and the low metabolic rate prevents quick repair of any damage done to the tendon, which may be further aggravated by activity prior to full recovery.

1.2.4 Tendon function and biomechanics

Historically, tendons were simply described as transmitting forces from muscle to bone, allowing for motion of the bone. More recently, research has focused on the effect of tendon elasticity on motion.

a) Elasticity

Tendons function as springs, which are defined by deforming when a force is applied and recoiling to the resting shape when the force is released. During deformation, the material of a spring stores energy in the form of elastic strain energy, which is released when the spring recoils. The amount of energy that is stored depends on the stiffness of the material and the deformation of the material. Furthermore, the spring

cannot produce additional energy, it may only release the energy loaded by the external source. While this action of a spring appears relatively simple, the springing action of tendons serves many functions, including conservation of energy, amplification of muscle power output, and attenuation of muscle power input [75].

First, in birds and moderately large animals, including humans, tendons decrease the amount of metabolic energy required for locomotion. For example, the Achilles tendon undergoes stretch and recoil as the ankle flexes and extends, allowing the muscles to remain at a constant length, thus decreasing the amount of work required by the muscles [76,77]. This action may also benefit animals when swimming or flying, as the tendons may lead to energy savings as the fins, tails, or wings undergo repeated acceleration and deceleration [78]. However, this benefit is lessened in smaller animals, as their Achilles tendons undergo very little stretch, requiring the muscles to do more work [79].

Second, tendons have accelerated recoil and can amplify muscle power, which is beneficial in many ways, including enhanced jumping ability. When muscles contract quickly, they exert less force [80], but tendons are able to amplify muscle power output by storing the muscle work slowly and releasing it rapidly. The energy released by the tendon is roughly equal to the overall amount of work done by the muscle during contraction, but it is released in a shorter amount of time. Because power = work/time, this leads to power output that exceeds the capacity of the muscle [75]. Since tendons can recoil faster than muscles are able to shorten [81], animals are able to maximize the power from the muscle and thus jump higher and further [82-84]. This amplification of muscle power in jumping has been observed in many animals, including frogs [84,85],

bushbabies, [86], birds [87], and humans [88]. Additionally, this muscle power amplification can be observed in accelerating animals, such as turkeys [89] and horses. For example, in horses, the power output of the biceps brachii is amplified more than 50 times during rapid bursts [90].

Tendons also contribute to the attenuation of mechanical power produced by muscles. Studies on isolated muscle-tendon units and *in vivo* studies have both revealed that when muscle-tendon units are rapidly stretched, the tendon may stretch, while the muscle remains at either the same or a reduced length [91,92]. This stretching of the tendons likely plays a protective role for the muscles, which are susceptible to damage when fibers are actively lengthened [93,94]. However, because tendons are springs, they are unable to absorb the energy during stretch, only store it temporarily. Similar to the power amplification by tendons, this temporary storage of the power allows the muscle to absorb the energy more slowly, leading to an overall absorption of energy beyond the muscle's maximum capacity for energy absorption [75].

It is important to note that while the elasticity of tendons is primarily beneficial, the work they do may lead to overheating. Approximately 93% of the work done by tendons during stretching is returned during recoil, with the remaining 7% being dissipated as heat [78]. Because tendons have very little vascularity, this heat is not easily dissipated, and when an animal performs excessive repeated movements, this may lead to heat damage of the tendon [81,95].

b) Stiffness

The stiffness of an object describes its rigidity, or the extent to which it resists deformation in response to an applied force. Stiffness can be measured by calculating the slope of a force-displacement curve and represents the ratio of force applied to the tendon to its elongation in response to the force. Tendon stiffness is dependent upon the location and function of the tendon and may be influenced by tendon length and cross-sectional area (CSA). For example, a shorter tendon with a larger CSA would likely have greater stiffness, but the actual relationship between tendon morphology and stiffness is unclear [96]. In order to determine tendon mechanical properties independent of the geometric characteristics, Young's modulus can be calculated. Young's modulus is calculated by dividing tensile stress by tensile strain in the linear region of the stress strain curve and provides a measure of stiffness normalized to tendon CSA and length [97].

Stiffness is an important factor in the mechanical properties of the tendon, having a significant influence on force transmission and muscle power. To demonstrate this, Bojsen-Moller, et al. [98] investigated the relationship between the mechanical properties of tendon and muscle performance of the vastus lateralis muscle-tendon unit. Participants performed squat jumps and the researchers found correlation between the tendon stiffness and the power, force, and velocity of the jumps. This indicated that muscle output is positively correlated to the tendon stiffness, and the group hypothesized that increased stiffness allows for more effective force transmission. Therefore, an optimal level of tendon stiffness is essential for effective muscle-tendon interactions.

Tendons may undergo remodeling to ensure that the stiffness is optimized for that muscle-tendon unit, as studies have shown increased tendon stiffness in response to long term exercise [99-101]. Reeves, et al. [101] conducted a study investigating the effect of strength training on patellar tendon stiffness and found that leg extension and leg press exercises increased tendon stiffness by 65%. This increased stiffness resulted in a reduction in tendon elongation and strain, which decreases the possibility of tendon injury. While tendon stiffness does increase with exercise, it is unknown whether this change in stiffness is due to changes in tendon dimension, material properties, or both [99,102,103].

c) Strain

Strain is a measure of deformation of the tendon. Because tendons are viscoelastic tissue, they are sensitive to different strain rates. At low strain rates, the tendons absorb more energy but are less effective at transferring loads, while at high strain rates, they are less deformable but are more effective at transferring loads [104].

Strain is typically presented as a stress-strain curve, which depicts the amount of deformation (strain) at specific levels of tensile loading (stress). Stress (σ) is defined as the ratio of force (F) to the CSA (A) ($\sigma = F/A$). Stress-strain curves provide many important details about the mechanical properties of the tendon and have four distinct regions (Figure 1.3):

1) Toe region - the tendon is strained up to 2%. In this region, the crimp pattern of the tendon is stretched out. This crimp pattern provides insight into the

mechanical properties of the tendon, as tendons with a small crimp angle fail before those with a larger crimp angle. Differences in the crimp pattern exist in different types of tendons and different sites within the same tendon [105].

2) Linear region - the tendon is strained up to 4%, resulting in a loss of the crimp pattern. The Young's modulus is calculated from the slope of this region and represents the stiffness of the tendon.
3) Microscopic tearing - the tendon is strained above 4%.
4) Macroscopic failure – the tendon is strained beyond 8-10%. If the tendon undergoes further stretching, rupture will occur [63]. However, some tendons may be able to withstand much greater stresses, including the avian flexor tendon, which can be stretched up to 14% [106].

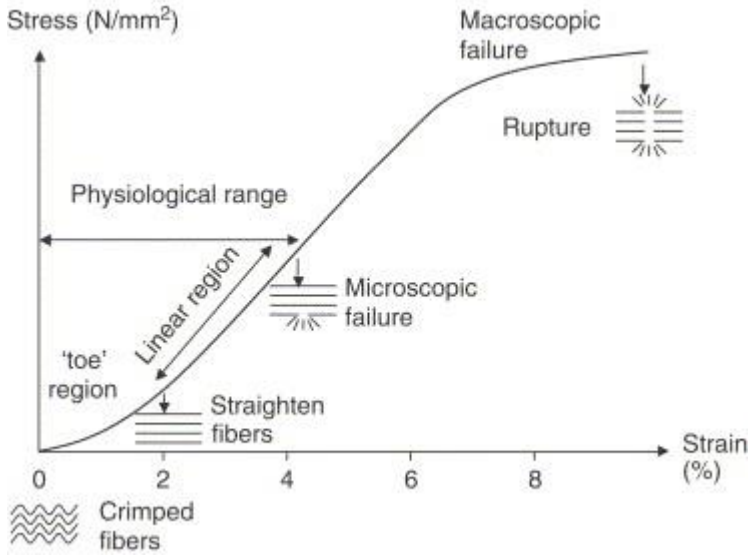


Figure 1.3. Tendon stress-strain curve (Reproduced from Wang [107] with permission from Elsevier).

Previous studies have been done on various tendons in both humans and animals and have found great variation in mechanical properties according to tendon location and

age. For example, the human patellar tendon has a Young's modulus of 660 ± 226 MPa in young donors and 504 ± 222 MPa in older donors [108]. Conversely, the anterior tibialis tendon has a Young's modulus around 1200 MPa [109].

1.3 Human tendon pathology

1.3.1 Pathology

Tendinopathy (*tendo*– = tendon, *–pathy* = disease) is characterized by activity-related pain, focal tendon tenderness, and intratendinous imaging changes [110] and typically results in changes in the histological, mechanical, and molecular properties of a tendon. Much debate has existed in the classification and nomenclature of tendinopathies. "Tendinitis", or inflammation of the tendon, has been commonly used to describe tendon disorders; however, after many histological studies, this term is now believed to be inaccurate. Puddu et al [111] originally used the term "tendinosis", or degradation of the tendon, to describe the tendon pathology. Kannus and Jozsa [69] later conducted an intensive study of human tendon histology and supported the use of tendinosis, finding that pathological tendons presented with degenerative, rather than inflammatory changes. Other studies have found that inflammation is infrequent and typically only occurs in cases of tendon rupture [112-115]. Therefore, tendinopathies are now widely regarded as "tendinosis" rather than "tendinitis".

a) Histological changes

Macroscopically, the degenerated areas of tendons appear soft, gray or yellow, and non-glistening [116]. Using light microscopy, tendinosis appears as changes in collagen, ground substance, and tenocytes. Collagen fibers lose their normal crimping pattern and undergo separation, losing their proper orientation. They may also exhibit decreased fiber diameter and density [104]. Furthermore, microtearing may occur throughout the tendon and an increase in the mucoid ground substance may exist in pathological tendons [117,118].

Cellularity and tenocyte morphology are variable in tendinosis. Pathological tendons may have tenocytes which are overly abundant, have rounded nuclei and an abundance of cytoplasm or they may be acellular and appear necrotic or apoptotic [104]. While there is variation in cellularity, an increase in vascularity is a hallmark feature in tendinosis. However, there is rarely an infiltration of lymphocytes, macrophages, or neutrophils [104].

Histological assessment is a standard method of evaluation for tendon research in both humans and animals [119]. The most widely accepted method of histopathological quantification is the Bonar score; however much variation exists in the characteristics analyzed as part of the score. For example, Maffulli, et al. [113] assessed fiber structure, fiber arrangement, rounding of the nuclei, regional variations in cellularity, increased vascularity, decreased collagen stainability, hyalinization, and GAG content on a scale from 0 to 3, with 0 being normal and 3 being the most abnormal. In contrast, Cook, et al. [120] condensed the analysis to only 4 categories: tenocyte morphology, ground

substance, collagen arrangement, and vascularity. Again, these categories were scored from 0-3, with 0 being normal and 3 being abnormal. Scott, et al. [121] assessed tenocyte morphology, tenocyte proliferation, collagen organization, GAGs, and neovascularization on a scale of 0 to 4, with 0 being normal and 4 being abnormal. Most recently, Fearon et al. [122] have conducted a study analyzing the variations in tendon histological assessment and evaluated the tendon cell morphology, collagen arrangement, cellularity, vascularity, and ground substance. These characteristics were graded from 0 to 3 with 0 being normal and 3 being abnormal. In addition, this study improved the standardization of the Bonar scale by investigating which area of the tendon should be assessed. Previous studies simply reported that the most pathological region of the tendon was assessed, which is difficult to reproduce. The study conducted by Fearon et al. evaluated which of the assessed characteristics should be used to define the most pathological region and concluded that scores measuring pathology were highest when assessing the area of worst cell morphology or collagen disruption. Furthermore, the study included a more specific description of how the tendon should be assessed by defining both the magnification and number of fields of view that should be analyzed for each category.

b) Mechanical changes

The mechanical properties of tendons are directly related to the arrangement of collagen fibers within the tendon [35]; therefore, it is expected that the altered collagen structure and arrangement in tendinosis would result in altered mechanical properties [107]. Studies analyzing the mechanical properties of tendinopathy are more limited than

those focusing on the histological presentation. Arya and Kulig [97] performed a study using real-time ultrasound imaging and dynamometry to assess the stiffness, Young's modulus, stress, strain, and CSA of pathologic Achilles tendons. They found that tendinopathic tendons had decreased stiffness and Young's modulus, and increased CSA. While an increased CSA is typically an indication of increased strength, the decreased Young's modulus and stiffness of these tendons indicate alterations in the tendon composition and structure. Specifically, the increased CSA is the result of several factors, including accumulation of water and ground substance [123], increased separation, crimping, and tearing of type I collagen, and increased levels of type III collagen [124]. Together, these changes lead to an increased CSA while weakening the mechanical properties of pathologic tendons.

c) Molecular changes

No definitive summary of gene expression in tendinopathy has been developed; however, many studies have had common findings when investigating mRNA expression in healthy versus pathological tendon tissue. Two of the most commonly studied genes are collagen and matrix metalloproteases (MMPs). Collagen is the major constituent in tendon tissue, with type I collagen being dominant. Type III collagen is typically present in low quantities, but in pathological tendons, both type I and type III collagen are increased [125-128]. MMPs are a group of zinc and calcium dependent endopeptidases which are responsible for ECM remodeling. They can be divided into four main groups: collagenases, which cleave types I, II, and III collagen; gelatinases, which cleave type IV

and denatured collagens; stromelysins, which degrade proteoglycans, fibronectin, casein, and types III, IV, and V collage; and membrane-type MMPs [129]. In tendons, MMP-1, -2, -3, and-13 have been found to be altered in tendinopathy. Of these, MMP-1 and -13 are collagenases, MMP-2 is a gelatinase, and MMP-3 is a stromelysin. MMP-1 and MMP-13 are upregulated in tendionpathy and tendon tears [130,131]. MMP-2 is also upregulated in tendinopathy [125,127,128,132], but may be upregulated or downregulated in tendon tears [133]. MMP-3 is downregulated in tendinopathy and tendon tears [125-128,130-132,134,135]. Therefore, the four groups of MMPs may have different effects on the tendon.

The activity of MMPs is inhibited by tissue inhibitors of metalloproteases (TIMPs) and the balance between MMP and TIMP activity regulates the remodeling of tendons. TIMPs inhibit MMPs by binding to the active site of MMPs, thus preventing the MMP from cleaving their particular substrates [136,137]. There are four types of TIMPs, TIMP-1, -2, -3, and -4, and all have been found to be downregulated in tendinopathy, with variable levels in tendon tears [127,131,133,138].

Transforming growth factor β (TGFβ) has been implicated in tendinopathy, as it is known to be a mediator of mechanically induced collagen synthesis in many different cell types [139-143]. In addition, connective tissue growth factor (CTGF) may play a role similar to TGFβ [144,145]. Studies typically examine the effect of loading on TGFβ and CTGF, with some reporting decreases in both TGFβ and CTGF [146], some finding increased levels of TGFβ [147,148] and CTGF [149], and others reporting no changes in TGFβ [150] or CTGF [148,150] following loading. Heinemeier et al. [146] speculate that these differences are due to short-term versus long-term loading, and hypothesize that

initial increases in TGFβ and CTGF exist, but then reduce once a steady state is reached. Therefore, short-term loading would result in increased levels and long-term loading would result in normal or decreased levels.

Vascularity of tendons varies by region on the tendon [151-156], but is typically minimal because of the few metabolic requirements of tendons [104]. The formation of new blood vessels, or angiogenesis, is controlled by the balance between stimulatory and inhibitory molecules, vascular endothelial growth factor (VEGF) and endostatin, respectively [157,158]. In normal adult tendons, endostatin, the anti-angiogenesis factor, is predominantly expressed [159], while fetal tendons express more VEGF [156,160]. Endostatin is a 20 kDa, C-terminal fragment of type XVIII collagen, which acts in several ways to inhibit angiogenesis, including inhibiting proliferation and migration of endothelial cells required for new vessels [161] and inhibiting VEGF signaling [162]. VEGF is a family of growth factors that result from alternative splicing of the VEGF gene. These growth factors work through a tyrosine kinase pathway to initiate angiogenesis. Because neovascularization is observed in tendinosis [163], increased levels of VEGF and decreased levels of endostatin are associated with degenerative tendons. Additionally, VEGF may have another role in tendon degeneration, as it is able to upregulate the expression of MMPs and downregulate the expression of TIMPs, leading to increased collagen degradation [164-166]

Scleraxis (Scx) has recently been identified as a regulator of embryonic tendon [167-169] by encoding for a transcription factor present in tendon progenitor cells during development through adulthood [168,169]. In tenocytes, Scx regulates transcription of type I collagen [170]. Further studies have revealed that scleraxis is coordinately

expressed in tendons after injury in animal models [171-173] and levels are increased following mechanical loading [174,175] and decreased following tendon unloading [174]. Therefore, scleraxis may play an important role in the adaptation to mechanical loading in tendons.

In addition to the aforementioned genes, inflammatory molecules and pathways may also have a role in tendinosis. Although tendinosis is degeneration rather than inflammation of the tendon, many groups have reported the presence of inflammatory markers, typically in the very early stages of pathology. Examples of inflammatory mediators are leukotrienes and prostaglandins, which are lipid molecules with autocrine and paracrine signaling capabilities. These molecules have many effects throughout the body; however, the effect most relevant to tendinopathy is the regulation of inflammation. Prior to examining the effects of inflammatory mediators in tendinopathy, it is important to understand their synthesis pathways (Figure 1.4). To begin, phospholipids are hydrolyzed by phospholipase A_2 (PLA_2), releasing arachidonic acid and lysophospholipids. Arachidonic acid is then converted to either endoperoxides or arachidonic acid 5-hydroperoxide (5-HPETE) by cyclooxygenase (COX) or 5-lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP), respectively. Endoperoxides are then converted to prostaglandins and 5-HPETE is converted to leukotrienes. A specific prostaglandin of interest is prostaglandin E_2 (PGE_2), which is also a potent inhibitor of type I collagen synthesis, leading to catabolic effects on the tendon structure [176-178].

There is a lack of consensus regarding any of these mediators and intermediate molecules in tendon pathology. Many *in vitro* studies have reported increased levels of

these molecules after mechanical deformation, including cytosolic and secretory PLA_2 [179], COX-1 and -2 [179,180], PGE_2 [180,181], 5-LO, and leukotriene B_4 (LTB_4) [182]. In addition, Fu, et al. [183] examined human tendinosis biopsies and *in vitro* tendinosis cultures and found increased expression of COX-2 and PGE_2. Other studies, however, have reported no increases in PGE_2 with *in vivo* microdialysis [184] or in cultured tendinosis specimens [185]. These contradictory findings contribute to the ongoing debate of inflammation versus degradation in tendinopathy and may be due to different processes occurring at different time points or locations of pathology in the tendons.

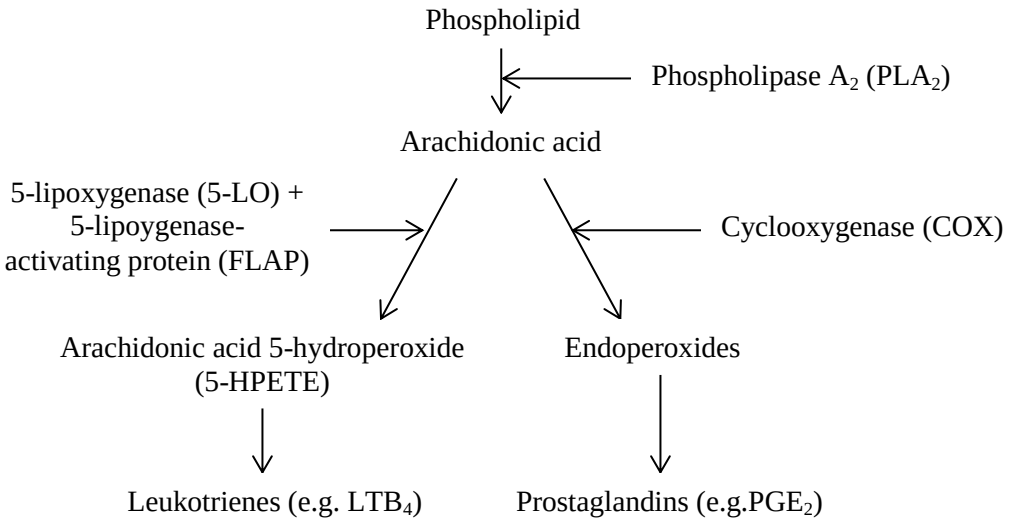


Figure 1.4. Synthesis pathway of inflammatory mediators.

Cytokines assosicated with inflammation, specifically interleukins (IL) and tumor necrosis factors (TNF), have also been investigated in tendinopathies. These cytokines are part of intricate inflammatory signaling pathways and also impact the prostaglandin pathway, as IL and TNF increase COX, and thus PGE_2 levels [186]. There is much debate over inflammatory cytokines and tendinosis, with some studies not detecting inflammatory cytokines in chronic tendinosis [127,132] and others reporting increased

levels of inflammatory cytokines during the three hours following mechanical loading. However, these levels decreased toward baseline at 12 hours post-loading [187], leading to speculation that inflammatory factors increase briefly following loading before returning to baseline.

Substance P (SP) is a neuropeptide that is produced by tenocytes [188] and its receptor, neurokinin-1 receptor (NK-1R) is found in blood vessel walls, nerves, and tenocytes in tendon [188]. SP has been associated with inflammation [189], angiogenesis [190], and cell proliferation [191,192] and the levels of SP and NK-1R have been shown to be increased in tendinosis. Because of the known effects of SP and NK-1R, it is thought that they are involved in angiogenesis and pain signaling in tendinosis [193]. While it is unknown whether the increased levels of SP and NK-1R are a cause or effect of tendinosis, a recent study has revealed that increased SP production occurs after the tendon undergoes mechanical loading; however, these elevated levels exist prior to tendinosis-like changes of the tendon. In this study, SP was noticeably elevated after one week of exercise, while tendinosis-like changes did not appear until at least three weeks of exercise [194]. This finding points to SP being a potential cause of tendinosis rather than an effect. In addition, Fong et al. [195] used human tenocytes in an *in vitro* study examining the effects of SP. They found an increase in type I collagen remodeling and increased levels of MMP-3, TIMP-1, and type III collagen in cells stimulated by SP, which supports the hypothesis that SP plays a role in the development or progression of tendinosis. While there is consensus among the current studies, the investigation of the role of SP in tendinosis is still in its infancy and many more studies are required before any definitive conclusions can be drawn.

1.3.2 Etiology

Understanding the etiology of tendinopathy is crucial in developing interventions that can be utilized prior to the onset of symptoms or tendon rupture. While little is known in this area of tendon research, several different factors have been identified and investigated. There is consensus that mechanical overuse is typically required for tendinopathies to exist; however, the definition of overuse is variable among individuals and may not be a factor in all cases, as sedentary individuals may also experience tendinopathy. Additionally, most agree that mechanical overuse is not the only factor contributing to tendinopathy, which warrants the investigation of all factors that may be involved, either independently or in combination. Chronic overuse injuries, including tendinopathies, are frequently the result of an interaction between intrinsic and extrinsic factors. While there is no specific combination for any tendinopathy, there are several common intrinsic and extrinsic factors that may have an effect on the pathophysiology of tendon disorders.

a) Intrinsic factors

Intrinsic factors are typically a result of the genetic makeup that may predispose an individual to tendinopathies. For example, variants of the Col5A1 [196], tenascin C [197], and MMP-3 [198] genes have been associated with increased risk of Achilles tendon injuries. Intrinsic factors might also be expressed as a phenotype. For example, in the legs, misalignments and leg length discrepancy may contribute to tendinopathy.

Hyperpronation is a common misalignment of the foot that is the result of excessive calcaneal eversion and it produces a whipping or bowstring action on the Achilles tendon [199]. This action places eccentric load on the medial side of the Achilles tendon, which may predispose it to injury [200-203]. In addition, hyperpronation may contribute to pathology of the posterior tibialis and patellar tendons as well as the iliotibial band. Patellar tendinopathies may also develop in individuals with long patellar tendons, high riding patella, or lateral displacement of the patella [204]. Muscle weakness [205-207], decreased flexibility, and joint hypermobility [208-210] have also been speculated as factors contributing to tendinopathies; however, it is unknown if these are causes or consequences of musculoskeletal injuries.

Because females often have weaker musculoskeletal systems that are less able to absorb repetitive loads, gender has been implicated as a factor contributing to tendinopathy [204]. However, in studies of patellar and Achilles tendinopathies in adolescents and adults, nearly two times as many males experienced tendon pathology [211-213].

Age is also linked to tendinopathy with both younger and older tendons being more likely to develop pathology. Because young tendons are small and unable to withstand large stresses [214], overloading the tendons may lead to tendinopathy [215]. Older tendons have increased stiffness and decreased ultimate strain and load, which decrease the tendons ability to absorb and recover from loading. These mechanical changes in older tendons are likely a result of a decrease in proteins required for collagen alignment [215] and changes in collagen cross-linking [216] and may be amplified by decreased vascularity [69,217].

Diseases affecting collagen, such as Ehlers-Danlos syndrome, Marfan syndrome, and Menke kinky hair syndrome increase the likelihood of developing tendinopathy, and individuals with these conditions are typically discouraged from participating in sports that may lead to overuse. Other diseases, including systemic lupus erythematosus [218], rheumatoid arthritis [219], glycogen storage disease [220], psoriasis [221], and vitamin C deficiency [30], may also predispose a tendon to pathology or rupture.

b) Extrinsic factors

Extrinsic factors are defined as all factors acting externally on the human body [204]. In tendinopathies, the extrinsic factors typically include an excessive load, training errors, or changes in the environment. The amount of load required to become excessive varies among individuals and may be considered excessive in terms of training type, volume, intensity, or frequency. Sudden increases or changes in activity have been associated with pain in the patellar [222], Achilles [199], and rotator cuff tendons [223]. This may be due, in part, to reactive oxygen species, which are oxygen molecules with an unpaired electron that combine with similar nitrogen molecules and begin to induce cellular and matrix damage [224]. During exercise, the formation of reactive oxygen species is increased [204], and cell death may increase in the presence of these reactive species [225]. While these molecules cause damage within the tendon, regular exercise routines increase antioxidants, allowing the tendon to adapt to the increased levels of reactive species since antioxidants inhibit the formation of reactive oxygen species. However, sudden changes or increases in exercise may occur faster than the tendon is

able to adapt, leading to increased levels of these harmful molecules, and exposing the tendon to greater damage [224]. Therefore, slower increases or changes in training may help prevent the onset of tendon pathology.

Tendons normally experience strain, or deformation, as a result of muscle forces, ground reaction forces, and interaction with surrounding structures. Tendons experience tensile (pulling), compressive (pushing), or shear (sliding) strains, with tensile being the most common in tendons that transmit large loads [226]. Most researchers attribute pathology to repeated strain that is less than the force required for rupture. This repeated strain damages collagen and blood vessels, decreasing the metabolic capacity of the tendon [204]. Because the tendon is designed to withstand large-magnitude loads, it normally recovers after loading by manufacturing and organizing the ECM. One hypothesis of tendinopathy is that the amount of strain exceeds the tendon's capacity to repair [227].

While tensile strains are typically the normal strain acting upon tendons, compressive and shear strains are frequently a result of impingement of the tendon, which has been reported to cause tendinopathy in the rotator cuff, Achilles, and patellar tendons. While impingement has been suggested as a cause for rotator cuff tendinopathy, contradictory viewpoints have been proposed, and thus decrease the likelihood of this hypothesis [228]. Similarly with the patellar tendon, it was hypothesized that the inferior pole of the patellar impinges on the tendon during knee flexion [229], but Schmid, et al. [230] found that patellar impingement is not likely a factor in patellar tendinopathy. However, the Achilles tendon may be impinged by the calcaneus, as the shape of the calcaneus affects the morphology of the Achilles insertion [231], especially during

dorsiflexion when the tendon bends near its attachment, resulting in the surface of the tendon pressing against the bone [232]. Therefore, any abnormalities in the shape of the calcaneus can affect the Achilles tendon during this motion.

Training errors have been reported to be present in 60-80% of overuse injuries [233] and include excessive distance, accelerated intensity progression, and hill workouts [204]. Monotonous training (e.g. distance running) increases the risk of tendon injuries and should instead be supplemented with other forms of exercise, such as biking or swimming. Improper technique and fatigue may also contribute to training errors, as frequently repeating technique errors can lead to tendon overuse and training while fatigued decreases the muscles' ability to absorb repetitive stress, causing other structures, including the tendons, to carry excessive stresses.

Improper equipment also contributes to tendinopathies of several different tendons, depending upon the type of training. Examples include the following: improper footwear leading to leg malalignment; tennis rackets being too stiff or strung to tightly transmitting an increased force to the arm and shoulder; hand paddles in swimming increasing resistance on the rotator cuff; and bicycle seats being too low, increasing knee flexion [16].

Several environmental factors may contribute to tendon pathology, including extreme temperatures, humidity, and altitude. High temperatures and humidity can lead to fatigue of the muscle-tendon units, while low temperatures may inhibit contractility, thus decreasing the ability of muscle-tendon units to absorb shock [204].

1.3.3 Pathogenesis

While many potential causes and risk factors of tendinopathy have been suggested, the pathogenesis remains unclear. Several theories of pathogenesis have been proposed and built upon one another. As early as 1978, Burry [234] suggested that changes in the tendons arose from tendon lesions that had not properly healed. Later, Leadbetter [227] and Khan et al. [115] hypothesized that tendinosis is a result of increased demands on the tendon with inadequate repair and progressive cell death, which implicated overuse in the pathogenesis. Kibler [235] and Sorosky [236] attempted to explain the inadequate repair as a response to tissue overload, along with a decrease in the number of cells. However these theories, along with Murrell's theory of apoptosis of tencoytes leading to degeneration [237-239], failed to account for the areas of hypercellularity within tendinopathy. Murrell's theory did, however, suggest oxidative stress, areas of cartilage development, and activation of MMPs as having a role in tendinopathy. Along with this theory, repetitive tensile strain [180], decrease in tendon load (stress-shielding) [240], contractile tension overloads [241], and compression [242] were all suggested as stimuli for tendon inflammation or degeneration. Others have suggested that hypoxia, increased vascularity [243], and increases in pro-inflammatory neuropeptides such as SP [119,186] may be involved in the pathogenesis. While the proposed causes of tendinopathy are widespread, Fu, et al. [244] suggest that they all be combined into a three stage pathogenesis: injury, failed healing, and clinical presentation (Figure 1.5).

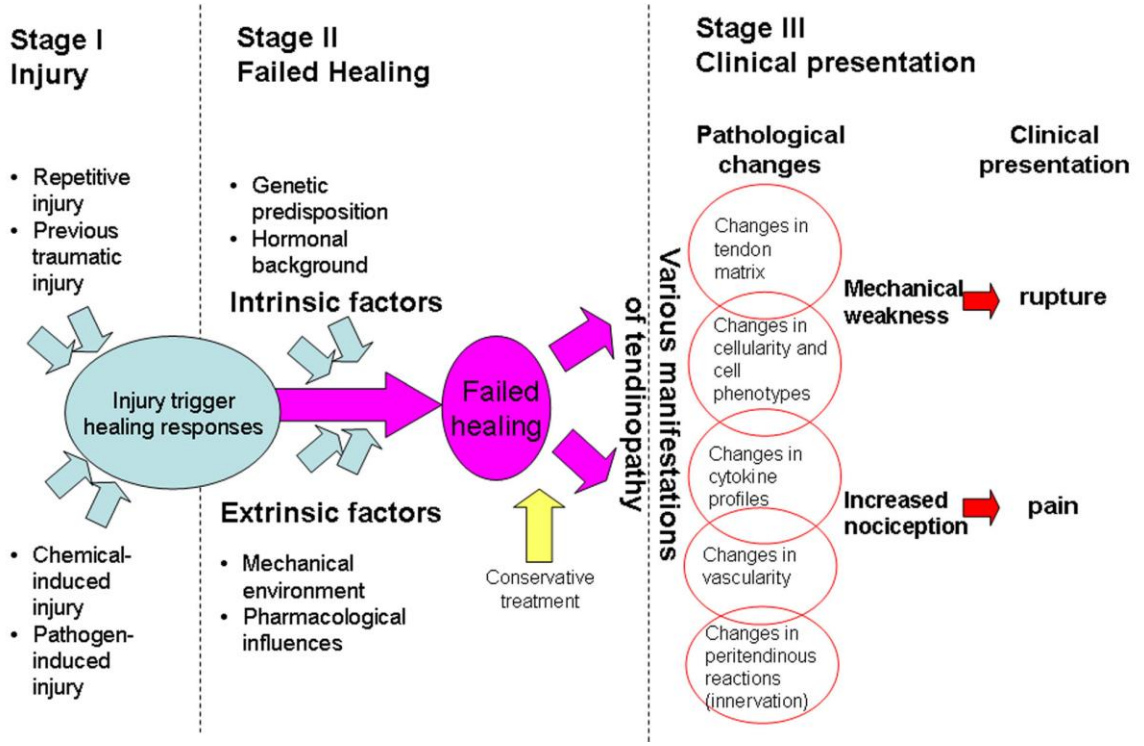


Figure 1.5. Failed healing theory for the pathogenesis of tendinopathy (Reproduced from Fu, et al. [244] with permission from BioMed Central).

In this theory, injury may be a result of chemicals, such as fluoroquinolones or oxidative damage, previous traumatic injury that has not healed, or repetitive injury. They hypothesize that mechanical weakness and tendon pain are not significant during this stage and at this point, the tendon may be able to heal without progression. The second stage, failed healing, may be due to a number of factors, including mechanical overuse, genetic pre-disposition, hormones, or pharmacological use. Finally, this combination of injury and failed healing leads to the clinical presentation that may be diagnosed by a clinician. While this theory remains vague in the exact contributing factors, it provides a rational progression of the pathology that may exist in human tendons.

1.3.4 Prevalence

The incidence of tendinopathy is quite high in both recreational and competitive athletes, and, though rare, may also occur in sedentary individuals. The actual prevalence in each tendon is variable and highly dependent on the individual's activity type and levels. For example, patellar tendinopathy has a high prevalence in athletes participating in sports involving jumping, with approximately 45% of elite volleyball and 32% of elite basketball players being diagnosed with patellar tendinopathy [245]. Achilles tendinopathy is the most prevalent lower extremity tendinopathy in the general population, with a 5.9% lifetime incidence among sedentary people [246]. This percentage drastically increases to 50% in elite endurance athletes [246]. Lateral epicondylalgia, or "tennis elbow", is prevalent in tennis players, while medial epicondylalgia is prevalent in throwers and golfers. Lateral epicondylagia occurs in 1-3% of the general population [247] and 9-35% of all tennis players [248]. Additionally, rotator cuff tendinopathy is quite common in throwing sports and swimming, and may involve either the supraspinatus, infraspinatus, teres minor, or subscapularis tendons, with the supraspinatus being the most commonly injured [249]. Rotator cuff tendinopathy is responsible for 85% of shoulder pain incidence in patients [250] and has had increasing prevalence in elite swimmers from 3% in 1974 to 69% in 2010 [251]. Other common sites of tendon overuse include the posterior tibialis and iliotibial tract [252].

1.3.5 Treatment

Many options have been proposed for the treatment of tendinopathy, but there is a lack of consistency in the reported efficacy. These options range from very conservative treatments to highly invasive procedures and include exercise, shockwave therapy, injections, and surgical techniques.

a) Eccentric exercise

The most conservative, and arguably the most effective, treatment option is eccentric exercise. Eccentric exercise consists of lengthening the muscle-tendon unit and combines stretching and strengthening. These exercises are thought to assist tendon remodeling by promoting collagen fiber cross-linking within the tendon [253,254]. However, the actual mechanisms involved in this treatment are largely unknown. Eccentric loading produces pain, and as pain decreases through adaptation or strengthening, load is increased to re-establish the pain; therefore, it has been suggested that eccentric exercise leads to progressive habituation of painful stimuli [255-258]. Others have attributed the effect to decreased stiffness in the tendon following eccentric loading [259]. Although these exercises have revealed improvements in both athletic and sedentary patients [260-267] with no adverse effects [254], further studies are required to identify the adaptations of the tendons during eccentric exercises.

Concentric exercises, which consist of shortening the muscle-tendon unit, have also been suggested as a treatment option for tendinopathy. However, eccentric exercises

have been found to be more effective than concentric exercises in improving the pain, dexterity, and agility in patients with tendinopathy [268-271].

b) Extra-corporeal shockwave therapy

Extra-corporeal refers to a procedure being performed outside of the body; therefore, this treatment option consists of shockwaves that are generated outside of the body. These shockwaves are either high or low energy pulses produced for short lengths of time. Low-energy treatment is typically administered once a week for three or four consecutive weeks without anesthesia, and high-energy therapy is only administered one time and requires local or general anesthesia [272]. These energy pulses break the sound barrier, creating shockwaves, which exert mechanical pressure and tension forces on the tissue. The waves leave bubbles, referred to as cavitation bubbles, which then collapse, creating secondary energy waves called microjets, which also apply mechanical force to the tissue. This therapy, which derived from the use of extracorporeal shockwave lithotripsy in the treatment of kidney stones, can be used to promote healing in both bone and connective tissue. As with eccentric exercise, the mechanisms are poorly understood, but this treatment is believed to stimulate interstitial and extracellular responses leading to tissue regeneration [273,274]. Similar to all tendinopathy topics, no consensus about shockwave therapy has been reached, with some studies reporting that shockwave therapy had favorable outcomes comparable to eccentric exercise [253,275,276] and others finding no benefit of shockwave therapy [277]. However, studies typically find success rates of 34-91%, with great variation in outcomes existing between different tendons [274].

c) Injections

Numerous agents are being injected into tendons with increased frequency in attempts to treat tendinopathy, but the mechanisms of their use are poorly understood and clinical studies are very limited. Several different injections have been used, including corticosteroids, sclerosing agents, aprotinin, and platelet-rich plasma. The rationale and the efficacy of these injections vary.

Corticosteroids- Corticosteroids have been tested in several studies, with some researchers investigating their effect because they may reduce edema and inflammation of the tendon [278]. However, this contradicts the belief that tendinopathy is the result of degenerative changes, making the rationale behind the use of steroid injections questionable. Others simply include steroid injections into clinical studies because they are often used to treat musculoskeletal pathologies [279]. In the patellar tendon, these injections lead to short-term improvement of tendinopathy, but the improvement diminished over time, having no beneficial long-term effect [279-281], which corresponds with the expected result of injecting them into degenerative tendons. Furthermore, corticosteroids may have negative side effects, as *in vitro* studies have found that exposing tenocytes to corticosteroids has negative effects on matrix synthesis and cell viability [282-285].

Sclerosing agents (Polidocanol)- Sclerosing injections involve introducing a chemical irritant (polidocanol) into the tendon to destroy the neovessels and nerves in an

attempt to alleviate the pain associated with tendinopathy. This treatment is derived from its use in treating varicose veins and telangiectasies (small, widened blood vessels on the skin) [286,287]. In tendinopathy pilot studies, two to four ultrasound-guided polidocanol injections led to a reduction in pain associated with tendinopathy [288,289]. In a subsequent study, researchers identified nerves lying closely to neovessels in tendinopathy biopsies and found that providing small amounts of local anesthesia into areas of the tendon with neovascularization temporarily relieved the pain [290]. Therefore, it was concluded that the neovessels and their associated nerves contribute to the pain associated with tendinopathy and sclerosing the areas containing these neurovascular bundles would decrease tendinopathy pain. Clinical assessments are limited, but studies have revealed that these injections do reduce the tendon pain [291,292]. However, Willberg, et al. [293] found that sclerosing injections were not as beneficial as other methods of destroying the neovascularization, such as arthroscopic shaving of the region of neovascularization. Also, is important to note that these injections may not improve the underlying pathology, and thus the degeneration leading to a decrease in the overall health of the tendon may continue. In addition, a risk of complications may exist, such as necrosis of the tendon due to a disturbance of the blood flow and nervous system.

Aprotinin- Aprotinin is a strong inhibitor of MMPs [294-298]; therefore, injecting this into the tendon may decrease the excessive collagenases that likely contribute to the ongoing pathology in patients [299]. Injections of aprotinin have been used for the treatment of tendinopathy since the early 1970s [300]. More recently, others have

examined its efficacy and potential risks and found favorable outcomes following injection [279,301-304]. However, anaphylaxis and other allergic reactions are potential side effects that may limit the use of aprotinin [305,306], with itch and rash being quite common following injection [304]. Because studies examining its effect are limited, more research must be conducted to determine if the benefits outweigh the potential risks of injection [307].

Platelet-rich plasma- Platelet-rich plasma (PRP) is prepared by separating PRP from platelet-poor plasma and red blood cells of autologous whole blood. PRP has been suggested to aid wound healing since the 1980s [308] and has recently been investigated in tendon healing [309-314]. The granules in PRP release many cytokines, including platelet-derived growth factor (PDGF), TGFβ, and VEGF [312-314], which may result in the production of proteins responsible for matrix synthesis and proliferation [309]. While initial studies using PRP to treat tendinopathies reported reduction in pain at both short-term and long-term follow-ups [309,311-314], de Vos, et al. [315] found no improvement in pain or activity following PRP injections. Many of the studies reporting a positive outcome had very small sample sizes, which may have affected results; therefore, additional randomized, controlled studies with larger sample sizes are required before determining the effectiveness of PRP injections as a treatment option.

Dry needling and autologous blood- Dry needling is performed by repeatedly passing a needle through the pathological region of the tendon. This technique is believed to disrupt the collagen fibers and stimulate bleeding and an inflammatory response. It is

hypothesized that this reaction of the tendon leads to strengthening of the tendon through the formation of granulation tissues as a result of the inflammatory response [316]. Autologous blood may be used in conjunction with dry needling with a rationale similar to the use of PRP, as the injection of autologous blood is thought to result in collagen regeneration and stimulation of an organized angiogenic response [315]. Studies examining autologous blood injections have reported decreased pain and symptoms resulting from lateral epicondylalgia [316,317]. Studies on the combination of dry needling and autologous blood are scarce, but James et al. [318] did report that the combination was successful in reducing the tendon thickness, the area of tendinosis, and the number of interstitial tears, thus showing promise as a treatment for tendinosis.

d) Surgery

Surgery may be used for treatment to remove fibrotic adhesions, remove or debride areas of failed healing, restore vascularity, and stimulate cells to initiate protein synthesis and promote healing [69,272,319]. Many surgical techniques have been described for different tendons, but two of the primary techniques are multiple percutaneous longitudinal tenotomy and open tenotomy. Multiple percutaneous longitudinal tenotomies are performed in patients who have isolated tendinopathy with a well-defined lesion < 2.5 cm long and no pathology of the paratenon [320]. This operation may be ultrasound guided to confirm the location of the tendinopathy [320-322], which is first identified by locating the area of maximum swelling or tenderness. For example, in the Achilles tendon, an incision is made at the identified location and is

followed by four additional incisions 2 cm medial and proximal, medial and distal, lateral and proximal, and lateral and distal to the site of the first incision [320]. These incisions are believed to improve circulation of the tendon, allowing for repair of the pathological region [68,323]. An open tenotomy may also be performed and requires ultrasound guidance to determine the proper location of the incision [320-322]. Once visible, the abnormal tissue is excised and the paratenon may or may not be stripped [324-330]. The outcome of surgery is highly variable, with a review of papers finding positive outcomes of surgery on patellar tendinopathy between 46% and 100% [331]. Most authors report favorable results following surgery in Achilles tendinopathy [114,325,332]; however, these results are not always observed in clinical practice [333], with some patients never regaining full strength and endurance [320]. Coleman et al. [331] described poor scientific methodology in reporting the outcome of tendons after surgery, with the highest success rates being described in papers with the poorest methodology. Therefore, the long-term outcome of surgery is still not fully identified.

1.4 Models of tendinopathy

In order to further the understanding of the causes, pathology, and potential treatments of tendinopathy, suitable animal models are required. While studies could be performed in humans, appropriate analyses are typically not plausible. Human tendons may be assessed by obtaining samples via biopsy [334], during surgery [69,134,335,336], or harvesting samples post-mortem [69,335]; however, the number of samples is typically small and appropriate control samples from normal tendons cannot be obtained since a

biopsy of normal tendon will likely lead to tendinopathy. Instead, many studies have been conducted using *in vitro*, *ex vivo*, and *in vivo* animal models, which allow for invasive studies of the tendon, its surrounding tissues, and the genes involved in the pathology. *In vitro* studies are frequently used to examine the molecular aspects of tendinopathy by investigating the local effects of mechanical or chemical factors upon tendon cells. Whole tendons may be removed post-mortem from animals or humans for *ex vivo* studies, and *in vivo* studies are performed to enable researchers to examine tendons in their natural environment, which accounts for systemic effects from the whole organism.

1.4.1 *In vitro* models

In vitro tendon models consist of studying cellular responses to stimuli, either mechanical strain or xenobiotics, which are thought to be involved in the pathogenesis of the condition. In order to apply the mechanical stimuli, tenocytes are exposed to cyclical stretching and the effect of cellular deformation on the production of potential cellular and molecular mediators is examined [182]. This model allows researchers to explore cellular process, such as DNA synthesis, mitosis, gene expression, and cell differentiation [181,337-347]. Over time, these studies have improved as the cells are arranged and stretched in a way similar to the shape, alignment, and stretching that occurs *in vivo* [180], which is necessary as changing the shape of the cell may lead to different cellular responses [347]. By using cyclical stretching, studies have revealed molecular changes that are associated with inflammation, including increased levels of PGE_2, COX-1 and -2, $cPLA_1$, and $sPLA_2$ [179-181].

The tendon cells may also be exposed to xenobiotics such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). When exposed to corticosteroids, the tendon cells resembled fibrocartilage [348]. The corticosteroids also affected matrix synthesis by suppressing proteoglycan production and collagen synthesis, reduced the overall cell number, and decreased cell viability [282-285]. NSAIDs inhibited tendon cell migration and proliferation, reduced GAG synthesis, and upregulated MMP expression [349-351].

1.4.2 *Ex vivo* models

Ex vivo models for tendinopathy preserve the relationship between the cells of the tendon and the ECM, providing a more natural environment without reducing the control over experimental conditions. Similar to the *in vitro* models, cyclical loading is frequently used to assess the effect of repetitive loading on the tendon. This method consists of applying a fluctuating force to the tendon, which has resulted in a decrease in ultimate force [352]. Additionally, the loading has led to increased cellular turnover and collagenase levels, which was interpreted as degradation [353]. However, use of this model does not allow for accurate detection of cell signaling due to the lack of vascularity and systemic signaling molecules. Also, mechanical strain differences between the grips and midsubstance have been found, which may be due to grip slippage or stress concentration effects at the grip edges [352].

In addition to cyclical loading, creep loading has been used to mimic sustained loading tendon injuries. This technique involves the application of a continuous load that

results in continuous deformation of the tendon specimens. The results of creep loading are similar to those of cyclical loading, specifically with higher initial strains, which lead to more damage and earlier failure of the tendon [354].

Because tendinopathy can also occur in inactive individuals, *ex vivo* stress deprivation has been examined. The stress deprivation led to decreased tensile modulus and ultimate tensile stress, but had little effect on collagen [355]. Because human tendinopathy involves collagen changes, the use of stress deprivation models should be limited.

1.4.3 *In vivo* models

Examining tendons *in vivo* is essential in developing a comprehensive understanding of the tendon. Because human studies are typically not feasible, animal models represent the most plausible method of conducting *in vivo* research of the tendon. Several animal species may be used for studies of tendinopathy, including non-human primates, horses, goats, dogs, rabbits, rats, and mice [146,356-373], but selection of the most appropriate animal is necessary. To do this, many factors must be taken into account including cost, availability, and ease of handling. For example, non-human primates would be ideal because they are the most similar to humans; however, their ethical protection, lack of availability, and high cost attenuate their use in tendon research [110]. Large animals, such as horses and goats, have naturally occurring tendinosis [374,375], but their cost and ease of handling frequently outweigh the benefit of their use. Rabbits may be used because of their mild-temperament and ease of handle; however, they are more expensive than rodents and are susceptible to life-threatening injury when

frightened [110]. Rats and mice are the most frequently used animals in the study of tendinopathy because they have short gestation periods, rapid growth rates, and short life spans, in addition to being low cost and having mild temperaments [110]. Once the appropriate animal model is chosen, *in vivo* analysis can be performed, typically via the use of chemical or mechanical tendon intervention.

a) Chemical intervention

Various chemicals have been injected into animal tendons, with compounds having variable results. These injections include cytokines, prostaglandins, fluoroquinolones, and most frequently, collagenase. Stone, et al. [376] injected species-specific cytokines into rabbit patellar tendons, which led to an initial increase in cellularity, followed by a return toward baseline after 16 weeks. However, these injections caused no matrix damage or collagen degradation, and thus all damage was reversible.

Repeated injections of prostaglandins into tendons have been shown to initiate degeneration of the tendon in both rat Achilles tendons [377] and rabbit patellar tendons [378]. This was observed as fibrosis of the paratenon, adhesions, and intra-tendinous degeneration after five weeks of one injection per week in rat Achilles tendons [377]. A similar effect was observed in rabbit patellar tendons injected with PGE_2 once a week for four weeks. These tendons had hypercellularity, loss of normal tissue architecture, focal areas of degeneration, and decreased collagen fibril diameter [378].

Fluoroquinolones, which have been linked to tendon rupture in humans [379-382] have been given in oral doses to rats, with the Achilles tendons being examined for damage. Kato [383] found that a single administration of pefloxacin or ofloxacin caused inflammatory cell infiltration and disorganization of the collagen bundles of the Achilles tendon, with the tenocytes undergoing cell death. After replicating this study with many other fluoroquinolones [384-386], variability in their effects has been observed. While some of the compounds had no effect on the tendon, pefloxacin and fleroxacin led to pathological changes, including breakdown of collagen fibrils and degenerative changes of the tenocytes [385,386].

Because tendinopathy is a degenerative disorder, the breakdown of collagen is required in an animal model. In order to mimic this, bacterial collagenase has been utilized in many studies to induce degeneration of the collagen, and thus the tendon. Foland [387] was one of the first to inject collagenase into tendons, and did so into the superficial digital flexor tendon of horses. This led to lesions and increased levels of type III collagen. Others have used collagenase injections in the rat supraspinatus tendon, resulting in increased cellularity and vascularity, along with collagen disorganization [365]. Fu [388] examined the effect of collagenase on the patellar tendon and found inflammation, as well as matrix calcification at 16 weeks post-injection. Lui, et al. [389] performed a similar experiment but analyzed the tendons for a longer time and found that the tendons had healed from the collagenase injection at 32 weeks post-injection. Because of the drastic effect on the tendon, collagenase injections are frequently used as models of tendon injury [390-393]; however, because the tendon can recover from the

injection, their effect is acute rather than the chronic tendinopathy that humans experience.

b) Mechanical intervention

Because tendinopathy typically occurs in athletes and active individuals [246], it is widely believed to be a result of overuse. In animals, fatigue loading has been used as a model for investigating the impact of damage accumulation on the tendon. Fatigue loading has been used in rat flexor digitorum longus [394] and patellar tendons [394,395] and consists of cyclical loading of the tendon. These studies reported changes in the tendon following fatigue loading, including an increase in types I, III, and V collagen levels, fiber deformations, microstructural damage, and variable changes in levels of MMP-13 and IL-1β, with higher strains causing an upregulation and lower strains causing a downregulation.

Electrical muscle stimulation is frequently used to cause flexion and extension movements, and thus loading of the tendon [396]. This method has been used on a wide variety of tendons and animals, including the triceps surae muscle of rabbits [396] and rats [397] and the flexor digitorum profundus muscle of rabbits [149,398-400]. The outcomes of these studies are widespread and conflicting, with some reporting degeneration with increased vascularity and inflammatory cells [396], increased tearing of the tendon [398,399], and increased cellularity with disorganization of the collagen [397], while others reported no increase in vascularity or inflammatory cells [149] and no changes in tendinopathy-related mRNA levels [400]. Because of these contradictions,

electrical muscle stimulation must be further investigated before it can be considered an acceptable animal model for tendinopathy.

Treadmill running has become a commonly used method of mechanical overuse in attempts to develop animal models of tendinopathy. Soslowsky, et al. [401] were the first to demonstrate the potential for treadmill running to induce tendinopathy by running rats on a treadmill at 17 m/min on a 10° decline for 1 hour/day, 5 days/week for 4, 8, or 16 weeks. Using this protocol, changes of the supraspinatus tendon became evident, including increased cellularity and deformation of the cell shape, which remained consistent from 4 to 16 weeks of treadmill running. Following this study, many other groups replicated the downhill running protocol and all found evidence of tendinosis in the supraspinatus tendon [121,402-406]. This downhill running protocol failed to affect the Achilles tendon [371]. However, a later study utilized enforced bipedal downhill treadmill running, and although this is not the natural gait of a rat, it did induce damage in the Achilles tendon [372].

Glazebrook et al. [370] altered the supraspinatus tendinopathy protocol using uphill rather than downhill treadmill running and found disorganization of collagen fibers with increased cellularity and vascularity in the Achilles tendon. These results were supported by Silva, et al. [373] showing tendinopathic changes in the Achilles tendon in response to uphill treadmill running at 27 m/min for 4-16 weeks. More recently, however, Heinemeier, et al. [146] replicated the uphill running protocol while including more outcome measures and found no pathological changes of the Achilles tendon. She reported that running had a beneficial effect on the tendon by improving the mechanical properties of the Achilles tendon.

In an attempt to understand tendinopathy in sedentary individuals, disuse has been examined in rat Achilles tendons [407]. Rats were tail-suspended for five weeks, which did cause changes in the collagen; however, this study had many confounding variables, so no conclusive results can be deduced.

1.5 The Achilles tendon

In humans, tendinosis can occur in many different tendons and can result from a wide variety of factors. Additionally, factors that impact one tendon may have no effect on another. This is evidenced by certain animal models (e.g. downhill treadmill running) causing tendinosis in some tendons, but not others. Therefore, each tendon must be individually examined. One of the most frequently pathological tendons in humans is the Achilles tendon, and thus examining this tendon is integral in treatment and prevention of injury.

1.5.1 Anatomy and function

The Achilles tendon, which connects the gastrocnemius and soleus muscles to the calcaneus, is the strongest tendon in the human body. The two bellies (lateral and medial) of the gastrocnemius join the deeper soleus muscle approximately 12-15 cm from the insertion into the calcaneus [202,408,409]. The tendons of the two muscles fuse, forming one tendon, approximately 5-6 cm from the insertion. From that point, the tendon rotates about 90°, with the medial portion rotating posteriorly and the lateral portion rotating

anteriorly. The Achilles tendon inserts on the posterior surface of the calcaneus, leaving space between the tendon and bone, which is occupied by the retrocalcaneal bursa [231].

There is no synovium surrounding the Achilles tendon. Instead, the paratenon consists of two layers: a deep layer that lies against the epitenon and a superficial layer that lies against the crural fascia (deep fascia of the leg). The two layers of the paratenon are connected via the mesotenon, which carries blood and lymph vessels. The Achilles tendon can stretch to 4-5% beyond its original length without damage, but when stretched more than 8%, it is likely to rupture [410,411].

The primary function of the Achilles tendon is locomotion and understanding this function must be preceded by a description of the gait cycle. The gait cycle consists of two phases for each leg: the stance phase, during which the foot is in contact with the ground, and the swing phase, when the foot is off the ground and swinging forward. The stance phase is further divided into three phases: contact, midstance, and propulsion. The contact phase consists of the time the heel makes contact with the ground to the remainder of the foot touching the ground. Midstance beings once the entire foot is touching the ground, and propulsion lasts from the time the heel leaves the ground until the toe leaves the ground. The swing phase is also separated into phases: forward swing and foot descent. The first phase is the forward swing, which occurs as the foot is carried forward, with the knee flexed (bent) and the foot dorsiflexed (toes are brought toward the shin). Next is the foot descent, at which point the foot is positioned for weight bearing and the muscles stabilize the body to absorb the shock of heel contact [412]. Once the heel contacts the ground, the stance phase begins again. The gait cycle is dependent upon the gastrocnemius-soleus muscles that act via the Achilles tendon [202,410,411,413],

which controls the midstance and propulsion intervals of the stance cycle [412]. During normal walking, it is estimated that the muscle tension on the Achilles tendon at the end of the stance phase is 250% of the body weight. While running, this increases to six to eight times the body weight and approaches the ultimate strength of the tendon [414-417]. Furthermore, any abnormalities in the foot positioning during the gait cycle can add extra forces upon the Achilles tendon.

1.5.2 Etiology and pathology

Achilles tendinopathy has been identified in 30-50% of all injuries in athletes [204] and occurs relatively frequently in military personnel around the world [418,419]. This pathology may also occur in sedentary individuals [246] and can also present as rupture, which is common in both runners [246,420] and athletes involved in contact sports, such as football [421]. Achilles tendinopathy is not limited to any age group, but is most prevalent between ages 21 and 60 [422].

Because of its poor vascularization, the Achilles tendon has limited healing capabilities. Conversely, the muscles are able to recover and heal quickly after training, which leads to an imbalance between the force the muscle can exert and the force the tendon can transmit. This result of excessive training is considered to be the main cause of Achilles tendinopathy. In addition, different activities expose the tendon to various stimuli, including tension, torsion, vibration, reduction of blood flow, and heat generation, all of which may be harmful to the tendon. Tension on the Achilles tendon exists during the propulsion phase of the gait cycle. Torsion and vibrations are induced

by a whiplash effect during the stance phase of the gait cycle and may be amplified by certain surfaces, such as artificial turf. All of these factors may contribute to reduced blood flow by constricting the blood vessels within the tendon. Finally, heat generation may also contribute to Achilles tendon pathology. Because of the poor vascularization, heat dissipation is inhibited and the tendon may overheat, leading to damage of the structure [412].

These stimuli may have an effect on the Achilles tendon, the paratenon, or both, with certain areas of the tendon being more susceptible to damage than others. Pathology of the Achilles tendon presents similar to all tendon pathologies as previously described. The mid-substance of the Achilles tendon is most susceptible to damage, which appears as degeneration. At the insertion into the calcaneus, inflammation of the bursa may exist, leading to painful adherences around the tendon and bony attachment. Pathology of the paratenon is typically inflammation, and thus referred to as "paratenonitis". This pathology is histologically identified by the proliferation of fibroblasts, infiltration of inflammatory cells, and fibrinous exudate [124,423,424]. In addition, deformities of the posterior tuberosity of the calcaneus may contribute to Achilles tendon pathology. This disorder is referred to as Haglund's deformity and may be congenital or a result of wearing certain footwear, such as hockey skates or high-heeled shoes [423]. Haglund's deformity leads to compression of the Achilles tendon and may cause a cycle of further enlargement of the calcaneal tuberosity which further irritates the Achilles tendon [202,409,423,425].

A final form of Achilles tendon pathology is tearing, or rupture. Normal Achilles tendons are unlikely to tear, as most tears occur following degeneration. However,

overuse is not a necessary precursor to tearing [69,426]. Tears may be partial, with incomplete disruption of fibers, or total, which span the entire cross-section of the tendon. The causes of Achilles tendon tearing are unknown, but many have been speculated, including the following: direct trauma, degeneration, recurrent microtrauma, corticosteroids, fluoroquinolones, chronic renal failure, rheumatoid arthritis, systemic lupus erythematous, and diabetes mellitus [117,202,427,428].

1.5.3 Symptoms and diagnosis

The symptoms accompanying Achilles tendinosis are variable and subjective. Different symptoms may exist, depending on the type and extent of the pathology present. For example, individuals with tendinitis at the calcaneal insertion describe pain as they exercise, which worsens when jumping or running uphill. They also feel pain with palpation and have visible inflammation in the region of insertion. Patients with acute paratenonitis experience crepitus, or popping sounds, while moving the foot and have difficulty moving the ankle after resting. Chronic paratenonitis is identified with pain during palpation, swelling along the tendon, and pain decreasing during activity, but increasing after the activity has ended. Tendinosis is unique, as it is typically asymptomatic and only becomes painful with the presence of microscopic tearing or the onset of inflammation of the paratenon or retrocalcaneal bursa. Symptoms for all Achilles tendinopathies may become amplified by changing a training routine, changes in weather, or sickness such as flu or infection [199,429].

Imaging is essential in the diagnosis of Achilles tendinopathy. While radiographic imaging is not always ideal in diagnosis, it can be used to identify Haglund's deformity and insertional tendinitis, as well as tendon calcifications and ossifications, which are sometimes present in tendinopathy [430-433]. Ultrasound is frequently used in the diagnosis of Achilles tendinopathy, as it can be used to find focal lesions within the tendon; however, it is inaccurate when differentiating partial ruptures from focal areas of tendinosis [434]. Magnetic resonance imaging (MRI) is likely the most accurate way of diagnosing and differentiating between the tendon pathologies. This imaging allows physicians to clearly view the insertion and origin along with the overall anatomy, and is very sensitive to pathological changes [412].

1.5.4 Treatment

Many treatment options for tendinopathy have been proposed, but few have undergone appropriate investigation with clinical trials [254,435]. This lack of data limits the knowledge required to adequately treat patients. For example, NSAIDs are frequently assumed to be a relevant treatment; however, this treatment would only be beneficial in inflammatory tendinopathies, not the more prevalent degenerative pathology [436]. Therefore, before making assumptions about appropriate treatments, it is important to fully understand the current treatment options.

Treatment options are divided into conservative management or surgical treatment, and most have insufficient evidence to be considered effective. Using braces or splints has been shown to be ineffective as a treatment option, as their use did not alter

pain, symptoms, or quality of life [437-440]. Orthotics have also been suggested, but there is not enough evidence to support this treatment option [440,441].

Extracorporeal shock wave therapy is being used more frequently as a treatment option [272]. This treatment utilizes acoustic waves that may be high or low energy, which are believed to stimulate soft-tissue healing and inhibit pain receptors. There is insufficient evidence to support this therapy, as outcomes are inconsistent [275-277]. Rompe, et al. [276] compared extracorporeal shock wave therapy to eccentric exercise and found that both reduced pain but there was no difference in outcome measures between the two therapies. Later, they investigated the combined effect of eccentric exercise and extracorporeal shock wave therapy and found that the combination decreased pain more than eccentric exercise in isolation; however, they did not examine the combined effect versus the effect of extracorporeal shock wave therapy in isolation [275]. In contrast, Costa et al. [277] found that extracorporeal shock wave therapy provided no pain relief after three months of treatment.

Dermal patches and injections have also been tested in an effort to treat Achilles tendinosis. Patches of glyceryl trinitrate, a prodrug of endogenous nitric oxide [442], have been applied to the Achilles tendon to increase levels of nitric oxide around the tendon. Studies have shown that inhibition of nitric oxide leads to a reduction in the synthesis, contraction, and amount of collagen [443] and a reduction in the CSA and ultimate load in tendons [444]. Therefore, it may be inferred that nitric oxide stimulates collagen synthesis and is necessary for maintenance of normal mechanical properties of the tendon. Paoloni, et al. [445] reported a reduction in pain and tenderness after 12 and 24 weeks of use of the glyceryl trinitrate patch, but the patch also caused side effects

including rash and headache. Several injection therapies have been tested, including corticosteroids, sclerosing therapy, aprotinin, and platelet-rich plasma [200,281,440,446-449]. Studies on these injections are very limited and none of the injections have been definitively supported or refuted in the treatment of Achilles tendinopathy.

While the previous options lack evidence or support, eccentric exercise has been shown to be successful [267,275,441,450]. This exercise provides a strong, controlled, mechanical force to the tendon by lengthening the muscle, which is generally achieved by dropping the heel over a step. As the patient adapts to the exercise, the load is gradually increased by adding weight or using weighted gym equipment. This therapy has been shown to decrease pain and is associated with very little risk if progression is gradual and monitored by a health professional [440].

Contrary to conservative management of tendinopathy, surgical treatment may be used to remove adhesions, remove or debride areas of failed healing, restore vascularity, and potentially stimulate cells to resume their normal activities [272]. Several surgical techniques are used, including removing hypertrophic regions of the paratenon [11,325-330], removal of abnormal tissue through a central longitudinal tenotomy [324-330], or multiple percutaneous longitudinal incisions in the area of tendinosis [320]. Surgery requires a long recovery and gradual return to activity, and thus is typically used as a last resort if other options failed at alleviating the symptoms.

1.6 Summary and aims

1.6.1 Tendinopathy summary

Tendinopathy is a common pathology that may exist in both active and sedentary individuals. While it was once thought to be a result of inflammation of the tendon, or "tendinitis", many studies have revealed that tendinopathies are actually due to tendon degeneration and should be referred to as "tendinosis". This condition is characterized by activity-related pain, focal tendon tenderness, intratendinous imaging changes, and typically results in changes in the histological, mechanical, and molecular properties of the tendon. Many tendons are susceptible to tendinosis and each is likely affected by different factors, either extrinsic, intrinsic, or a combination of both. Although the condition is quite common, knowledge concerning the pathology is very limited. This limited knowledge is primarily a result of tendinosis being difficult to study in humans; therefore, animal models that mimic the human condition are required. The variation in etiology among tendons requires that models be developed for each tendon. Models have been established for some tendons, such as the supraspinatus; however, a consistently reliable model of Achilles tendinosis has yet to be established.

1.6.2 Dissertation overview

The purpose of this dissertation is to investigate the effects of an extrinsic factor, an intrinsic factor, and a combination of both in an attempt to develop an acceptable

animal model of Achilles tendinosis that mimics the human condition. To do this, an extrinsic factor, uphill treadmill running, and/or an intrinsic factor, intratendinous collagenase injection, was introduced to rodents. This research expands upon previous research in several ways. First, greater incline and running speeds were used. Second, these studies investigate the combination of intrinsic and extrinsic factors, which has only been reported once in the literature [391]. Finally, rats selectively bred for high capacity running (HCR) were used.

a) High capacity running rats

HCR rats are derived from a founder population of genetically heterogeneous N:NIH rats that have been artificially selected for intrinsic aerobic endurance running capacity. Prior to endurance testing, rats were acclimated to the treadmill by gradually increasing the running speeds from 10 m/min for 5 minutes on a 15° incline to 15 m/min for the same duration and at the same slope. The treadmills were equipped with an electric shock grid that delivered a mild shock when the rat rested at the base of the treadmill. This assisted the acclimation by encouraging rats to run rather than be shocked and also played a role in the determination of aerobic capacity.

During the second week, the rats were evaluated for maximal running endurance capacity for five consecutive days. To perform this test, rats began running on a slope of 15° at 10 m/min. The speed of the treadmill was increased by 1 m/min every 2 minutes until the rat was exhausted. Exhaustion was defined as the third time the rat remained on the shock grid for 2 seconds.

After completing the running trials, the 13 highest and 13 lowest capacity rats of each gender were randomly bred. The offspring underwent the same treadmill acclimation and running capacity trials and were bred in the same manner. By the sixth generation, the high capacity rats ran 839 ± 21 m before exhaustion, while the low capacity rats ran 310 ± 8 m. As a point of reference, the founder population ran 355 ± 11 m before exhaustion [451]. This selective breeding has continued for many generations and the difference between high- and low-capacity endurance continues to grow (Figure 1.6).

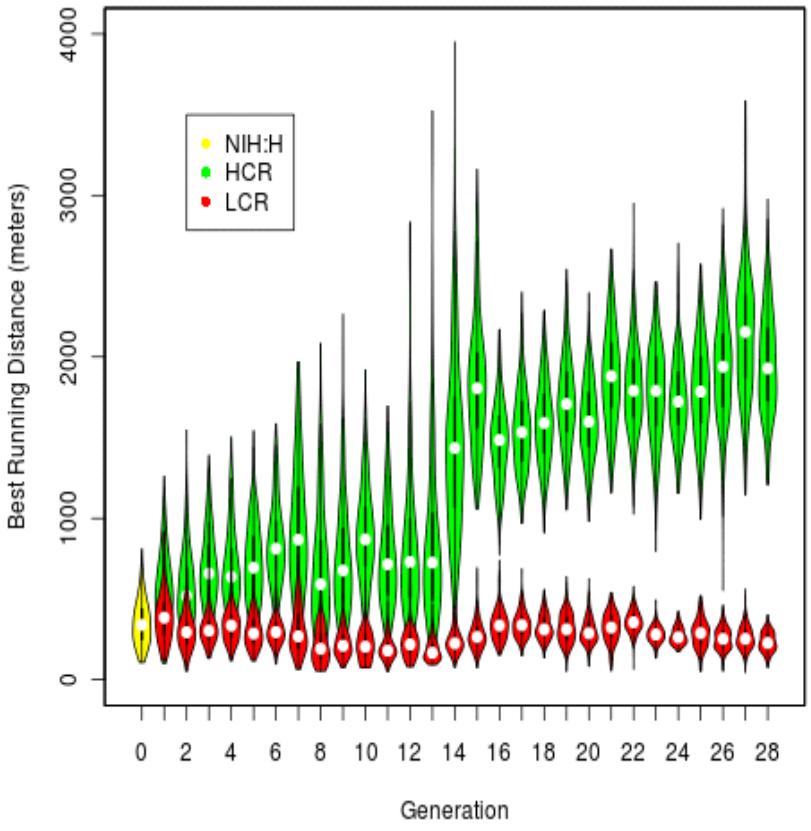


Figure 1.6. Progression of difference between high and low capacity runner rats in running distance during untrained phenotype endurance testing. (Reproduced with permission from the laboratory of Jun Li, University of Michigan).

HCR rats were selected for two of the studies included in this dissertation. Because of their increased aerobic capacity, they are able to run at greater speeds for longer durations than standard rats. This is essential in studies using treadmill running in an attempt to stimulate mechanical overuse. In the wild, rats naturally locomote approximately 8 km/night [452]; therefore, using rats that are able to run longer distances may allow mandatory treadmill running that approaches the distances run in the wild, and thus are more likely to result in mechanical overuse affecting the Achilles tendon.

b) Extrinsic factor

For the studies of this dissertation, mechanical overuse via uphill treadmill running is used as the extrinsic factor in attempts to create an animal model of Achilles tendinosis. Although electrical muscle stimulation has been suggested as a mechanical overuse model, studies using this method to examine the Achilles tendon are limited. In contrast, numerous investigators have attempted to use treadmill running as a means of mechanically overloading the rodent Achilles tendon [146,369-373]. Some success has been reported in terms of inducing histopathological and mechanical changes consistent with human Achilles tendinosis [370,372]; however, these have not been replicated and, more recently, have been contradicted, with Heinemeier, et al [146] reporting that uphill treadmill running is beneficial to the Achilles tendon. Therefore, treadmill running was used for this dissertation in attempt to further investigate these studies. Specifically, uphill treadmill running was used instead of downhill, as uphill running has been reported as more successful in inducing changes to the Achilles tendon.

c) Intrinsic factor

Collagenase injection directly into the Achilles tendon was chosen as the intrinsic factor for the studies included in this dissertation. Collagenase catalyzes the hydrolysis of collagen and has elevated expression in human tendinosis [370]. Intratendinous injection of collagenase into the Achilles tendon results in an acute and intense inflammatory reaction (tendinitis) followed by progressive tendon healing [121,390-392]. Therefore, collagenase injection was used to create an initial injury that could be intensified by repetitive use.

1.6.3 Aims

The overall aim of this dissertation was to investigate the individual and combined effects of treadmill running (extrinsic factor) and collagenase injection (intrinsic factor) in creating pathology within the rat Achilles tendon consistent with Achilles tendinosis in humans. Specifically, we assessed the effect of uphill treadmill running and collagenase injection on the histological, mechanical, and molecular properties in rats.

The general aims of this dissertation are to investigate:

1. The effect of an extrinsic factor (uphill treadmill running) on the rodent Achilles tendon;
2. The effect of an intrinsic factor (collagenase injection) on the rodent Achilles tendon; and

3. The combined effect of an extrinsic (uphill treadmill running) and intrinsic (collagenase injection) factor on the rodent Achilles tendon.

CHAPTER TWO: THE EFFECT OF UPHILL RUNNING ON THE HISTOLOGICAL APPEARANCE OF THE ACHILLES TENDON IN HIGH CAPACITY RUNNING RATS

2.1 Introduction

Despite consistent identification of the pathology underlying Achilles tendinopathy, little is known about the pathological process/es taking place within the tendon. This limited knowledge has restricted treatment options, with clinical management presently being more of an art than a science. In order to address this void, a suitable animal model of Achilles tendinosis is required. As knowledge regarding human Achilles tendinosis is currently centered around its histopathological features, a suitable animal model is one in which the histopathological features of the injured animal Achilles tendon replicate those observed in the human condition.

Treadmill running represents a potential means of repetitively loading tendons in rats to induce histopathological changes. Although established for the generation of supraspinatus tendinosis [401], treadmill running has had variable success in developing tendinosis-like changes in rat Achilles tendons [146,369-373]. Soslowsky and colleagues [371] used the same downhill (10º decline) treadmill running program (17 m/min, 1 hr/d, 5 d/wk for up to 16 weeks) as they used to induce supraspinatus tendinosis in an unsuccessful attempt to induce mechanical and geometric changes within the rat Achilles tendon. A possible explanation for the lack of an effect may be that downhill running in quadrupeds results in a forward shift of the center of mass [453] resulting in increased

forelimb loading (and elevated subacromial compression) combined with a relative decrease in hindlimb loading.

Glazebrook et al. [370] replicated the same running program as Soslowsky and colleagues [371], but furthered the work by running rats uphill (10º incline) rather than downhill for up to 12 weeks. Uphill running requires the calf muscles (and other antigravity muscles) to contract concentrically to raise the center of mass with each step. The net result may be increased Achilles tendon loading as the increased muscle forces are transmitted to the skeleton. Glazebrook et al. [370] showed uphill running resulted in histological changes consistent with human Achilles tendinosis, including a reduction in collagen organization and an increase in tenocyte number [370]. However, the latter observations were not replicated by Heinemeier et al. [146] who completed a comprehensive study using the same uphill running program, but with the modification of increased running speed (20 m/min).

The aim of this study was to build upon these previous studies and investigate whether uphill treadmill running at a higher incline (15º) and speed (up to 30 m/min) creates histopathological changes within the rat Achilles tendon consistent with Achilles tendinosis in humans. Specifically, we assessed the effect of uphill treadmill running on Achilles tendon collagen arrangement, tenocyte morphology, cellularity, and vascularization in rats selectively bred for high-capacity running.

2.2 Methods

2.2.1 Animals

Twenty-six male HCR rats (age = 24.8 ± 3.2 weeks; weight = 374.0 ± 30.3 g) were acquired from the University of Michigan (Ann Arbor, MI) and acclimated for 2 weeks. HCR rats have been artificially selected for aerobic capacity from a founder population of genetically heterogeneous N:NIH rats [451]. Animals in the current study were from the 26th generation of HCR rats, with this strain of rat being used due to their known ability to run long distances. Rats in this study ran an average distance of 1.7 ± 0.1 km within 64.8 ± 1.1 min on a treadmill when phenotyped as untrained, young adults.

All animals were maintained under standard conditions and provided *ad libitum* access to food and water. All procedures were performed following *a priori* approval from the Indiana University Institutional Animal Care and Use Committee.

2.2.2 Treadmill running

Animals were randomly divided into two groups: cage control (n = 11) and running (n = 15). Rats in the cage control group maintained normal cage activity throughout the duration of the study. Rats in the running group ran 5 days/week for 9 weeks on a treadmill at a 15° incline. Rats were acclimated to the treadmill starting with 5 minutes at 10 m/min. The duration and speed of running were gradually increased throughout weeks 1 and 2 until the rats were running for 60 minutes at 25 m/min (Table

2.1). The duration was kept constant for the remainder of the study while speed was progressively increased up to 30 m/min by the final week of running. Treadmills were equipped with an electric shock grid set to 10 Hz. If rats remained on the shock grid for more than three seconds, they were manually prodded back onto the treadmill or removed from the shock grid and placed onto the treadmill. If this occurred more than five times, the rat was removed from the treadmill and allowed to rest until the running session the following day.

Table 2.1. Running protocol used for the running group of rats.

		Duration (min)	Speed* (m/min)
Week 1	Day 1	5	10
	Day 2	5	10
	Day 3	5	10-15
	Day 4	5	10-25
	Day 5	30	15-25
Week 2	Day 6	45	15-25
	Day 7	50	15-25
	Day 8	55	15-25
	Day 9	60	15-25
	Day 10	60	15-25
Week 3		60	15-25
Week 4		60	20-25
Week 5		60	20-25
Week 6		60	20-25
Week 7		60	20-27.5
Week 8		60	20-27.5
Week 9		60	20-30

*Hyphenated values in the speed column represent the gradual increase in speed at the beginning of each running session. Running began at the lower speed and was increased by 1 m/min each minute until the higher speed was achieved. Rats ran at this higher speed for the remainder of the duration of that running session.

2.2.3 Histology

Animals were euthanized after 9 weeks of the running regimen and one of their Achilles tendons was harvested and fixed in 10% neutral buffered formalin for 48 hours before being transferred to 70% ethanol. Tendons were embedded in paraffin and 6 μm thick midsubstance sagittal sections were cut using a microtome and stained with hematoxylin and eosin.

Prior to staining, acid alcohol was prepared by combining 2,000 mL 70% ethanol with 10 mL hydrochloric acid, and ammonia water was prepared by combining 2,000 mL distilled water with 6 mL 0.3% ammonium hydroxide. Hematoxylin (#01560 - Harris' Hematoxylin) and Eosin (#01600 - Eosin) were obtained from Surgipath (Leica Biosystems; Nussloch, Germany). A Shandon Linistain GLX (ThermoFisher Scientific; Waltham, MA) was used for hematoxylin and eosin staining using the following protocol: First, sections were soaked in three baths of xylene for five minutes each. Next, they were dehydrated in 100% ethanol twice for three minutes each, 95% ethanol for three minutes, 70% ethanol for three minutes, and then placed into water for one minute. Sections then went through a series of eight hematoxylin stains, spending 30 seconds in each stain bath for a total of four minutes in hematoxylin. They were dipped into water for 30 seconds, and then quickly dipped into acid alcohol four times. Sections were placed into water for 30 seconds, ammonia water for 30 seconds, then water for 30 seconds. Next, the slides were dipped into two eosin baths for 30 seconds each, totaling one minute in eosin. Sections then went through another round of dehydration, spending 30 seconds in 70% ethanol, one minute in 95 % ethanol, and two periods of three minutes

in 100% ethanol. Finally, sections were placed into xylene for three minutes. Following staining, slides were dipped into fresh xylene for three minutes and cover slipped with eukitt, a xylene-based mounting media. Sections were then viewed on a Zeiss Axiophot light microscope and tendon damage assessed using a modified Bonar histopathology scale [120,121,454].

The most pathological region of the tendon was determined based upon collagen arrangement under polarized light. In this region, tendons were graded for collagen arrangement (one field of view at 100x), tenocyte morphology (four fields of view at 200x), cellularity (one field of view at 100x), and vascularity (up to 10 fields of view at 400x). A greater number of fields were viewed for characteristics requiring assessment at higher magnification in an effort to grade a similar total area of tissue for each outcome. The characteristics were graded between 0 (normal) and 3 (maximum pathology) (Table 2.2) [122]. The sum of all 4 categories gave a completely normal tendon a score of 0 and a tendon with maximum damage a score of 12. Samples were randomized and graded by two independent blind examiners. Discrepancies in scoring were resolved by a discussion, with a third examiner being consulted when consensus could not be reached.

2.2.4 Statistics

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 19.0; IBM) software, with tests being two-tailed with a level of significance set at 0.05. Kolmogrov-Smirnov and Levene tests were used to test for the presence of a normal distribution and homogeneity of variance, respectively. Mann-

Whitney U tests were used to compare histopathological scores between groups because the data did not meet the assumptions required for parametric statistics. Kappa statistics were calculated to measure the inter-rater agreement.

Table 2.2. Semiquantitative scale used to grade tendons.

Characteristic	Grade 0	Grade 1	Grade 2	Grade 3
Collagen arrangement	• Tightly cohesive, well demarcated bundles • Smooth, dense homogeneous polarization pattern • Normal crimping	• Separation of individual fiber bundles • Demarcated bundles • Non-homogeneous polarization	• Separation of fibers • Loss of demarcation of bundles • Expansion of tissue • Clear loss of normal polarization pattern	• Marked separation of fiber bundles • Complete loss of architecture
Tenocyte Morphology*	• Elongated, spindle-shaped nuclei • No obvious cytoplasm	• Ovoid to round nuclei • No conspicuous cytoplasm	• Round and slightly enlarged nuclei • Small amount of visible cytoplasm	• Large, round nuclei • Abundant cytoplasm • Formation of lacunae
Cellularity	• Many discrete cells	• Hypercellular (> 30 nuclei/field of view) • Cells in runs	• Areas of hypo (< 20 nuclei/field of view) and hypercellularity	• Mostly acellular
Vascularity	• Inconspicuous blood vessels between fiber bundles	• < 2 clusters of vessels	• 2-3 clusters of vessels	• > 3 clusters of vessels or pathological avascularity

*If >20% of nuclei in the field of view receive a higher score, the tendon receives the higher score (ie- if 75% of the field of view is grade 1 and 25% is grade 2, the tendon will receive a 2 for this category).

2.3 Results

Animals in the running group ran on the treadmill an average of 51.2 ± 7.5 km during the study (Figure 2.1 and Table 2.3). Tendons from the cage control and running groups were similar on gross histological appearance (Figure 2.2). Kappa statistics ranged from 0.60 to 0.78 for each assessed characteristic (Table 2.4). There were no group differences within any of the individual histopathological categories assessed (all $P > 0.16$) or for total histopathological score ($P = 0.13$) (Table 2.4).

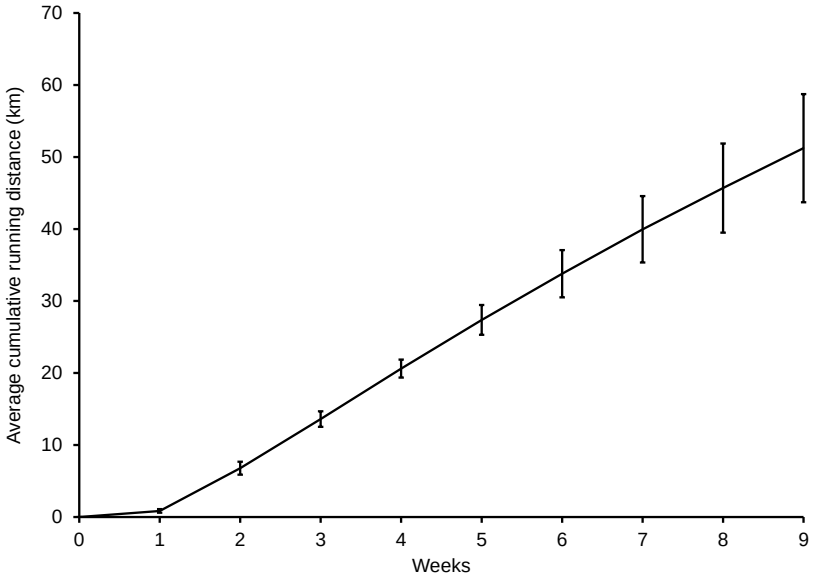


Figure 2.1. Cumulative distance ran on the treadmill by rats in the run group. Error bars represent standard deviation.

Table 2.3. Weekly running distances by rats in the run group. Distances are presented in km and maximum represents the maximum possible distance for that week.

Rat	Week									Total
	1	2	3	4	5	6	7	8	9	
1	0.95	6.43	6.73	5.19	6.27	5.32	6.47	7.27	6.59	51.19
2	0.84	5.20	7.00	7.03	5.88	4.47	5.04	3.73	7.98	47.16
3	0.68	5.37	6.89	6.88	6.09	4.33	7.89	7.54	4.68	50.35
4	0.92	5.40	7.15	6.83	7.30	6.12	7.62	7.98	4.88	54.19
5	0.93	6.48	7.23	7.43	7.43	7.43	7.62	6.80	4.04	55.36
6	0.73	6.44	7.14	6.76	6.83	7.25	6.99	7.40	7.83	57.36
7	0.95	6.48	7.23	7.43	7.43	7.43	8.09	7.98	8.02	61.02
8	0.95	6.48	7.23	7.43	7.43	7.43	8.09	8.09	8.70	61.80
9	0.95	5.85	4.99	6.82	3.45	3.49	1.57	3.03	3.15	33.27
10	0.95	5.65	6.90	6.95	6.92	6.55	4.85	2.48	1.50	42.74
11	0.95	6.48	7.23	7.43	7.43	7.18	3.42	1.07	2.80	43.96
12	0.00	4.11	7.10	7.43	7.43	7.43	4.27	3.75	5.24	46.74
13	0.92	6.35	7.23	7.43	7.43	7.43	6.47	5.40	4.90	53.54
14	0.95	6.48	6.35	7.03	7.07	7.30	6.19	6.40	5.85	53.61
15	0.91	5.85	6.05	7.13	7.13	7.03	8.04	7.02	7.00	56.15
Maximum	0.95	6.48	7.23	7.43	7.43	7.43	8.09	8.09	8.73	61.86

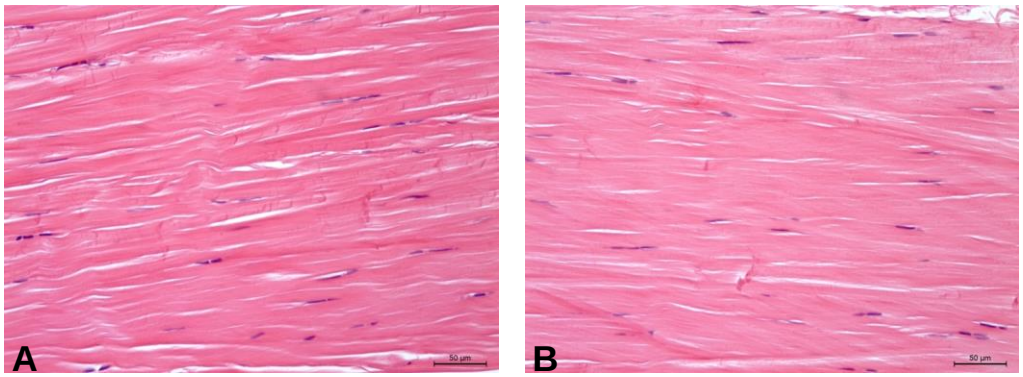


Figure 2.2. Representative photomicrographs of the Achilles tendon. A) run group and B) control group tendons. Note the uniform appearance of tightly packed, well-aligned collagen fibrils with interspersed, spindle-shaped tenocytes aligned parallel to the fibrils in the tendons from both groups (Stain = Hematoxylin & Eosin).

Table 2.4. Differences in individual histopathological categories and total histopathological score in Achilles tendons. Differences are shown as mean ± SD.

Histopathological characteristic	Cage control	Run	Kappa statistic
Collagen arrangement	1.91 ± 0.83	1.70 ± 0.70	0.78
Tenocyte morphology	0.91 ± 0.70	0.78 ± 0.60	0.60
Cellularity	1.37 ± 0.50	1.00 ± 0.60	0.78
Vascularity	0.91 ± 0.83	0.78 ± 0.95	0.78
Total score	*5.09 ± 1.58*	*4.26 ± 1.68*	-

2.4 Discussion

Histopathological evaluation of the tendon specimens failed to differentiate between the control rats and the running rats. These data suggest that uphill treadmill running in rodents may not be a suitable animal model for the study of human Achilles tendinosis. Achilles tendinosis in humans is characterized by tissue degeneration with a failed reparative response [69,115,455]. These changes are identified histologically as collagen fiber disorganization, hypercellularity with atypical tenocyte proliferation and

morphology, and neovascularization [69,117,456]. We did not observe changes in these or other individual histopathological categories for human Achilles tendinosis in the current animal study.

Uphill treadmill running in rats has had variable success in producing tendinosis-like changes in the Achilles tendon, with some investigators reporting preliminary tendinosis-like changes [369,370,373] whereas others reported no evident pathology [146]. The current study used a running program (15° incline with speeds of up to 30 m/min) seemingly more intense than these previous studies, but was unable to find histopathological evidence of Achilles tendinosis. These findings support those of Heinemeier et al. [146] who found that an uphill running program had no effect on the histological appearance and actually improved some mechanical properties of the rat Achilles tendon. These cumulative data suggest that uphill treadmill running in isolation in rats is unable to induce the same histopathological changes as observed in human Achilles tendinosis. Ng et al. [372] recently described a unique bipedal running model wherein rats ran at 17 m/min on a treadmill at a 20° decline, but with the animals in an upright posture. This model resulted in a decrease in Achilles tendon mechanical properties as well as histological changes associated with human Achilles tendinosis; however, the model has yet to be replicated.

One of the strengths of this study was the age of the animals which were older than those used in previous studies. The use of older animals may facilitate the development of tendon pathology if it occurs. Another strength of this study was the use of animals selectively bred for aerobic capacity. The use of HCR rats enabled us to run the animals at a greater incline and at a faster pace than in previous studies, with the

intent that these parameters would potentiate the generation of Achilles tendon degeneration. However, this study strength may also be a weakness as the selective breeding of the animals for aerobic capacity may also have led to the development of a tendon phenotype that enhanced tendon resistance to degeneration. Similarly, this study was limited by its relatively small number of animals and limited outcome measures. It is not likely that increasing the sample size would have altered the study conclusions as the total histopathological score in treadmill ran rats in the current study were actually about 25% better than in cage controls. A final limitation of the current study was the relatively prolonged treadmill acclimation period which lasted 2 weeks and subsequent relatively short period (7 weeks) of running at full speed and duration. These factors may have potentiated tendon adaptation to running and/or limited the ability to produce detectable pathology.

In summary, we were unable to identify histopathological changes in the Achilles tendon of rats that ran uphill on a treadmill. These cumulative data suggest that uphill running in isolation in rats is unable to induce the same histopathological changes as observed in human Achilles tendinosis.

CHAPTER THREE: THE EFFECTS OF UPHILL RUNNING AND COLLAGENASE INJECTION ON THE ACHILLES TENDON IN HIGH CAPACITY RUNNING RATS

3.1 Introduction

Recent decades have witnessed significant advances in the understanding of tendon pathologies. It is now generally accepted that the predominant pathology underlying chronic tendon injuries is degeneration (tendinosis), as opposed to the historically assumed inflammation (tendinitis) [69,111,457]. Tendinosis is characterized histologically by a loss of collagen fibril alignment coupled with increased collagenase activity, atypical fibroblast proliferation and neovascularization. The ultimate consequence is mechanical weakening and a subsequent propensity for the afflicted tendon to rupture. Various animal models have been developed to advance our understanding of tendinosis [458]. These include models for supraspinatus [459], elbow common extensor origin [460] and patellar tendinosis [461], with each model replicating certain histopathological and mechanical features of human tendinosis. To date, no accepted small animal model exists for Achilles tendinosis.

Numerous attempts have been made to develop an animal model of Achilles tendinosis. The most common approaches have been to inject the tendon with collagenase or mechanically overload the tendon via treadmill running. Collagenase catalyzes the hydrolysis of collagen and has elevated expression in human tendinosis [462]. However, intratendinous injection of collagenase into the Achilles tendon results in an acute and intense inflammatory reaction (tendinitis) followed by progressive tendon healing [390-

393]. This does not replicate the degenerative pathology (tendinosis) observed in the human Achilles tendon. Some success at replicating human Achilles tendinosis has been reported with mechanical overloading of rat Achilles tendons via forced treadmill running [370,372]; however, these studies have yet to be replicated and more recently it was reported that a similar treadmill running protocol was actually beneficial to the tendon [146]. Furthermore, the first study of this dissertation found no effect of running on the Achilles tendon.

A potential reason for the variable success of previous efforts to develop an animal model of Achilles tendinosis may be that only part of the etiological cause was introduced. The development of Achilles tendinosis is multifactorial and thought to result from a combination of extrinsic and intrinsic factors. Extrinsic factors (such as mechanical overuse) are most commonly indicted, but the development of Achilles tendinosis in some individuals while others with equivalent loading are spared indicates intrinsic factors also play a role. With this multifactorial etiology in mind, it was our hypothesis that in order to develop an animal model of Achilles tendinosis a combination of an intrinsic and extrinsic factor for Achilles tendinosis needs to be introduced. While uphill running in isolation failed to create a pathology in the Achilles tendon in the first study of this dissertation, this method of overuse was used in the current study with a different rationale. In the previous study, no initial injury existed, whereas in this study, an intial injury is introduced and treadmill running represents the failed healing stage in the pathogenesis of tendinopathy. The intrinsic factor (intratendinous collagenase injection) will generate matrix changes, while the extrinisic factor (mechanical overuse via treadmill running) will restrict repair of the initial matrix changes and cause

progression to a degenerative lesion. This dual method has previously been used to advance the pathology in a rat model of rotator cuff disease [365,463].

The aim of the current study was to investigate the individual and combined effects of collagenase injection (intrinsic factor) and treadmill running (extrinsic factor) in creating pathology within the rat Achilles tendon consistent with Achilles tendinosis in humans. Specifically, we assessed the effect of collagenase injection and uphill treadmill running on the histological, mechanical, and molecular properties in rats selectively bred for high-capacity running.

3.2 Methods

3.2.1 Animals

Eighty-eight HCR rats (36 female and 52 male; age = 35.5 ± 7.9 wk) were obtained from the University of Michigan (Ann Arbor, MI) and acclimated for a minimum of 2 weeks. Animals were from the 26th, 27th and 28th generations of HCR rats, and ran an average distance of 1.8 ± 0.3 km within 68.1 ± 7.5 min on a treadmill when phenotyped as untrained, young adults. Animals for each outcome measure were age-, gender- and generation-matched, and were maintained under standard conditions with *ad libitum* access to food and water. All procedures were performed following approval from the Indiana University Institutional Animal Care and Use Committee.

3.2.2 Intrinsic factor

All animals had their right Achilles tendon injected with collagenase at baseline to induce preliminary tendon changes (Figure 3.1). Collagenase was prepared by dissolving 100 mg of bacterial collagenase type I (Sigma-Aldrich, Inc., St. Louis, MO) in 10 mL of phosphate buffered saline and sterile filtering through a 0.22 µm filter. With the animal under inhalation anesthesia, 30 µL (0.3 mg) of collagenase was injected percutaneously into the right Achilles tendon 2 mm proximal to the calcaneus using a 50 µL syringe equipped with a 22G needle (Hamilton Company, Reno, NV). A one-time intratendinous dose of 0.3 mg of collagenase was chosen based on previous work showing this dose consistently induces matrix changes, including an initial acute and intense inflammatory reaction coupled with collagen disruption followed by progressive tendon healing [390-393]. Left Achilles tendons were not injected and served as non-injected controls. All animals were returned to their home cage upon recovery from anesthesia.

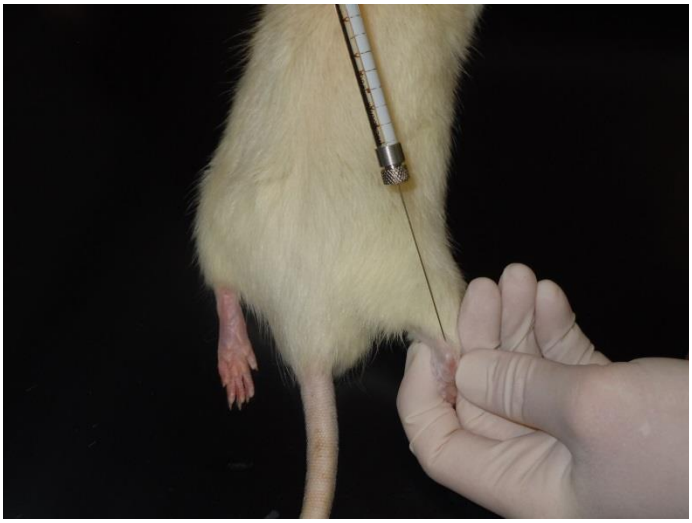


Figure 3.1. Collagenase injection into the Achilles tendon.

3.2.3 Extrinsic factor

Animals were randomly divided into treadmill running and cage control groups at nine days post-collagenase injection. A delay between the introduction of the intrinsic and extrinsic factors was permitted to allow the initial collagenase-induced inflammation to subside leaving a mechanically-compromised tendon with matrix changes. The treadmill running group ran on a treadmill at a 15° incline, 5 days/week for either 4 or 10 weeks (Table 3.1). In animals that ran for 10 weeks, speed and duration were gradually increased during the initial 2 weeks until they were running at 20 m/min for the maximum duration of 60 minutes, as previously described [122]. Thereafter, speed was increased every second week to a maximum of 30 m/min by the 10^{th} week of running. Animals in the 4 week running group were exposed to a more vigorous running regimen in order to potentiate tendon damage in a shorter period. These rats ran for 60 minutes at 15 m/min by the end of the first week. Thereafter, speed was increased every week to a maximum of 25 m/min by the 4^{th} week of running. Cage control animals were restricted to cage activities for either 4 or 10 weeks.

3.2.4 Histopathological analysis

Achilles tendons from 24 male rats (4 wk: n = 5 treadmill running and 4 cage control; 10 wk: n = 8 treadmill running and 7 cage control) were harvested and fixed in 10% neutral buffered formalin for 48 hours before being transferred to 70% ethanol.

Table 3.1. Running protocol used for the running group of rats.

		4 week		10 week	
		Duration (min)	Speed (m/min)	Duration (min)	Speed (m/min)
Week 1	Day 1	15	15	5	10
	Day 2	20	15	5	10
	Day 3	45	15	5	10-14
	Day 4	60	15	5	10-18
	Day 5	60	15	30	15-25
Week 2	Day 6	60	15-20	45	15-20
	Day 7	60	15-20	50	15-20
	Day 8	60	15-20	55	15-20
	Day 9	60	15-20	60	15-20
	Day 10	60	15-20	60	15-20
Week 3		60	15-25	60	15-25
Week 4		60	20-25	60	15-25
Week 5		-	-	60	20-25
Week 6		-	-	60	20-25
Week 7		-	-	60	20-27.5
Week 8		-	-	60	20-27.5
Week 9		-	-	60	20-30
Week 10		-	-	60	20-30

Tendons were embedded into paraffin and thin (6 μm) midsubstance sagittal sections were cut using a microtome and stained with hematoxylin and eosin. Sections were viewed on a Zeiss Axiophot light microscope and assessed for damage using a modified Bonar histopathology scale [120,121,456]. The most pathological region of the tendon was determined based upon collagen arrangement using polarized light. In this region, tendons were graded for collagen arrangement (one field of view at 100x), tenocyte morphology (four fields of view at 200x), cellularity (one field of view at 100x), and vascularity (up to 10 fields of view at 400x). A greater number of fields were viewed for characteristics requiring assessment at higher magnification in an effort to grade a similar total area of tissue for each outcome. The characteristics were graded between 0 (normal) and 3 (maximum pathology). The sum of the four categories gave a completely

normal tendon a score of 0 and a tendon with maximum damage a score of 12. Samples were randomized and graded by two independent blind examiners. Discrepancies in scoring were resolved by discussion, with a third examiner being consulted to assist in achieving consensus.

3.2.5 Mechanical testing

Whole hindlimbs were removed from 28 male rats (4 wk: n = 8 treadmill running and 6 cage control; 10 wk: n = 8 treadmill running and 6 cage control) and stored at -20°C. Hindlimbs were thawed at room temperature on the day of testing with all testing being performed at a constant room temperature of 22˚C. Muscle-Achilles tendon-bone units were harvested by dissecting the calcaneus and transecting the mid-belly of the gastrocnemius-soleus muscle complex. Muscle fibers were stripped away to leave the gastrocnemius-soleus epimysium and the paratenon was removed from around the Achilles tendon. The muscle-Achilles tendon-bone units were fixed to an electromagnetic mechanical actuator (Bose® ElectroForce® 3200 series; Bose Corporation, Minnetonka, MN), with the calcaneus embedded in a fixture containing a low-melting point alloy (Bismuth Alloy; Small Parts, Inc., Miami Lakes, FL) and gastrocnemius-soleus epimysium glued between two pieces of sandpaper with cyanoacrylate and clamped between the serpentine jaw faces of a tissue grip. Achilles tendon width and thickness were measured optically, and tendon cross-sectional area was estimated using elliptical geometry ($\text{CSA} = \frac{\text{Tendon width} * \text{Tendon thickness}}{4} * \pi$). Tendons were pulled to failure in displacement control at a constant rate of 0.5 mm/s. During testing, force and

displacement data were collected at 200 Hz. The location of failure was observed, and the mechanical properties of ultimate force (N), stiffness (N/mm) and energy to failure (mJ) were obtained. In addition, ultimate stress (MPa) was derived by dividing ultimate force by tendon cross-sectional area.

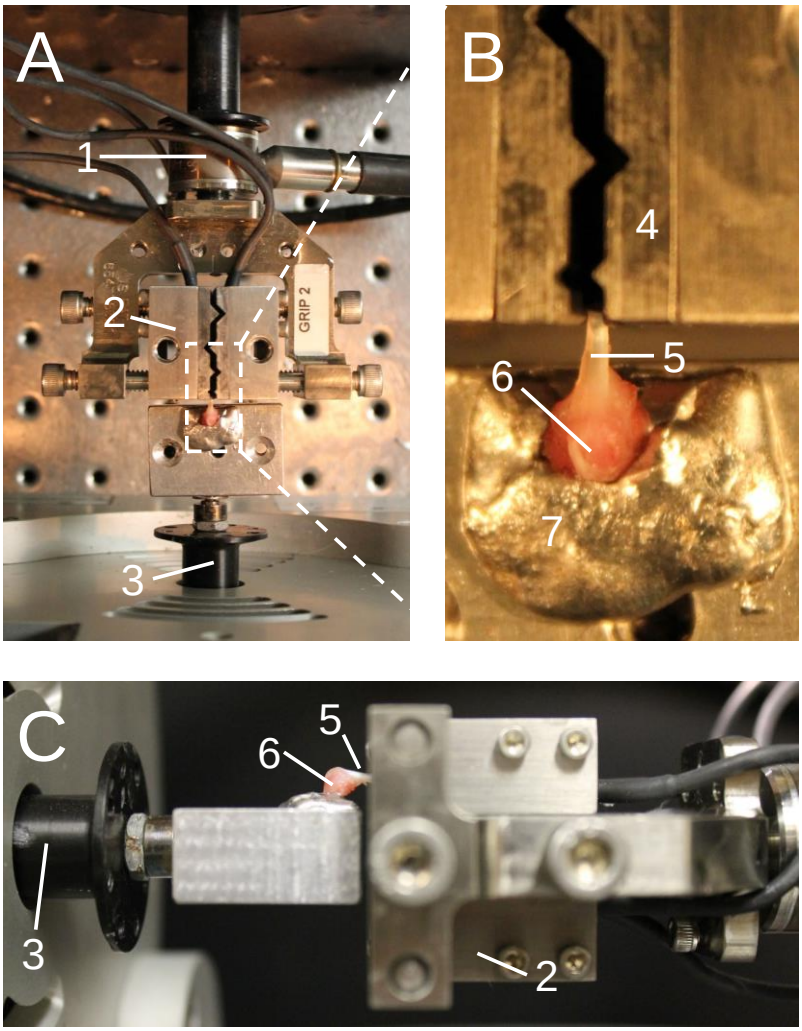


Figure 3.2. Set-up for the mechanical testing of rat Achilles tendons. A) View from above. B) Magnified view of the muscle-tendon-bone complex from above. C) Side view. 1— load cell (225N); 2— titanium tissue grips; 3 — computer controlled actuator; 4 — serpentine grip surfaces; 5— Achiles tendon; 6 — calcaneus fixed at 90° to Achilles tendon; 7— Wood's low melting point alloy used to for the calcaneus.

3.2.6 Gene expression

Achilles tendons from 36 female rats (4 wk: n = 9 treadmill running and 9 cage control; 10 wk: n = 9 treadmill running and 9 cage control) were removed and prepared for quantitative real-time PCR (qRT-PCR). Tendons were ground in liquid nitrogen into a fine powder using a mortar and pestle. The frozen tendon powder was further homogenized in Trizol® (Invitrogen, Carlsbad, CA) using a Tissue-Tearor™ (BioSpec Products, Inc., Bartlesville, OK). Total RNA was extracted from the Trizol-tendon powder samples with chloroform and precipitated with isopropanol. The RNA pellet was further processed using the RNeasy Plus Mini Kit (QIAGEN, Inc., Valencia, CA) and RNA quantity and quality were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). First strand cDNA synthesis was performed with the First-Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK).

qRT-PCR primers and probes were obtained from Assays-on-Demand™ (Applied Biosystems, Carlsbad, CA) for the following genes: Col1a1 [collagen, type I, alpha a] (Rn01463848_m1), Col3a1 [collagen, type III, alpha 1] (Rn01437681_m1), Mmp13 [matrix metallopeptidase 13] (Rn01448194_m1), Scx [scleraxis] (Rn01504576_m1), and Il1a [interleukin 1 alpha] (Rn00566700_m1). TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) was used for amplification in a Mastercycler ep realplex^2 real-time PCR system (Eppendorf, Westbury, NY). The qRT-PCR amplifications were performed in a sealed 96-well optical plate with the following reaction conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 sec followed by 60°C for 1 min. GAPDH (Rn99999916_m1) was used as an endogenous

control. Levels of target gene mRNA were expressed as fold change using the ΔΔCt method relative to expression from non-injected tendons from cage control animals.

3.2.7 Statistics

Statistical analyses were performed using IBM SPSS Statistics (v20.0; SPSS Inc., Chicago, IL) software and were two tailed with a level of significance set at 0.05. Two-way one-repeated-measure analyses of variance (ANOVA) were used for comparisons, with the intrinsic factor (collagenase injected vs. non-injected) being the within-animal and the extrinsic factor (treadmill running vs. cage control) being the between-animal independent variables. In the event of a non-significant ANOVA interaction, main effects for each independent variable were explored. Gene expression data were log transformed prior to analysis. Kappa statistics were calculated to measure the inter-rater agreement.

3.3 Results

Animals in the 4 and 10 wk treadmill running groups ran an average total distance of 22.6 ± 1.9 km and 58.3 ± 9.7 km, respectively (Figure 3.3; Table 3.2; Table 3.3). There were no body mass differences between treadmill and cage control animals at either 4 or 10 weeks (all $P > 0.11$; *unpaired t-test*). There were no statistical interactions between the intrinsic and extrinsic factors on any outcome measure (all $P > 0.11$), indicating treadmill running and collagenase injection had independent effects.

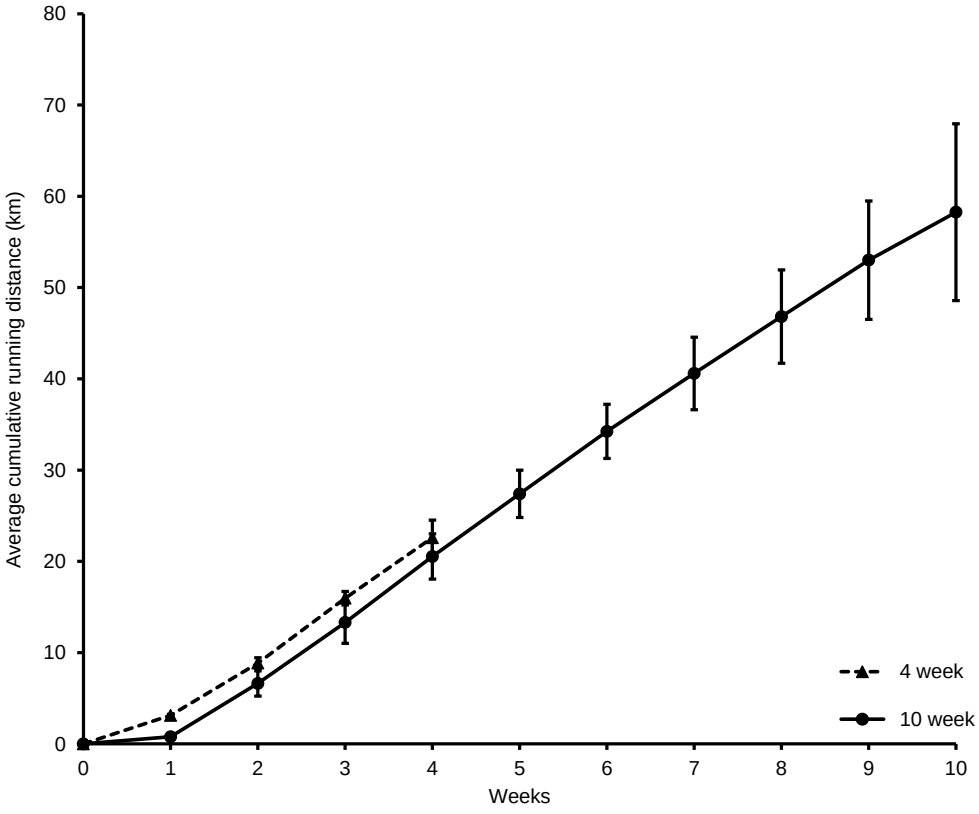


Figure 3.3. Cumulative distance ran on the treadmill by rats in the run group. Error bars represent standard deviation.

Table 3.2. Weekly running distances by rats in the 4 week run group. Distances are presented in km and maximum represents the maximum possible distance for that week.

Rat	Week				Total
	1	2	3	4	
1	3.15	5.93	7.35	8.02	24.45
2	3.15	5.93	7.35	0.00	16.43
3	3.15	5.93	7.23	7.43	23.73
4	3.15	5.93	7.23	7.43	23.73
5	3.15	5.93	7.23	7.43	23.73
6	3.15	5.93	6.98	7.30	23.35
7	3.15	5.93	7.23	7.18	23.48
8	3.15	5.93	7.23	7.30	23.60
10	3.15	5.93	6.48	6.68	22.23
11	3.15	5.93	6.23	2.96	18.26
12	2.63	5.34	7.10	6.55	21.61
13	3.15	3.41	6.60	7.05	20.21
14	3.15	5.93	7.23	7.18	23.48
15	3.15	5.93	7.23	7.43	23.73
16	3.15	5.93	7.23	6.80	23.10
17	3.15	5.93	7.23	6.93	23.23
18	3.15	5.93	7.23	7.43	23.73
19	3.15	5.93	7.23	6.30	22.60
20	3.15	5.93	7.23	7.18	23.48
22	3.15	5.53	7.23	6.68	22.58
23	3.15	4.93	7.23	7.18	22.48
24	3.15	5.93	7.23	7.43	23.73
Maximum	3.15	5.93	7.25	7.43	23.76

Table 3.3. Weekly running distances by rats in the 10 week run group. Distances are presented in km and maximum represents the maximum possible distance for that week.

Rat	Week										Total
	1	2	3	4	5	6	7	8	9	10	
1	0.95	5.85	4.99	6.82	3.45	3.49	1.57	3.03	3.15	0.00	33.27
2	0.16	5.33	6.63	7.23	7.43	7.43	7.82	7.82	8.73	7.23	65.77
3	0.16	5.33	6.13	5.85	6.68	7.43	6.99	7.82	4.19	7.04	57.59
4	0.95	5.65	6.90	6.95	6.92	6.55	4.85	2.48	1.50	0.00	42.74
5	0.55	5.23	5.76	7.23	7.18	7.30	8.09	7.54	7.08	7.79	63.72
6	0.38	5.33	6.63	7.23	7.43	7.18	6.52	4.93	3.49	0.47	49.56
7	0.38	1.17	2.60	6.85	7.43	7.18	5.53	5.62	6.33	2.80	45.87
8	0.95	6.48	7.23	7.43	7.43	7.18	3.42	1.07	2.80	0.00	43.96
9	0.88	5.33	6.51	7.23	7.43	7.30	7.68	5.07	6.63	2.89	56.91
10	0.95	6.48	7.23	7.43	7.23	7.23	7.23	7.39	7.43	8.73	67.28
11	0.95	6.48	7.23	7.43	7.23	7.23	7.23	7.39	7.43	8.73	67.28
12	0.00	4.11	7.10	7.43	7.43	7.43	4.27	3.75	5.24	0.00	46.74
13	0.95	6.48	7.23	7.43	6.98	6.98	7.23	7.14	7.18	8.73	66.28
14	0.95	6.48	7.23	7.43	7.23	7.23	7.23	7.39	7.43	8.73	67.28
15	0.95	6.48	7.23	7.43	7.23	7.10	7.23	6.39	6.80	7.68	64.48
16	0.92	6.35	7.23	7.43	7.43	7.43	6.47	5.40	4.90	0.00	53.54
17	0.95	6.48	7.23	7.43	7.23	7.10	7.23	7.39	7.43	8.73	67.16
18	0.95	6.48	7.23	7.43	7.23	7.10	7.23	7.39	7.43	8.73	67.16
19	0.95	6.48	6.35	7.03	7.07	7.30	6.19	6.40	5.85	0.00	53.61
20	0.95	6.48	7.23	7.43	7.23	7.10	7.23	7.39	7.30	8.73	67.03
21	0.95	6.48	7.23	7.43	5.93	5.93	5.93	7.43	7.43	8.73	63.42
22	0.95	6.48	7.23	7.43	5.93	5.93	5.93	7.43	7.43	8.73	63.42
23	0.91	5.85	6.05	7.13	7.13	7.03	8.04	7.02	7.00	0.00	56.15
24	0.95	6.48	7.23	7.43	5.93	5.93	5.93	7.43	7.18	8.28	62.72
25	0.95	6.48	7.23	7.43	5.93	5.93	5.93	7.43	7.43	8.73	63.42
Maximum	0.95	6.48	7.23	7.43	7.43	7.43	8.09	8.09	8.73	8.73	70.59

3.3.1 Histopathological appearance

There were no obvious qualitative differences between Achilles tendons from cage control and treadmill running animals at 4 weeks, whereas collagenase injected tendons at the same time point demonstrated hypercellularity with rounded tenocytes and increased vascularity (Figure 3.3A). Collagenase injected tendons showed higher total histopathological scores than non-injected tendons at 4 weeks ($P < 0.01$) (Figure 3.3B). There was no effect of treadmill running on total histopathological score at 4 weeks ($P = 0.41$) (Figure 3.3B), and neither collagenase injection ($P = 0.29$) or treadmill running ($P = 0.71$) influenced total histopathological score at 10 weeks (Figure 3.3C). Scores for individual histopathological characteristics are shown in Table 3.4.

3.3.2 Mechanical properties

During testing, all tendons failed proximal to their insertion into the calcaneus without bone avulsion. There was no effect of treadmill running on tendon cross-sectional area or mechanical properties at either 4 or 10 weeks (all $P > 0.33$; Figure 3.4). Collagenase injection increased tendon cross-sectional area by 56% and 53% at 4 and 10 weeks, respectively (all $P < 0.01$; Figure 3.4A, B). At 4 weeks, collagenase injected tendons had 15% and 42% lower ultimate force and stress than non-injected tendons, respectively (all $P < 0.05$; Figure 3.4C, E). Collagenase injected tendons also had 42% lower energy to failure ($P < 0.05$; Figure 3.4G) than non-injected tendons at 4 weeks; however, there was no effect of collagenase at 4 weeks on stiffness ($P = 0.31$, Figure 3.4I). At 10 weeks, collagenase injected tendons had 13% and 35% less ultimate force

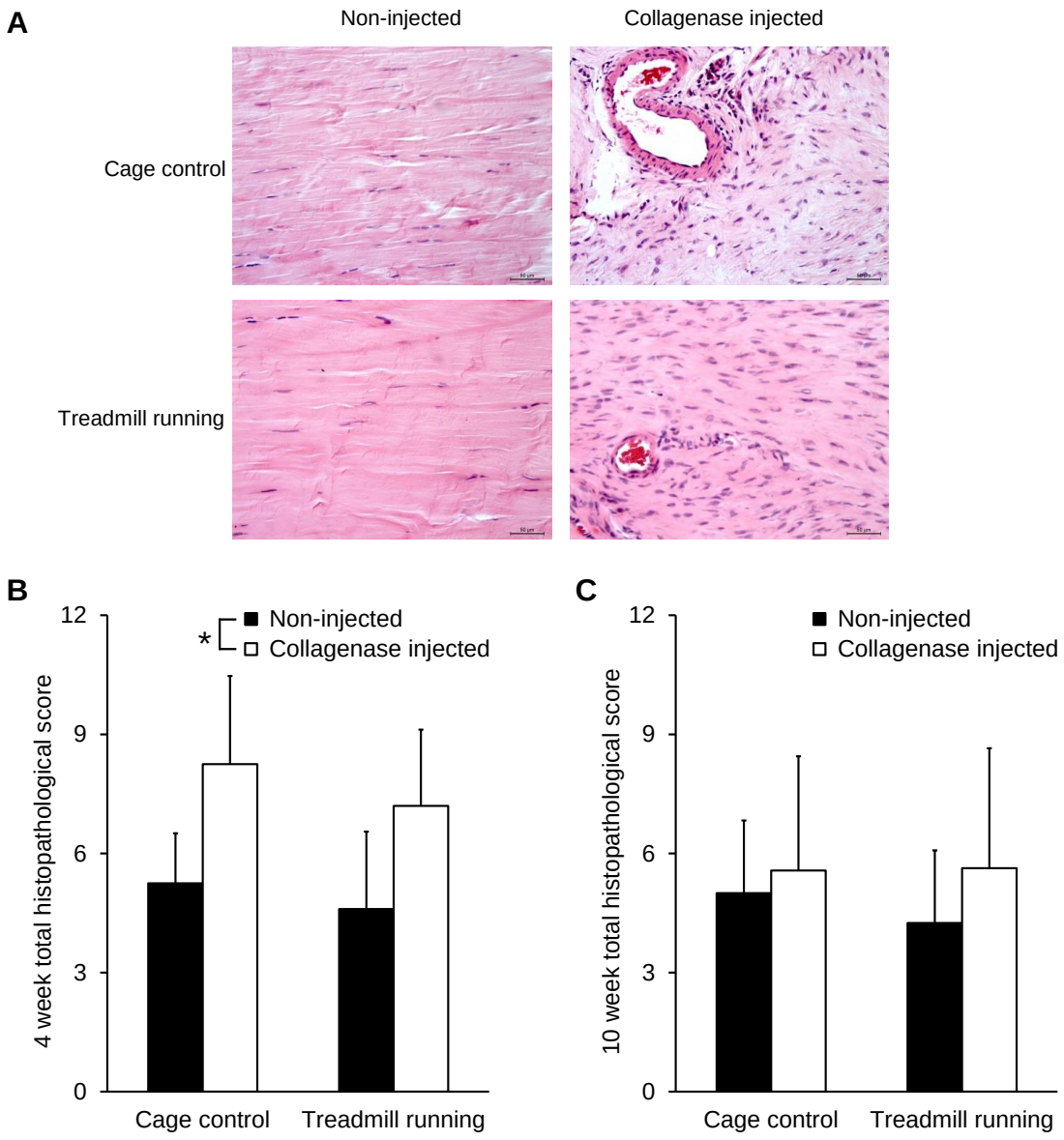


Figure 3.4. Collagenase and treadmill running effects on the histological presentation of the Achilles tendon. A) Representative photomicrographs of Achilles tendons in the 4 week experimental group. Note the well-aligned collagen fibers and elongated tenocyte nuclei in the non-injected tendons. Collagenase injected tendons had notable changes with highly unorganized collagen fibers, hypercellularity, rounded tenocytes, and increased vascularity. B) Average total histopathological scores of tendons in the 4 week group. C) Average total histopathological scores of tendons in the 10 week group. Bars in C and D represent mean ± SD. There was no collagenase injection x treadmill running interaction at either time point (all $P > 0.85$). There was no main effect for treadmill running in the 4 week group ($P = 0.58$) or main effects for either collagenase injection ($P = 0.31$) or treadmill running ($P = 0.43$) in the 10 week group). *Collagenase injection main effect ($P < 0.01$).

Table 3.4. Collagenase and treadmill running effects on histopathological characteristics. Scores are presented as mean ± SD.

	Histopathological characteristic	Cage control		Treadmill running		Kappa statistic	P-values		
		Non-injected	Collagenase injected	Non-injected	Collagenase injected		Collagenase effect	Running effect	Interaction effect
4 week									
	Collagen arrangement	2.25 ± 0.50	2.25 ± 0.50	1.20 ± 0.84	2.20 ± 0.45	0.67	0.15	0.07	0.15
	Tenocyte morphology	1.25 ± 0.96	1.50 ± 1.00	0.80 ± 0.45	1.60 ± 0.89	0.92	0.06	0.74	0.29
	Cellularity	1.25 ± 0.50	1.75 ± 0.50	1.20 ± 0.84	1.40 ± 0.55	0.75	0.36	0.41	0.69
	Vascularity	0.50 ± 1.00	2.75 ± 0.50*	1.40 ± 1.34	2.00 ± 1.41*	0.92	0.03	0.9	0.15
	Total score	5.25 ± 1.26	8.25 ± 2.22*	4.60 ± 1.95	7.20 ± 1.92*	-	0.01	0.41	0.81
10 week									
	Collagen arrangement	1.71 ± 0.95	1.86 ± 0.90	1.63 ± 0.52	1.50 ± 0.93	0.70	0.98	0.36	0.72
	Tenocyte morphology	0.71 ± 0.49	1.00 ± 1.00	0.75 ± 0.46	1.13 ± 0.83	0.40	0.19	0.79	0.86
	Cellularity	1.43 ± 0.53	1.29 ± 0.76	1.00 ± 0.53	1.50 ± 0.76	1.00	0.50	0.64	0.23
	Vascularity	1.14 ± 0.69	1.43 ± 1.51	0.88 ± 0.99	1.50 ± 1.20	0.75	0.23	0.84	0.65
	Total score	5.00 ± 1.83	5.57 ± 2.88	4.25 ± 1.83	5.63 ± 3.02	-	0.29	0.71	0.66

*Collagenase injection main effect ($P < 0.03$)

and stress than non-injected tendons, respectively (all $P < 0.04$; Figure 3.4D, F). There was no effect of collagenase at 10 weeks on energy to failure ($P = 0.31$, Figure 3.4H); however, collagenase injected tendons had 13% less stiffness than non-injected tendons ($P = 0.04$, Figure 3.4J).

3.3.3 Gene expression

The effects of collagenase injection and treadmill running on gene expression are shown in Figure 3.5. Expression levels of Il1a at 10 weeks were undetectable. Treadmill running decreased Col3a1 and MMP13 expression at 4 weeks (all $P < 0.05$; Figure 3.5C, E). There was no effect of treadmill running on any other genes at 4 weeks or any genes at 10 weeks (all $P = 0.06$ to 0.98). Collagenase injection increased expression of Col1a1, Col3a1, Mmp13 and Il1a at 4 weeks (all $P \leq 0.03$; Figure 3.5A, C, E, I). MMP13 remained elevated in collagenase injected tendons at 10 weeks ($P = 0.03$; Figure 3.5F). There was no effect of collagenase on Col1a1 or Col3a1 at 10 weeks (all $P \geq 0.08$; Figure 3.5B, D), or Scx at either 4 or 10 weeks ($P > 0.15$; Figure 3.5G, H).

3.4 Discussion

The results of this study indicate that combined introduction of intratendinous collagenase injection and uphill treadmill running is unable to create lasting pathology within the HCR rat Achilles tendon consistent with chronic Achilles tendinosis in humans. Collagenase injection induced molecular, histopathological and

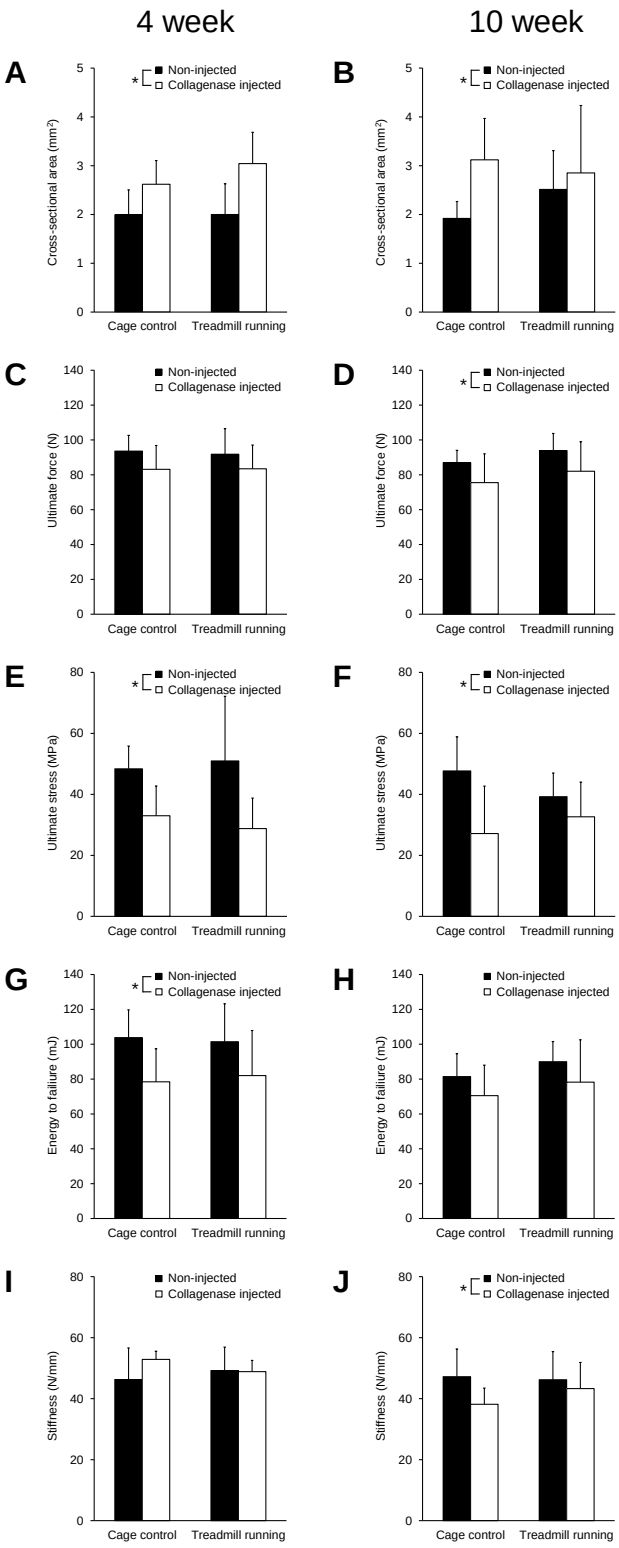


Figure 3.5. Collagenase and treadmill running effects on the mechanical properties of the Achilles tendon. Bars represent mean ± SD. *Collagenase injection main effect ($P < 0.05$).

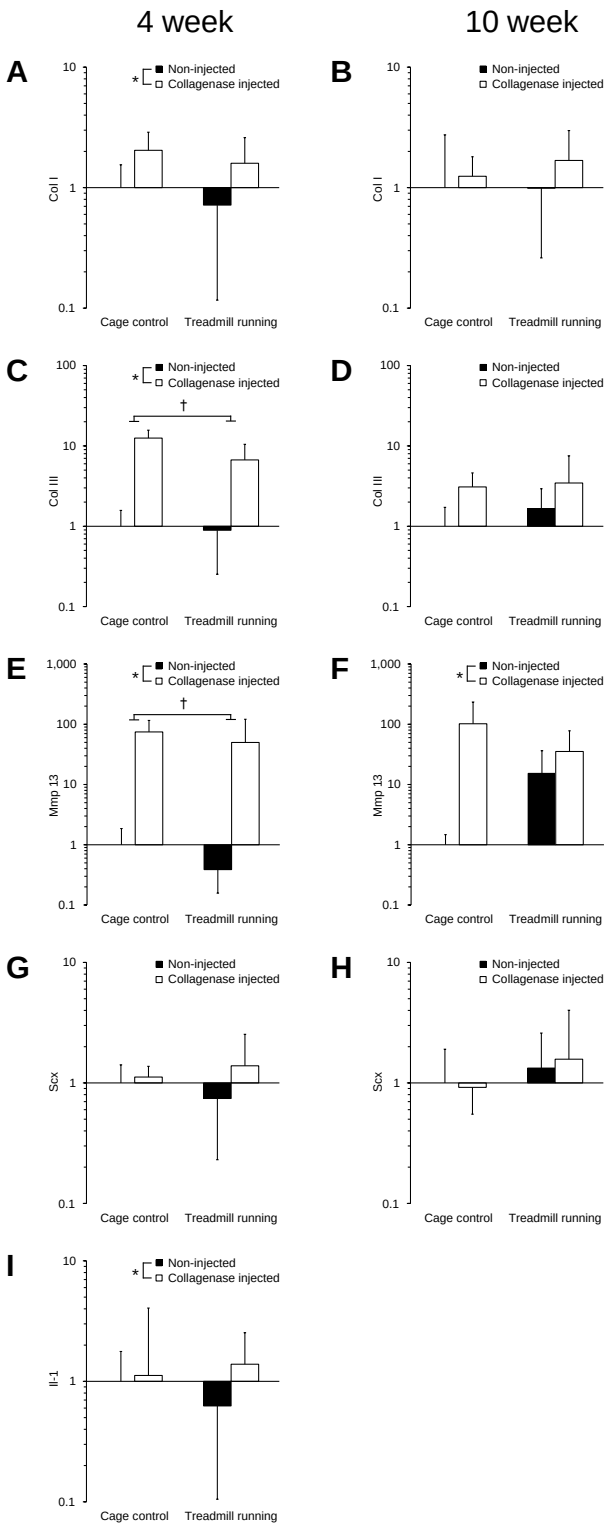


Figure 3.6. Collagenase and treadmill running effects on the gene expression of the Achilles tendon. Levels of total RNA presented as fold expression relative to non-injected cage control rats. Bars represent mean ± SD. *Collagenase injection main effect ($P < 0.03$). †Treadmill running main effect ($P < 0.05$).

mechanical property changes within the Achilles tendon when assessed at 4 weeks post-injection.The mechanical changes persisted at 10 weeks following injection; however, the histopathological and majority of the molecular changes associated with collagenase injection were no longer present at 10 weeks. Treadmill running did not exacerbate the collagenase-induced changes as there were no statistical interactions between collagenase injection and treadmill running. In fact, treadmill running had minimal effect on the Achilles tendon, with the only measureable effects of running being reductions in Col3a1 and Mmp13 expression at 4 weeks. These early gene expression changes did not translate into running-induced histopathological or mechanical property changes. Overall, these data suggest that intratendinous collagenase injection had early, partially-reversible effects on the Achilles tendon of rats selectively bred for high-capacity running which were not influenced by treadmill running.

The current data contrast the reported combined effects of collagenase injection and treadmill running in a rat model of rotator cuff disease [365,463], but generally support those of Godbout et al. [391] who showed voluntary wheel running beginning 7 days following collagenase injection had no additive effect on rat Achilles tendon mechanical properties when assessed at 28 days post-injection. Intratendinous collagenase injection in the current study induced early tendon degradation, evident by the presence of hypercellularity with rounded tenocytes, increased vascularity and mechanical weakening of the tendon at 4 weeks post-injection. These histopathological and mechanical changes were coupled with elevated expression of markers for inflammation (Il-1a) and tendon remodeling (Mmp13, Col1a1 and Col3a1). As the histopathological changes were no longer evident at 10 weeks post-injection, the early

collagenase injection effects were partially reversible with the pathology not progressing to replicate Achilles tendinosis as observed in humans. The reversibility of intratendinous collagenase injection effects in the Achilles tendon is consistent with previous reports [390-393]. Superimposition of uphill treadmill running had no synergistic or even additive effect on the histopathological or mechanical properties of collagenase-injected Achilles tendons, suggesting running was unable to sufficiently overload the intrinsically compromised tendons.

Numerous investigators have previously investigated treadmill running as a potential means of mechanically overloading the rodent Achilles tendon [122,146,369-373]. Some success has been reported in terms of inducing histopathological and mechanical changes consistent with human Achilles tendinosis [370,372]; however, these data have not been independently replicated and, in one instance, have been contradicted by more recent comprehensive data showing treadmill running actually had beneficial tendon effects [146]. The absence of a treadmill running detrimental effect on the Achilles tendon may relate to the innate ability of rodents to run. Rats are habitual runners, and run in excess of 8 km/day in the wild and in voluntary wheel-running studies [452,464]. The preference of rats to run may make it difficult to induce overuse tendon injuries as their musculoskeletal systems may be inherently adapted to the loads associated with running [465]. Inherent adaptation may have been exaggerated in the current study by the use of HCR rats which have been selectively bred for aerobic capacity. The use of HCR rats enabled us to run our animals at a greater incline (15°) and faster pace (up to 30 m/min) than in previous studies, with the belief that these parameters would potentiate the generation of Achilles tendon degeneration. However,

the study strength of using HCR rats may also be a weakness as the selective breeding of our animals for aerobic capacity may also have led to the development of a tendon phenotype that further enhanced tendon resistance to degeneration

It is possible that the relatively long delay (9 days) between collagenase injection and commencement of treadmill running contributed to the absence of an interaction between the two interventions. The delay was introduced to allow the initial inflammation (tendinitis) response to intratendinous collagenase injection to subside so that mechanical loading was being performed on a relatively non-inflamed, but intrinsically compromised tendon. Unfortunately, the delay may have ultimately reduced the ability to observe an interaction between the intrinsic and extrinsic factors for tendon degeneration. This hypothesis is partly support by Godbout et al. [391]. In addition to exploring the effects of voluntary wheel running beginning 7 days following collagenase injection, Godbout et al. [391] explored the effect of running commenced immediately following injection. Combining collagenase injection with immediate access to a running wheel, Godbout et al. [391] observed elevated markers of inflammation after 3 and 7 days, and reduced Achilles tendon mechanical properties at 28 days compared to when the interventions were introduced in isolation. These data suggest running commenced earlier after collagenase injection may be more effective at advancing Achilles tendon degeneration. However, this requires further investigation as it remains unclear whether the larger decline in mechanical properties with combined introduction of collagenase injection and wheel running in Godbout et al.'s [391] study was due to tendinosis or persistent tendinitis as histopathological analyses were not performed and tendons were not followed beyond 4 weeks of intervention.

The present study had a number of strengths with regards to the development of a suitable animal model of Achilles tendinosis. It intrinsically degraded one Achilles tendon in relatively old animals (age = 35.5 ± 7.9 wk) prior to running the animals at a relatively steeper incline and speed than used in previous studies. The injection of one tendon per animal enabled within-animal comparisons to be performed, while the use of relatively older animals may have potentiated the development of tendon degeneration. Despite these strengths, the study did have limitations beyond those already discussed. In particular, the relatively prolonged treadmill acclimation period of 2 weeks after collagenase injection and subsequent relatively short period (8 weeks) of running at full speed and duration may have facilitated tendon adaptation to running and/or limited the ability to progress the initial collagenase-induced pathology. An additional limitation may have been the lack of a control injection in the contralateral limb. Khan, et al [378] conducted a study utilizing injections into tendons and included three control groups: no injection, saline injection, and needlestick only. None of these treatments resulted in inflammatory or degenerative changes with light microscopy; therefore, neither the volume of the collagenase nor the needlestick is likely the cause of the collagenase effect in injected tendons. The current study was also limited by relatively small numbers of samples for each outcome measure and the use of animals of different sexes for different outcome measures (although all analyses were performed on sex-matched samples).

In summary, the results of this study indicate that combined introduction of intratendinous collagenase injection and uphill treadmill running is unable to create pathology within the HCR rat Achilles tendon consistent with Achilles tendinosis in humans.

CHAPTER FOUR: THE EFFECTS OF UPHILL RUNNING AND COLLAGENASE INJECTION ON THE ACHILLES TENDON IN SPRAGUE DAWLEY RATS

4.1 Introduction

Although several studies have reported pathological changes in the Achilles tendon following treadmill running [369,370,373], the previous studies in this dissertation did not result in similar changes. The initial study was completed to determine if mechanical overuse at higher speeds and greater incline than previous studies could produce pathological changes in the histology of the Achilles tendon in HCR rats. Similar to Heinemeier, et al. [146], this protocol did not induce histopathological changes in the Achilles tendon.

To build upon this initial study, the individual and combined effects of an extrinsic and intrinsic factor were investigated. This protocol was designed to model the three-stage process of pathogenesis of tendinopathy described by Fu, et al. [244]. This hypothesis includes an initial injury followed by failed healing, which eventually results in clinical presentation of tendinopathy. While this process seems to provide an accurate theory for the human pathology, studies investigating this process in animal models are limited. Godbout, et al. [391] conducted a study utilizing voluntary wheel running with collagenase injection in rats, but this method has yet to be replicated. Therefore, we performed intratendinous collagenase injections to mimic the initial injury, followed by uphill treadmill running to contribute to the failed healing stage. Additionally, the mechanical and molecular changes were investigated along with the histological

characteristics in order to provide a more complete description of the effect of this protocol. These outcome measures revealed that the combination of uphill treadmill running and collagenase injection did not induce pathology in the HCR rat Achilles tendon similar to human tendinosis.

The aim of the current study was to investigate the individual and combined effects of collagenase injection and uphill treadmill running in creating pathology within the rat Achilles tendon consistent with Achilles tendinosis in humans. The effects of these factors were analyzed by observing the histological properties of Sprague Dawley Achilles tendons. The negative results of the previous studies required further investigation of the limitations of the protocols in order to determine if these limitations were preventing a positive result. The limitations of HCR rats, a long delay between collagenase injection and treadmill running, and treadmill acclimation following collagenase injection were addressed in the present study by using a different strain of rats and adjusting the protocol to minimize any limitations present in the previous studies.

4.2 Methods

4.2.1 Animals

Twenty-five 12 week-old, virgin, female Sprague Dawley rats (weight: 210.3 ± 8.0 g) were acquired (Harlan Laboratories, Indianapolis, IN) and acclimated for one week. All animals were maintained under standard conditions and provided *ad libitum*

access to food and water. All procedures were performed following *a priori* approval from the Indiana University Institutional Animal Care and Use Committee.

4.2.2 Treadmill acclimation

Following the one week acclimation period, all rats underwent 5 days of treadmill acclimation, which began with rats running for 20 minutes at 17 m/min and a 15° incline. Duration was increased by 10 minutes each day, reaching 60 minutes on day 5. The speed and incline were kept constant at 17 m/min and 15°.

4.2.3 Intrinsic factor

At the conclusion of running on the final day of treadmill acclimation, all rats received a collagenase injection in their right Achilles tendon to induce preliminary tendon changes. Collagenase was prepared by dissolving 100 mg of bacterial collagenase type I (Sigma-Aldrich, Inc., St. Louis, MO) in 10 mL of phosphate buffered saline and sterile filtering through a 0.22 μm filter. With the animal under inhalation anesthesia, 30 μL (0.3 mg) of collagenase was injected percutaneously into the right Achilles tendon 2 mm proximal to the calcaneus using a 50 μL syringe equipped with a 22G needle (Hamilton Company, Reno, NV). A one-time intratendinous dose of 0.3 mg of collagenase was chosen based on previous work showing this dose consistently induces matrix changes, including an initial acute and intense inflammatory reaction coupled with collagen disruption followed by progressive tendon healing [390-393]. Left Achilles

tendons were not injected and served as non-injected controls. All animals were returned to their home cage upon recovery from anesthesia.

4.2.4 Extrinsic factor

Animals were randomly divided into treadmill running (n = 12) or cage control (n = 13) groups after 3 days of normal cage activity following the collagenase injections. Rats in the treadmill running group ran 5 days/week for 8 weeks, while rats in the cage control group maintained normal cage activity for 8 weeks. On day 1 of the running protocol, rats in the treadmill running group ran for 45 minutes at 17 m/min and a 15° incline. On day 2, the duration was increased to 60 minutes, where it remained for the rest of the study. Speeds were increased by 1 m/min every week throughout the 8 weeks of the protocol, reaching 24 m/min in the final week (Table 4.1). Treadmills were equipped with an electric shock grid set to 10 Hz. If rats remained on the shock grid for more than three seconds, they were manually prodded back onto the treadmill or removed from the shock grid and placed onto the treadmill. If this occurred more than five times, the rat was removed from the treadmill and allowed to rest until the running session the following day.

4.2.5 Histopatholgical analysis

All animals were euthanized after eight weeks of the running regimen and Achilles tendons were harvested and fixed in 10% neutral buffered formalin for 48 hours

before being transferred to 70% ethanol. Tendons were embedded into paraffin and 6 μm midsubstance sagittal sections were cut using a microtome. Sections were stained with hematoxylin and eosin as previously described and additional sections from each tendon were stained with alcian blue and counterstained with nuclear fast red.

Table 4.1. Running protocol used for the running group of rats.

			Duration (min)	Speed (m/min)
Acclimation	Week 1	Day 1	20	17
		Day 2	30	17
		Day 3	40	17
		Day 4	50	17
		Day 5*	60	17
Protocol	Week1	Day 6	45	17
		Day 7	60	17
		Day 8	60	17
		Day 9	60	17
		Day 10	60	17
	Week 2		60	18
	Week 3		60	19
	Week 4		60	20
	Week 5		60	21
	Week 6		60	22
	Week 7		60	23
	Week 8		60	24

*Intratendinous collagenase injections were given following running

Prior to staining, the alcian blue and nuclear fast red counterstain reagents were prepared. To prepare the 1% alcian blue, 97 mL of distilled water was combined with 3 mL glacial acetic acid, and then 1 g of alcian blue, 8Gx was added. This mixture was stirred overnight and adjusted to pH 2.4 by adding glacial acetic acid. Sodium hydroxide was added if the reagent became too acidic. Once the pH was correct, the reagent was filtered before use. The 0.1% nuclear fast red was prepared by dissolving 5 g aluminum

sulfate into 100 mL distilled water, followed by slowly adding 0.1 g nuclear fast red. The mixture was heated to a boil and cooled, then filtered. A grain of thymol was added as a preservative.

After preparing the reagents, sections were de-waxed and re-hydrated, then placed in the alcian blue solution for 90 minutes. Next, the sections were washed in running tap water for two minutes, and then rinsed in distilled water. Sections were then counterstained in the nuclear fast red solution for eight minutes and washed in running tap water for one minute. Sections were dehydrated through two changes of 95% and 100% ethanol and cleared in xylene twice. Finally, slides were coverslipped with resinous mounting medium.

All sections were viewed on a Leica DM3000 light microscope (Leica Microsystems; Wetzlar, Germany). In addition to the four characteristics previously described, ground substance pathology was determined from observation of sections stained with alcian blue (Table 4.2). The characteristics were graded between 0 (normal) and 3 (maximum pathology). The sum of all five categories gave a normal tendon a score of 0 and a tendon with maximum damage a score of 15.

Table 4.2. Semiquantitative scale used to grade tendon ground substance.

Characteristic	Grade 0	Grade 1	Grade 2	Grade 3
Ground substance	• No stainable ground substance	• Stainable mucin between fibers • Discrete bundles	• Stainable mucin between fibers • Loss of demarcation of bundles	• Abundant mucin throughout the section • Inconspicuous collagen staining

4.3 Results

Animals ran an average total distance of 41.8 ± 3.5 km during this study (Figure 4.1; Table 4.3). There were no obvious differences between Achilles tendons from cage control and treadmill running animals; however, collagenase injected tendons had notable hypercellularity with rounded tenocytes and increased vascularity (Figure 4.2A), as well as increased ground substance (Figure 4.2B). These observations were confirmed quantitatively with collagenase injected tendons having higher scores than non-injected tendons in all of the categories assessed (all $P \leq 0.001$). Additionally, the total histopathological scores of collagenase injected tendons were higher than non-injected tendons ($P < 0.001$). There was no effect of treadmill running on the individual (all $P > 0.25$) or total histopathological scores ($P = 0.37$), nor were there any interaction effects (all $P > 0.19$ (Figure 4.2C). Scores for individual histopathological characteristics are shown in Table 4.4.

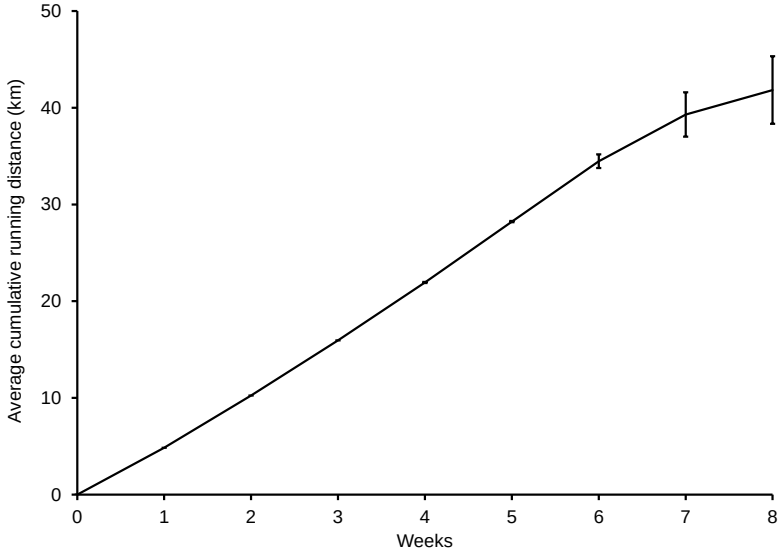


Figure 4.1. Cumulative distance ran on the treadmill by rats in the run group. Error bars represent standard deviation.

Table 4.3. Weekly running distances by rats in the run group. Distances are presented in km and maximum represents the maximum possible distance for that week.

Rat	Week								Total
	1	2	3	4	5	6	7	8	
1	4.85	5.40	5.70	6.00	6.30	6.05	2.88	0.31	37.48
2	4.85	5.40	5.70	6.00	6.30	4.29	1.38	0.48	34.40
3	4.85	5.40	5.70	6.00	6.30	6.38	3.91	1.56	40.10
4	4.85	5.40	5.70	6.00	6.30	6.60	6.90	3.36	45.11
5	4.85	5.40	5.70	6.00	6.30	6.60	6.90	0.96	42.71
6	4.85	5.40	5.70	6.00	6.30	6.60	4.49	1.03	40.36
7	4.85	5.40	5.70	6.00	6.30	6.60	5.87	5.88	46.59
8	4.85	5.40	5.70	6.00	6.30	6.60	3.45	2.76	41.06
9	4.85	5.40	5.70	6.00	6.30	6.60	6.90	3.60	45.35
10	4.85	5.40	5.70	6.00	6.30	5.39	4.60	3.72	41.96
11	4.85	5.40	5.70	5.80	6.30	6.60	4.95	2.64	42.23
12	4.85	5.40	5.70	6.00	6.30	6.60	5.75	4.08	44.68
Maximum	4.85	5.40	5.70	6.00	6.30	6.60	6.90	7.20	48.95

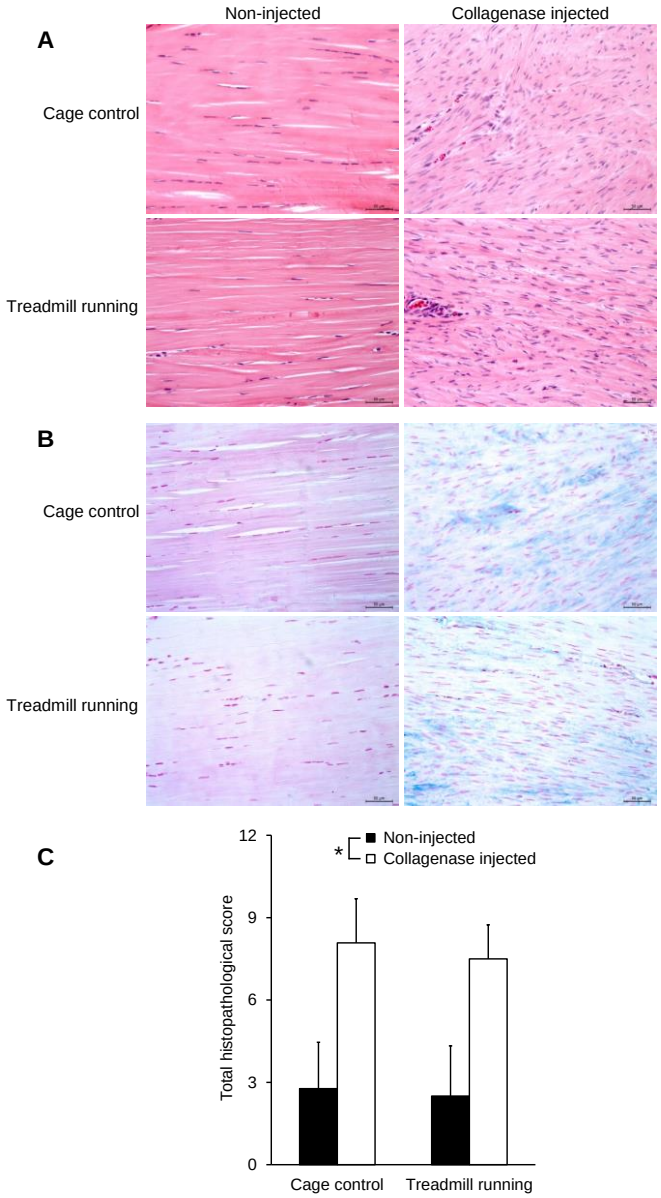


Figure 4.2. Collagenase and treadmill running effects on the Achilles tendon. A) Representative photomicrographs of Achilles tendons. Collagenase injected tendons had notable changes with highly unorganized collagen fibers, hypercellularity, rounded tenocytes and increased vascularity (H&E, 200x). B) Representative photomicrographs of Achilles tendons stained to observe ground substance within the tendon. Note the minimal staining in the non-injected tendons and abundant staining in collagenase injected tendons (Alcian blue, 200x). C) Average total histopathological scores of tendons. Bars represent mean ± SD. There was no collagenase injection x treadmill running interaction (P = 0.82) or main effect for treadmill running (P = 0.36). *Collagenase injection main effect ($p < 0.001$).

Table 4.4. Collagenase and treadmill running effects on histopathological characteristics. Scores are presented as mean ± SD.

Histopathological characteristic	Cage control		Treadmill running		P-values		
	Non-injected	Collagenase injected	Non-injected	Collagenase injected	Collagenase effect	Running effect	Interaction effect
Collagen arrangement	0.77 ± 0.93	2.08 ± 0.86*	0.92 ± 0.67	2.25 ± 0.62*	<0.001	0.56	0.94
Tenocyte morphology	0.77 ± 0.60	1.69 ± 0.95*	0.75 ± 0.75	1.17 ± 0.58*	0.001	0.25	0.19
Cellularity	0.54 ± 0.78	1.31 ± 0.48*	0.42 ± 0.79	1.25 ± 0.45*	0.001	0.58	0.88
Vascularity	0.69 ± 1.18	3.00 ± 0*	0.42 ± 0.51	2.83 ± 0.39*	<0.001	0.26	0.78
Ground substance	0.23 ± 0.44	1.85 ± 1.14*	0.08 ± 0.29	1.75 ± 1.22*	<0.001	0.63	0.92
Total score	*3.00 ± 1.73*	*9.92 ± 2.53**	*2.58 ± 1.73*	*9.25 ± 1.86**	<0.001	0.36	0.82

*Collagenase injection main effect ($P \leq 0.001$, as determined by two-way, one-repeated ANOVA)

4.4 Discussion

Similar to the previous studies, neither treadmill running nor the combination of treadmill running and collagenase injection resulted in any histopathological changes. Collagenase injection in isolation resulted in significantly higher histopathological scores than control tendons; however, previous research has found that this effect would have healed over time rather than progress into the chronic pathology observed in humans.

This study was conducted to address the suspected limitations of the former studies. One limitation of the previous studies was the use of HCR rats, whose selective breeding may have led to the development of a tendon phenotype that further enhanced resistance to degeneration. In order to address this limitation, this study was conducted using Sprague Dawley rats rather than HCR rats; however, 8 weeks of treadmill running in the present study had no histopathological effects on collagenase injected tendons in Sprague Dawley rats, suggesting that the strain of animals used in the current study may not completely explain the absence of significant findings in the previous studies. However, there may be slight differences between the two strains of rats as significant differences existed in each of the graded characteristics of Sprague Dawley rats, but not HCR rats. Ultimately, it appears difficult to sufficiently run rats to induce measureable Achilles tendon damage, with the threshold between beneficial and detrimental Achilles tendon loading with treadmill running apparently being beyond reach even in intrinsically compromised tendons.

Another limitation of the previous study was the long delay of nine days between collagenase injection and the commencement of treadmill running. In the present study,

the 8 weeks of treadmill running began only three days following collagenase injection, yet this did not create any histopathological effect on the Achilles tendon.

Finally, the relatively prolonged treadmill acclimation period of two weeks after collagenase injection followed by only two or eight weeks of running at full speed and duration used in the previous study may have facilitated tendon adaptation to running and/or limited the ability to progress the initial collagenase induced pathology. In the present study, this was addressed by acclimating the Sprague Dawley rats to the treadmill for one week prior to collagenase injection and subsequently increasing them to full speed and duration on the fourth day after collagenase injection. This adjustment also failed to create an interaction effect on the histological presentation of the Achilles tendon.

In summary, we were unable to identify histopathological changes in the Achilles tendon of Sprague Dawley rats following the introduction of both an intrinsic and extrinsic factor. This finding may be a consequence of the inability to mechanically overload tendons with forced treadmill running, even those tendons that have been intrinsically compromised by previous intratendinous collagenase injection.

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

5.1 Dissertation summary

Studies in this dissertation investigated the effects of exercise and collagenase on the rodent Achilles tendon in attempt to develop an animal model of Achilles tendinosis. Previous studies have reported conflicting results using exercise, specifically treadmill running, as a means of inducing Achilles tendinosis in rodents. The initial study of this dissertation found no histopathological changes in the Achilles tendon of HCR rats following nine weeks of mandatory uphill treadmill running. Subsequent studies included an initial intratendinous collagenase injection to induce an initial injury that may be exacerbated by mandatory uphill treadmill running in both HCR and Sprague Dawley rats. Light microscopy, mechanical testing, and molecular analysis all revealed a damaging effect of the collagenase injection. While this collagenase effect was apparent, it was not consistent with human pathology. In addition, these effects did not consistently remain at 10 weeks, which may have been a result of progressive healing rather than persistent damage. In contrast, the effects of treadmill running were minimal, as the extrinsic factor had no effect on the histology or biomechanics and only the expression of two genes. Furthermore, these effects appeared beneficial rather than destructive. Additionally, there were no interaction effects between collagenase injection and uphill treadmill running in any of the outcome measures which were analyzed.

5.2 Strengths and limitations

Several strengths exist in the studies of this dissertation. First, these studies were performed in two different strains of rats, as well as rats of both genders and various ages. This variety helps to eliminate uncertainty that the lack of degeneration may be due to the age, gender, or strain of the rats used in these studies. Also, several durations of treadmill running and cage activity were used, including 4, 8, 9, and 10 weeks, which allows the effects of the treadmill running and collagenase to be examined at multiple time points. In addition, the series of the studies allowed limitations from one study to be adjusted in the subsequent studies in order to minimize the possibility that these limitations were the cause of negative outcomes. Finally, these studies contribute to the ongoing search for an acceptable animal model of Achilles tendinosis. While there were no positive outcomes of these protocols, the results provide researchers with knowledge that may contribute to an eventual successful model.

While many strengths exist, there are acknowledged limitations within the studies of this dissertation. The most recognizable limitation is the difficulty of mandatory treadmill running. Typically, rats began the protocol running for the daily duration with little to no rest on the electric shock grids; however, after approximately seven weeks, the majority of the rats required manual prodding or removal from the shock grid and placement on the treadmill. In addition to lessening the overall time spent running, the manual prodding often resulted in an altered gait, which may have prevented the appropriate stresses from acting upon the Achilles tendon.

The use of male and female rats may have contributed to the limitations of the studies. The conclusions of the studies in this dissertation compared outcome measures performed in one gender to outcome meaures performed in another gender; therefore, gender may have played a role in the results. However, we do not believe this influenced conclusions from the studies as minimal changes were observed as a result of treadmill running, whether in isolation or combined with collagenase injection.

The daily distances run by the rats in these studies exceeded the distances of previous studies, but may not have been great enough to induce damage to the tendons. Rats naturally run long distances in the wild, and thus distances exceeding what rats normally run may be required for overuse; however, the distances in these studies do not likely reach the status of overuse. It is important to note that using mandatory treadmill running to achieve these long distances would require a large amount of time daily, which may outweigh any benefits of using the model.

Finally, the use of rodents rather than large animals may contribute to the lack of running effect on the Achilles tendon. Alexander [78] described that one function of tendons is reduction of work required of the muscle, but this effect is lessened in smaller animals. Biewener and Blickhan [79] examined stresses acting on the gastrocnemius and plantaris tendons of kangaroo rats when hopping and found that the stresses are much lower than the stresses in wallabies, which demonstrate a similar hopping gait. Because the kangaroo rat tendons experience less stress, the tendons stretch very little while the muscles undergo substantial lengthening and shortening as the ankle bends and extends. In larger animals, including humans, the tendons do more stretching and recoiling, likely making the tendons more susceptible to damage than the tendons of smaller animals.

While this is suggested as a limitation, little research exists on this effect. Also, valid rodent models have been established for other tendons, which further negate the hypothesis of this effect being a limitation. In addition, using large animals to maximize tendon stresses would add to the difficulty and expense of investigating Achilles tendinopathy.

5.3 Future directions

These studies found that neither the individual nor the combined effects of uphill treadmill running and intratendinous collagenase injections resulted in an animal model of Achilles tendinosis that mimics the human condition. Future research must focus on replicating models that other laboratories have reported as successful or conducting new studies with alterations to protocols that have been unsuccessful, including those included in this dissertation.

One model that requires further investigation is rodent bipedal downhill treadmill running proposed by Ng, et al. [372]. This method involves suspending the upper bodies of rats from harnesses while running, ensuring that all loading is placed upon the hind limbs. The initial study reported histological and biomechanical changes which resembled the pathological changes of human Achilles tendinosis. While this model interferes with the natural gait of the rat, it may allow insight into the molecular changes occurring during the pathological process and may allow for pharmaceutical interventions to be tested. However, follow-up studies are lacking, and thus the credibility of this model is not confirmed.

The studies of this dissertation may also be adjusted in several ways in the search for an appropriate animal model. First, rather than mandatory treadmill running, running wheels could be provided in the rat cages and voluntary wheel running may be measured. This may increase the distance the rats run during the duration of the study and may approach distances run in the wild. However, this may be limited by pain from the injections prohibiting rats from running. Godbout, et al. [391] found in a four week study that animals receiving injections ran on a wheel significantly less than non-injected rats during the first week after injection, but all rats ran similar amounts during the final three weeks. Additionally, it is unlikely that rats would voluntarily run to the point of overuse. If voluntary running is unsuccessful, a combination of voluntary wheel running and mandatory treadmill running could be tested.

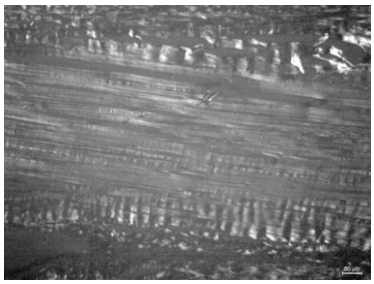
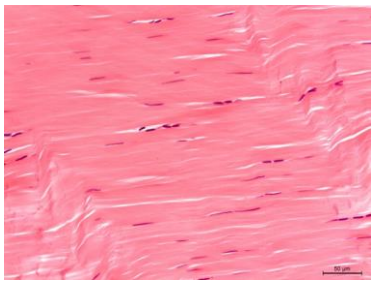
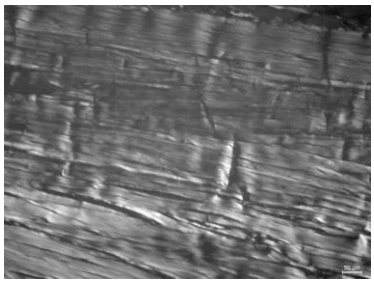
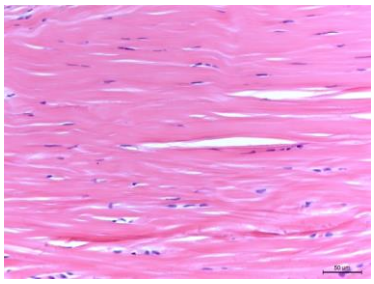
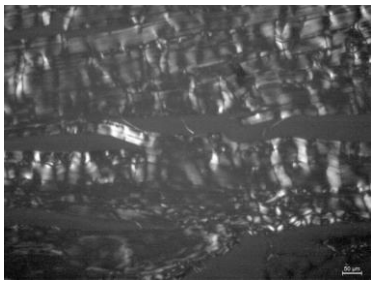
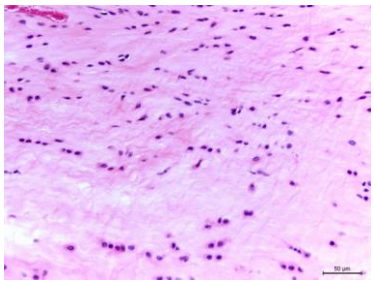
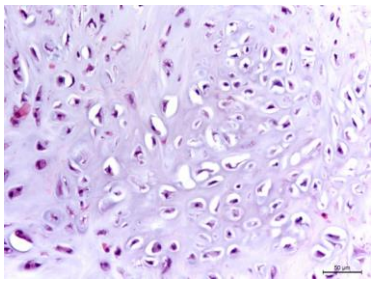
If all running is shown to be unsuccessful, intratendinous injections may be combined with electrical muscle stimulation, which produces flexion and extension, and thus loading of the tendon. Because animals are under anesthesia during this stimulation, the amount of loading can be controlled and optimized, allowing for a greater potential for overuse to occur.

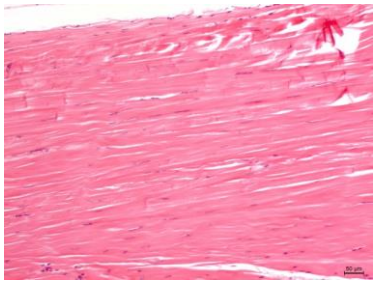
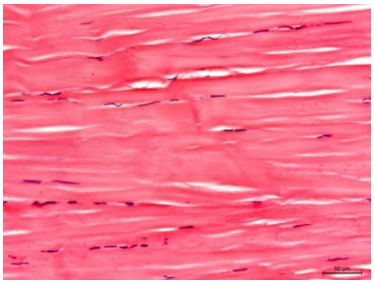
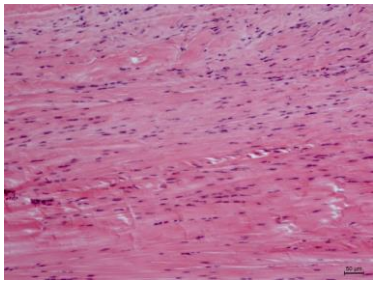
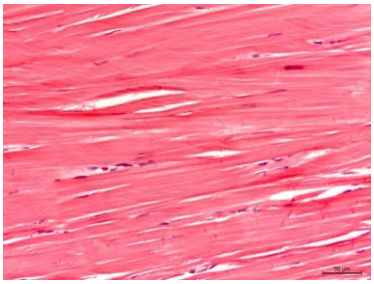
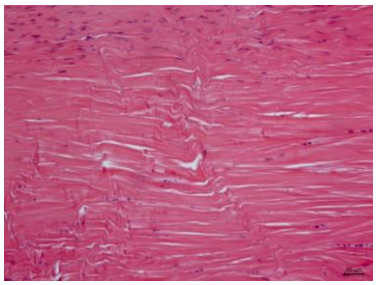
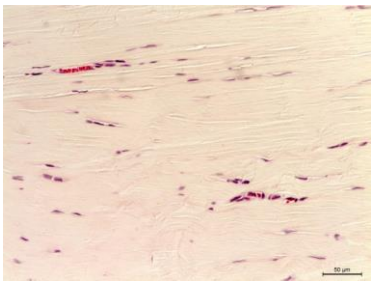
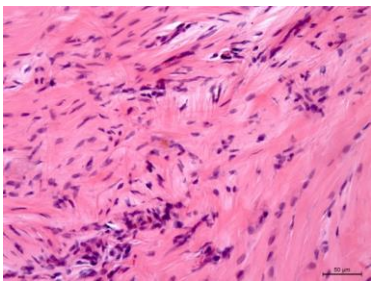
In addition to adjustments of the extrinsic factor, the intrinsic factor may also be altered. One change may be to increase the number of collagenase injections by giving a second collagenase injection at the midpoint of the study. This would likely reverse any healing that had begun, but may be detrimental to the tendon and induce rupture. Also, different injections may be used in attempts to induce degeneration. For example, preliminary studies injecting prostaglandins into the tendon have reported an initial effect of acute inflammation which progressed to fibrosis of the paratenon and intratendinous

degeneration after receiving weekly PGE_1 injections for 35 days [377]. One downfall of testing other injections is the limited number of existing studies. Because of the lack of use, initial studies would have to be performed in order to establish proper dosage amounts and schedules.

In conclusion, this dissertation provides insight into the individual and combined effects of uphill treadmill running and intratendinous collagenase injection on the rodent Achilles tendon. The outcomes of these studies, while negative, provide further evidence of the complexity that exists in tendon pathology. Although these protocols did not produce the desired effect on the Achilles tendon, they may be altered in ways to contribute to an acceptable animal model of Achilles tendinosis.

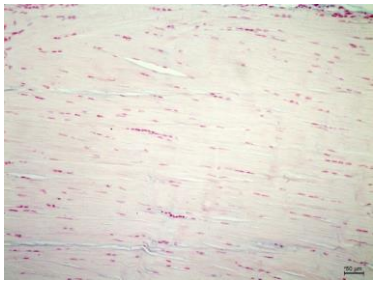
APPENDIX: REPRESENTATIVE PHOTOMICROGRAPHS OF THE GRADED HISTOLOGICAL CHARACTERISTICS

	Collagen arrangement	Tenocyte morphology
Grade 0		
Grade 1		
Grade 2		
Grade 3		

	Cellularity	Vascularity
Grade 0		
Grade 1		
Grade 2		
Grade 3	No tendons received this grade	

Ground substance

Grade 0



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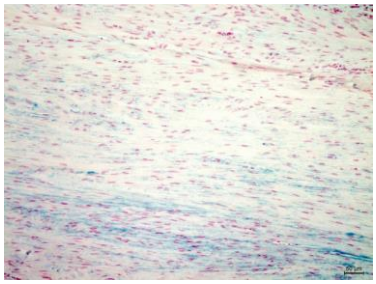

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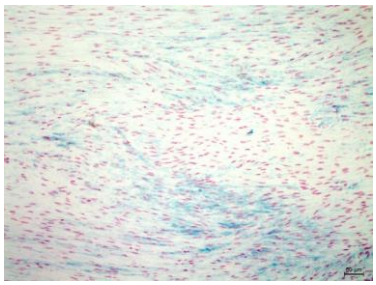

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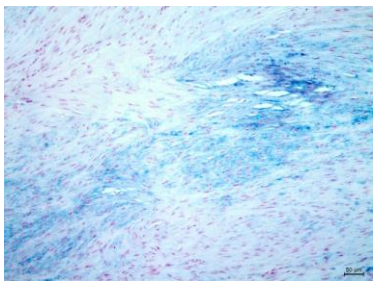

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Presentations

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Dirks, RC; Ellis, SN; Clifton, AM; Fuchs, RK; Robling, AG; Warden, SJ. Muscle forces directly influence bone adaptation. Presented at the Annual Conference of the American College of Sports Medicine, June 1, 2011.

Truitt, WA; Johnson, PL, Dietric, AD; Minic, PE; Fitz, SD; **Deal, R**; Shekhar, A. Anxiety-like responses induced by orexin A in the BNST are attenuated by NMDA antagonism. Presented at the Indiana Society for Neuroscience meeting, October 30, 2009.

Dirks, RC. The effect of ontogenetic changes on the passive buoyancy of the freshwater turtle, *Graptemys geographica*. Presented at the Butler University Undergraduate Research Conference, April 18, 2008.

Abstracts

Dirks RC, Fearon AM, Scott A, Galley MR, Koch LG, Britton SL, Warden SJ (2013) The effects of uphill treadmill running and collagenase on rodent Achilles tendons [Abstract]. British Journal of Sports Medicine. 47(9): e2.

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