AN IN VITRO EXPLANT MODEL OF OVERUSE TENDINOPATHY. THE EFFECTS OF CYCLIC LOADING AND INFLAMMATORY MEDIATORS ON MECHANICAL AND COMPOSITIONAL PROPERTIES OF TENDONS.

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ABSTRACT

AADITYA DEVKOTA: An In Vitro Explant Model Of Overuse Tendinopathy. The Effects of Cyclic Loading And Inflammatory Mediators On Mechanical And Compositional Properties of Tendon.
(Under the direction of Paul Weinhold)

Overuse injuries are common clinical problems, athletically and occupationally, with estimates that nearly 50% of all injuries are overuse. Though a major clinical issue, the underlying etiology of tendon overuse injuries is unknown. Currently, the prevailing thought centers on microdamage accumulation. This is where loading (well below failure levels) causes damage at the fibrillar level. When this damage outpaces the tendon's ability to repair itself, then tendinopathy is thought to occur.

The elastic limit beyond which this microdamage occurs has long been accepted at 4% strain. The first objective was to examine this idea utilizing newer methodologies by first loading tendons to various subfailiure strain levels, then loading to failure after rest. The elastic limit approached the failure strain limit (16%), implicating cellular responses as the driving factor in tendinopathy.

To examine overuse tendinopathy, the next objective was to develop and characterize a model to apply and monitor complex, cyclic loadings to viable tendon. This device was able to significantly reduce the mechanical properties of loaded tendons (relative to control) as well as monitor this damage (strain accumulation).

With a model established, cyclic loadings were performed across varying periods to assess cellular responses' role on tendon's mechanical and biochemical characteristics. Compositional changes, as well as pronounced magnitude and duration dependant effects in collagenase, cellularity and the inflammatory mediator PGE_2 were observed. Significant loss of material properties were also observed with respect to loading magnitude.

To determine the magnitude effect of these responses and separate it from duration dependence, an experiment with four levels of loading was performed across three days. This duration proved too short for compositional changes, however, significant magnitude dependant cellular responses were observed in regards to collagenase, cellularity and PGE_2.

PGE_2, long hypothesized to contribute to tendinopathy development, was examined across cyclic loadings. PGE_2 treatment caused decreased stiffness, resulting in increased strain and cellular responses. COX inhibitors were effective at reducing PGE_2, as well as its responses. This study suggests PGE_2 may contribute in driving healthy tendons into tendinopathy, however this response is reducible with COX inhibitors which otherwise showed no negative effects in an explant model.

To my family and friends

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid (all-cis-5,8,11,14-eicosatetraenoic)
COX	Cyclooxygenase (EC 1.14.99.1)
DMEM	Dulbucco's Modified Eagle Medium
DMEM-H	Dulbucco's Modified Eagle Medium (High Glucose)
DVRT	Differential Variable Reluctance Transducer
E	Elastic Modulus (Young's Modulus)
ELISA	Enzyme-Linked Immunosorbent Assay
GAG	Glycosaminoglycan
Hypro	Hydroxyproline
LDH	Lactate Dehydrogenase
LVDT	Linear Variable Differential Transducer
MMP	Matrix Metalloproteinase
MPa	Megapascals $(kg*m/s^2)/m^2 \times 10^6$
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide
NSAID	Non-Steroidal Anti-Inflammatory Drug
PGE_2	Prostaglandin E2
sGAG	Sulfated Glycosaminoglycan
TLD	Tissue Loading Device
VDA	Video Dimension Analyzer
VSA	Video Strain Analysis

LIST OF SYMBOLS

σ	Stress (Pascal $kg*m/s^2)/m^2$)
ε	Strain (Unit-less m/m)
μ	Micro (x 10^{-6})

CHAPTER 1

INTRODUCTION

Overuse injuries comprise a significant portion of tendon injuries, both athletically and occupationally. Though overuse injuries account for nearly 50% of all injuries, the etiology of tendon overuse injuries is still poorly understood. The issue is compounded with seemingly conflicting diagnosis. Clinical symptoms such as pain and swelling are inconsistent, but the diagnosis is often the same. Furthermore, nomenclature contradiction arises from diagnosis made to specify tendonitis (inflammation) or tendinosis (no inflammation), which is not suggested without a biopsy and histopathologic confirmation. To curtail further confusion, the general aetiologic term of overuse tendinopathy is advised until such confirmation.

The classical elucidation of the etiology centers on the concept of micro-damage to the collagen fibers with each loading. The classic elastic limit, the strain level beyond which non-recoverable damage will occur, has long been accepted as 4%. The first objective showed no such limit at slow loading rates, implying an increased role of the cellular response in tendinopathies. The tendon is a robust biological tissue with adaptive responses that must also be considered when attempting to assess the damage source. Many studies have shown mechanical load to play a major role in the development of tendinopathic characteristics through such responses. Responses to loading include an increase in the release of collagenases as well as various cytokines and inflammatory mediators.

The first overuse tendinopathy studies were expansions of general tendinopathy studies, performed across numerous cycles to determine the basic mechanical properties of tendon to repetitive loading. While much was gained from this approach, it was performed on cadaver tendons and thus neglected a key component of overuse tendinopathy, the cellular response. The next line of studies took the opposite approach and loaded tendon fibroblasts on deformable substrates and observed a host of cellular responses to loading. The limitation of this type of study lies in the inability to account for the matrix and it's interaction with the cells. The latest approach, which brings the cells and the matrix together, is in vivo loading. Rabbit and rat models have been developed that have shown numerous cellular responses as well as matrix changes as a result of repetitive loading. An in vivo human model has also shown some cellular responses to loading. However, it has been suggested that due to the complexity of in vivo loading, it is necessary to develop in vitro models if one wants to determine precise causative loading effects on tendinopathy.

In order to determine how particular loading characteristics contribute to overuse injury, in vitro explant models can be used to apply controlled cyclic loads to tissue segments maintained in culture. Major deficiencies in the systems currently available necessitated the development of a new loading device and damage monitoring system. The next objective of this project was to fully develop and calibrate a novel device and assess its performance, while developing a video strain analysis system to monitor midsubstance strain.

After establishing the system's ability to damage the tendon through cyclic loading while monitoring its deteriorating mechanical characteristics, the next major goal was to establish the ability of this device to damage the tendon through cyclic loading in a manner that establishes the damage to be more than would be expected with simple material fatigue.

Damage beyond fatigue was observed and was assumed to have been facilitated by the cellular responses of the tendon to the loading. Matrix ground substance turnover was noted, while matrix remodeling agents and inflammatory mediators were detected in load and duration dependant manners. The device had been established to not only damage the tendon through cyclic loading, but elicit various cellular responses that are consistent with past studies that likely damage the tissue further. Next, it was necessary to determine the timing of these responses and determine their responses based on various loading levels. It was determined that many of these presumably causative cellular responses are elevated very quickly and that they are all very highly magnitude dependant.

Having established a cyclic loading model which produced fatigue damage, elicited various noxious cellular responses and saw interactions between the damage and cellular responses, a model sutiable for studying tendinopathy was created. The final step was to isolate one of these responses, the inflammatory mediator Prostaglandin E2 (PGE_2), which has been linked both directly and indirectly with various noxious responses in our previous experiments as well as in past studies. This was done by both adding exogenous PGE_2 and inhibiting it with non-steroidal anti-inflammatory drugs (NSAIDS). NSAIDS are a common treatment for overuse tendinopathy even though their full effects have not been completely established and recent studies have shown potential health hazards. PGE_2 treatment was seen to cause a decrease in stiffness, resulting in increased strains and increased cellular responses. Trends for collagen and GAG decreases were also seen. COX inhibitors were found to be highly effective in reducing all of these responses, implying a potential therapeutic role for COX inhibitors in preventing repetitively loaded tendons from developing into overuse tendinopathy.

CHAPTER 2

BACKGROUND REVIEW

2.1 Tendon Anatomy & Physiology

2.1.1 Function

Tendons are force transmitting connective tissues that attach muscles to bones and facilitate active joint motion. Tendons are structurally and compositionally similar to ligaments, which connect bone to bone. Each muscle has two tendons, one at the origin (proximal bone) and one at the insertion (distal bone). Consequently, each tendon has two attachments, one to the bone called the osteotendinous junction (OTJ) and one to the muscle called the myotendinous junction (MTJ). The main function of tendon is to transmit the force generated by the muscles across joints, thereby creating the angular motion that is responsible for all human motion.

The tendon also possesses many secondary functions such as making movement more energy efficient. This is accomplished by limiting the lengthening of muscles, tissues with high energy demands. Tendons also satisfy kinematic requirements by allowing muscles to be located further away from joints they cannot move across. Furthermore, tendons allow single muscles to control multiple joints through pulleys. They also absorb shock by damping sudden motion which limits damage to muscles and limits sudden, volatile motor stimuli (Jozsa and P. 1997). A final role of the tendon is to store elastic energy, which can be

converted to kinetic energy by elastic recoil, another key feature in energy conversation for movement.

2.1.2 Structure and Organization

Tendons are bright white in color and fibroelastic in texture. Inter-tendon size and shape variability is quite stark, with a range of sizes and possible shapes including rounded cords, strap-like bands, flattened ribbons and fan shaped. Tendons used in powerful, resistive forces (quadriceps contractions) have short and broad tendons, while tendons used for subtle, delicate movements such as finger flexion are long and thin. A host of structures are associated with tendon to facilitate motion in the intended path and reduce friction. These include fibrous sheaths, reflection pulleys, synovial sheaths, peritendinous sheets and bursae. Few tendons actually have a tendon sheath, most have only a paratenon.

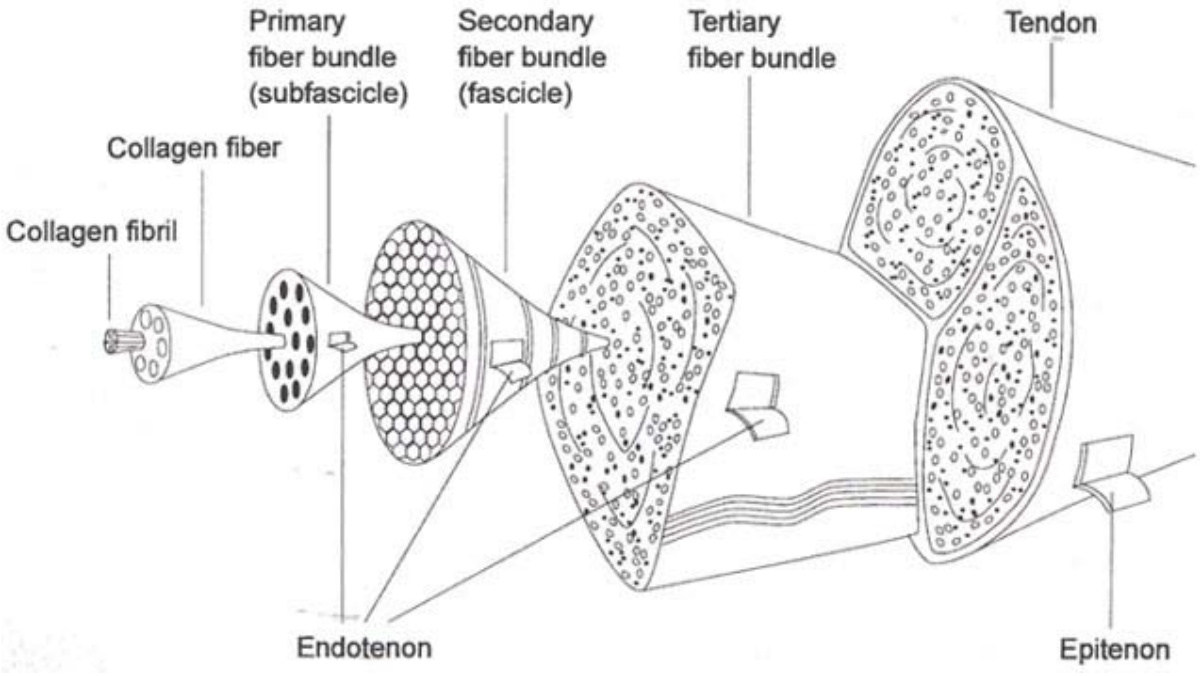

Figure 1: Hierarchal Structure of Tendon (Jozsa and P. 1997).

Below the paratenon is a highly organized tendon assembly (Figure 1), whose outermost layer is called the epitenon, is contiguous with the paratenon. The epitenon is an 8-10nm thick layer of dense collagen fibrils in various orientations. Contiguous with the epitenon, the next internal layer of tendon is the endotenon. The endotenon surrounds several fiber bundles (or fascicles) to form the tertiary fiber bundle which varies from a diameter of 1mm to 3mm. These fascicles, average diameter 150μm to 1000μm, are also surrounded by the endotenon. Within these fascicles are numerous subfascicles, also surrounded by the endotenon. Within each subfascicle are the individual collagen fibers, the basic unit of the tendon (Jozsa and P. 1997).

The collagen fibrils are long rod shaped structures composed of numerous triple-helix polypeptide chains. The chains are surrounded by a thin layer of proteoglycans (PGs) and glycosaminioglycans (GAGs). The molecule is roughly 300nm long and 1 – 2nm wide. Each molecule overlaps its neighbors by 67nm or roughly 25% and is often referred to as the quarter-staggered array. This 67nm region is comprised of an overlap region of 40% and a hole region of 60% (Silver *et al.* 2003). Theses fibril are surrounded by more PGs and GAGs that are different from those surrounding the alpha chains (Jozsa and P. 1997).

2.1.3 Composition

Tendons are primarily composed of type I collagen, with trace amounts of type III and V (Silver *et al.* 1992) and some elastin fibers. The remaining components are the ground substances (proteoglycans, glycosaminoglycans and glycoproteins), fibroblasts and a few inorganic substances.

Collagen comprises 90% of the total protein content and 60 – 75% of the tissue dry weight. The other protein, elastin, comprises 2% of the tendon dry weight and its purpose is unknown. It has been suggested to play a role in the recovery of the wavy fiber configuration after stretch (Butler *et al.* 1978). Procollagen is the precursor to the main tendon component, collagen fibrils, and is synthesized in cells with non-helical amino and carboxyl terminals which are cleaved just prior to microfibril arrangement. Type I collagen is composed of two α_1 chains and one α_2 chain. The sequence of the alpha chains consists of a repeating series of glycine and two other amino acids (Gly-X-Y). Glycine, being the smallest amino acid, allows the helix to form by being placing it in the interior of the axis. Proline, hydroxyproline and hydroxylysine are amino acids that predominately fill the remaining positions. Proline and hydroxyproline give collagen fibrils their strength. Consequently, regions devoid of these amino acids give collagen its flexibility. Regions with the sequence Gly-Pro-Hypro have the greatest rigidity (Silver *et al.* 2003) and are the basis for the great strength of the collagen fibril.

Each helix chain contains roughly 290 hydroxyproline residues which help stabilize the helix by forming water-bridges (Jozsa and P. 1997). This regularity of hydroxyproline, coupled with the fact that it is found predominately in collagen, makes it a convenient marker for collagen content. The stability of collagen makes it resistant against denaturing by all tissue proteases except for collagenase. The other major amino acid, hydroxylysine, is essential in the formation of intermolecular cross-links, which along with the helix chain strength, is the basis for tendon strength (Jozsa and P. 1997).

The next major components of tendons are proteoglycans and matrix glycoproteins. Proteoglycans (PGs) are composed of a protein core and glycosaminoglycan (GAG) side

chains. They are large, hydrophilic molecules that can hold water 50X their weight. They provide the tendon with resistance to compressive forces. Small PGs have short proteins with one or two GAG chains (dermatan sulfate) attached and are found predominately in tensional areas of tendons. Large PGs have longer core proteins with multiple GAGs (chondroitin sulfate) attached and are found predominately in pressure zones of tendon (Merrilees and Flint 1980). Glycoproteins are smaller macromolecules with various functions, not all of which have been established (Jozsa and P. 1997). Some established glycoproteins include, fibronectin, thrombospondin and laminin. Their roles include binding macromolecules such as calcium and cells together, as well as to each other.

The inorganic component of tendon, roughly 0.2% of the dry weight, include copper, manganese, magnesium, cadmium, cobalt, zinc, nickel, lithium, lead, fluoride, phosphor and silicon. Their roles are various, some yet unknown, but some well defined ones include copper's role in cross-linking, manganese's role in molecule synthesis and calcium's role in signaling (Minor 1980).

The final component of the tendon is the cell, or the internal fibroblast. Fibroblasts comprise 95% of the total cellular component of tendons. The remainders are chondrocytes at insertion sites, synovial cells in the tendon sheath, vascular cells and macrophages /inflammatory cells in other in some pathologic conditions. Fibroblasts are long (80 - 300μm) cells with numerous processes that attach to the extracellular matrix. Originally thought to be inert, the fibroblasts' oxygen requirements rival those of cardiac muscles cells on a per/cell basis (Peacock 1959).

2.1.3 Mechanical Properties

The basis for determining the material properties of tendons still remains in vitro failure tests. This is performed at a constant deformation rate while monitoring the load and deformation levels. The resulting force-elongation curve provides structural properties of the tendon such as stiffness, maximum load at failure, maximum strain at failure, etc. However, to compare tendon properties one must normalize for specimen geometry, that is determine the material properties of the tendons. This is achieved by dividing the force by the cross sectional area of the tendon and the elongation by the initial length, leaving a stress-strain curve (Figure 2). Mechanical properties of tendon can be determined after this. Average maximum stress of tendons ranges from 45 – 125 MPa, while the average strain at failure ranges from 9 – 35% strain and elastic moduli of 1.2 – 1.8 GPa (Buckwalter *et al.* 2000).

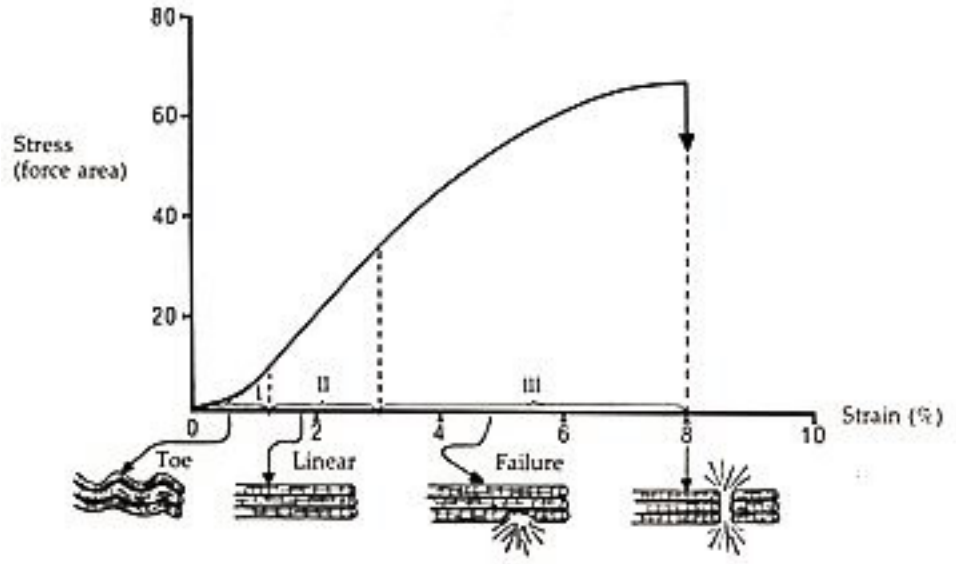

Figure 2: Typical Stress-Strain curve of tendons.

The classical stress-strain curve of tendon is divided into four distinct regions. The first region, called the toe region, is the strain required to straighten the crimp pattern of resting tendons and orient the fibers to the loading direction. This portion, roughly 0 – 2%

strain, is fully recoverable (Butler *et al.* 1978). The second portion, the linear region, ranges from 1.5 – 4% strain (Viidik 1973; Butler *et al.* 1978). This portion is equivalent to the elastic region of synthetic materials, meaning it is non-damaging and the strain is completely recoverable. The next region, the failure region, is where non-recoverable damage to individual fibrils occurs as they begin to shear past each other and cross links break in an unpredictable fashion (Butler *et al.* 1978; O'Brien 1992; Buckwalter *et al.* 2000). This continues until enough fibrils and cross-links are damaged to cause macroscopic failure, which commences around 8-10% stain (Butler *et al.* 1978; O'Brien 1992).

Along with the properties exhibited from a single loading, tendons display a host of time and history dependant properties (viscoelastic properties). Tendon displays classic creep and stress relaxation properties. Under constant load (well below failure level), tendon have been shown to fail by creep (Wang and Ker 1995). Physiologic consideration for creep is applicable in events such as carpel tunnel syndrome (Smutz *et al.* 1995). However, creep also has advantages as it decreases muscle fatigue by allowing the muscle to shorten during isometric contractions (Buckwalter *et al.* 2000). Another viscoelastic behavior of tendon is that of preconditioning, a phenomenon where the first few cycles of load induce considerable hysteresis, relative to subsequent loading cycles and is the basis for warming-up before exercising (Buckwalter *et al.* 2000). Another time dependant effect of tendons is that of strain-rate dependence. Increasing the strain rate increases the stiffness and the failure strength of tendons (Standish *et al.* 2000).

2.2 Tendon Loading & Injury

Various activities cause diverse stresses and strains on the tendon as they transfer the load generated by the muscle to move the bone and perform the activity. The typical range of stresses seen by tendons is 1/3 - 1/2 of its failure strength (Buckwalter *et al.* 2000). Maximal tendon load is seen during eccentric muscle contraction and, in the case of the Achilles, can be to up to 15 times body weight. Typical strains are in the range of 1-3% (Butler *et al.* 1978; Jozsa and P. 1997; Buckwalter *et al.* 2000). These stresses and strains vary significantly by the tendon, the activity, predisposition and preparation. Little data exists clinically of the tendon-muscle unit, but parallels can be drawn from the knowledge acquired from animal models. The tendon is not an inert cable as originally thought. The adage that hyperfunction leads to hypertrophy and hypotrophy leads to atrophy exist in the tendon as well, however in a narrower scope than for bone and muscle (Jozsa and P. 1997).

2.2.1 Disuse and Immobilization

Disuse and immobilization are well studied aspects of tendon response. Animal studies of MTJ immobilization have demonstrated a significant decrease in muscle cell/tendon connections as well as decreased GAG content, increased collagen type III, resulting in decreased failure strength (Tidball 1991; Jozsa and P. 1997). Similar compositional changes have also been observed in humans (decreased GAG and increased type III collagen), leading many to speculate decreased tendon strength in humans as well (Jozsa and P. 1997).

The tendon proper displays a variety of responses to load deprivation. Tenocytes show signs of necrosis and hypoxia such as mitochondrial and nuclei shape alterations (Jozsa and P. 1997). The enzymes that catalyze collagen and their mRNA decrease. Immobilization causes human tendons to exhibit decreased tensile strength, elastic stiffness, total weight and energy to failure (Amiel *et al.* 1982; Woo *et al.* 1982; Tipton *et al.* 1986; Jozsa and P. 1997; Maganaris *et al.* 2004). Biochemically, immobilization causes increased collagen turnover, reduced cross-linking, collagen disorientation, reduction in cross-linking and decreased GAG content (Akeson 1961; Jozsa and P. 1997; Maganaris *et al.* 2004). Animal models have generally agreed with these results, showing decreased ultimate strength and modulus, however data on differences in collagen fibril size, diameter, density and organization is contradictory (Yamamoto *et al.* 1993; Hannafin *et al.* 1995; Yamamoto *et al.* 2000; Lavagnino *et al.* 2005). Increases in MMP-1 expression has also been shown to be significant with stress depervation in animals (Lavagnino *et al.* 2003).

Disuse causes many degenerative mechanical and biochemical changes in tendon. Comparing treatment groups to unloaded controls must first be carefully examined. A more appropriate model for normalizing data may be untreated contra-lateral limbs with in vivo work, or moderately loaded controls with in vitro works.

2.2.2 Regular Activity

During regular activity, that is low loads within 1-3% strain with only a few repetitions, no changes are expected in tendon. However, changes are possible through a host of intrinsic and extrinsic factors that can drastically alter the load/strain creating hypertrophic or atrophic conditions. Even two individuals performing the same activity can

elicit varying loading conditions on the same tendon. Some important intrinsic factors include malalignment, leg length discrepancy, muscle weakness, decreased flexibility, joint laxity, gender, age, weight as well as various predispositioning diseases (Jozsa and P. 1997). A common example is of malaligned loading caused by foot hyperpronation. This is caused by excessive pronation of the subtalar joint in the stance phase, resulting in a whipping or bowstring action on the Achilles. This not only leads to excessive loads on the Achilles, creating excessive stresses and strains there, but it can affect the lower leg and knee joints as well (Jozsa and P. 1997). Extensor tendons of the hand are also more likely to rupture in patients with rheumatoid arthritis because of bone spurs that frequently form and the added friction associated with them (Standish *et al.* 2000).

Extrinsic factors that can affect how load affects tendon includes excessive magnitude loads, types of movements, speed of movements, number of repetition, footwear, surface, training errors, environmental factors as well as poor equipment and technique. All the factors indicated above can affect the loading on the tendon and cause an otherwise regular (or non-injuring activity) to turn into an injury even though it has been estimated that normal activities never exceed ¼ of the tendon's ultimate tensile strength (Walker *et al.* 1964; Elliott 1965).

Regular use can include repetitions, provided they are accompanied with appropriate rest periods. No histological or cross-sectional area changes of Achilles tendon were seen in exercised rats, but the tensile strength was significantly increased (Jozsa and P. 1997). Exercising rabbits has shown the Achilles tendon to increase its CSA as much as 25% relative to unloaded controls. These tendons also showed an increase in wet weight and fibril thickness (Jozsa and P. 1997). Similar increases in the CSA have been shown by ultrasound

of race horses during training (Gillis *et al.* 1993). However, 1/3 of the horses developed signs of tendonitis during this period. Findings such as these demonstrate the difficulty in determining the proper level of exercise, even for well understood and meticulously monitored subjects such as racehorses. The proper combination of loading and rest required for myotendinous hypertrophy, or regular loading, without developing degenerative conditions is not easily determinable and still requires considerable investigation.

2.2.3 Strenuous Use

Beyond regular use, loading to levels that may cause damage is strenuous use. This region does not have specific stress or strain limits and differs across individuals as well tendons. It is the maximal level that individuals can voluntarily load their tendons. This is reached mainly in forced eccentric exercises against external loads or with fast aggressive movements.

Mechanical and biochemical data is scarce because subjects are not willing to donate otherwise healthy tendon, but few animal studies exist. Maximally contracted rabbit fusiform muscles and pinnate muscles, showed stresses of 25 MPa and 15 MPa in their respective tendons (Elliott 1965). Estimates made on patellar tendons of subjects in non-injurious settings of kicking are 26 MPa (Standish *et al.* 2000), while estimates of weightlifters maximum stress at spontaneous PT rupture is 30 MPa (Standish *et al.* 2000). Spontaneous ruptures were rare before 1950, but are now common in many developed countries (Jozsa and P. 1997), likely because of the increase in recreational activity. The most frequently rupturing tendon is the Achilles, and 75% of those are sports related, while only 2% of other tendon ruptures are sports related (Jozsa and P. 1997). Classifying these

ruptures as spontaneous may be a little naive because histological assessment often displays considerable degenerative conditions that likely contributed to the failure (Jozsa and P. 1997; Buckwalter *et al.* 2000).

No studies exist of otherwise normal tendons that have been aggressively loaded only a few times that exhibit biochemical or biomechanical changes. Most of these injuries, as stated before, are athletic, which likely involves overuse and preexisting degenerative pathologies. Furthermore, age has been shown to be a direct factor in ruptures (Jozsa and P. 1997; Buckwalter *et al.* 2000), further enforcing the role of degenerative changes in otherwise spontaneous ruptures. Ruptures that are not spontaneous or degeneration-induced have been observed, but in most cases they have been found to be a result of failures in the neuromuscular protective mechanisms (Jozsa and P. 1997).

Given these findings, many researchers believe that even aggressive loadings (voluntary) are all well within the capacity of the tendon, and if given time before reapplying force, tendons will recover (Standish *et al.* 2000). If the loadings are reapplied too quickly however (that is, overused), then degenerative conditions will ensue in the tendon and it will be more susceptible to failure, even at sub maximal loads.

2.3 Response to Cyclic Load

It has been suggested that though the metabolic rate of the tendon is slow, repetitive loadings (or exercise) over an extended time period can cause degeneration and inflammation via biochemical, mechanical or most likely, a combination of the two pathways (Jozsa and P. 1997; Khan *et al.* 2002; Wang 2005). Up to a certain level this exercise can be beneficial, however if extended too long it begins to have negative effects on the tendon (Archambault

et al. 1995; Wang *et al.* 2006). Given this difficulty in ascertaining the level at which exercise becomes detrimental, an examination of the response to cyclic loading is appropriate. Various changes in the mechanical properties, cellular expressions and composition have been observed in response to cyclic load or exercise. These responses have been obtained from in vivo and in vitro studies, as well as cadaveric tendons and fibroblasts. These study designs are each unique in their advantages and limitations which must be considered when attempting to compare between the study designs.

2.3.1 Mechanical Changes

Basic material properties of tendon are determined by in vitro ramp or cyclic loading. It has been suggested that tendons are similar to bones in that they have a complex breakdown and remodeling process (Archambault *et al.* 1995; Jozsa and P. 1997; Ker 2002). It must be noted that in vitro models do not accommodate the classic inflammatory repair process because they have no access to inflammatory cells usually supplied by vasculature. However, the repair process is slow and thus if the studies are not extended then they still provide invaluable information about the basic material properties of tendon.

Though tendon has a repair process that may potentially be similar to bone, it has some distinct differences. Endurance tests of human tendons have shown tendon to be devoid of an endurance limit, that is as stress decreases, the tendon always fails with more cycles (Schechtman and Bader 1997). Utilizing a converse study design, frequency was found to be unrelated to failure time in human tendons (Schechtman and Bader 2002), however a similar study in wallaby tendon saw increases in frequency leading to shortened failure time (Wang *et al.* 1995). A study of various wallaby hind limb tendons (Ker *et al.* 2000) showed the

fatigue life of the tendons to vary by orders of magnitude, however it is correlated with in vivo tissue stress. When tendons were cyclically loaded at their mean isometric muscle contraction force, the fatigue life was similar for all of the tendons. It was suggested that this is a tendon design criterion, that is, under stress conditions related to real life, the time to rupture is the same for all tendons (Ker 2002).

Because tendons are constantly undergoing remodeling, to fully understand the effects of cyclic load on tendon properties, exercise of healthy tissue is required. Among the first animal exercise studies examining tendon showed rabbit Achilles tendon to increase in ultimate tensile stress after 40 weeks of training relative to control (Viidik 1967; Viidik 1969). A rat exercise study on treadmill showed decreases in the elastic modulus and ultimate tensile stress on the supraspianatus tendon after only 4 weeks (Soslowsky *et al.* 2000). After 8 weeks however, the ultimate tensile stress remained lower than control, but there was a significant increase in the cross sectional area. Similar results were also observed in rat Achilles tendon exercised for 16 weeks (Sommer 1987).

The loss of ultimate tensile stress is either due to damage; that is, breaking of collagen fibrils or cross-links, or an artifact due to the increase in cross sectional area of the tendon. The artifact can exist if the failure load increases, but concurrent relative increase in cross sectional area occurs, resulting in lower ultimate stress. This was seen in studies of exercised swine flexor and extensor tendons (Woo *et al.* 1980; Woo *et al.* 1981). Their maximum failure load was increased significantly, but their cross sectional area increased much more (25% vs. 164%) causing a steep decrease in failure stress. Functionally, if the area increase does not cause impingement or other gliding issues, then the stress decrease is functionally irrelevant if failure load increases. Whether this is unintentional or part of normal

remodeling is unclear (Archambault *et al.* 1995; Ker 2002). Specific analysis of the structural content and organization is required to assess whether this remodeling response to loading was beneficial or detrimental.

Conversely, the effects of stress deprivation are quite clear. Stress deprivation in various animal studies has shown significant decreases in the weight, stiffness and the ultimate tensile strength of the tendon (Tipton *et al.* 1975; Amiel *et al.* 1982; Woo *et al.* 1982; Tipton *et al.* 1986). A final note when comparing the mechanical response to exercise is that of age. Studies of mice have shown significant increases in fibril size, fibril packing density, ultimate strengths, cross sectional area and stiffness, (Michna 1984; Michna 1987; Michna and Hartmann 1989) however similar studies of rats have led authors to conclude that the differences are similar, but substantially less (Enwemeka *et al.* 1992; Archambault *et al.* 1995; Jozsa and P. 1997). When exercising animals, loading is always external and difficult to control. It has been suggested that, to understand tendinopathy, load itself must me more closely monitored (Archambault *et al.* 1995).

2.3.3 Biochemical & Structural Changes

Histopathologic studies of tendons symptomatic for overuse tendinopathy have revealed various changes in the ultrastructure composition and organization. Marked increases in vascularity with a lack of obvious organization of the blood vessels is often seen (Williams 1986; Nelen *et al.* 1989; Messner *et al.* 1999). Most vascularity increases have been observed in tendon lesions and little data exists on their changes in overuse tendinopathies (Archambault *et al.* 1995). The possible role of vascularity in tendinosis examined in horses (Kraus-Hansen *et al.* 1992), has shown tendinosis like degeneration to

develop with diminished blood supply. This idea has been long postulated for the human Achilles tendon, which shows injury most often at the site with the most limited vascularity (Jozsa and P. 1997; Kjaer 2004).

This role of vascularity changes in tendinopathic conditions implies a critical role of cells, or more specifically, cellular responses in tendinopathy. Cellular responses have been suggested to be the key factor that drives loading to cause tendinopathy (Leadbetter 1992). In preparation for this role, fibroblasts may proliferate slightly in response to strain (Yang *et al.* 2004). More detailed analysis of tendon fibroblasts have shown them to not proliferate with load alone, but only when growth factors such as platelet derived growth factor (PDGF-BB) or insulin-like growth factor-I (IGF-I) are combined with load (Banes *et al.* 1995). IGF-I, the aforementioned growth factor that increases cellular proliferation has also been shown to increase in exercised rats (Hansson *et al.* 1988). Rats exercised into overuse show marked cellular proliferation (Soslowsky *et al.* 2000), however this increase was not normalized by the cross-sectional area increase of the tendon, so it is unclear whether the tendon has more cells relative to it's size. Finally, studies of exercised rats have shown quiescent tenocytes to turn into active fibroblasts (Zamora and Marini 1988).

Fibroblasts respond in a variety of ways to mechanical stimulus that could drive the tendon into tendinopathy like conditions. A postmortem study of asymptomatic superficial digital flexor tendons (SDFT) of racehorses showed a significant increase of sulfated GAG in the portion of the tendon usually associated with damage, relative to other parts of the same tendon (Birch *et al.* 1998). Also, separate studies have shown damaged and visibly degenerated horse SDFT to exhibit significant increases in GAG content (Kobayashi *et al.* 1999). Since GAGs, or specifically the proteoglycans they are a component of, also have a

role in fibril formation and organization, an abnormal GAG composition may be a good indicator of tendinopathy. Along with PDGF-BB and IGF-I, other growth factors such as interleukin -1β (IL-1β) and transforming growth factor β1 (TGF-β) are load inducible in human fibroblasts (Jozsa and P. 1997; Archambault *et al.* 2002; Tsuzaki *et al.* 2003; Kjaer 2004). Growth factors have many roles in remodeling and if expressed excessively, could lead to tendinopathy.

The cellular responses likely to have the greatest affect in driving the tendon towards tendinopathy are the expression, activation and synthesis of MMPs. Immobilization results in pre- and post-translational decrease in MMP levels (Kjaer 2004). However, cyclic loading has been shown to both induce (Archambault *et al.* 2001; Archambault *et al.* 2002; Tsuzaki *et al.* 2003) and inhibit (Lavagnino *et al.* 2003; Arnoczky *et al.* 2004) MMP expression. The induction cyclic loading mechanism suggests that MMPs are released and activated due to loading, and then these MMPs break down the collagen, leading to tendinopathy. This can be aided greatly by various cytokines and inflammatory mediators, which themselves increase with loading (Kjaer 2004; Sharma and Maffulli 2005; Wang *et al.* 2006). Conversely, the MMPs suppression mechanism suggests that loading collagen suppresses MMPs, but if that collagen is damaged then it no longer transmits load and thus acts like unloaded collagen and attached cells release MMPs, again leading to tendinopathy. A study of rat tail tendons showed MMP expression to be dependant on very low levels of load magnitude and frequency (Lavagnino *et al.* 2003). During short, non-damaging tests MMP expression was completely eliminated with only 1% strain at 0.17 Hz. Furthermore, the addition of Cytochalasin D, an actin disruptor, completely eliminated all inhibitory effects

previously seen, implying MMP expression is modulated by a mechanotransduction pathway through the cytoskeleton.

The release of MMP through damaging loading, whether directly or indirectly, causes the degeneration of collagen, the main component of tendon. Histopatholigic studies have shown collagen disorganization and disruption as a prevalent findings in overuse tendinopathies (Nelen *et al.* 1989; Enwemeka *et al.* 1992; Gillis *et al.* 1993; Astrom and Rausing 1995). Because treatment and biopsies are not done prior to damage, the early stages of tendon degeneration have not been examined in human tendons. However studies of over-exercised animals have shown performance decrease and frayed collagen (Barbe *et al.* 2003), as well as disorganized and damaged collagen consistent with human tendinopathy (Soslowsky *et al.* 2000), both by eight weeks. A study of over-exercised avian tendon also found consistent increase of collagen disruption and turnover, as well as decreased amounts of pyridinoline cross links (Curwin *et al.* 1988). Studies of positive exercise (those that caused increases in structural and material properties) show increase in collagen content (Woo *et al.* 1980; Woo *et al.* 1981; Michna 1984; Michna and Hartmann 1989). Exercise in humans have also shown a significant increase in type-I collagen through microdialysis (Langberg *et al.* 1999; Langberg *et al.* 2001). Also, positively exercised tendons show better alignment of collagen fibrils through birefringence (Jozsa and P. 1997). Overall, studies that overload and damage tendons tend to exhibit decreased collagen content and organization, while those that are exercised but not overloaded tend to exhibit an increase in collagen content and show better organization. Collagen, the most abundant protein in tendon, is modulated by load and is an important indicator of tendinopathy (Jozsa and P. 1997).

Overloaded and damaged tendon can often exhibit infiltration of inflammatory cells, edema as well as fibrosis (Backman *et al.* 1990; Soslowsky *et al.* 2000; Wang 2005). This is a common clinical observation, but again, it must be noted that clinical samples are from ruptured or damaged tendons. The inflammatory cells and edema are usually a result of paratenonitis or rupture, as histopathologic studies have failed to find inflammatory cells in non-rupture tendinopathy (Astrom and Rausing 1995; Jozsa and P. 1997; Sharma and Maffulli 2005; Wang *et al.* 2006).

Though inflammatory cells are not found in tendinopathy, inflammatory mediators are frequently observed. A microdialysis study of peritendinous space of the Achilles tendon showed significant increases of PGE_2 in response to exercise (Langberg *et al.* 2003). Furthermore, this load induced PGE_2 was inhibited with the administration of COX-2 inhibitors. Though it is not determinable from this study what cells caused this response, studies of tendon fibroblasts in culture suggest their role. Mechanically loading fibroblasts repeatedly has shown an increase in production of PGE_2 as well as another inflammatory mediator, LTB_4 (Almekinders *et al.* 1995; Wang *et al.* 2003; Li *et al.* 2004). PGE_2 has also been shown to be load inducible in a dose dependant manner in periodontal ligament fibroblasts (Yamaguchi *et al.* 1994). Loading tendon fibroblasts has also shown the increase of COX-2 (Archambault *et al.* 2002; Tsuzaki *et al.* 2003). PGE_2 has been shown to cause localized degeneration as well as affect cellular proliferation and collagen synthesis, implicating it's likely role in tendinopathy (Cilli *et al.* 2004; Khan *et al.* 2005). In other tissue such as bone, these inflammatory mediators play a key role in general remodeling, as well as remodeling after damage (Radi and Khan 2005). Their role in tendon is not currently known and needs to be further examined.

Tendons respond robustly to cyclic loading by releasing cytokines, inflammatory mediators and matrix metalloproteinases which can modulate extra-cellular content, organization and composition. Long term repetitive loading and the potential overproduction of such enzymes can cause the tendon to degenerate even if the loading level is well within the mechanical limit of tendon. Coupled with extrinsic factors such as age and genetics, over time this can manifest as tendon overuse injuries, even in sedentary people.

2.4 Tendon Overuse Injuries (Clinical)

Tendons become overused when they are repeatedly loaded in the strenuous range (4-8% strain). This level of loading causes microdamage, which by itself is insignificant, but if it accumulates because of repetitive loadings at a rate greater than the tendon's ability to repair itself, it will cause a degenerative state and make the tendon susceptible to injury. This damage can manifest itself as collagen fiber or cross-link damage, causing the fibers to slide past each other, denature, become enflamed and become painful. Clinically, this could be diagnosed as tendonitis, peritendinitis, tenosynovitis, insertis, tendinous bursitis, apophystisis, or a combinitaion of these injuries (Hess *et al.* 1989). It has been suggested that these terms are often contradictory and their pathogenesis poorly understood, possibly resulting in misdiagnosis (Wang *et al.* 2006). These misdiagnoses occur because though there is swelling, histopathology often shows a lack of inflammatory cells that tendonitis implies (Astrom and Rausing 1995). Therefore, it is suggested that these terms not be used until histopathological confirmation, and instead the general descriptor overuse tendinopathy be used (Maffulli *et al.* 1998; Paavola *et al.* 2000; Riley 2004; Wang *et al.* 2006). The

epidemiology of overuse tendinopathies lends itself a good division between upper extremities and lower extremities, which are generally work and sports induced, respectively.

2.4.1 Occupational Overuse

Occupational overuse injuries focus mainly on the upper extremities, with the humeral epicondyles, wrist, forearm and hand common sites at greatest risk, mainly in manual workers who do repetitive motion. A retrospective study (Kivi 1984) of nearly 3100 Finnish laborers with repetitive occupational tasks observed 93% of all injuries to be on the upper limbs. Peritendinitis, tenosynovitis and tendonitis were present in 58% and epicondylitis in 24% of all cases. The majority of the cases were of females (69%) with the greatest relative risk (4-5x) of musculoskeletal disorders in the textile, leather and food industries relative to other industries. The highest relative risk for musculoskeletal disorders came within the butchers, packers and workers of the food industry, who were 8 – 12 times more likely to have disorders relative to other occupations. A review of the US Department of Labor, Bureau of Labor Statistics by (Gordon *et al.* 1995) showed 60% of all new occupational illnesses in 1992 were associated with repetitive motion. Similar to the Finnish data, high incidence of injuries were observed in industries which require great manual labor such as meat packing plants (14% of all workers) and automobile plants (9% of all workers). An interesting note however, was that there was great increases in "safer" jobs as indicated by the early 10x increase in incidence of carpel tunnel syndrome over the previous 10 years. This is thought to be related to increased computer use, where across the same period, workers that used computers rose from 25% to nearly 50%. In a separate study, of the 76% of musicians that have suffered performance-impairing medical problems, 2/3 were

musculotendinous (Jozsa and P. 1997). These types of data provide direct evidence on the causative effects of repetitive occupational tasks on upper-extremity overuse injuries. However, whether it is an effect solely of repetitive mechanical load or whether it is due to chronic degenerative changes remains unclear and requires further investigation.

2.4.2 Athletic Overuse

Over the past 50 years, there has been a dramatic increase in the number of athletic overuse injuries treated, with some estimates now suggesting over 50% of all sports injuries being of this variety. Though the increase in clinical treatment has been significantly greater, likely due to the increase of recreational activity in developed countries, direct scientific evidence of this increase is still inconclusive (Jozsa and P. 1997). These injuries occur mainly in the lower extremity, because of the necessity of running, both as an exercise and as training in specific athletic events.

Yearly incidence of running injuries is estimated at between 25-50%, and of these, 50% - 75% are overuse injuries within the tendons of the knee extersor apparatus and the Achilles tendon, and less so posterior tibial tendonitis (Jozsa and P. 1997). Furthermore age and gender play a significant role in the epidemiology of sports-induced overuse injuries. Age is a factor, not only because of degenerative changes in the tissue, but because of the nature of older patients to be more likely involved in endurance, rather than contact sports. Historically, men have shown more overuse injuries, however, the rise of incidence in women that has been seen lately leads to the suggestion that this is mainly because more men participate in athletic activities then women.

Sports related overuse injuries are numerous and often specific for certain sports. Soccer, long-distance running and volleyball are sports where the knee (patellar tendon insertion) is the major area of overuse, with Jumper's knee and Osgood-Schlatter disease very common clinical issues (Jarvinen 1992). Shin splints are a common overuse injury in long distance runners, tri-athletes, and cross country skiers. Lateral epicondylitis of the humerus, or tennis elbow, has been reported to be the prevalent tennis injury, accounting for 30-40% of all tennis injuries (Jarvinen 1992). Injuries of the shoulder, most commonly impingement syndrome or rotator cuff tendonitis involving the supraspinatus tendon is most prevalent in swimming, baseball, volleyball and tennis. It should be noted that because of the demand of winning on athletes, these injuries are often ignored or "played through" leading a predominance of the overuse injuries to be likely due to an accumulated degenerative condition.

2.5 Etiology of Overuse Injury

As noted earlier, tendons display four distinct regions during loading. The first two regions (toe and linear, up to 4% strain) are completely recoverable and thus no damage occurs. The third region (4-8% strain) is the area of microdamage, while strain beyond that results in acute damage. The microdamage, though insignificant by itself, can accumulate due to repetitive loadings at a rate greater than the tendon's ability to repair itself. However, how this microdamage actually occurs is debatable. Most theories center on the cells reacting to collagen damage:

> The structure of the tendon is disrupted micro- or macroscopically by this repetitive strain (i.e., collagen fibers begin to slide past one another, causing breakage of their cross-linked structure, and denature, causing inflammation, edema, and pain.) (Josza, 1997, p164).

While this idea of cells reacting to the damage is likely the key contributor to the impending pathologic condition, it disregards the ability of the cells to cause the damage. Cells not only react to damage, but also react to the load itself. Stretching of fibroblasts in culture has shown the release of various cytokines, inflammatory mediators and enzymes that potentially play a significant role in the pathology of overuse tendinopathy. This leads to the possibility of the cells damaging the tendon with its response, if it is too great. However, accurately translating tissue strain to cellular strain is not currently feasible, so the level of this cellular response cannot be directly compared to the in vivo condition.

The clinical diagnosis of overuse injuries is usually only done after the patient has experienced pain or decreased function. There is likely a long history in the tendon beyond the recent events that caused the pain when the patient first seeks help. This history could include anything form localized degeneration to acute injuries. Therefore the first clinical diagnosis can range from tendinosis to tendonitis, peritendonitis, tenosynovitis, insertitis, bursitis, apophysitis, or any combination of the above.

A main distinction between the various diagnoses is the presence (or lack) of inflammatory cells. Tendinosis (no inflammation) is the term used for general tendon degeneration. It is this degeneration that leaves the tendon susceptible to the eventual damage. Tendonitis (inflammation) is an inflammatory state with inflammatory cells, edema etc. associated with gross remodeling in response to severe, usually acute damage. It is suggested that tendon overuse injuries are caused by tendinosis, but can be enhanced by tendonitis. They are separate entities that can occur together (Backman *et al.* 1990; Leadbetter 1992; Jozsa and P. 1997) which has led to many misdiagnoses.

2.5.1 Tendon Degeneration ("-osis")

Though the prevalence of tendinosis is well known, the pathogenesis is not well understood. In a retrospective study of healthy, urban Hungarians older than 35 with no prior evidence of tendon problems, signs of degeneration were seen in 1/3 of the patients (Jozsa and P. 1997). This led the authors to conclude that tendon degeneration is very common, and is a function of age. Furthermore, it is asymptomatic and no data exists to correlate degeneration versus previous occurrences of tendon disorders. The asymptomatic nature of tendinosis is reiterated by a second study of nearly 900 spontaneous tendon ruptures, in which nearly 2/3 had no previous symptoms such as tenderness, stiffness or pain (Jozsa and P. 1997). Furthermore, none of the spontaneous ruptures exhibited any inflammatory cell infiltration. Tendinosis have a range of clinical classifications for the type of degeneration thought to be predominant: hypoxic, myoxid, hyaline, fibrinioid and fatty degenerations.

Etiologically, the reason for tenocyte and collagen degeneration that leads to tendinosis is unknown. Recent evidence suggests one reason may be tissue hypoxia as overuse is likely to damage blood vessels (Archambault *et al.* 1995). Lesions, focal areas of concentrated growth, exhibit significant vascular proliferation. Conversely, tendon degeneration regions often show empty spaces and reduced vascularity (Zamora and Marini 1988). In horses, tendinosis like conditions have been induced by limiting the blood supply (Kraus-Hansen *et al.* 1992). A reduced blood supply is suspected to be the cause of the decrease in tenocyte viability, which in turn could initiate tendinosis (Kraus-Hansen *et al.* 1992; Archambault *et al.* 1995; Jozsa and P. 1997). Furthermore, the hypoxia could result from exercise-induced hyperthermia. Intratendoinous temperatures of 45ºC have been measured in exercising horses, while in vitro fibroblast death is seen above 42.5ºC. The

authors suggest that since blood often cools tissue, the lack of blood vessels could lead to tendinosis, especially when combined with exercise.

Though repetitive motion induced cellular and molecular responses are thought to be important in the development of tendinosis, their underlying mechanisms remain unclear. Load inducible cellular responses that likely play a key role in driving tendon degeneration are cytokines, inflammatory mediators and MMPs (Leadbetter 1992; Wang *et al.* 2006). MMPs are the only proteins used to remodel the collagen matrix, and thus their potential role in tendinopathy is direct and obvious. MMPs are load and damage inducible, leading to the possibility of a positive feedback mechanism which would exacerbate the tendinopathy. Injection studies of collagenase have shown tendinosis like degeneration, similar to that observed during tendon injury, to ensue (Williams *et al.* 1984; Stone *et al.* 1999). Cytokines and inflammatory mediators act indirectly through a host of cascades that can lead to tendinopathy. These agents, which are load inducible, include IL-1β, PDGF-BB, IGF-I, TGF-β, LTB_4 and PGE_2. Injection models of both cytokines and inflammatory mediators have shown tendinosis type degeneration (Stone *et al.* 1999; Khan *et al.* 2005). Though their exact mechanism in tendinosis is unclear, it is evident that that these cytokines and inflammatory mediators (and possibly other markers) alter cellular signaling and induce tendon degeneration.

2.5.1.1 Hypoxic Degeneration

Hypoxic degeneration is defined as tendinosis caused by the lack of oxygen. It is the most common form of tendinosis in all tendons except the Achilles, patellar and quadriceps tendons. In a study of ruptured tendons that showed degeneration, hypoxic degeneration was

observed in 44%, while only 10% was observed in controls (Kannus and Jozsa 1991). This type of degeneration is evident within the tenocytes and the collagen fibers.

Microscopic observations note hypoxic degeneration to exhibit edema and small areas with no cells. Larger patches with a complete loss of tendon structure and no cells stainability are also sometimes seen. Metabolically, tenocytes show decreased oxidative enzyme activity and increased hydrolytic enzyme activity. Pyknosis of the nuclei is seen while the mitochondria swells (Jozsa and P. 1997). Collagen organization shows decreased anisotropy, loss of periodicity and loss of birefringence. Collagen fiber degeneration is also visible in the form of fragmentation, fraying and longitudinal splitting, giving the tissue a streaked, irregular appearance (Jozsa and P. 1997).

2.5.1.2 Mucoid Degeneration

Another common form of tendinosis is mucoid degeneration, where semisolid nodules are noted. It is the most common form of degeneration in the Achilles tendon and was found in 21% of all tendon degeneration and only 9% of the controls (Kannus and Jozsa 1991). Visually, the tendon loses its bright white luster and has a soft grayish-white appearance. Tenocytes change from their normal elongated shape and become more rounded, with decreased nucleus/cytoplasm ratios and increased number of vacuoles (Jozsa and P. 1997). Collagen fibers are usually thin, frayed, longitudinally split and have lost their periodicity. The tendon also exhibits increased extracellular proteoglycan and glycosaminoglycan content.

2.5.1.3 Hyaline, Fibrinoid & Fatty Degeneration

The remaining three most common form of tendinosis are hyaline, fibrinoid and fatty degeneration. Hyaline degeneration is charachterized by the appearance of nodules of

homogenous translucent material. Macroscopically, tendons become yellowish, stiffer and less elastic. The collagen fibers show increased thickness, fibril diameter variability is reduced and the cells-matrix ratio decreases. Fibrinoid degeneration is charachterized by the appearance of a noticeable fibrous lesion. The tissue becomes imbedded with fibrinogen, fibrin and fibronectin. Tenocytes become necrotic, collagen fibers denature and homogenate and the adjacent area can become surrounded by reactive cells (plasma cells, lymphocytes, etc.) (Jozsa and P. 1997). The final form is fatty degeneration, characterized by lipid accumulation. The various forms of this include fine droplet, masked lipidosis, lipid accumulation in tenocytes and tendolipomatosis. In extreme cases the lipid cells can form long, complex three-dimensional chains between the collagen fibrils, whereas lipid cells are rarely found in healthy tendon (Jozsa and P. 1997).

2.5.2 Tendon Inflammation ("-itis")

When patients seek treatment for their tendons, they sometimes exhibit the classic signs of inflammation: heat, redness, swelling and pain. This classic wound healing is a response to acute tissue loss or damage and results in inflammation, repair and remodeling. The inflammatory phase starts at day one and lasts four to seven days. The repair phase lasts a further two weeks and the final phase, the remodeling lasts a further three plus weeks. Depending on the type of damage, the remodeling phase may last up to one year.

The inflammation phase begins immediately as the surrounding fluids, blood and plasma from damaged vessels seep into the damaged area. Platelets regulate clotting while fibrin and fibronectin create cross-links with collagen to control local bleeding. Chemotatic agents such as histamines, fibronectin and bradykinin induce vascular permeability,

prostaglandin release and cellular infiltration by leukocytes, monocytes and macrophages ensues, usually within the first 24 hours. Early in the response, PGE1 increases vascular permeability and PGE_2 attracts leukocytes, while later they both may initiate early repair and ongoing inflammation (Jozsa and P. 1997). As the inflammatory cells remove the injured area of necrotic tissue and debris, the repair stage begins.

The repair phase begins with hypercellularity of fibroblasts, myo-fibroblasts and endothelial cells. Growth factors release by macrophages and platelets stimulate cellular reproduction and migration. Angiogenesis is seen and the cells begin to lay ground substance and extracellular matrix to replace the fibrin clot. GAGs and type three collagen are present in the early stages, but collagen production soon switches to type I (Leadbetter 1992), usually by the end of the second week and continues for about a week more.

The remodeling phase begins by slowly decreasing the amount of inflammatory cells, decreasing angiogenesis rate and deceased matrix synthesis. The scar density increases as fluids are slowly removed. The amount of GAGs decrease and mainly type I collagen is left. Collagen maturation and alignment is seen by six months and fibroblasts revert to fibrocytes. As the response completes the newly formed section of tendon can have drastically different material composition and properties as the rest of the tendon and can be 30% weaker, depending on the healing response (Leadbetter 1992).

This inflammatory response affects certain well-vascularized areas of certain tendons, but mostly it is seen in the peritendinous structures. Actual tendonitis is rare and usually the observed inflammation is peritendonitis or tenosynovitis. This peritendinous inflammation can become self-perpetuating and secondarily involve surrounding structures, that is cause tendonitis. Peritendonitis and tendinosis have been shown to be separate entities (Backman

et al. 1990; Leadbetter 1992; Jozsa and P. 1997). Tendonitis is rarely the cause of overuse tendinopathy though it can be seen in conjunction with tendinosis (Leadbetter 1992; Jozsa and P. 1997; Khan *et al.* 2002). Though there is not inflammation in tendinosis, the release of load inducible inflammatory mediators such as prostaglandins, leutrokines and cytokines may drive the tendon into tendinosis (Khan *et al.* 2005; Wang *et al.* 2006).

2.6 Arachidonic Acid Cascade

Arachidonic acid is an essential, polyunsaturated, 20-carbon omega-6 fatty acid. The source of arachidonic acid is exogenous supply or dietary linoleic acid that is esterified into the phospholipids of the cell membrane. Upon an external stimulation, such as an inflammatory response, G proteins initiate phospholipid hydrolysis to release arachidonic acid into the cytoplasm. Phospholipase A2 (PLA) achieves this directly, while phospholipase C and phospholipase D require further enzymes to produce free arachidonic acid. The amount of free arachidonic acid in resting conditions is very low and thus exogenous diffusion is negligible. However, studies with artificially introduced exogenous arachidonic acid have shown that, if present, these will be absorbed and metabolized by the cells and that they will also inhibit any unnecessary phospholipid hydrolysis from the cell membrane (Brash and Ingram 1986). Once free, the arachidonic acid in the cytoplasm has three fates: esterification into the cell membrane (storage), diffusion outside the cell or metabolism.

Once outside the cell, newly freed arachidonic acid (and exogenous arachidonic acid) has been suggested to have several roles, even without enzymatic modifications. One such role is to activate leukocyte NADPH oxidase, which reduces molecular oxygen to superoxide and induces respiratory bursts (Badwey *et al.* 1981). Arachidonic acid has also been shown

to affect the activity of several ion channels such as mechanosensitive potassium, calcium and gap junctions (Brash 2001). It has also been shown to block cellular proliferation and drive cells into apoptosis (Surette *et al.* 1996). The lack of specificity of arachidonic acid is suggested to be a result of its simple structure and the existence of many close chemical analogues. The effects of arachidonic acid are wide ranging, but the concentration levels required are 1000 – 100,000 times those of its metabolites such as prostaglandins and leukotrienes (Brash 2001). Furthermore, platelets esterify arachidonic acid even in environments with metabolizing agents such as cyclooxygenase and lipoxygenase present suggesting that the role of free, unmetabolized arachidonic acid is very limited (Neufeld *et al.* 1983).

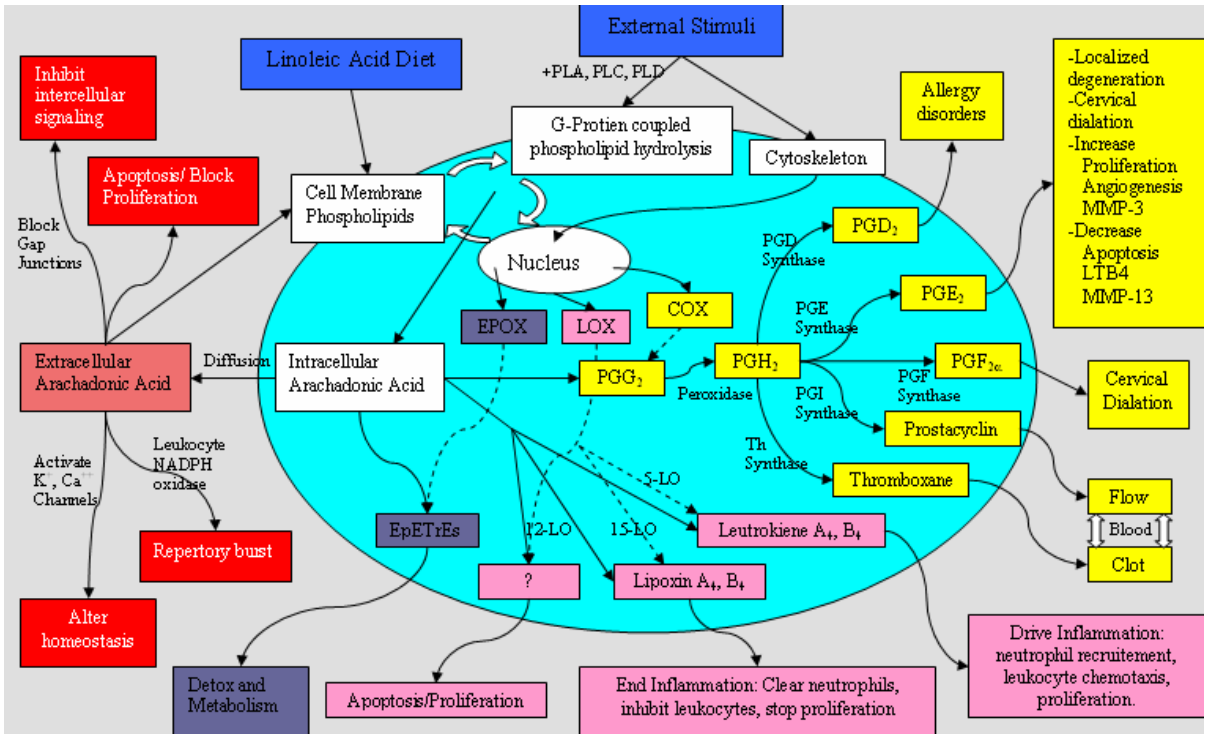

Figure 3: Arachadonic Acid Pathway

The final, and most important, fate of arachidonic acid is the metabolism of several hormones with wide ranging effects. The metabolism pathway is driven by one of three main enzymes: cytochrome P450, lipoxygenase and cyclooxygenase. The cytochrome P450 enzyme produces epoxyeicosatrieonic fatty acids (EpETrEs) which have varied responses such as detoxification. The lipoxygenase and cyclooxygenase enzymes produce eicosanoids such as leukotrienes, prostanoids and thromboxanes. These are metabolites with a host of autocrine and paracrine responses in the management and regulation of inflammation and its symptoms.

2.6.1 Lipoxygenase Pathway

Arachidonic acid is oxidized by one of three main isoforms of lipoxygenase: 5-Lipoxygenase (5-LO), 12-Lipoxygenase (12-LO) and & 15-Lipoxygenase (15-LO), numbered for the steric position they oxidize to form 5, 12 or 15-hydroperoxyeicosatetraenoic acid (HPETE). HPETEs then are converted to lipoxins, 12-HETEs and leukotrienes of the 4 series, which are both pro and anti-inflammatory.

There is limited knowledge about the 12-LO pathway, but it is primarily expressed in the pituitary gland, leukocytes, platelets and the lungs (Yoshimoto *et al.* 1990). Fluctuations in the levels of 12-LO causes apoptosis and high levels have been observed in breast cancer (Natarajan *et al.* 1997). Its main role lies in regulating cellular concentrations, either through apoptosis or through proliferation, during inflammation and normal maintenance for various tissues.

The 15-LO pathway produces the lipoxins (A_4 and B_4). Lipoxins are anti-inflammatory eicosanoids that signal the end of inflammation. They are known to inhibit

leukocyte-mediated injury, stimulate macrophage clearance of apoptotic neutrophils, suppress inflammatory cytokines, modulate cytokine regulated MMP activity, and inhibit cellular migration and proliferation (McMahon and Godson 2004). A potential therupatic role of lipoxins is protecting from leukocyte-mediated tissue injury and is a large area of current research (McMahon and Godson 2004).

The most studied area of lipoxygenase is the 5-LO pathway that produces the 4 series leukotrienes. 5-LO is activated by a protein bound to the nuclear membrane, 5-lipoxygenase activating protein (FLAP). The resulting leukotrienes act mainly on G protein coupled receptors and sometimes on peroxisome proliferator-activated receptors (PPARs). They all play a role in the inflammatory process. Leukotriene B4 (LTB4) stimulates aggregation and degranulation of neutrophils, supports chemotaxis of leukocytes, modifies cellular proliferation and triggers pro-inflammatory responses (Jampilek *et al.* 2006). Leukotrienes are involved intimately in asthma and other allergic reactions. Anti-leukotrienes, drugs that block leukotrienes synthesis or antagonize their binding sites, were once heavily researched but their negative effects on the liver has curtailed their research (Reinus *et al.* 2000). Currently anti-leukotrienes are used only for psoriasis medication and asthma (Jampilek *et al.* 2006).

2.6.2 Cyclooxygenase Pathway

Arachidonic acid can be metabolized by cyclooxygenase enzymes to form prostanoids. The rate limiting step of prostanoid synthesis is driven by the three isoforms of cyclooxygenase to oxidize free arachidonic acid into PGG2. Peroxidase then immideately

reduces the PGG2 into PGH2. Then several synthases, specific to cell types, convert the PGH2 into the various prostanoids: thromboxanes, prostacyclins and prostaglandins.

The cyclooxygenase enzyme has two established isoforms (COX-1 and COX-2), and a third, recently described isoform (COX-3). All three catalyze the identical reaction, with the same kinetic rates, however their active sites are distinct and independent (Claria and Romano 2005). COX-1 is the constitutive form and is produced at low levels by most cell types. COX-2 is the inducible form that is not found in most resting cells, but is heavily expressed in the presence of cytokines, growth factors, endotoxins and other external stimulations. COX-3 is a variant of COX-1 isoform, in which the first intron is not spliced during transcription. The isoforms share nearly 80% homology, and their major difference is the amino acid at position 523: isolucine in COX-1 and valine in COX-2 (Claria and Romano 2005).

Following the action of the cyclooxygenases and peroxidase to convert arachidonic acid into PGH2, specific synthases, convert the PGH2 into thromboxanes, prostacyclins, and prostaglandins D, E and F. Thromboxanes are produced only in platelets, while prostacyclins are produced only by endothelial cells. Thromboxanes and prostacyclins are antagonist, homeostatic agents that control blood flow and clotting. However, prostaglandins are produced in most cell types. The opening of the endoperoxide ring of PGH_2 at c-9 and c-11 and subsequent attachment of functional groups at those sites produce the prostaglandins. A ketone at C-9 and hydroxyl at C-11 produces prostaglandin E_2, while the converse hydroxyl at C-9 and ketone at C-11 produces prostaglandin D_2. Reduction with NADPH leads to hydroxyl groups at both C-9 and C-11 and produces prostaglandin $F_{2\alpha}$. The nomenclature of the subscript 2 signifies the two double bonds (C-5/6 and C-13/14) and the alpha signifies

stereochemistry of O-9. Prostaglandins primary role lies in mediating inflammation, but they are also known to facilitate calcium movement, hormone regulation and control cell growth.

All prostanoids have short life spans and are immideately transported out of the cell through prostanoid transporters. Their autocrine and paracrine responses are conveyed through G protein coupled receptors and somtimes PPARs. Nine specific GPCRs have been identified: DP-1 and DP2, EP1 through EP4, FP, IP and TP for PGDs, PGEs, PGFs prostacyclins and thromboxanes, respectively. Depending on cell type, receptors may inhibit or stimulate cAMP formation, or may activate a phosphatidylinositol signal pathway leading to intracellular calcium release (Narumiya *et al.* 1999).

2.7 Clinical Treatment

The pathogenesis of tendon overuse injury is still unknown, leading to a debatable treatment methodology. Further debate ensues because chronic cases are treated as inflammation even though histopathology clearly reveals a lack of inflammatory cells (Astrom and Rausing 1995). Few studies have thoroughly examined the efficacy of empirically based treatment protocols, even though most treatments are based on these observations (Jozsa and P. 1997; Khan *et al.* 1999). The most common treatment methods are rest, controlled exercise, pharmaceutical agents, a host of physical modalities and surgery.

2.7.1 Rest

The first treatment option is to discontinue the overuse, or rest because tendinosis is a degenerative condition brought on by overuse. There is likely structural damage in the form

of collagen fiber tears and cross-link breaks at this stage (Khan *et al.* 1999; Wang *et al.* 2006). Rest is intended to allow the tendon time to heal itself because until now, the damage rate has been greater then the repair rate. However, the slow metabolic rate of tendon, almost 1/10 the oxygen consumption of muscle, means it will likely require a much longer healing time than most doctors prescribe or patients allow. If the clinical condition is not severe, sometimes reduced activity level is prescribed when rest should always be prescribed first. Rest is meant as the cessation of the exercise, and not immobilization which causes atrophic conditions.

Treatment of tendinosis is usually sought after the occurrence of pain or performance loss. Because tendinosis is a largely asymptomatic degenerative condition, the first indications of pain is likely observed well into the degenerative state (Leadbetter 1992). In mild cases, that is without other compounding injuries, pain is therefore not a good indicator of damage and tissue repair may require months, instead of the weeks (Khan *et al.* 1999). Tenocytes are suggested to require roughly 2 to 3 weeks to fully initiate a tissue response (Jozsa and P. 1997), therefore it is advised that at least this amount of rest be afforded the tendon before gradual exercise is introduced again.

2.7.2 Exercise

It has been suggested that extrinsic factors such as improper training and equipment can cause overloading of tendons, leading to tendinopathy. Before resuming exercise, these factors should be checked, and if necessary, corrected with proper braces, equipment or shoes. After a few weeks of rest to initiate the repair process, it is suggested that strengthening and stretching exercises, particularly eccentric exercise be performed (Clement

et al. 1984; Stanish *et al.* 1986). Various clinical studies have shown aggressive loading to relieve symptoms, as well as effectively help in healing (Clement *et al.* 1984; Alfredson 2003). While the exact mechanism that facilitates this healing is not know, it has been suggested to be a result of increased load magnitude and frequency inducing an increase of tenocytes metabolism (Kannus *et al.* 1997).

Controlled stretching is likely to increase collagen synthesis and improve fiber alignment, resulting in better mechanical strength of the healing tendon. Collagen unstressed during the proliferative and remodeling phase are less organized and weaker (Sharma and Maffulli 2005). Furthermore, repetitive motion has been shown to induce a host of cellular responses such as DNA increase, protein synthesis and cellular proliferation, even at low loading rates.

2.7.3 Pharmaceutical Treatments

Often, if rest and controlled exercise aren't sufficient or the pain is too severe, pharmaceutical treatments are pursued. The most common pharmacologic agents used in the treatment of tendinosis are non-steroidal anti-inflammatory drugs (NSAIDS). Other pharmaceutical treatments, not all currently used, include corticosteroids, aprotinin, heparin, sclerosing agents and ground substance.

2.7.3.1 NSAIDS

The oldest synthetic drug, and the one often given credit for beginning the pharmaceutical industry is Aspirin, an NSAID. The history goes back as far as the 5th century BC when Hippocrates noted the use of willow bark extract to ease aches and pains and reduce fever. Over time this was extracted and isolated, but side effects such as gastric

problems and even death made this drug unsafe. German scientists at Bayer managed to change a hydroxyl group to an acetyl group which instantly reduced the side effects. It has proceeded to become the oldest and one of the most successful drugs, but the mechanism of action did not become apparent until the 1970s (Vane and Botting 1992).

Studies by Vane (Vane and Botting 1992) showed that Aspirin and other anti-inflammatory medications worked by inhibiting cyclooxygenase. A host of drugs with similar roles existed or were then manufactured, including other salicylates (like Aspirin), arylalkonic acids (like Indomethacin) and profens (such as Ibuprofen). More potent versions like oxicams (Pirixocam) soon were manufactured, but these drugs began to show side effects of Dyspepsia, gastric ulcers and diarrhea. It was later discovered that there were many isoforms of COX and that these NSAIDs blocked all of them. The discovery of COX-2 led to the development of COX-2 specific NSAIDs (COXIBS) such as Celecoxib, Rofecoxib, Valdecoxib and others. As long term clinical trials with these drugs became available, many years after the drugs were already being prescribed, they tended to show a potential risk of heart attacks when taken chronically. Certain Coxibs have been both banned and voluntarily withdrawn amidst pending litigation. Finally, acetaminophens, long known to have fever reducing and analgesic effects are labeled NSAIDs but their ability to inhibit COX-1 or COX-2 has not been shown. Recently discovered COX-3 however has been shown to be effectively inhibited by acetaminophens (Chandrasekharan *et al.* 2002).

Prostaglandins have many effects, one of which is to play an extensive role in the inflammation driven remodeling and repair process of musculoskeletal tissue such as bone (Radi and Khan 2005). It is blocking this action that makes NSAIDs anti-inflammatory, and

combined with the fact that inflammatory mediators are seen in tendonitis as well as tendinosis is the basis for NSAID use in tendinopathy.

Theoretically, these should have no affect on tendinosis, a non-inflammatory condition. NSAIDs also have analgesic effects that may make patients ignore pain and further damage the tissue, suggesting many to call for more investigation of their use (Almekinders 1990). Furthermore, the effects of NSAIDs in tendinosis have not been systematically evaluated, and the few studies that have examined it have found no beneficial effects (Jozsa and P. 1997; Almekinders and Temple 1998). However, even though an inflammatory cell cascade is not present, there are several load induced inflammatory mediators, cytokines and growth factors expressed by the intrinsic fibroblast in tendinosis (Almekinders *et al.* 1995; Wang *et al.* 2006). Affecting alternate mechanisms such as these remains a likely method of action for NSAIDs (Khan *et al.* 1999). In one such example, NSAIDs block PGE_2 which has been shown to increase collagenase production, increase cell death and create tendinopathy (Khan *et al.* 2005).

2.7.3.2 Other Pharmaceutical Agents

Other pharmaceutical agents used include corticosteroids, aprotinin, heparin, sclerosing agents and ground substance. Corticosteroids are quite controversial and not used as much currently because tendinosis is a non-inflammatory condition and they have been shown to inhibit collagen synthesis and decrease the strength of the tendon (Clement *et al.* 1984; Jozsa and P. 1997; Wang *et al.* 2006). Corticosteroids work further upstream than NSAIDs, and their full effects are even less understood. Furthermore, they have been established to cause cell death and atrophy in injection studies (Nirschl 1992; Khan *et al.* 1999). Other pharmaceutical agents used include the protease inhibitor aprotinin and the

anticoagulant heparin. Injections of ground substance, mainly glycosaminoglycans, as well as sclerosing agents have shown some positive results (Alfredson 2003).

2.7.4 Physical Modalities

Phyisiotherapists have also employed a wide range of physical modalities such as ultrasound, laser photo-stimulation, deep heat, pulsed magnetic/electromagnetic fields and electrical stimulation. The intended purposes of these modalities are similar to those seen in various other tissues such as to disrupt the affected region and promote remodeling through the mechanical effect of high-frequency sound waves or increase local blood flow and heat (Wang *et al.* 2006).

Ultrasound has been shown to increase collagen synthesis by tendon fibroblasts and increase the tensile strength in animal models (Jackson *et al.* 1991; Enwemeka *et al.* 1992). Laser photostimulation has been shown to significantly increase collagen concentrations, which combined with ultrasound and electric stimulation was shown to increase the biomechanics of rabbit tendon (Gum *et al.* 1997; Reddy *et al.* 1998). Bipolar radiofrequency treatment has been suggested because of its ability to stimulate blood vessel formation and regulate vascular endothelial growth factor (VGEF), α-V-integrin and other growth factors while not compromising the strength of the tendon (Tasto *et al.* 2003; Silver *et al.* 2004).

Because NSAIDs are thought to provide mainly analgesic effects and may have unintended side-effects, extracorporeal shock-wave treatment has been suggested because it too has established analgesic effects but little reported side-effects (Speed 2004; Wang *et al.* 2006). All physical modalities of treatment are controversial because they are mainly

adaptations from other tissues and their long term effects have not been clinically established in tendon (Rivenburgh 1992).

2.7.5 Surgery

In most cases of tendinosis, surgery is not recommended because its benefits have not been systematically documented. Chronic cases of tendinosis which are not responsive to non-operative treatments and still exhibit pain and performance loss are the majority of cases accepted for surgery. The goals for surgery include inducing scar repair, removing affected portions of the tissue, encourage revascularization, relieve excess pressure and overload and repair/replace if there is rupture.

The main method utilized is to excise the affected sites on the tendon after a longitudinal tenotomy (Nelen *et al.* 1989; Leadbetter *et al.* 1992; Alfredson 2003). Another common method is to make multiple percutaneous incisions to promote blood circulation, enhance oxygen uptake and induce macrophage migration (Wang *et al.* 2006). Grafts and tendon transfer are always options for severe cases. The main hope of the surgery is to induce an inflammatory healing response. This inflammatory response would induce macrophages which remove damaged cells and matrix as well as release growth factors. The resulting fibroblast proliferation and collagen synthesis likely drives the repair process (Wang *et al.* 2006). Even though there is a prevalent surgery history, good results of surgery are poorly documented histologically and little data exists to suggest that normal tendon properties are ever reached (Burks *et al.* 1990; Leadbetter *et al.* 1992). It has been suggested that the postoperative healing response and careful rehabilitation, and not the surgery itself is

the cause of the tendon improvement (Leadbetter *et al.* 1992; Khan *et al.* 1999). Therefore, in most cases tendinosis (barring rupture), non-operative measures are the gold standard.

2.8 Overuse models

To better understand the etiology of overuse tendinopathy many models have been examined. Various in vitro and in vivo animal models, as well as a human model have been developed. The various models offer great insights into the possible factors affecting overuse tendinopathy, however they also contain some shortcomings. Improvements to existing models or an improved model, specifically in controlling loading, would be beneficial in better understanding tendon overuse injuries (Archambault *et al.* 1995; Jozsa and P. 1997).

2.8.1 In Vitro Overuse Models

Understanding overuse injuries of tendons first requires the understanding of the basic effects of cyclic load on tendon. To this effect, the most basic model of overuse injuries is that of the time-dependant response of tendon to load. In the same manner all basic material properties of tendons were developed, isolated specimens are cyclically stretched by external force on material testing systems while the specimen deformation and applied force are recorded. This model has adequately demonstrated the accumulation of fatigue microdamage by verifying the existence of time dependant properties of stress-relaxation, creep and mechanical hysteresis in tendon (Rigby *et al.* 1959; Viidik 1973; Butler *et al.* 1978; Schechtman and Bader 1997; Ker *et al.* 2000; Schechtman and Bader 2002). However, the biggest limitation of this model is the use of tendons devoid of cellular activity, a potentially significant contributor to overuse injuries.

The converse model of tendon overuse injury is that of cyclically loading cells in culture. Because cells likely play a significant role in overuse injuries through the modulation of repair and remodeling, their response to cyclic load must be examined. The cellular model consists of stretching fibroblasts on deformable substrates in culture and examining their response. This model has demonstrated that cells express mRNA and proteins of various agents in response to cyclic load. Some common expressions that likely play a role in the development and modulation of injury are cytokines, inflammatory mediators, growth factors and proteases (Banes *et al.* 1995; Banes *et al.* 1999; Tsuzaki *et al.* 2000; Archambault *et al.* 2001; Tsuzaki *et al.* 2003; Yang *et al.* 2004). The biggest limitation of this model arises from the lack of an extracellular matrix. The equivalent tissue strain cannot be determined, nor can the effects of the cellular responses on the matrix.

The last in vitro model, tissue explants, attempts to bring together the effects of cyclic loading on the tissue as well as the cellular response. This model incorporates the use of a tissue loading device, usually custom designed, to cyclically load viable explants. This model allows for the examination of loading and stress deprivation on various cellular expressions and matrix composition (Slack *et al.* 1984; Hannafin and Arnoczky 1994; Hannafin *et al.* 1995; Tanaka *et al.* 1995). Because the loading is controlled, the various features of loading, that is, magnitude, frequency and duration can be controlled and their effects on tendon strength, matrix composition and cellular responses can be observed (Arnoczky *et al.* 2002; Lavagnino *et al.* 2003; Arnoczky *et al.* 2004). The main limitation of explant models thus far is the inability to mechanically damage tendons through sufficient loading. This is a limitation of existing devices, not of the explant model in general.

However, a limitation of all explants is the inability to account for damage and responses associated with in vivo motion such as those around joints and pulleys.

2.8.2 In Vivo Overuse Models

The models used most often however are animal exercise models, usually consisting of treadmill exercise. Such models have been used for the Achilles, patellar, digital flexor, digital extensor and supraspinatus tendons of either rats, rabbits or swine. As discussed earlier, such models have been used to show atrophy-like disuse characteristics, as well as exercise induced hypertrophy in the form a collagen, GAG, MMP and stress change (Viidik 1969; Tipton *et al.* 1975; Woo *et al.* 1980; Amiel *et al.* 1982; Sommer 1987). The Achilles, patellar and flexor tendons are suitable for overuse studies from the knowledge that, anatomically, the rat is very similar to humans. However, extensive work to detail the kinematics of rat tendon during free locomotion for comparison has not been made and most of these models are not quantifiable (Archambault *et al.* 1995).

The first quantifiable model examined the controlled motion of an anaesthetized rabbit Achilles tendon on a kicking machine (Backman *et al.* 1990). Degenerative changes were seen visibly in the form of nodules, increased vascularization, edema, and infiltration of inflammatory cells. Models of this type are beneficial because the loading can be indirectly controlled and the animal will not have the ability to distribute its weight to compensate. Another model is that of the rat supraspinatus tendon, determined thorough a comparative examination of rat and human anatomy (Soslowsky *et al.* 1996). The importance of this model is that it is the first such model for a tendon that is susceptible to injury by intrinsic factors such as pinching, making it suitable for rotator cuff overuse studies. This model is

however, a treadmill exercise model of the rat and thus quadruped vs. biped differences must be noted.

The final sets of animal models are those which use in-vivo measurement techniques to monitor tendon during overuse injury. The first set of these methods are invasive and include the implantation of a pressure-sensitive transducer, a buckle transducer sensitive to flexion, measuring intratendinous pressure with fiber optic cables, a length measuring device or a force transducer (Meyer *et al.* 2004). These methods are good for direct measurement of forces, but questions have arisen about the damage incurred during the implantation and that they have not been successfully used for long (>24hr) term studies. A second set of models are non-invasive and examine tendon properties ultrasonically just after exercise. This method is particularly good for determining the area change and detecting the onset of tendinopathy (Gillis *et al.* 1993).

Because animal exercise models can be costly and are often inconsistent, an alternative approach studied has been animal injection models (Wang *et al.* 2006). These models include the injection of collagenase, cytokines and inflammatory mediators. The collagenase injection studies create tendon degeneration, but seem to recreate the tendon healing response to traumatic injury, whereas tendinosis is a slow degenerative condition (Riley 2004; Sharma and Maffulli 2005). Cytokine injections are usually done by injecting a mixture of cytokines, growth factors and other unknown agents that are derived from synovial fibroblasts in response to an inflammatory agent (Stone *et al.* 1999). Tendon degeneration is observed, but the complete composition of agents injected is unknown and is derived from a process that is not repetitive loading, the cause of tendinosis. The final injection model is that of prostaglandins. Since these inflammatory mediators are load

induced and acts upstream to collagenase and other factors in tendinosis, it may be a good model. Peritendinous rat Achilles tendon injection of PGE1 has shown fibrosis and intra-tendinous degeneration (Sullo *et al.* 2001). Direct injection of PGE_2 was shown to cause degenerative conditions in rabbit patellar tendons as well (Khan *et al.* 2005). Of the injection models, the final approach of injecting upstream factors directly observed in tendinosis is likely the best approach, but it may be improved by adding other load-inducible factors such as IL-1β and TNF-α if micro-injuries undergo similar healing response to traumatic injuries (Wang *et al.* 2006).

A final model that must be noted, though it has not been used for overuse studies yet, is the human model. All animal model listed above are possible for application on humans, but are well beyond regulations for human subjects. One type of in vivo human studies that is currently being examined is that which measure the intratendinous fluid via a micro-catheter during specific exercises (Langberg *et al.* 1999). There are limitations on the exercise that subjects can perform as well as the ability to collect only fluids and not tissue samples, but consistent results of increases in collagen turnover and PGE_2 have been seen with this approach (Langberg *et al.* 2001; Langberg *et al.* 2003).

CHAPTER 3

AIMS

3.1 Aim 1 – Elastic Strain Limit

The elastic strain limit of tendons has long been established at 4% strain. This is the level beyond which irrecoverable damage occurs. However, these studies were performed with older methodologies that may have negatively influenced the results. To reassess the elastic limit more accurately with newer gripping and strain monitoring methodologies, tendons will be loaded to subfailure strains, allowed to rest for 5 minutes, and then loaded to failure.

Hypothesis:

Strain levels significantly greater than previously thought, will be required to cause irrecoverable damage to the tendons.

3.2 Aim 2 – Tendinopathy Model

The effect of cellular responses on the extracellular matrix and the inherent complexities of in vivo loading require the development of an in vitro explant model to precisely model the cyclic loading level effect on tendon material and compositional properties such that tendon degeneration can be studied. A new pneumatic tissue loading device (TLD) and a high-speed real time video strain analysis (VSA) system will be

developed, calibrated, have their performance characteristics established and tested for the ability to create and monitor damage.

Hypothesis:

1) The new TLD will be capable of applying repeatable, damaging cyclic loads while maintaining the viability of the tissue, allowing for short term overuse tendinopathy studies.
2) The new VSA system will be capable of measuring midsubstance strain, giving a more accurate representation of tissue damage without compromising tissue viability. And, this can be done during cyclic loadings within tissue culture.

3.3 Aim 3 – Tissue Response

With an overuse tendinopathy model established, the next aim is to utilize the model to perform a cyclic loading study and examine the biochemical and biomechanical response of tendon. A fixed number of cycles (86,400) will be varied across two periods (Short – 1day, Long – 12 days) at two loading levels (Low – 3 MPa, High – 12 MPa). Composition, sGAG and collagen, will be measured as well as cellular responses (LDH, PGE_2 and collagenase). Strain analysis during loading, as well as failure testing after the loading will help in monitoring the mechanical properties of the tendons.

Hypothesis:

1) Composition – The low loading groups will not cause any damage and thus will not exhibit compositional changes. The short duration will not cause measurable compositional changes, but the short-high group will cause damage. The high group across the longer period will cause increase in GAG and decreased collagen

content, signs of tendinosis, because of the extra time. Minimal viability changes will be seen, possibly a decrease in the high-long group.

2) Cellular responses – Again, the low loading groups will not cause significant changes in the cellular responses because the loading will not be damaging. The high groups however, will both exhibit increased PGE_2, LDH and collagenase with the long period group exhibiting a greater response in each instance.
3) Mechanical Properties – The low loading groups are not intended to be damaging and thus we expect minimal changes in any mechanical property in these groups. The high short group will exhibit increased damage in the form of reduced stiffness, increased strain as well as lower failure strength. Again, we expect the high long group to exhibit a similar but more pronounced response.

3.4 Aim 4 – Magnitude Response

Having determined the tendon response to load and how this changes across time, the next aim is to establish the load magnitude effect of this response. A similar loading model will be utilized to load tendons to the same number of cycles but across four loading levels (0 MPa, 3 MPa, 12 MPa and 18 MPa). This study will be performed across a period of 3 days. This period should be long enough to allow time for responses, but yet short enough to eliminate any duration dependant effects.

Hypothesis:

1) Composition – A magnitude dependant sGAG increase will be seen, while collagen decrease will be seen in the highly loaded groups (12 and 18 MPa) but

not the 3 MPa or unloaded groups. There will be no viability changes in the 0 and 3 MPa groups with a possible decrease in the 12 and 18 MPa groups.

2) Cellular Responses – PGE_2 levels will be highly magnitude dependant as will cell death and collagenase levels. Overall, the 3 MPa group will be very similar to the 0 MPa group, if not slightly better.

3.5 Aim 5 – COX Inhibion

An expected consistent response in our tendinopathy model is the load-magnitude dependant PGE_2 response. A known inflammatory mediator with both positive and negative affects, it is also a source of clinical focus as its inhibitors are prescribed for overuse tendinopathies. Furthermore PGE_2 may be linked to various other responses we monitor (GAG, collagenase, cellularity, collagen content). NSAIDs are prescribed in tendinopathy even though a full inflammatory process does not exist. To examine the their effects in tendinosis, selective and non-specific COX inhibitors, as well as exogenous PGE_2, will be applied to cyclically loaded tendons. This will be done with the same model with a fixed number of cycles, across six days and three loading levels (0 MPa, 3 MPa and 12 MPa).

Hypothesis:

1) Composition – The groups with the COX inhibitors will see reduced changes in the sGAG content and collagen content relative to untreated but loaded groups. The converse will hold for the exogenous PGE_2 groups, which will see a more pronounced magnitude dependant sGAG increase and collagen decrease than the untreated but loaded group.

2) Cellular Response – The COX inhibitor treated groups will decrease the PGE_2 levels in the media as well as the LDH and collagenase levels at all of the loading levels relative to control. Any magnitude dependant responses will be diminished. The converse will hold true in the exogenous PGE_2 groups with the greatest effects seen in the highly loaded groups.

3) Mechanical Properties – The COX inhibitor treated groups will show increased mechanical properties relative to the control groups. The exogenous PGE_2 treated groups will show the converse, decreased mechanical properties relative to the control groups.

CHAPTER 4

ELASTIC STRAIN LIMIT

Devkota AC & Weinhold PS, 2003. Mechanical Response of Tendon Subsequent to Ramp Loading to Varying Strain Limits. Clinical Biomech (Bristol, Avon) 18, 969-974.

4.1 Abstract

Objective: To determine the *in vitro* elastic limit of avian flexor tendons utilizing more current methodologies.

Design: Assess the mechanical changes between subfailure and subsequent failure ramps at various loading levels.

Background: Currently accepted values of elastic strain limits were determined utilizing older methodologies. Consequently, reported values are fairly small and imply matrix damage occurs with small strains.

Methods: Flexor tendons were loaded *in vitro*, to various subfailure strain levels between 1% and 14%, allowed to rest for 5 minutes, then taken to failure. Suture markers, across the midsubstance, and cryo-grip displacement were monitored for strain using a video strain analysis system and a linear variable displacement transducer, respectively. The mechanical changes between the various subfailure and failure ramp loadings were assessed.

Results: Varying strains of subfailure ramp loading did not influence (P>0.05) the ultimate tensile failure strength, elastic modulus, strain at failure, or strain energy density of tendons.

In addition, residual strain after subfailure loading was not significant, nor was it influenced by the level of the subfailure loading.

Conclusions: Tendon behaves elastically under ramp loading to significantly higher strains (nearly failure) than previously reported (4%).

Relevance

This study has found strain thresholds required to cause matrix damage to be significantly higher than previously thought, implying that matrix changes to acute loading events are more a result of the cellular response to the loading event. The clinical relevance is that the clinician may have a greater opportunity to prevent matrix changes than once thought by biochemically altering the cellular response.

4.2 Introduction

Acute strains and cumulative trauma injuries to tendon are a common and growing clinical problem having debilitating effects on an individual's activity level and quality of life. While the pathophysiology following these injuries is complex, it is believed that loading of the tissue beyond specific mechanical limits can cause irreversible damage to the extracellular matrix, resulting in a permanent change in the mechanical characteristics of the tendon. Furthermore, resident cells of the tissue and infiltrating cells must react to the damage of the tissue with a wound healing response in order to repair the injury (Leadbetter 1992). The specific mechanical limits that cause damage to tendon have been largely inferred based on single tensile loading tests conducted to failure, while few studies (Rigby *et al.* 1959; Abrahams 1967) have examined how the mechanical response of tendon changes with loading to subfailure levels. Such information would help identify the mechanical thresholds (strain), at which matrix damage begins to occur in tendon. In addition, with the increased interest in examining how cell viability and metabolism of cultured tissue explants or cells grown on deformable substrates are affected directly by controlled mechanical stimuli (Banes *et al.* 1995; Hannafin *et al.* 1995), it is important to understand how matrix damage strain thresholds relate to cell response strain thresholds.

Classic tendon mechanics studies (Rigby *et al.* 1959; Abrahams 1967) have suggested that loading beyond 4% strain can cause permanent deformation and a reduction in strength and stiffness; factors indicative of matrix damage. However, the methods used in these studies may have adversely influenced these results. The contact extensometers used for deformation assessment and the mechanical techniques employed to clamp the tendons are susceptible to slippage and stress concentrating effects. In contrast, recent studies of

ligament (Panjabi *et al.* 1996) where subfailure stretches of 80% of the failure deformation have been applied to bone-ligament-bone complexes, has shown an absence in reductions in strength, deformation at failure, and energy at failure, while an increase in stiffness was reported. In addition, assessments of the deformation response at low load levels (Panjabi *et al.* 1996) found that such deformations were significantly increased in the ligaments treated with subfailure stretching.

In an effort to reexamine the results of the classic tendon studies while utilizing more current testing methodologies, the following study was carried out to characterize mechanical changes in avian digital flexor tendon with subfailure ramp loading to varying strain limits. In this study, the more current methodologies of cryo-clamping (Riemersa and Schamhardt 1982) and video strain analysis (VSA) (Woo 1982; Lam *et al.* 1992; Derwin *et al.* 1994) were employed to minimize grip slippage and stress concentrating effects. The main objective of this study was to evaluate changes in permanent deformation, elastic modulus, ultimate tensile strength, energy to ultimate strength, and low load-level deformations after ramp loading is applied to tendon to the following strain limits: 1,2,3,4,6,8,10,12, and 14%. It is hypothesized that permanent deformation will increase while strength and stiffness measures will decrease with subfailure ramp loading, but that these changes will begin to appear at higher strain limits then previously reported.

4.3. Methods

4.3.1 Specimen Information

Freshly severed feet from unprocessed 45-day old Rock Cornish chickens were acquired from a local poultry processing plant, then subsequently cleaned and frozen at -20°C. The feet were thawed at a later date (1 – 2 months) and the flexor *digitorum profundus* tendon was isolated from the middle digit of each foot and frozen at -20°C (for 24 – 48hours) in saline soaked gauze prior to testing. The rationale for this specimen comes from its anatomic similarity to human flexor tendons (Lindsay and Thomson 1960; Farkas *et al.* 1974) and its prevalence in tissue explant studies of tendon injury (Manske and Lesker 1984; Hannafin *et al.* 1995; Tanaka *et al.* 1995). On the day of testing, tendons (average length 85mm) were thawed and duplicate area measurements were made along the middle third of the tendon by an area micrometer after applying a 0.12MPa compressive stress for 2 minutes (Butler *et al.* 1984). The final reported area of the tendon is the average of the two measurements.

4.3.2 Instrumentation

Tendons were clamped by means of liquid nitrogen fed cryo-clamps similar in design to that previously described (Riemersa and Schamhardt 1982), and mechanically loaded by a servo-hydraulic materials testing system (8500 Plus, Instron Corporation, Canton, MA, USA). Tendons were attached with the grip-grip displacement set at 38mm. Two black #5-0 sutures were stitched transversely, approximately 13mm apart at the longitudinal center of the tendon for midsubstance strain analysis. The suture displacement was monitored by the VSA system (Devkota and Weinhold 2001) and the grip displacement was monitored by the LVDT within the actuator of the Instron system. The load level, monitored by a 250lb load

cell (Sensotec Inc., Columbus, Ohio, USA), and the two displacements were collected simultaneously at 60Hz for all mechanical tests.

4.3.3 Mechanical Testing (Elastic Strain Limit)

Mechanical testing was preceded by first pretensioning the tendon at 0.25 N, then preconditioning with 10 cycles of a 0.5Hz, symmetric triangular waveform with amplitude of 0 to 0.5% grip strain. The specimens were again pretensioned at 0.25 N, and then taken to a predetermined grip strain-level (1,2,3,4,6,8,10,12, &14) by a 1% strain/second linear ramp. A subsequent -1% strain/second ramp brought the tendon back to the original rest state, where it was wrapped in saline soaked gauze and allowed to rest for 5 minutes to recover from any viscoelastic effects. After the rest period, the specimens were pretensioned at 0.25 N, and then taken to failure by the same 1% strain/second linear ramp.

From the predetermined strain-limited and failure ramp loadings of each specimen, the following material and structural properties were evaluated based on suture marker (midsubstance) strains, grip strains and load: max load, max displacement, ultimate stress, strain at ultimate load, energy density at ultimate load, stiffness, elastic modulus by linear regression of data between load limits of 25-75% of the ultimate load, residual strain 5 minutes after strain-limited ramp loading at 1MPa stress level, and change in strain between strain-limited/failure ramp loadings within a specimen at a 10MPa stress level.

4.3.4 Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's mean comparison testing was utilized to evaluate differences in mechanical properties at each strain limit. Two-way repeated measures ANOVA (the repeated factor being readings from the strain-limited

& failure ramp loadings within a specimen) was utilized to evaluate the residual strain and change in strain at 10MPa parameters. A significance level of 0.05 was used for these tests.

4.4. Results

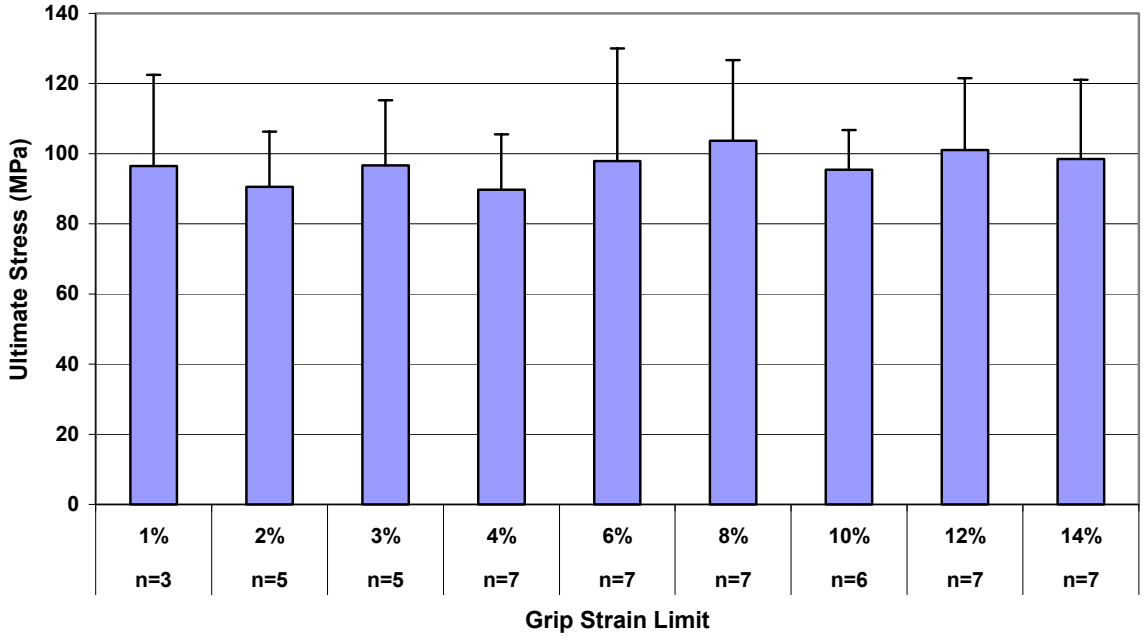

Figure 4: Ultimate tensile failure strengths of avian flexor digitorum profundus tendons subjected to varying strain-limits of a single subfailure ramp loading were not found to differ with strain-limit level (ANOVA, P>0.05).

Varying the strain-limit (1-14%) of the subfailure ramp loading applied to the tendon did not influence (P>0.05) the ultimate tensile failure strength of the tendons (Figure 4). In addition, the elastic modulus based on midsubstance or grip strain (Figure 5) was not found to differ (P>0.05) with the level of the strain-limit applied. The elastic modulus based on the midsubstance strain was found to be significantly stiffer then the modulus based on the grip strain (P<0.05). Paired strain-limit/failure stress-strain curves (marker displacement) for individual specimens tested to strain limits of 1, 6, and 12% (grip strain) show the similarity between the strain-limit and failure curves (Figure 6). The average stress level achieved in the tendon with each strain-limited subfailure ramp loading is shown (Table 1).

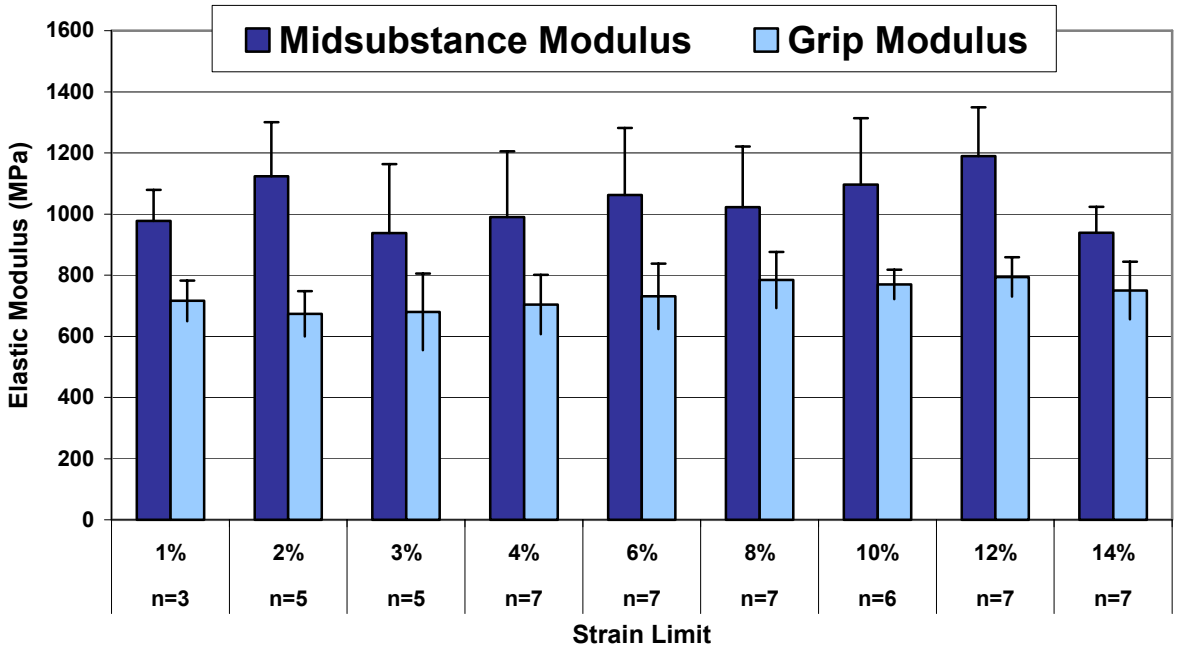

Figure 5: Elastic moduli based on the midsubstance or grip strain of avian flexor digitorum profundus tendons subjected to varying strain-limits of a single subfailure ramp loading were not found to differ with strain-limit level (ANOVA, P>0.05). Elastic moduli based on midsubstance strain were found to be significantly stiffer then the moduli based on grip strain (paired t-test, $P<0.001$).

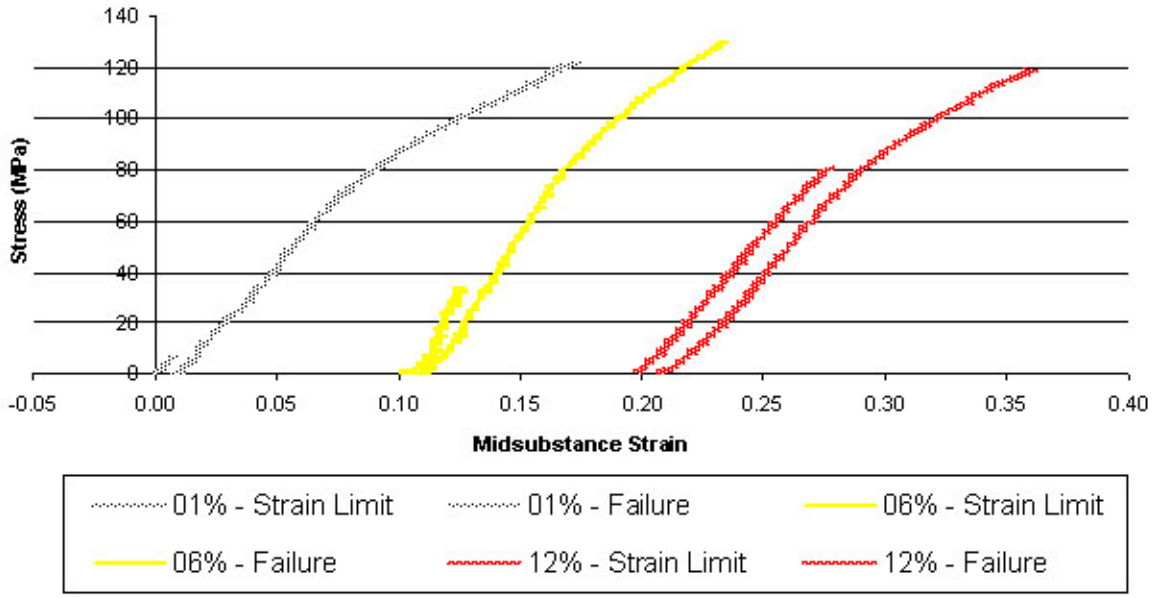

Figure 6: Examples of paired midsubstance strain-limit/failure stress-strain (VSA) curves for individual specimens tested to grip strain limits of 1, 6, and 12% (shown up to max stress). Strain levels of curves have been artificially shifted for clarity of presentation. Note, grip strain is greater than midsubstance strain.

The midsubstance residual strain 5 minutes after each strain-limited subfailure ramp loading (Table 1) was not found to differ from zero ($P>0.05$), and this strain was not found to differ with the level of the strain-limit applied ($P>0.05$). However, there was a minor change ($P<0.001$) in midsubstance strain between the strain-limited and failure ramp loadings when 10MPa of stress was applied (Table 1), but this change in strain was not influenced by the level of the strain-limit applied. The midsubstance and grip strains at failure (ultimate stress) were not influenced by the level of the strain-limit applied (Table 1). Finally, the strain energy density at failure was not influenced by the level of the strain-limit applied (Table 1).

All failures were fairly instantaneous (no subfailures) and occurred between the grips. Most failed in the midsubstance between the sutures, but some did fail in the other region (between suture and grip). No differences or patterns were observed in relation to the failure site and the strain levels. Overall averages at failure across all groups were 96.7 MPa for max stress, 16.4% for grip strain and 12.2% for midsubstance strain. One specimen did fail during the 14% ramp loading, and its data was discarded.

Table 1: Mechanical properties (mean (SD)) of tendon based on tensile ramp loading to a specific grip strain limit or on subsequent test to failure.

Limit Values		Mechanical Properties				
Strain Limit (%)	Stress at Strain Limit (MPa)	Midsubstance Residual Strain After Strain Limit Test (%)	Δ Midsubstance Strain at 10MPa between Limit & Failure Test (%)	Midsubstance Strain at Failure (%)	Grip Strain at Failure (%)	Strain Energy Density at Failure (mJ/mm^3)
1 (n=3)	2.9 (2.9)	-0.3 (0.7)	Not Available	13.5 (4.4)	16.8 (2.1)	8.9 (3.3)
2 (n=5)	5.8 (1.8)	0.3 (0.4)	Not Available	10.5 (3.1)	16.8 (2.4)	8.8 (3.2)
3 (n=5)	16.0 (4.8)	0.1 (0.7)	0.6 (0.5)	13.7 (3.1)	16.8 (1.7)	8.7 (2.5)
4 (n=7)	20.7 (7.1)	0.4 (0.8)	0.4 (0.7)	12.2 (3.8)	15.4 (2.5)	7.4 (2.7)
6 (n=7)	30.9 (9.2)	0.3 (0.9)	0.6 (1.0)	12.3 (3.0)	16.4 (2.5)	8.8 (4.4)
8 (n=7)	50.8 (13.3)	-0.3 (0.5)	0.1 (0.4)	11.9 (3.1)	16.6 (2.2)	9.5 (3.2)
10 (n=6)	62.6 (9.2)	0.6 (0.5)	0.6 (0.7)	12.2 (3.6)	15.7 (1.0)	7.8 (1.1)
12 (n=7)	73.8 (10.2)	0.3 (0.4)	0.6 (0.4)	11.6 (3.6)	16.9 (1.8)	8.9 (2.9)
14 (n=7)	87.7 (7.8)	0.3 (0.6)	0.1 (0.8)	13.0 (3.4)	16.2 (2.8)	8.2 (3.8)

-None of the mechanical properties differed with strain limit level (ANOVA, $P > 0.05$).
-Residual strain was not significantly different than zero (RM-ANOVA, $P > 0.05$)
-Change in midsubstance strain at 10MPa between limit & failure test differed from zero (Repeated Measures ANOVA, $P < 0.001$) independent of strain level.

4.5 Discussion

A study was carried out utilizing cryo-clamp and VSA testing methodologies to characterize mechanical changes to tendon with subfailure ramp loading to varying strain limits in order to better characterize the strain thresholds at which matrix damage occurs in tendon. Contrary to our stated hypothesis, the current study was unable to demonstrate a decrease in the strength and stiffness properties (Figure 4 & Figure 5) or an increase in permanent (residual) strain (Table 1) with ramp loading to strain limits ranging from 1-14%. In addition, no change in failure strain or strain energy density (Table 1) was found with ramp loading to the specified strain limits. These results are in contrast to classic early tendon biomechanical studies that have suggested that strength and stiffness decline with loading beyond a strain limit of 4% (Rigby *et al.* 1959) and permanent deformation can begin to occur with strains exceeding 4% (Abrahams 1967).

It is believed that the difference in results between this study and past studies is largely related to the mechanical clamping methodologies used in past studies that may have exaggerated damage to the tissue due to local stress concentration effects near the grips. While it is possible that these differing results may be due to species specific effects, this would appear to be unlikely due to the similarity in the mechanical properties of avian tendon to human tendon. The ultimate stress (96.7MPa), grip elastic modulus (735.7 MPa) and strain at failure (16.3 %) of avian tendon reported in the current study compare relatively well with human patellar tendon studies that have reported values of 65-111 MPa, 612-660 MPa and 13-26% for the ultimate stress, modulus and failure strain, respectively (Butler *et al.* 1984; Butler *et al.* 1986; Johnson *et al.* 1994). The inference that stress concentrating effects near the grips are the cause of the difference is supported by recent studies of ligament (Panjabi *et*

al. 1996) that have reported an absence of reductions in strength, deformation at failure, and energy at failure following subfailure stretches of up to 80% of the failure deformation on bone-ligament-bone complexes. In addition, in this ligament study an increase in deformation was observed at low-level loads (5-50% of failure) in ligament receiving the subfailure stretch. Similarly, in the current study we saw a minor increase (Table 1) in strain at 10MPa loading after the initial ramp loading. However, as this increase in strain was not found to be dependent on the level of the strain-limited ramp loading, it would appear that this strain effect might be a tissue conditioning effect rather than a damaging effect. In addition, due to the small size of this strain change and the more dynamic adaptability of the length of the myotendinous unit, it is questionable if this strain change has physiological significance.

The current study has significance in that it suggests 1) tendon behaves as an elastic material to higher strains (i.e. nearly to failure) then previously reported and 2) a decline in mechanical properties due to matrix damage from a single loading stimulus is less likely to occur than previously thought. Readers are advised however, that this does not imply *in vivo* tendons or their matrix cannot be damaged by subfailure stretches. Tendons *in vivo* wrap around structures such as bones and pulleys, and are therefore exposed to compressive and shear loads that this *in vitro* study does not take into account. These loads vary not only between species, but also across tendon sites, and would have a definite effect on *in vivo* damage. Therefore, the results should be used in analyzing the basic matrix tensile characteristics of tendons in general.

A limitation of the current study is that it focuses on the mechanical changes of tendon alone while damage may also occur at the myotendinous and osteotendinous

junctions. An additional limitation of the study is the form of the loading stimulus: a single ramp loading at a strain rate slower than that which occurs in many injury situations. The selected strain rate was a compromise to achieve a fairly sensitive strain resolution within the dynamic capabilities of the VSA system. Additionally, a preponderance of the literature has been performed at strain rates similar to that selected. While mechanical changes of tendon with cyclical loading is likely of greater significance to the majority of clinical injuries to tendon, it was believed that a fundamental understanding of the mechanical changes as a result of a single ramp load was required to more fully investigate the response to cyclical loading. The limited studies that have examined the effect of cyclical loading on tendon have shown declines in strength and stiffness with increasing load cycles (Wang *et al.* 1995; Schechtman and Bader 1997).

The decision to utilize cryo-clamps for testing necessitated a compromise be made between how well the environmental conditions could be altered to mimic physiological temperature and hydration conditions. In this study, spray irrigation and wrapping the tissue in saline-soaked gauze was utilized to maintain hydration during the rest period. The temperature of the tissue near the grip edge was found to be approximately 10° C during testing. While temperatures effects on the viscoelastic properties of the tissue are debatable, most studies have observed no significant differences in the load elongation curve for a single loading for temperature ranges between 0°C & 55°C (Rigby *et al.* 1959; Hasberry and Pearcy 1986; Lam *et al.* 1990). In addition, the tendons were frozen twice before testing for practicality purposes. Effects due to this were examined by evaluating the mechanical properties of once versus twice frozen specimens in our lab and no significant differences were found. In addition, past studies (Smith *et al.* 1996) have found little difference in

properties between samples frozen once and up to five times before testing. A final consideration in examining the lack of differences in mechanical properties is in reference to the number of specimens tested per strain-limit group. While the power of statistical tests may be improved with additional specimens per group, it is unlikely that this would uncover any physiologically significant differences in the strain-limit groups due to the proximity of the means of the mechanical properties of each group.

Limitations withstanding, these results suggest that a single tensile subfailure stretching does not directly cause significant matrix damage. These results also suggest that the cellular response to a loading stimulus may not be due to damage of the matrix, but due to tendon cells directly responding to the loading stimulus itself such that their cellular response causes a decline in the mechanical properties of the matrix. The clinical significance of this is that a loading event may not instantaneously cause damage to the matrix, and therefore the clinician may have a greater opportunity to prevent matrix changes by biochemically altering the cellular response to a loading event.

4.6 Acknowledgements

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CHAPTER 5

OVERUSE TENDON EXPLANT MODEL

Devkota AC & Weinhold PS, 2005. A Tissue Explant System For Assessing Tendon Overuse Injury. Med Eng Phys. 27(9), 803-808.

5.1 Abstract

Tendon overuse injuries are common athletic and occupational problems. When studying mechanisms that cause these injuries, inherent complexities associated with controlling in vivo loading necessitates alternative approaches such as in vitro organ culture. Current devices for loading explants in organ culture, whether custom-built or commercial, have various deficiencies in their loading capability, control mechanism and strain assessment. To overcome these shortcomings, an advanced tissue loading device with video strain analysis capabilities was developed for investigating overuse injuries and its performance/calibration were evaluated. Two tests were used to assess the ability of the system to create and monitor mechanical changes with overuse. Overuse loading significantly increased strains and decreased strength, showing the ability of this system to create and monitor tissue damage. Furthermore, the device design allows for its use in a standard incubator. Coupled with custom loading and data collection programs, this system is suitable for long-term overuse injury studies.

5.2 Introduction

The etiology of tendon overuse injury is such that repetitive loadings considerably below failure levels is believed to cause micro-damage that exceeds the basal repair ability of the tissue. To better understand the mechanisms of tendon injury, many models have been studied. Original studies examined the effects of force on tendon healing in vivo after acute injury (McDowell and Snyder 1977; Woo *et al.* 1981), while more recent animal model studies have been successful in creating overuse-like injuries (Soslowsky *et al.* 2000). A common difficulty in these models has been in controlling the in vivo loading.

Because of the inherent complexity of controlling loading in an in vivo environment, additional studies have exposed cultured tendon cells to controlled substrate strains as a means of studying overuse injury (Almekinders *et al.* 1995). The main deficit of this approach lies in its' inability to account for the interaction between the cells and the extracellular matrix. The native matrix's role on the cellular response and the cellular role in matrix modification cannot be assessed through such models.

An attractive alternative approach to determine how particular loading characteristics contribute to overuse injury is to apply controlled cyclical loads to tissue segments maintained in culture (explants). Early studies using this approach have used custom-built displacement controlled devices to examine the influence of load or stress-deprivation on cell proliferation and matrix synthesis (Slack *et al.* 1984; Hannafin and Arnoczky 1994). Recent studies using such devices have examined effects of amplitude and frequency on various biochemical expressions (Lavagnino *et al.* 2003). A deficit of these displacement-controlled devices is that grip slippage can adversely influence the application of controlled strains to the tissue. Furthermore, the method does not fully allow for tissue creep, a factor that has

been suggested to be an important mechanism of overuse injuries (Goldstein *et al.* 1987). Commercial load-controlled devices used to load tendon in culture (Tanaka *et al.* 1995) have been limited by the amount of force they could produce. Studies examining higher levels of cyclic loads have been performed on nonviable tendons (Wang *et al.* 1995; Schechtman and Bader 2002; Wren *et al.* 2003), while tissue culture studies have largely focused on the response of tendon to low-level loading(Slack *et al.* 1984; Tanaka *et al.* 1995).

In an effort to investigate a tissue explant model for tendon overuse injuries, a portable load-controlled tissue loading device (TLD) with video strain analysis (VSA) capabilities was developed to be used within a standard incubator (Figure 7). The objective of this paper is to describe this device, its performance, calibration and preliminary tests.

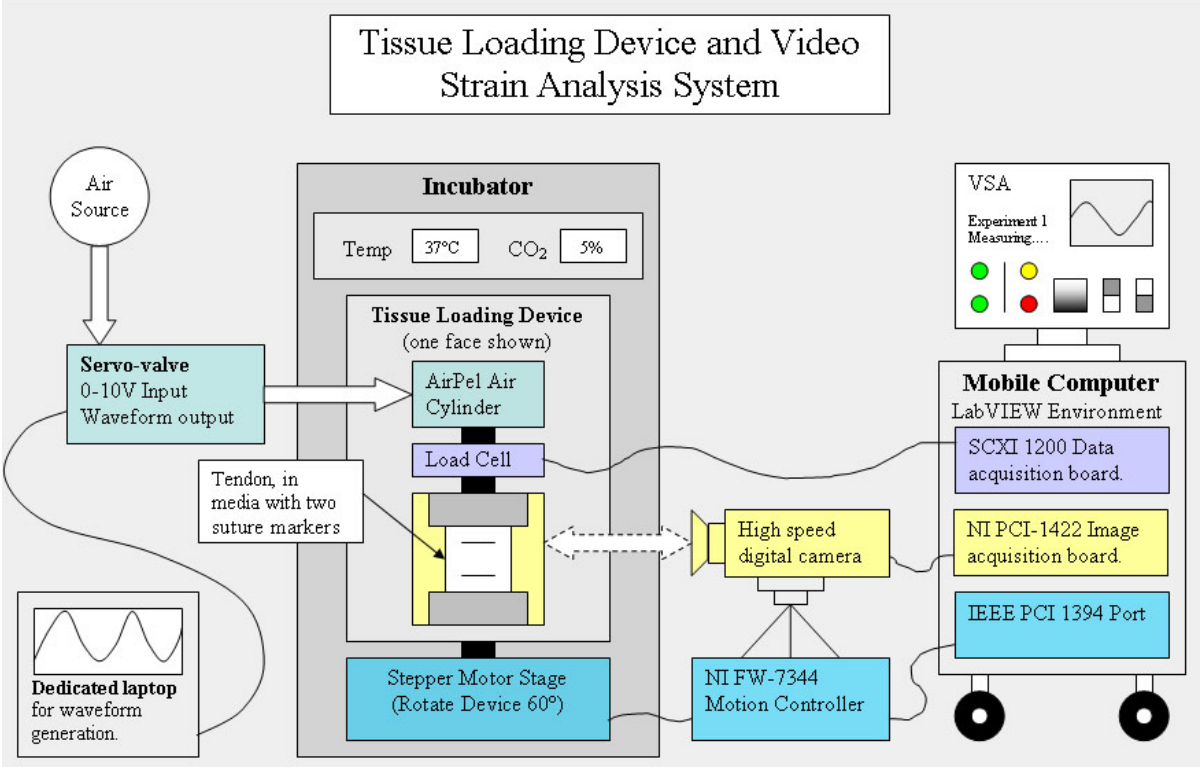

Figure 7: System Diagram of Tissue Loading Device (TLD) and Video Strain Analysis (VSA) System. The TLD sits in an incubator while a dedicated laptop controls the load waveform applied to the tissue. A mobile computer operates the video strain analysis system with the digital camera capturing tendon strain and the DAQ board capturing loading, as the motion controller rotates the six faces of the device in front of the camera for analysis.

5.3 Methods

5.3.1 Tissue Loading Device (TLD):

The TLD is one of two identical, hexagonal, 6-station pneumatic apparatuses (Figure 8). Filtered air is supplied to fifty-psi servo-valves (QB Series, Proportion-Air, McCordsville, IN) to produce pressure waveforms from input voltage waveforms. Air manifolds on each device distribute pressure to six air-cylinders (E16D1.0N, Airpot Corp, Norwalk, CT) through individual on/off valves. The piston rods of the air-cylinders extend into 50ml conical tubes filled with media, which house the tissue. The tissue, secured in stainless steel serpentine grips, attaches to the piston rod at the top and fixed posts at the bottom. A single thumbscrew fixes the tube to the device, expediting media replacement.

A custom LabVIEW (Version 6.0, National Instruments, Austin, TX) program on a dedicated laptop-computer generates input voltage waveforms by selection of the peak and trough pressures (MPa) and frequency (Hz) which are positive, sinusoidal and include an offset for the combined weight of grips and connectors. Waveforms correcting for differences between expected and observed loads, as measured directly by a load cell in series, have been pre-programmed for common loading regimens. Duration and average cross sectional area values complete the waveform selection. Three regimens (peak, trough, frequency and duration), time delay between them, and how often to repeat them are independently selectable for each device, allowing for automatic, complex loading scenarios. The voltage waveform is transmitted to the servo-valve by a 12-bit National Instruments (NI) card (DAQCARD-6062E). The range of the system is limited by the servo-valve at 0 – 61.384 N and the accuracy is limited by the air-cylinders at <0.245 N.

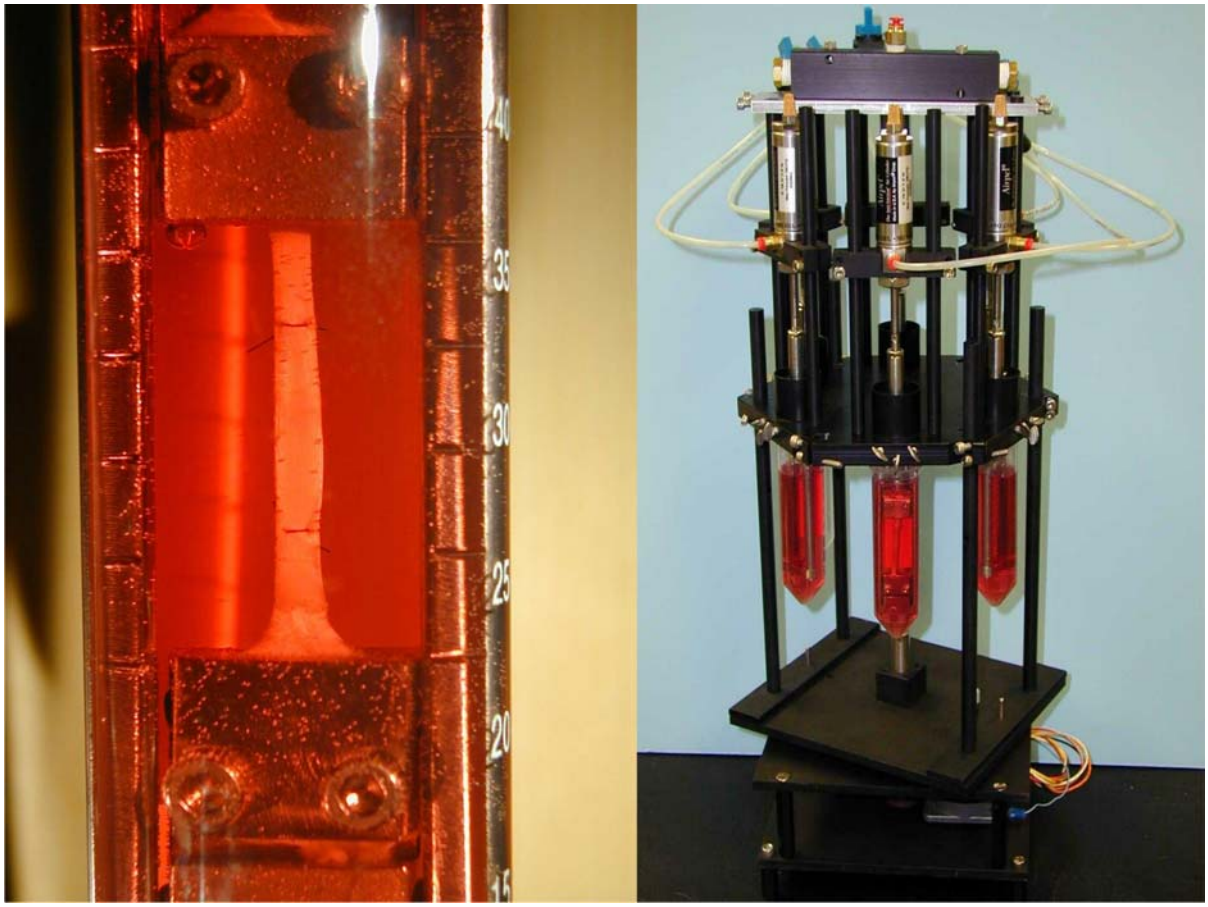
Figure 8: Close-up of gripped tendon with midsubstance suture markers, and tissue loading device on rotating stage.

The TLD sits on a platform connected to a stepper-motor (IS23002, MCG, Inc, Eden Prarie, MN) which allows rotation of the device for VSA of each specimen. A NI motion controller (FW-7344) supplies the command signal, also generated from LabVIEW.

5.3.2 Video Strain Analysis (VSA) System:

Midsubstance VSA assesses mechanical changes in explants during cyclic loading by measuring the displacement of sutures (Figure 8), stitched transversely approximately 13mm apart, at the longitudinal center of the tendon using a high-speed digital camera (Megaplus ES310, Roper Scientific, San Diego, CA) and an 8-bit NI image acquisition board (PCI-1422).

Image preprocessing, consisting of selecting a minimal region-of-interest and a binary look-up-table to threshold markers based on lighting (Figure 9), greatly increases overall speed. Tracking the first and last edges of the markers along a longitudinal user-drawn line of action allows real-time strain collection synchronously with load data. The optical zoom applied limits overall system resolution.

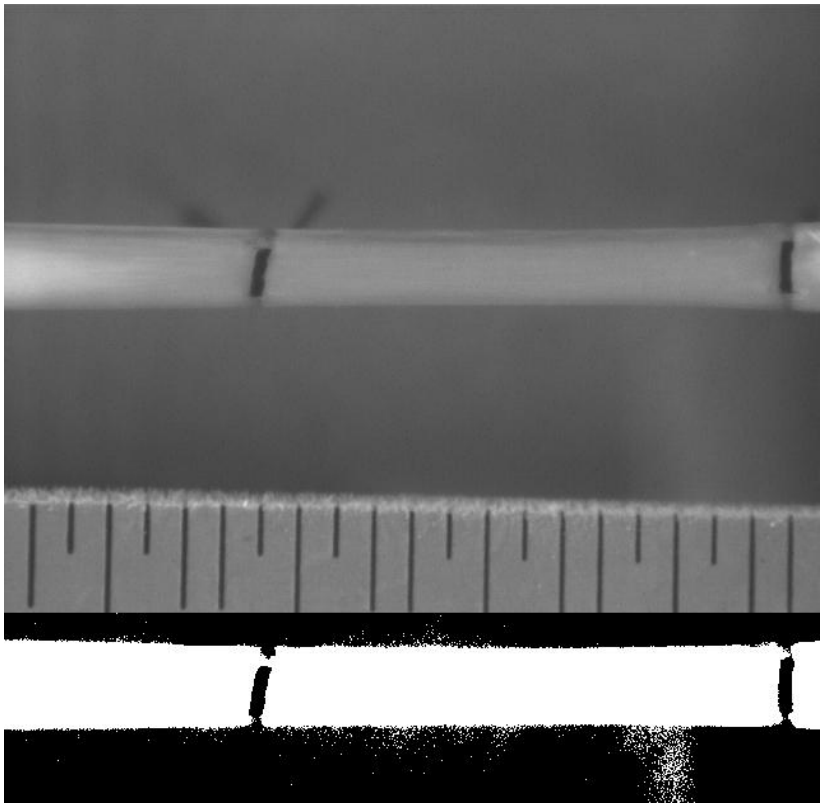

Figure 9: Image of tendon with calibration ruler from a failure test, and subsequent VSA thresholding of midsubstance suture markers.

5.3.3 Calibration of Tissue Loading Device:

The range and performance were determined by testing the static and dynamic capabilities of the servo-valves and air-cylinders with a load cell connected in series. Variability in the output signal with changes in the waveform or its individual parameters separately (frequency, peak load, trough load) were examined with nylon strings gripped in place of tendons. Frequencies of 0.25, 0.5 and 1 Hz, peak loads from 3 to 24 MPa and trough

loads of 0.25 and 1.0 MPa were assessed, as well as three waveforms: low load (0.25 – 3.0 MPa, 1.0 Hz), moderate load (0.25 – 12.0 MPa, 1.0 Hz) and high load (0.25 – 24 MPa, 1.0 Hz). Loads from 1 to 30 MPa were tested for the static condition. Furthermore, because all twelve air-cylinders have individual valves, the variability caused by altering the number of concurrently operating cylinders was also examined.

Once the range and performance capabilities of the TLD was determined, tendon was gripped and the input signal was manually modified until the output values reached the desired values for waveforms to be used. Frequencies from 0.25-2.0 Hz, a trough of 1.0 MPa and peaks from 1 to 30 MPa were tested. This process was repeated and the manually modified values required for the desired output values were averaged and recorded as preset values for common waveforms.

5.3.4 Calibration of Video Strain Analysis System:

VSA calibration is performed by placing an object with marks of known separation in the same plane as the tissue. A pattern-matching algorithm locates the tick marks, and the average distance, in pixels, between successive centroids divided by the known distance between them (mm) is the calibration factor (pixels/mm) for the square pixels of the system. To validate VSA performance, static and dynamic tests were performed using a digital caliper (CD-6CS, Mitutoyo, Japan; accuracy 0.02mm) and the linear variable differential transformer (LVDT), accuracy 0.05mm, of our materials testing system (8500+, Instron Corp., Canton, MA). Static testing was performed by zooming maximally at 50mm, then incrementally decreasing the caliper by 5mm (to 20mm) while recording the distance between caliper edges at each interval. Repeated trials with sutures (black, silk 9-0) affixed on each caliper edge were performed. Dynamic validation was performed by the VSA

measuring grip-to-grip displacement during a displacement-controlled ramp test on the Instron. Repeated trials with an initial gauge length of 13mm (average midsubstance gauge length) displaced 8% and 20% (expected failure range of tendon) at 1, 2 and 5% strain/sec were performed with VSA collection at 30Hz, the common range of typical systems (Woo 1982; Lam *et al.* 1992).

5.3.5 General Tests:

To characterize the system's ability to create and monitor mechanical changes with overuse loading, two tests were performed. The first was to determine the extent to which the system could alter the mechanical properties of tendons with overuse loading, and the second to demonstrate the ability of the VSA systems to measure strains accumulating throughout such experiments. Previously isolated avian flexor digitorum profundus tendons were thawed, mechanically gripped and placed in the TLD in saline. The gauge lengths (grip and midsubstance) were determined with 3.0 MPa tension after preconditioning for five minutes (0.25 – 3.0 MPa, 0.25Hz). Tendons were then loaded for 24-hours (0.25 – 12 MPa, 1.0Hz), after which final gauge lengths were measured with 3.0 MPa tension. Dynamic strains [(trough-peak)/trough] were monitored at 0, 4, 20 and 24 hours. Control tendons sat gripped in the TLD for 24 hours with no loading. Following the 24-hour periods, tendons were removed from the grips and frozen in saline-soaked gauze.

For failure testing, performed on the Instron, tendons were thawed and duplicate area measurements were made along the midsubstance (avoiding vascularizations) utilizing an area micrometer similar to that described elsewhere (Butler *et al.* 1984). The tendons were cryogenically gripped (Riemersa and Schamhardt 1982) just inside of where they were mechanically gripped by the TLD to ensure midsubstance failure. Failure testing was

preceded by pretensioning at 0.25N, preconditioning with 10 cycles of a 1% strain/sec havertriangle waveform at 0.50 Hz, and then pretensioning again at 0.25 N before applying a 1% strain/sec ramp until failure.

5.4 Results

5.4.1 Tissue Loading Device:

With one servo-valve controlling no more than six air cylinders at once, the variability in the output were minimal with changes in peak load, trough load, frequency and number of air-cylinders open in both the static and dynamic conditions. The difference in output from device one, with all six vs. zero cylinders open on device two, was less than 1% for both static and dynamic conditions. During static loading, the difference between observed and expected output changed steadily as the loading level increased, with a maximum difference of slightly less than 6%. The same characteristic was observed under dynamic conditions for the peak load, again with <6% difference. The same magnitude changes were observed in the trough load as well, but the small magnitude of the trough made percentages large (50-1500%). Changing the frequency (0.25 and 0.5 Hz) caused output changes of less than 1% throughout the loading range. At 1 Hz, the differences were slightly larger, but remained below 5% at 24MPa.

Holding the first device at one of three waveform while varying the waveform to the second device across all three waveforms, showed differences of <1% in the observed load on the first device. Finally, manually adjusted input signals produced repeatable (<1% error) output for load levels up to 24 MPa at the tested frequencies and waveforms. Load levels of 30 MPa or greater were possible only with the low frequency (0.25 Hz) signals.

5.4.2 Video Strain Analysis:

Static validation tests determined the average percentage error in length measurements across all 5mm increments to be within 0.26%, while individual errors across all trials ranged from 0 – 1.59%. The average coefficient of variance of the repeated measurements at each gauge length was 1.45% (range: 1.29-1.6%). Dynamic tests saw percentage errors rise to 51% at the smallest strain values, but decrease substantially thereafter as strain values increased (Figure 10). Overall, a raw strain error of 0.25% strain or less was maintained for 99% of all data points collected which coincides with the theoretical resolution of the VSA system (1/400 pixels = 0.0025 strain) for the number of pixels typically defined across the strain markers at expected zoom.

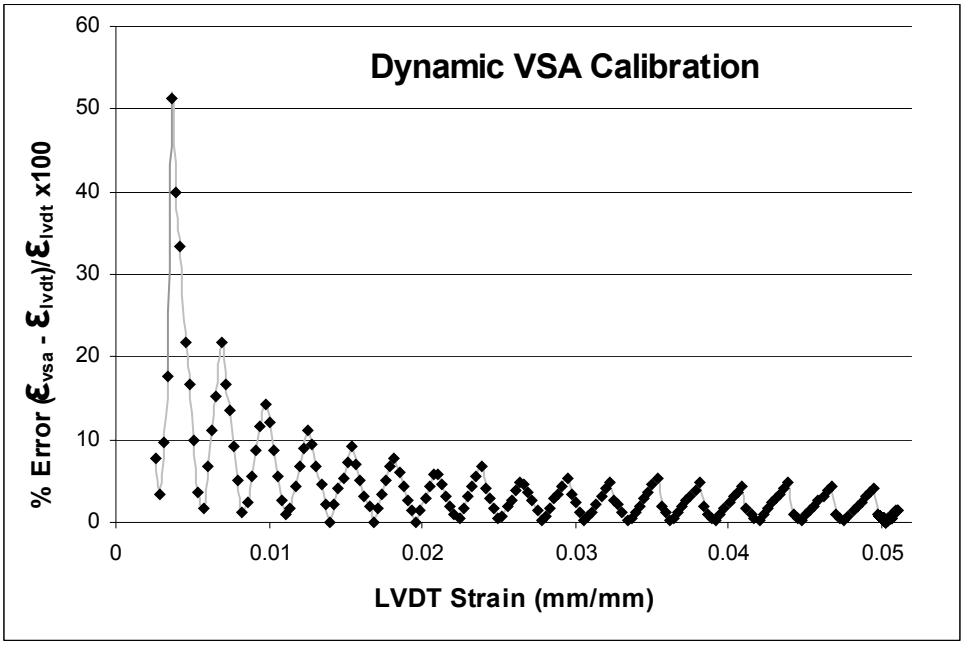

Figure 10: Dynamic calibration of VSA system showing VSA error across actuator strain.

5.4.3 General Tests:

The results of the damage assessment (Figure 11) experiment showed the treated tendons, 24 hours at (0 - 12MPa, 1.0 Hz), had a decreased ($P<0.05$, t-test) ultimate failure strength (104.04 ± 29.98MPa) compared to unloaded controls (126.04 ± 19.26MPa). The results of the grip and midsubstance strain measurement assessment experiment (Figure 12) showed a significant increase in the both the midsubstance ($P = 0.007$) and the grip ($P < 0.001$) gauge lengths after 24 hours of loading. The strain accumulated between the grips was also significantly ($P = 0.007$) greater than the strain accumulated between the suture markers as determined by paired t-tests. One-way repeated measures ANOVA across time showed dynamic suture strain to be different between 0 and 24 hours, and the grip strain at time 0 to be different from 4, 20 & 24 hours.

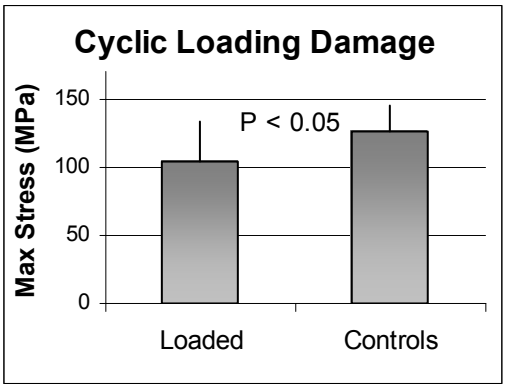

Figure 11: Significant difference in failure strength of tendons loaded with a cyclic waveform of 0.25 – 12.00MPa at 1.00Hz for 24 hours (N=16) versus unloaded controls (N= 11).

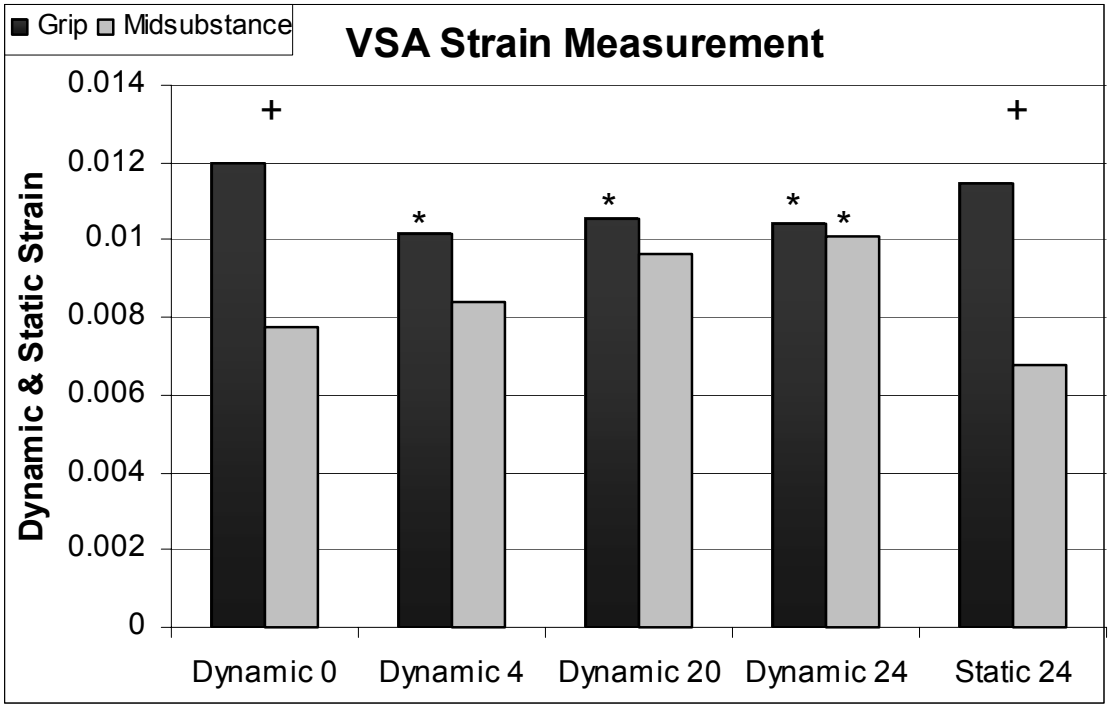

Figure 12: Dynamic [(Peak-Trough)/Trough) and static [(Post-Pre)/Pre] measurement of midsubstance and grip strains across a 24-hour, 12 MPa sinusoidal waveform. Note: * Significance from time 0; + Significance between midsubstance and grip.

5.5 Discussion

Much of the research on tendon overuse injuries has focused on the biochemical activity in response to cyclic loads while few studies have examined fatigue of viable tendons with sufficiently large loads. Existing studies were performed on large materials testing systems with non-viable samples (Schechtman and Bader 1997; Schechtman and Bader 2002; Wren *et al.* 2003) due to the unavailability of an appropriate device to load tendons to sufficient magnitudes in an in vitro tissue culture environment (Tanaka *et al.* 1995). Current devices, mostly displacement control, were designed for low load applications and usually operate off a single displacement actuator, which requires all tendons to be gripped at the same length and slip equally to achieve uniform loading. Furthermore, displacement controlled devices do not fully allow for creep, a substantial part of overuse injury (Wang *et*

al. 1995). The device described in this paper operates under load control, which is independent of grip slippage or tendon length and is capable of applying damaging loads that allow for creep to tendons in a tissue culture environment. Furthermore, tendons are bathed individually in media, allowing for intra-experiment comparisons while eliminating potential cross-contamination.

Calibrations of the device showed the difference between expected and observed output load changed with the level of loading and frequency in a complex manner. Due to this complexity, the input signal was manually adjusted until desired outputs were observed for the waveforms to be utilized. This step is required again only if changing the tissue of interest. This process, when repeated, showed low variability (<1%). These values were inputted into the waveform generation program and are used to load the tendons precisely. With the ability to apply controlled load levels up to 61 N, roughly 30 MPa, the ability to physically damage the tendon is in place.

To measure the tissue response to load throughout testing, a video strain analysis system was developed. The VSA system negates grip slippage issues in strain measurement, providing a more accurate representation of the tissue damage from the cyclic load. Our validation tests (Figure 10) demonstrated superior performance relative to commercially available systems(Lam *et al.* 1992). Additional advantages include the ability to operate at higher speeds, flexibility in programming and interfacing other control systems and reduced equipment costs. Other custom-developed VSA systems (Derwin *et al.* 1994) have reported higher resolutions and lower errors utilizing alternative image processing strategies, however, these systems could not process data in real-time.

Validation of the device required damage assessment tests. The failure tests demonstrated that the 24-hour cyclically loaded specimens were significantly weaker than unloaded tendons. These results attest to the device's ability to damage the tendon matrix, making it suitable for overuse injury studies.

A second validation was performed to evaluate mechanical changes during overuse loading by strain analysis. Measurable mechanical changes, in terms of accumulated strains and an increase in gauge length across both the midsubstance and the grips were seen. In addition, these two strains were significantly different, indicating the existence of stress concentrating effects from the mechanical grips. Some of this difference could be due to a change in geometry across the length of the tendon, but we believe this to be negligible because of the similarity of the two area measurements. This indicates that midsubstance strain, collected by the VSA system, should be measured when performing any damage assessment in the future because grip-to-grip strains include damage caused by gripping as well as slippage. If position control devices were used for loading, these gripping effects would effectively decrease the loading on the tendon. Without VSA analysis, this effect would be inaccurately expressed as overuse damage. Furthermore, the dynamic creep associated with repetitive loading is another factor that position controlled devices cannot account for, further reducing the loading. With load control, slippage and stress concentration effects do not decrease the loading level.

One limitation of the TLD is the gripping methodology. The difference in strain between the grips and midsubstance markers may be due to stress concentration effects at the grip edges or grip slippage. However, the significant midsubstance strain increase from the beginning to the end of the experiment indicates that fatigue damage or creep has occurred.

In failure assessment, cryo gripping was done just inside the TLD grip zones such that the area damaged by the original gripping was not tested, and no further stress concentrating effects were present.

It is believed that the described tissue loading device and video strain analysis system are valuable devices that will assist in studying tendon overuse injury. The ability to operate easily and efficiently within the confines of an incubator allows for long term viable tests, while the load control mechanism assures no reduction of applied load due to stress concentration or slippage at the grips. Furthermore, midsubstance strain analysis will measure damage resulting from the loading only and the replaced media can be individually analyzed for biochemical responses. It is believed that biochemical and biomechanical analyses gathered with the use of this system will provide useful information on the mechanisms of overuse injury in tendon.

5.6 Acknowledgement

Funding from a Whitaker Foundation Research Grant is gratefully acknowledged.

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CHAPTER 6

TISSUE RESPONSE TO CYCLIC LOAD

6.1 Abstract

Purpose: Tendon overuse injuries continue to be a major source of clinical concern, both athletically and occupationally. Studies have shown cyclic loading to cause material damage as well as induce biochemical responses in tendon. The purpose of this study was to examine the tissue response of tendon after applying a fixed amount of cyclical loading over varying durations.

Methods: Avian flexor digitorum profundus tendons were loaded (3 MPa or 12 MPa) to a fixed number of cycles across either one or twelve days *in vitro*. Biomechanical data were gathered during the loading, as well as during the subsequent failure testing. Cellular viability and death, as well as proteoglycan, collagen, collagenase and prostaglandin E_2 (PGE_2) activity measurements were obtained from tissue specimens and media samples.

Results: Significant strain accumulated during loading. Loading tissues at 12 MPa significantly reduced material properties at both 1 and 12 days as expected. The 3 MPa loading group also caused a reduction in properties, but only when applied across the 12 days. Cell death and collagenase activity increased significantly with increasing loading as well as with increasing duration. However, no differences were seen in cell viability or collagen content. Glycosaminoglycan content increased with load magnitude, while PGE_2

production was highly dependent on the magnitude of the loading as well as the duration across which it was applied.

Conclusions: Mechanical fatigue-induced material property changes were exhibited by the tendons in response to increased loading magnitude. However, as the same loading was applied over a longer period, most outcomes were magnified substantially, relative to the short duration regimens. This is presumably due to a complex cellular response to loading. A key contributor may be the inflammatory mediator, PGE_2, which exhibited large magnitude and duration dependent increases to cyclic loading.

6.2 Introduction

Overuse injuries comprise a significant portion of orthopaedic cases, both athletically and occupationally. Overuse injuries are believed to be caused by the accumulation of micro-damage to individual collagen fibers(Archambault *et al.* 1995; Maganaris *et al.* 2004). While the etiology is likely more complicated than simple material fatigue, micro-damage due to fatigue has been suggested to play a significant role in tendon overuse injuries(Slack *et al.* 1984; Maganaris *et al.* 2004). Conversely, inflammatory mechanisms have also been suggested(Archambault *et al.* 1995; Khan *et al.* 1999; Fu *et al.* 2002), as overuse injuries are often termed tendonitis. However, confusion arises because histological studies have not shown the presence of inflammatory cells such as leukocytes one would expect with classic inflammation(Astrom and Rausing 1995). However, *in vitro* studies of tendon fibroblasts in monolayer have shown substantial increases in levels of inflammatory mediators, such as prostaglandin E_2 (PGE_2), with mechanical loading(Almekinders *et al.* 1993; Almekinders *et al.* 1995; Tsuzaki *et al.* 2003; Wang *et al.* 2003; Li *et al.* 2004). The etiology of overuse injuries is likely comprised of a combination of both material fatigue and cellular responses which result in cytokine release, matrix metalloproteinase (MMP) production and matrix degradation(Ireland *et al.* 2001; Riley *et al.* 2002; Alfredson 2003; Tsuzaki *et al.* 2003; Tsuzaki *et al.* 2003).

Early studies of overuse injuries established relationships between various material parameters and the amount of damage accumulated in tendons(Goldstein *et al.* 1987; Wang *et al.* 1995; Schechtman and Bader 1997; Schechtman and Bader 2002). However, most of these studies were performed with non-viable tendons, which neglects a potentially

significant factor, the cellular response to loading. The cellular responses can aid the remodeling and repair, as well as exacerbate the damage (Archambault *et al.* 1995).

Studies investigating the cellular response to mechanical loading have predominately utilized an alternative approach involving application of mechanical loading to deformable substrates upon which cells have been seeded. While a variety of important biochemical responses such as PGE_2(Almekinders *et al.* 1993; Almekinders *et al.* 1995; Tsuzaki *et al.* 2003; Wang *et al.* 2003; Li *et al.* 2004), protein kinases(Arnoczky *et al.* 2002; Skutek *et al.* 2003) and MMPs(Archambault *et al.* 2002; Tsuzaki *et al.* 2003) production have been observed utilizing this approach, this model neglects the cell-matrix interaction. Furthermore, it may not be possible to accurately produce physiologic cellular strains. To address these issues, animal models have been proposed (Soslowsky *et al.* 1996). These *in vivo* models apply loads externally by means of treadmill exercise. With such approaches, potential external and internal adjustments such as weight shifting and load-redistribution may cause variability in the tendon loading.

To selectively study the effect of loading as a single causative or inductive factor for overuse injury these previous methodologies are inadequate. The use of live tissue explants however, would help fill the gap caused by the limitations of the *in vivo* models and cell culture studies(Slack *et al.* 1984). A device, capable of maintaining tissue viability and applying damaging cyclical loads was recently fully developed(Banes *et al.* 1999; Devkota and Weinhold 2005; Flick *et al.* 2006). To study the influence of the duration of cyclical loading on tissue mechanical properties and biochemical expressions, tendon explants were subjected to cyclical loading over varying periods. Since tendon overuse injuries are most likely due to both mechanical damage and the cellular response during loading and damage,

it was hypothesized that if a damaging loading of a fixed number of cycles were distributed across a longer period, the cellular responses would exacerbate the decline in mechanical properties relative to the same loading applied across a shorter period.

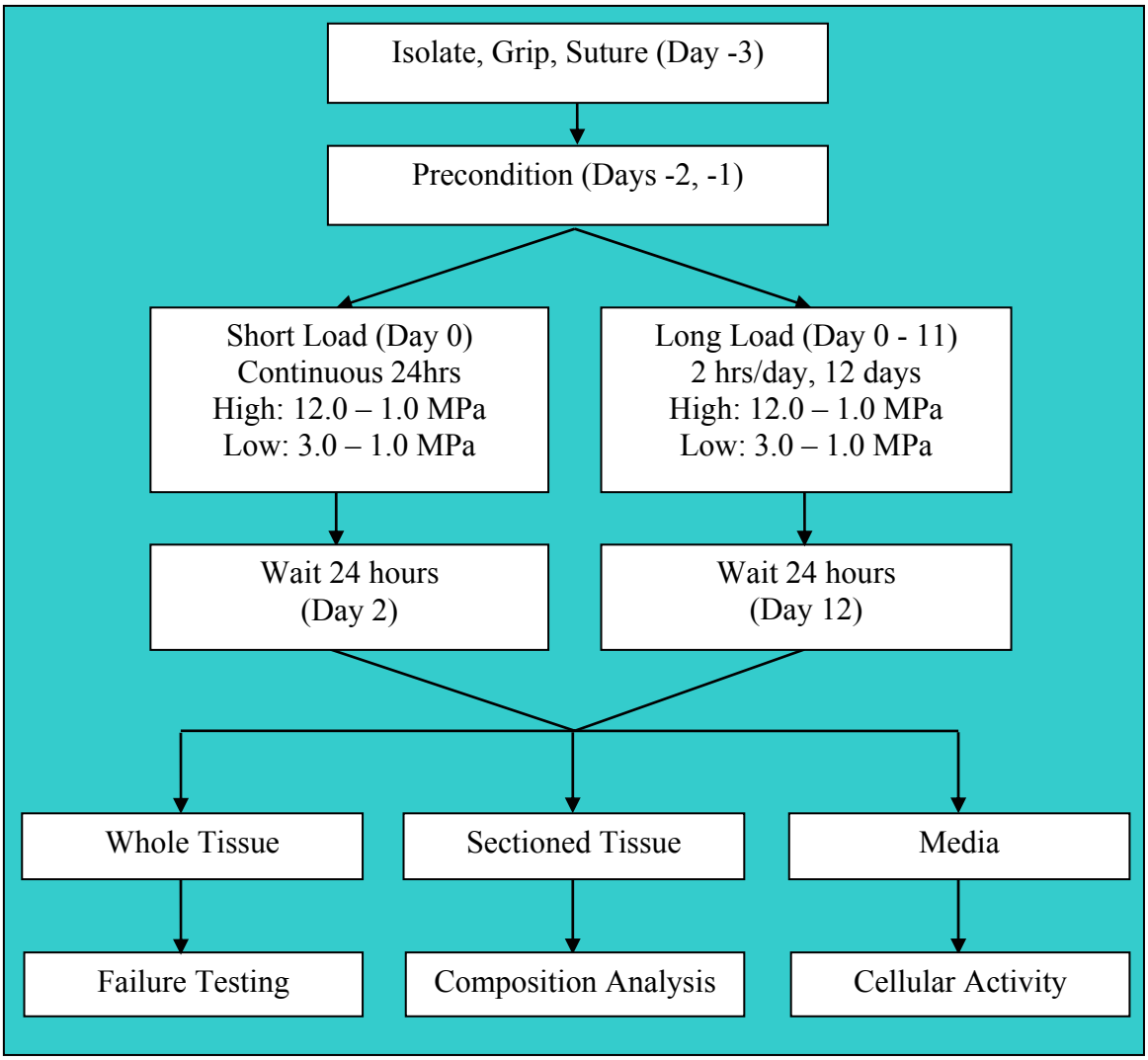


Figure 13: Methods Flowchart- The loading and assaying scheme for the flexor digitorum profundus tendons is shown. The main steps are isolation, preconditioning, loading, resting and processing.

6.3 Methods

Avian flexor digitorum profundus (FDP) tendons were isolated from the middle toes of 45-60 day old Rock Cornish hens. The complete middle FDP, from the distal insertion at the 4th phalanx to the union with the remaining flexor tendons just proximal to the claw, was isolated with average length of 85mm and area of 2mm^2. Tendons were mechanically gripped proximal to the most proximal vincula, exposing a 35mm (average) section superficial to the first three phalanges. Suture markers were stitched across the midsubstance for video strain analysis (VSA). Tissues were allowed 24 hours of rest (Figure 13) in tissue culture medium consisting of phenol-red free DMEM-H media supplemented with 20mM Hepes, 100IU/ml penicillin, 100µg/ml streptomycin, 2µg/ml amphoteracin B, 100µM ascorbate, 1mM sodium pyruvate, 4mM L-glutamine and 2% fetal bovine serum. The final media additive was 2µM arachidonic acid, a supplement to insure adequate substrate for the production of prostaglandins.

Following the rest period, two days of preconditioning ensued, with 2 hours of a sinusoidal waveform (3MPa peak, 0.5Hz) and 22 hours of rest applied each day. The loading regimen commenced the following day. All loading regimens consisted of equal cycles (86,400) of a sinusoidal waveform with a peak magnitude of either 12 MPa (High) or 3 MPa (Low) at 1.0 Hz. The loadings were applied for two durations: either 12 days (Long) or 1 day (Short). Following loading, tendons were afforded 24 hours of rest before some were sectioned for compositional analysis, while the others were frozen in saline-soaked gauze for failure testing at a later time. The loading regimens were applied via a custom-built load-controlled tissue loading device within an incubator(Devkota and Weinhold 2005). Fresh media were replaced one day before the loading regimen, the initial day of loading, and every

third day during the loading. A final media collection was performed after the 24 hours of rest, post-loading. All media were stored at -20ºC and analyzed later.

Midsubstance strain analysis was performed during the cyclic loading within the incubator. Both dynamic strains ((peak– trough)/ displacement) throughout loading and static strains, before and after loading, were measured. Static strains were measured with a 1.0 MPa tension load applied. All measurements were performed on midsubstance suture markers with a custom built VSA system(Devkota and Weinhold 2005).

The mechanical properties of the tendons were analyzed by performing failure testing on a servo-hydraulic materials testing system (Instron 8500+, Instron Corp, Canton, MA). The tendons were cryogenically gripped in custom-developed, liquid nitrogen-cooled grips similar to those described previously(Riemersma and Schamhardt 1982). Tendons were pre-tensioned with a 1.0 N load, and then preconditioned with 10 cycles of a 1% strain haversine waveform. The tendons were pre-tensioned again with 1.0 N, and then taken to failure with a 1% grip strain/second linear ramp. During this ramp loading, the load data and both displacement data (midsubstance and grip) were collected for structural property calculations. The grip and midsubstance displacement at time 0 (with 1.0 N pretension) were used as reference displacements for all strain measurements. Prior to failure testing, an area-micrometer(Butler *et al.* 1984) was utilized for material property calculations. As a control for the failure property evaluation, a separate group consisting of tendons frozen at -20ºC immediately following isolation was added. One-way and two-way analysis of variances (ANOVA) were utilized to determine significant differences within the groups for each of the properties investigated ($P<0.05$).

The sectioned tendons were assessed for their viability, collagen and proteoglycan contents. Viability was evaluated immediately following loading by the modification of an MTT assay(Klein *et al.* 1996). Briefly, tendon sections were incubated for 4hrs at 0.5mg/ml MTT. The tendons were then transferred to 2-methoxyethanol solution and shaken for 12 hours at which point the resulting supernatant fluid was assessed for absorbance at 570nm with background subtracted at 690nm. For compositional analysis, the remaining sections were digested with Papain (0.5mg/ml). The resulting digest was aliquoted and stored at -80ºC for collagen and proteoglycan content analysis.

A modified hydroxyproline content assay was performed on the digest to measure hydroxyproline, a collagen constituent of known proportions (Bergman and Loxley 1963; Riley *et al.* 1994). Briefly, digests hydrolyzed in 6M HCl at 105 ºC were treated with an oxidant and reagent combination and allowed to incubate, and then had their absorbance measured at 540nm. Hydroxyproline standards were also performed and the values were normalized by the wet weight of the tissue prior to digest.

A modified sulfated glycosaminoglycan (sGAG) content assay was performed on the digest to measure sGAG, the covalently attached glycoprotein chains of proteoglycans (Farndale *et al.* 1986; Riley *et al.* 1994). Briefly, digest samples were treated with 200µl of a color reagent (DMMB) and the resulting absorbance was measured at 540nm. Chondroitin sulfate was used for standards and the data were normalized by the wet weight of the tendon prior to digest.

Media were analyzed for cell death, PGE_2 and collagenase content. Cell death was quantified through a commercial kit (Cytotox 96, Promega, Madison, WI). The kit quantitatively measures lactate dehydrogenase (LDH), an enzyme released upon cell

lysis(Decker and Lohmann-Matthes 1988) through an enzymatic assay and is read on a platereader at 490nm. Cell death data are represented as percentage of total death, with 100% death being a group of fresh, unloaded tendons treated with a liquid nitrogen freeze-thaw process.

The levels of the inflammatory mediator, PGE_2, were quantified through a competitive immunoassay kit (PGE_2 - Monoclonal, Caymen Chemical, Ann Arbor, MI). Free PGE_2 in the media competes with supplied PGE_2 tracer for a limited amount of PGE_2 antibody. This antibody- PGE_2 complex binds to a plate pre-coated with goat anti-mouse IgG, and following washing and development, is read at 492nm in a platereader.

The amount of total collagenase in the media was measured by a modified Azocoll procedure(Chavira *et al.* 1984). Briefly, azocoll, a dye impregnated collagen complex was suspended at 5mg/ml in buffer with aminophelylmercuricacetate (APMA), an agent supplemented to activate latent collagenase. The insoluble Azocoll was transferred while stirring to samples and given 24hrs to process. The supernatant of the resulting mixture had its absorbance measured at 520nm.

6.4 Results

Significant static strain accumulated in all loading groups except the low short group (Figure 14). The accumulation was significant with respect to both magnitude and duration, as measured by a two-way repeated measures analysis of variance (RM-ANOVA). Dynamic strains increased by 0.01% (short) and 0.46% (long) for the high groups only. A two-way RM-ANOVA of these data showed significant magnitude and duration dependent strain increases, indicating a decrease in stiffness.

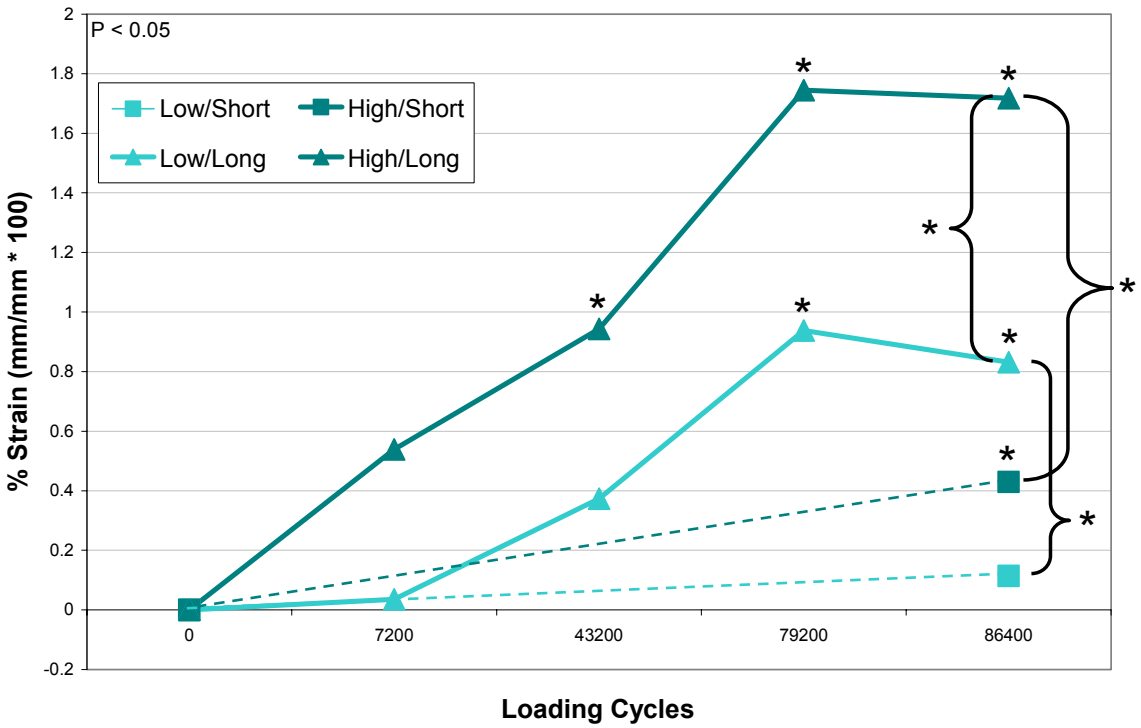


Figure 14: Strain, relative to time 0, is shown for the groups across cycles of loading with 86,400 cycles being completed in 1 or 12 days, for the short or long groups, respectively. Asterisks on points denote difference from time zero, while asterisks on brackets indicate difference between two groups. Note the dashed lines for the short groups, as they were continuously loaded and did not have intermediate measurements. Significance of $P<0.05$, as measured by a 2-way RM ANOVA.

Table 2: Mechanical failure data of control tendons and those subjected to one of four regimens. Within each test category, groups that do not share a common letter are significantly different as determined by a one-way ANOVA ($P<0.05$). Those categories marked with an asterisk (*) were measured at max load.

	Control		Low/Short		High/Short		Low/Long		High/Long	
Max Load (N)	359.25 ± 49.84	A	375.58 ± 42.24	A	267.60 ± 67.40	B	280.33 ± 85.04	B	251.97 ± 67.64	B
Max Stress (MPa)	143.62 ± 17.78	A	134.53 ± 16.35	A	96.21 ± 22.30	B	118.99 ± 32.57	A, B	104.48 ± 27.45	B
Grip Displacement* (mm)	6.10 ± 0.58	A	4.58 ± 1.20	B	4.28 ± 1.64	B	3.37 ± 0.71	B	3.79 ± 1.08	B
Midsubstance Displacement* (mm)	1.99 ± 0.39	A	1.93 ± 0.38	A	1.58 ± 0.48	A, B	1.33 ± 0.46	B	1.62 ± 0.49	A, B
Grip Strain* (mm/mm)	0.17 ± 0.02	A	0.15 ± 0.04	A, B	0.14 ± 0.06	A, B	0.12 ± 0.02	B	0.11 ± 0.03	B
Midsubstance Strain* (mm/mm)	0.14 ± 0.02	A	0.12 ± 0.02	A, B	0.10 ± 0.03	B	0.10 ± 0.03	B	0.11 ± 0.03	A, B
Grip Stiffness (N/mm)	67.25 ± 5.71	C	104.78 ± 19.60	A	77.84 ± 21.48	C	99.68 ± 21.70	A, B	81.27 ± 16.91	B, C
Midsubstance Stiffness (N/mm)	238.09 ± 44.39	A	292.97 ± 54.86	A	248.02 ± 50.17	A	283.83 ± 69.01	A	231.35 ± 79.50	A
Grip Elastic Modulus (MPa)	993.94 ± 103.5	B, C	1132.84 ± 181.0	A, B	844.3 ± 244.7	C	1238.91 ± 259.9	A	1032.79 ± 268.4	A, C
Midsubstance Elastic Modulus (MPa)	1313.98 ± 197.8	B	1620.62 ± 126.0	A	1288.8 ± 146.2	B	1623.51 ± 250.9	A	1510.58 ± 412.5	A, B
Energy (mJ)	1344.22 ± 324.1	A	1037.57 ± 322.2	A, B	720.56 ± 413.3	B, C	572.28 ± 242.2	C	584.65 ± 298.2	C
Energy Density (mJ/mm^3)	14.28 ± 3.06	A	12.26 ± 4.10	A, B	8.56 ± 4.65	B, C	8.25 ± 3.01	B, C	7.12 ± 3.58	C
Area (mm^2)	2.51 ± 0.25	B	2.80 ± 0.17	A	2.78 ± 0.24	A	2.36 ± 0.23	B	2.42 ± 0.21	B

Maximum stress was highly dependent on magnitude with the high groups being significantly weaker than the control group. The grip strain (LVDT) was highly dependent on duration with the long groups enduring significantly less strain before failure than the control. The energy density, the amount of energy the tendons can absorb before failing, was significantly reduced relative to control, in all groups except the low/short. A list of all mechanical failure properties (Mean ± St.Dev.) of the tendons, both structural and material, as compared by one-way ANOVA are presented in Table 2.

The levels of PGE_2 in the media increased significantly in all groups, relative to time, as measured by a two-way RM-ANOVA (Figure 15). The data are normalized relative to

levels at day 0 within the individual specimen. A large duration-dependent increase was seen as both the high/long and low/long groups released more PGE_2 into the media than their respective short groups. Magnitude dependence was also seen, as the high/long group was significantly greater than the low/long group.

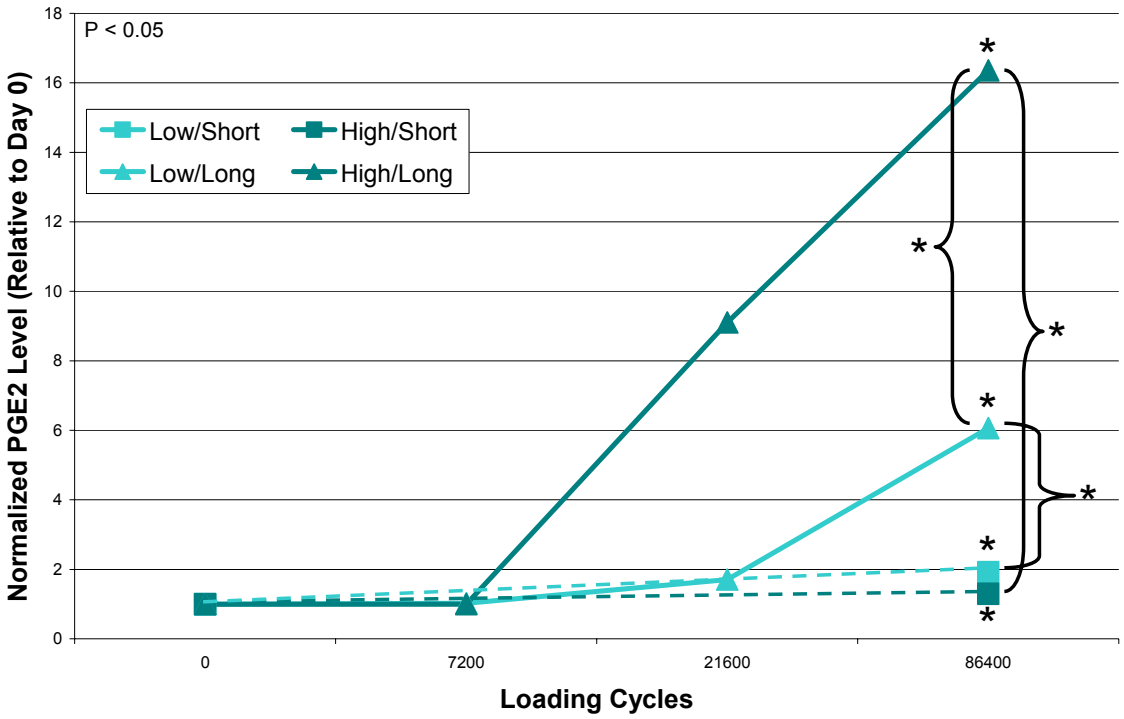
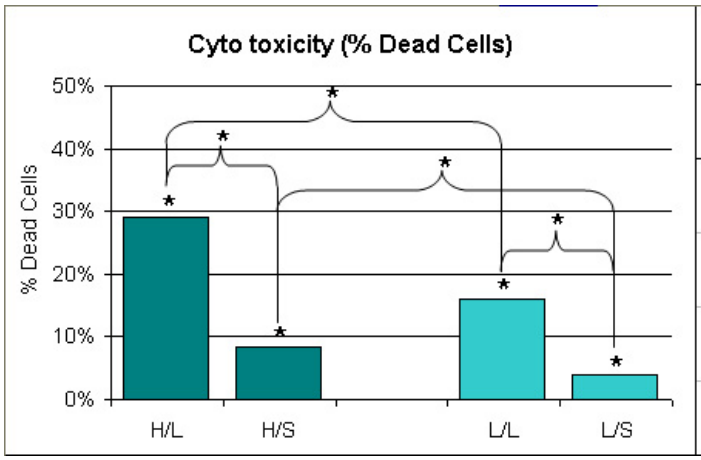


Figure 15: Relative increase in media PGE_2 levels, normalized by day 0 values, are shown across cycles of loading with 86,400 cycles being completed in 1 or 12 days, for the short or long groups, respectively. Asterisks on points denote difference from time zero, while asterisks on brackets indicate difference between groups. Note the dashed lines for the short groups, as they were continuously loaded and did not have intermediate measurements. Significance of $P<0.05$, as measured by a 2-way RM ANOVA.

Cellular turnover was indirectly measured by determining the tissue viability at the end of the experiment and comparing it to LDH levels, an indirect measure of cell death. Cell death increased significantly with the magnitude of the load, and the duration across which it was applied (Figure 16), as measured by a two-way RM-ANOVA. However, the total tissue

viability, as measured by the MTT assay, did not change (Figure 16). Both high groups showed more cell death than their respective low groups, while both long groups exhibited more cell death then their respective short groups.

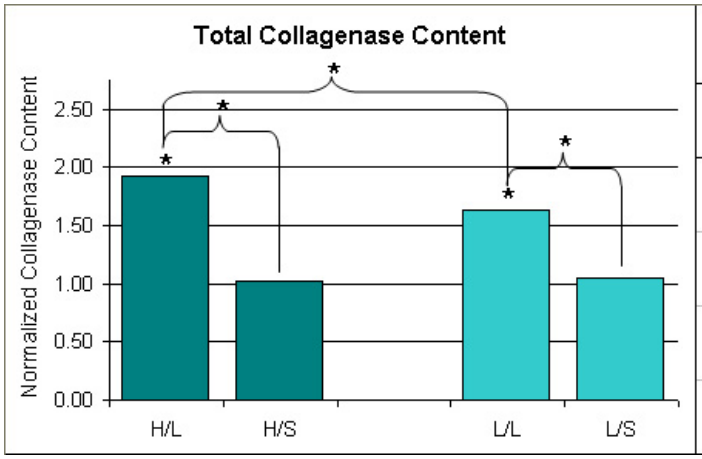


Cell Viability (Live Cell Count)

	Absorbance/mg dry weight	
High Long (HL)	146.23 ± 35.64	A
High Short (HS)	131.10 ± 28.64	A
Low Long (LL)	103.61 ± 30.15	A
Low Short (LS)	142.51 ± 59.31	A

Figure 16: Significant magnitude and duration dependent increases in cell death were observed, but no differences were seen in the total live cell count following loading. (a) Cell death, as measured by LDH released in media, is reported as the percentage of dead cells upon completion of loading. Normalization is relative to 100% death reported as LDH levels of tendons treated to a liquid nitrogen freeze-thaw process. (b) Cellular viability, as measured by a MTT assay, is reported as the absorbance of tissue segments immediately following loading, relative to the tissue weight. Letters beside normalized absorbance values denote a significant difference between groups without a common letter (Note: All "A's" represent no difference in viability). Significance of $P<0.05$, as measured by a 2-way RM ANOVA.

Collagen turnover was also assessed indirectly by measuring hydroxyproline and collagenase content. The amount of total collagenase increased significantly, with time, in all groups (Figure 17) with a large magnitude and duration dependent increase (two-way ANOVA). However, the hydroxyproline content did not change (Figure 17). The amount of proteoglycans, as measured by sGAG in the tissue segments, was not affected by duration of loading, but significantly increased with the level of loading (Table 3).



Collagen Content (Hydroxyproline)		
	µg/mg dry weight	
High Long (HL)	91.86 ± 0.66	A
High Short (HS)	95.22 ± 20.36	A
Low Long (LL)	92.71 ± 16.25	A
Low Short (LS)	82.08 ± 8.89	A

Figure 17: Significant magnitude and duration dependent increases in collagenase levels were observed, but no difference was seen in the total collagen content following loading. (a) Total collagenase in the media, as measured by an Azocoll assay, is reported as the relative increase in collagenase levels, normalized by values at day 0. (b) Collagen contents following loading, as measured by hydroxyproline quantification of digested tissue segments, are reported relative to the dry weight of the tissue segment. Letters beside hydroxyproline measurements denotes a significant difference between groups without a common letter. (Note: All "A's" represent no difference in collagen content between the groups). Significance of $P<0.05$, as measured by a 2-way RM ANOVA.

Table 3: Sulfated glycosaminoglycan content, as measured from digested tissue segments following loading, normalized by the dry weight of the tissue segment. Groups without a common letter are significantly different as determined by a 2-way ANOVA ($P<0.05$).

	Sulfated GAG µg/mg dry weight	
High Long (HL)	6.73 ± 0.89	A
High Short (HS)	6.63 ± 1.48	A,B
Low Long (LL)	4.91 ± 0.94	B,C
Low Short (LS)	4.20 ± 0.76	C

6.5 Discussion

Current descriptions of tendon overuse injuries have emphasized the role of the accumulation of fatigue micro-damage with cyclical overuse loading. However, how the cellular response to this cyclical load contributes to or lessens the fatigue-based decline in tissue properties is less clear. In the current study, it was hypothesized that the cellular responses would exacerbate the mechanical property changes with cyclical loading. In support of this hypothesis, it was found that strains, PGE_2 production, cell death, collagenase production and various mechanical property measures at a given magnitude of loading displayed a duration dependent effect.

The larger accumulation of static strains in the high/long versus the high/short regimens would suggest that a cellular response contributes to the observed tissue strains. A similar effect observed in the low loading regimens would suggest that a general effect related to the time in tissue culture may contribute to this response. However, the greater changes observed in the higher loading regimens would suggest that this is not the dominant factor. Additionally, there were no differences in cell viability between groups. The extra time for strain recovery during the unloading portions of the long regimens would suggest that observed differences in accumulated strain were also not a result of passive viscoelastic effects.

Dynamic strain assessments also support this concept of cellular response-induced property loss as the tissue was found to display decreased stiffness with the high/long regimen, compared to high/short. This decrease may be indicative of increased micro-damage or remodeling in the high/long regimens. Furthermore, the lack of differences in

dynamic strains between low/long and low/short would suggest this is not merely a time of tissue culture effect.

The failure mechanical property data were less definitive in providing evidence of a cellular response contributing to the decline in material properties. Various properties were either load-magnitude or duration dependent and not both. The high/long regimen did not consistently produce the most inferior properties as anticipated. However, this lack of difference may be related to a statistical difference in the area between tendons of the short and long duration loading regimens. A limitation to the methodology used in this study was that the area and gauge lengths used in failure material property calculations were measured after the cyclical loading regimens were applied. This was a design criterion elicited by the risk of contamination.

It is believed the further decline in mechanical properties with the longer loading regimens is due to the cellular responses to cyclical loading. A key factor in this cellular response may be the production of the inflammatory mediator, PGE_2. In agreement with previous studies(Almekinders *et al.* 1993; Almekinders *et al.* 1995; Tsuzaki *et al.* 2003; Wang *et al.* 2003; Li *et al.* 2004), load-induced PGE_2 production was observed. The PGE_2 levels increased in all groups and exhibited load-magnitude and duration dependence. Traditionally it has been suggested that this type of loading profile would put an individual at increased risk of clinically developing overuse tendinopathy. Previous studies have shown that high concentrations of PGE_2 can increase collagenase production in uterine cervical fibroblasts(Goshowaki *et al.* 1988) and inhibit collagen production in human tendon fibroblasts(Cilli *et al.* 2004). Furthermore, PGE_2 has been used clinically to promote cervical ripening, a process in which the extensibility of the collagen matrix of the cervix is increased

due to apparent collagen reorganization (Shi *et al.* 2000). Finally, recent studies have demonstrated that repeated exogenous administration of PGE_2 *in vivo* in the rabbit patellar tendon can lead to a tendon that appears degenerative under histological examination(Khan *et al.* 2005).

LDH measurements showed a steady, significant increase of cell death across all groups. Like PGE_2, the long groups displayed significantly more cell death than their respective short group. It is believed this difference is due to the lack of a response time for the short groups. The cell viability analysis showed no differences between the groups at the end of the experiment. Because cell death increased across time and the viability measures remained unchanged, a possible interpretation is that tendon fibroblasts proliferated accordingly in order to maintain live cell numbers. Previous studies have provided evidence that PGE_2 can stimulate fibroblasts to proliferate (Uribe *et al.* 1989; Martinez *et al.* 1997; Khan *et al.* 2005). In addition, the profile of cellular turnover paralleled the profile of PGE_2 production, suggesting a possible link between these responses.

Though high levels of collagenase were found (increase in all groups), no differences in the amounts of collagen were seen. It must be noted that the collagen measured can include both degraded and healthy collagen. Because total collagenase was measured (both active and latent) this overall response may be interpreted as the opportunity for high collagen turnover being present, if not occurring. The collagenase results agree with previous studies on the effects of prostaglandins, which have shown increases in MMP expression (Goshowaki *et al.* 1988; Ito *et al.* 2004; Kim *et al.* 2005) and coincide with our PGE_2 expression profile.

There are a few limitations in the present study however. For basic tissue maintenance, the media were replaced every three days. While this is a standard period, a majority of the responses exhibited duration dependence, suggesting that the accumulation of cellular responses in the media were likely highest just prior to change. Greater tissue effects are likely if the tissue were exposed to these higher cellular response concentrations for a longer period. Furthermore, the cellular responses may have been larger if more response time was provided. For the short groups, the media were collected after just two days (one day loading, and one day rest). This short time may not have been sufficient for the tissue to fully respond, and could partly explain why some of the high/short groups did not exhibit greater cellular responses. A further limitation may lie in dilution of the cellular responses in the media. While cell culture studies have volumetric cell to media ratios of roughly 1:50, the current study has a 1:500 tissue to media ratio. This is a design criterion that could be changed. Altering these factors would likely cause increases in cellular responses and concentrations which could potentially have had greater effects on the tissue, and thus failure mechanical properties, as well as collagen and sGAG content.

Overall, a large dependency in loading duration was observed in most of the responses. This is not expected for the mechanical measures with simple material fatigue, and is likely due to the cellular response of the living tissue. A key contributor in this response is likely PGE_2. Though debate is ongoing about the presence of inflammation with overuse tendinopathy, a large increase of this particular inflammatory mediator was measured. PGE_2 may contribute to the pathology of tendinopathy by stimulating cellular proliferation, partially decreasing collagen synthesis and increasing collagenase activity(Goshowaki *et al.* 1988; Uribe *et al.* 1989; Martinez *et al.* 1997; Khan *et al.* 1999; Ito

et al. 2004; Khan *et al.* 2005). In the loading regimens where PGE_2 production was increased, the profiles of observed responses seem to coincide with the reported cellular effects of PGE_2. The greater PGE_2, cell death and collagenase levels in the longer duration groups, especially the high/long group, suggest the potential significance of these cellular responses to overuse tendinopathies. To definitively assess if the cellular responses to cyclical loading are acting by way of a PGE_2 pathway, future studies will focus on applying inhibitors.

6.6 Acknowledgement

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CHAPTER 7

MAGNITURE RESPONSE TO CYCLIC LOAD

7.1 Abstract

The purpose of this research was to examine the biochemical response of avian flexor digitorum profundus tendon to repetitive cyclic loadings of various magnitudes. An in vitro explant model was utilized to apply four levels of sinusoidal load (0, 3, 12 and 18 MPa) at 1.0 Hz, 8 hrs/day for three days. The loading caused minimal compositional changes as collagen content did not change, but a trend for a load-magnitude based increase of glycosaminoglycan content was observed. Load-magnitude dependant increases ($P<0.05$) in collagenase, cell death and prostaglandin E_2 were observed. These data indicate that elevated cyclical mechanical loading of tendon quickly results in enhanced biochemical tissue responses indicative of tissue injury. More sustained cyclical loading over time may be required for these initial responses to induce more dramatic tissue changes as observed in clinical tendinopathy.

7.2 Introduction

Tendons are collagenous structures that transmit muscle force to bones across joints to provide motion. Similar to other connective tissues, tendons adapt their structure and composition in response to mechanical and biochemical stimuli. Tendon overuse injuries account for 30% of all running injuries and 40% of all tennis injuries (Sharma and Maffulli

2005). Though originally labeled tendonitis due to clinical symptoms of inflammation, histological studies have not shown classic inflammatory cells (Chard *et al.* 1994; Astrom and Rausing 1995). Cases of inflammatory cell infiltration in tendon are rarely observed without acute damage or inflammation of the surrounding tissue. Surrounding tissue inflammation and concurrent tendon degeneration are often separate entities (Backman *et al.* 1990; Leadbetter 1992). Tendon overuse injuries result from an accumulation of degeneration, or tendinosis (Archambault *et al.* 1995; Khan *et al.* 2002). The major causative factor of overuse tendinopathy is suggested to be mechanical loading, however the most detrimental aspect of this loading (magnitude, frequency, duration, etc) has not been established (Archambault *et al.* 1995; Wang *et al.* 2006).

The lack of mechanical load has detrimental and atrophic effects. Load deprivation causes tenocyte necrosis, hypoxia, increased collagen breakdown, reduced cross-linking, collagen disorganization and decreased glycosaminoglycan (GAG) content (Akeson 1961; Jozsa and P. 1997; Maganaris *et al.* 2004). Load deprivation induces the release of matrix metalloprotienases (MMP), growth factors, cytokines and inflammatory mediators (Lavagnino *et al.* 2003; Uchida *et al.* 2005). Animals models of stress deprivation have generally agreed with explant studies, showing decreased ultimate strength and modulus, however data on changes in collagen fibril size, density and organization are often contradictory (Yamamoto *et al.* 1993; Hannafin *et al.* 1995; Yamamoto *et al.* 2000; Lavagnino *et al.* 2005).

Mechanical loading has both positive and negative effects on the mechanical and structural properties on tendons. Histopathologic examination of degenerative horse tendons with overuse tendinopathy show GAG content increases (Birch *et al.* 1998; Kobayashi *et al.*

1999). Exercise animal models show positive effects such as increased failure strength and elastic modulus (Woo *et al.* 1980; Woo *et al.* 1981), as well as negative effects such as lower failure strength and elastic modulus (Sommer 1987; Soslowsky *et al.* 2000). It is proposed that up to a certain level exercise can be beneficial to the tendon, however if extended too long it begins to have negative effects especially in regard to fatigue damage accumulation (Archambault *et al.* 1995; Schechtman and Bader 1997).

With an improper balance of loading, the tendon may slowly degenerate into a tendinosis state. The precise etiology is thought to be due to several factors, chief among them are the nature of the loading and subsequent biochemical responses (Sommer 1987; Khan *et al.* 2002; Kjaer 2004; Riley 2004). Biochemical responses include apoptosis in degenerated tendons (Yuan *et al.* 2003) as well as immediately following high magnitude strain in healthy tendon (Scott *et al.* 2005). However, increase in cellularity (Banes *et al.* 1995; Soslowsky *et al.* 2000; Yang *et al.* 2004) and growth factors such as platelet derived growth factor BB, insulin like growth factor I, interleukin -1β and transforming growth factor β1 (Jozsa and P. 1997; Archambault *et al.* 2002; Tsuzaki *et al.* 2003; Kjaer 2004) are also observed in stretched fibroblasts. Mechanical loading induced over-expression of these growth factors as well as matrix synthesizing and degrading enzymes and inflammatory mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) have been observed and hypothesized to be involved in the development of tendinopathy (Almekinders *et al.* 1995; Wang *et al.* 2003; Li *et al.* 2004; Khan *et al.* 2005). Furthermore, injection of collagenase, cytokines and inflammatory mediators into tendon or peri-tendinous space have all independently shown tendinosis-like degeneration (Stone *et al.* 1999; Li *et al.* 2004; Riley 2004).

To better understand tendinopathy, it has been proposed that tissue responses as a function of load level must be more closely monitored (Archambault *et al.* 1995). Many load inducible responses such as growth factors, cytokines and inflammatory mediators have been characterized and proposed to play a role in tendinopathy, but their relationship to load levels have not been established. The objective of this study is to examine how biochemical responses indicative of tissue injury (PGE_2, collagenase and cellular viability, GAG and collagen content) are influenced by the magnitude of cyclical loading applied in a tendon explant model. The hypothesis is that these biochemical responses will increase with loading magnitude.

7.3 Materials and Methods

7.3.1 Specimen: Avian flexor digitorum profundus tendons were harvested from the middle toe of 50 – 60 day old Rock Cornish hen. All specimens were obtained from freshly severed feet from a local commercial poultry processing plant.

7.3.2 Loading Design: Tendons were gripped and placed on a load-controlled tissue loading device (TLD) (Devkota and Weinhold 2005). The TLD sits in an incubator and has the ability to produce significant fatigue damage through cyclic loadings while maintaining viability (Devkota and Weinhold 2005). An equilibration period of 72 hours was afforded the tendon following isolation. A preconditioning (3 - 1 MPa, 0.50 Hz sine wave) loading was applied for two hours at 24 and 48 hrs to discourage atrophic changes. After the equilibration period, tendons were loaded 8 hours, and allowed to rest for 16 hours each of the subsequent three days. The loadings were 1.0 Hz, sinusoidal waveforms with peaks of 18 MPa, 12 MPa, 3 MPa as well as an unloaded group (0 MPa).

7.3.3 Media Analyses: Media was replaced just prior to loading and sampled daily (5%). Also, 24 hours before loading started (48 hours into the equilibration period), the media was replaced to remove/minimize surgery-induced cellular responses. All media was stored frozen (-80ºC) and analyzed at a later time.

PGE_2 levels in the media were quantified by a commercial immunoassay kit (PGE2 EIA – monoclonal, Cayman Chemical, Ann Arbor, MI). Cellular death was quantified by measuring the levels of lactate dehydrogenase (LDH), a membrane protein released upon cell lysis by a commercial colormetric assay (Cytotox 96, Promega Corp, Madison, WI). Total collagenase protein levels were measured by quantifying the amount of dye released from a mixture of sampled media and Azocoll, a dye impregnated collagen (Chavira *et al.* 1984). Aminophelylmercuricacetate (APMA), a collagenase activating agent was added during the analysis. All media comparisons were made by a two-way (load magnitude x day) repeated measures analysis of variance (RM-ANOVA, $P < 0.05$).

7.3.4 Compositional Analyses: Following the third day of loading, all tendons were sectioned for compositional analysis and viability. Viability was evaluated immediately by a modified MTT assay (Klein *et al.* 1996). Briefly, tendon sections were incubated for 4hrs in 0.5mg/ml MTT, then transferred to a methoxymethanol solution and shaken to release the dye. Absorbance of the resulting solution was read at 570nm with background subtracted at 690nm. The remaining sections were digested with Papain (0.5mg/ml) and stored at -80ºC for collagen and proteoglycan content determination.

A modified hydroxyproline content assay was performed on the digest to measure hydroxyproline, a collagen constituent of known proportions (Bergman and Loxley 1963). Briefly, digests hydrolyzed in 6M HCl at 105 ºC were treated with an oxidant and reagent

combination and allowed to incubate, and then had their absorbance measured at 540nm. A modified sulfated glycosaminoglycan (sGAG) content assay was performed on the digest to measure sGAG, the covalently attached glycoprotein chains of proteoglycans (Farndale *et al.* 1986). Briefly, digest samples were treated with a color reagent (DMMB) and the resulting absorbance was measured at 540nm. Hydroxyproline and chondroitin sulfate standards were performed and all values were normalized by the wet weight of the tissue. All comparisons were made by a one-way ANOVA ($P < 0.05$, $n = 12+$).

7.4 Results:

7.4.1 PGE_2 Analysis: PGE_2 was found to be load inducible as all loading levels tested caused elevated concentrations in the media as compared to concentrations present during pre-loading (Figure 18). This increase from pre-loading levels was significant for the highly loaded groups (12 MPa and 18MPa) only. Both highly loaded groups also exhibited significantly greater PGE_2 levels than the low and unloaded groups at this time point. Basal PGE_2 levels (measured prior to loading) were in the range of 200 – 400 pg/ml.

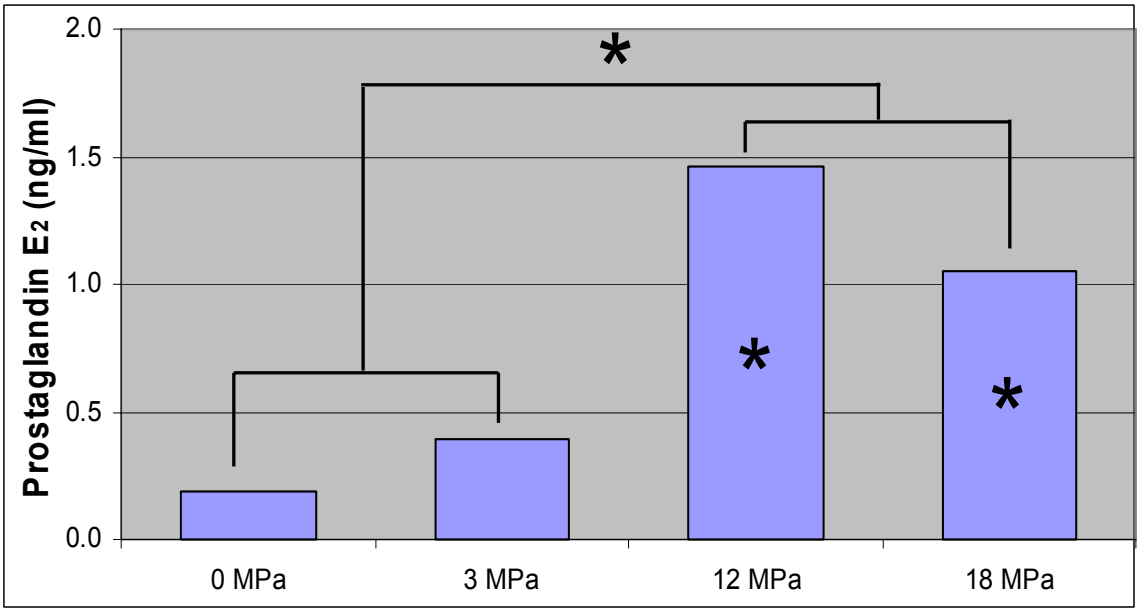
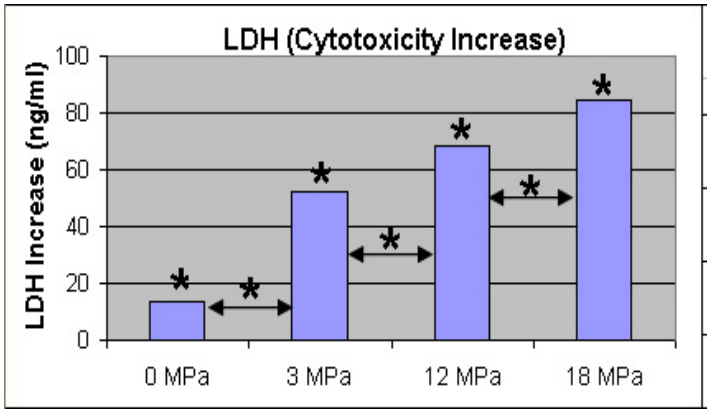


Figure 18: Raw levels of prostaglandin E2 in the media of tendons cyclically loaded for three days were load inducible and load-magnitude dependant. Asterisks on bars indicate a significant difference from the pre-loading level, while asterisks above bars represent significant differences between groups ($P < 0.05$).

7.4.2 Cellularity Analysis: Cell death, measured by LDH levels in the media, increased significantly in a time and magnitude dependant manner for all groups (Figure 2). A significant increase (relative to pre-loading) in the number of dead cells was seen by the end of the first day in the 18 MPa group, second day in the 12 & 3 MPa groups, and third day for the unloaded group. A significant load magnitude dependant increase was seen by the end of the third day (18 MPa > 12 MPa > 3 MPa > 0 MPa).

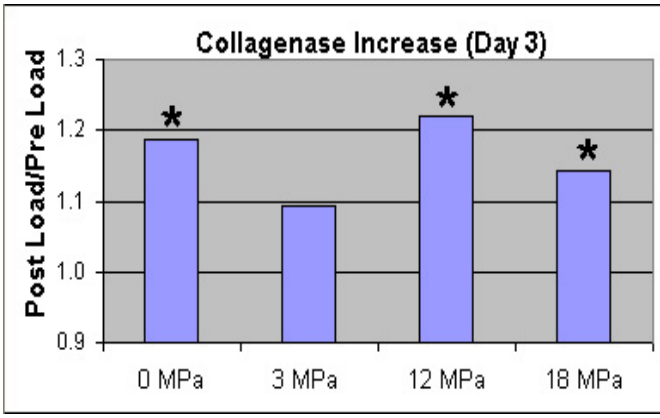
Cell viability, as measured by the MTT assay, showed minimal differences in the number of live cells (Figure 19). No groups were significantly different from the unloaded group. The only difference was the 18 MPa had greater viability than the 3 MPa group.



Cell Viability (Live Cell Count)		
Absorbance/mg dry weight		
0 MPa	114.17± 14.76	A, B
3 MPa	103.16 ± 19.13	A
12 MPa	109.95 ± 20.65	A, B
18 MPa	126.83 ± 12.38	B

Figure 19: Increase of LDH (cytotoxicity indicator) in the media of tendons cyclically loaded for three days and the relative similarity in cellular viability of tendons, indicating cellular turnover. Asterisks on bars indicate a significant difference from the pre-loading level, while asterisks above bars represent significant differences between groups. For viability, groups that do not share a common letter are significantly different ($P < 0.05$).

7.4.3 Collagen & Collagenase: Collagen content (Figure 3), measured from hydroxyproline, was not different between groups ($P = 0.312$). Collagenase measurements showed a significant increase by day three in the 18 MPa, 12 MPa and unloaded groups relative to day zero (Figure 20). This difference became significant after the second day for the 12 MPa group, while no differences were observed in the 3 MPa group across time.



Collagen Content (Hydroxyproline)		
	µg / mg dry weight	
0 MPa	38.39 ± 6.85	A
3 MPa	38.33 ± 8.53	A
12 MPa	34.07 ± 4.90	A
18 MPa	38.38 ± 6.12	A

Figure 20: Relative increase in collagenase levels in the media of tendons cyclically loaded for three days and the similarity of collagen content of the tissue. Asterisks above bars indicate a significant difference from pre-loading levels (Post/Pre). For collagen content groups that do not share a common letter are significantly different ($P < 0.05$).

7.4.4 Sulfated GAG Analysis: The sulfated glycosaminoglycan content in the tissue after loading showed a trend for load-magnitude dependence, as both highly loaded groups showed greater, but not significant ($P = 0.062$), GAG content compared to the low and unload groups (Table 4).

Table 4: Sulfated glycosaminoglycan content of tendon explants cyclically loaded for three days. Values were normalized by dry weight of tissue segment.

	sGAG (µg/mg)
0 MPa	7.221 ± 1.620
3 MPa	7.771 ± 2.678
12 MPa	10.278 ± 4.390
18 MPa	8.693 ± 2.847

.

7.5 Discussion:

Tendon overuse injuries are a major clinical problem and their etiology is thought to be due to a combination of the loading applied and the resulting biochemical responses. Responses that are potential candidates in the development of tendinosis include inflammatory mediators, particularly PGE_2 (Almekinders *et al.* 1995; Wang *et al.* 2003; Li *et al.* 2004; Khan *et al.* 2005). The current study found PGE_2 to be load inducible, which is consistent with results of past fibroblast and human micro-dialysis studies (Yamaguchi *et al.* 1994; Langberg *et al.* 1999; Langberg *et al.* 2003; Li *et al.* 2004). Though not directly comparable to the results of cell culture studies, the levels of PGE_2 measured during loading in our model are consistent with in vivo micro-dialysis studies of human tissue (Langberg *et al.* 2003). PGE_2 was also load-magnitude dependant, as the higher loading levels produced more PGE_2 than did the low and unloaded groups. This effect has not been shown in tendon tissue, but has been shown in cell culture studies of stretched fibroblasts of tendon and ligament (Yamaguchi *et al.* 1994; Wang *et al.* 2003). However, translating strains observed in cell culture models into tissue loads is difficult. Thus, the physiologic relevance of the effects observed in such cell culture studies is less clear. In particular, the established role of PGE_2 as an inflammatory mediator coupled with its increase with loading have prompted the consideration of excessive PGE_2 production to play a role in the pathology of overuse tendinopathy (Wang *et al.* 2006). This has led to several injection studies investigating the ability of PGE_2 or PGE_1 to initiate degenerative changes similar to overuse tendinopathy. While histological studies suggested these injections could initiate degenerative changes (Sullo *et al.* 2001; Khan *et al.* 2005), a recent study evaluating mechanical properties found structural strength and stiffness to increase after eight weeks (Ferry *et al.* 2006). Seemingly

contradictory, this improvement in properties would be consistent if PGE_2 plays a role in the activation of remodeling and the histological studies observed degenerative changes during this remodeling phase while the biomechanical study observed mechanical improvement at eight weeks, after some remodeling. The concept of PGE_2 having a role in activating tissue remodeling has precedence in studies of bone, where increased bone mass has been observed following prostaglandin injections (Jee and Ma 1997). While the putative activation of tendon remodeling by PGE_2 to repair load-induced microdamage would seem to be a positive effect, researchers have suggested that tendon may experience a transient period of weakness as the tendon remodels (Zamora and Marini 1988). However, excessive activation of remodeling by PGE_2 may actually make the tissue more susceptible to further injury when it is being repetitively loaded. A potential clinical impact of the observed load dependent increase in PGE_2 may be to alter the timing or dosage of NSAID administration in patients with symptoms of tendinopathy, based on their activity level.

The accumulation of glycosaminoglycans seen in the 12 MPa and 18 MPa groups is consistent with past studies which have seen such increases in degenerated tendons (Birch *et al.* 1998; Kobayashi *et al.* 1999), and decreases in immobilized tendons (Jozsa and P. 1997; Maganaris *et al.* 2004). Though these results weren't significant, a strong trend ($p=0.062$) was evident and may have been significant given more response time. This GAG response may be linked to the levels of PGE_2, as studies have shown NSAID treatment to decrease GAG content (Riley *et al.* 2001). The amount of GAG accumulation after only three days in culture was not expected, but warrants further investigation. Further examination of specific GAGs expressed may help elucidate the onset of tendinosis as increases in dermatan sulfate

may signify wound healing type response, while increases in hyaluronic acid or chondroitin sulfate may indicate a tendency for granulation (Kobayashi *et al.* 1999).

Though a trend for load-based GAG concentration increase was seen, no differences or trends were seen in the collagen content. This may be due to the short response time (3 days) afforded the tendons or the sensitivity of the collagen assay may not be sufficient. Reports on animal studies have shown differences in collagen density, organization and collagen content with loading (Woo *et al.* 1980; Woo *et al.* 1981; Astrom and Rausing 1995; Soslowsky *et al.* 2000). The current procedure analyzed total collagen so increases in type III collagen seen in tendinopathy (Riley *et al.* 1994; Birch *et al.* 1998; Archambault *et al.* 2001), may be balancing the decrease in type I, and resulting in no change in total collagen. To better gauge the current degenerative state of the tendon, assessment of organization and ratio of collagen type I to type III may be beneficial.

The levels of collagenase were found to increase with the highly loaded (18 MPa and 12 MPa) and unloaded (0 MPa) groups but not the low group (3 MPa). The up- regulation of collagenase in the unloaded group is consistent with studies of immobilization (Lavagnino *et al.* 2003; Uchida *et al.* 2005). MMP-1 has been found to be down-regulated with load (Lavagnino *et al.* 2003), while MMP-3 is up-regulated (Archambault *et al.* 2001; Tsuzaki *et al.* 2003) in past tendon studies. The 3 MPa group is likely the groups that best represents normal loading activity, or a non-damaging group (unpublished data), and this could explain the lack of collagenase or remodeling needed in the group. Cyclic loadings at 12 MPa has been previously shown to be damaging in our tissue explant system (Devkota and Weinhold 2005), therefore the highly loaded groups' increase in MMPs may be of MMP-3, the MMP upregulated during remodeling. However, like the collagen data, evaluating the specific

MMPs released would need to be performed to confirm this. Also, the collagenase response may be linked to the PGE_2 response as past studies have shown PGE_2 to increase collagenase production in uterine cervical fibroblasts (Goshowaki *et al.* 1988) and PGE_2 has been used clinically to promote cervical ripening, a process in which the extensibility of the collagen matrix of the cervix is increased due to apparent collagen reorganization (Shi *et al.* 2000).

Large increases in cell death were seen in a magnitude and duration-dependant manner in our cyclic loading model. Interestingly, the lack of change in viability between groups may suggests active cellular turnover. This inferred cell turnover appears to occur especially in the highly loaded groups, and is consistent with past studies of stretched fibroblast (Banes *et al.* 1995; Yang *et al.* 2004). This conjectured increase in cell turnover in the highly loaded groups could be linked to the load dependent PGE_2 response as previous studies have indicated that PGE_2 can stimulate cell proliferation in fibroblasts (Martinez *et al.* 1997). However, studies of tendon have previously shown increased apoptosis in tendinopathy as well as immediately following high magnitude loading (Yuan *et al.* 2003; Scott *et al.* 2005). Cell death with cyclical loading of tendon may have a role in the pathology of overuse tendinopathy as the release of cytosol proteinases or proenzymes may induce degeneration of the surrounding matrix. A limitation of the cell death and viability evaluations is that the assays used did not localize changes of these variables within the different regions of the tissue.

Significant changes in the tendon responses were seen after only three days with a cyclical loading explant model. Increases were observed in the collagenase levels, GAG content, cell death and PGE_2 content. Each of these biochemical responses are suggestive of tissue injury and have been previously hypothesized to play a role in the development of

tendinopathy (Akeson 1961; Archambault *et al.* 1995; Jozsa and P. 1997; Li *et al.* 2004; Khan *et al.* 2005; Wang *et al.* 2006). PGE_2, a response independently linked with the other responses and likely a key contributor to tendinopathy, was found to be inducible in a load-magnitude dependant manner. The clinical significance of these findings may lie in the potential modulation of NSAID administration during heavy exercise and rest for tendinopathy.

7.6 Acknowledgement:

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CHAPTER 8

COX INHIBITION

8.1 Abstract:

The following study was carried out to examine the hypothesis that chronic expressions of prostaglandin E2 (PGE_2) plays a role in the development of overuse tendinopathy. Avian flexor digitorum profundus tendons were cyclically loaded four hours/day in an explant model for six days with and without treatments of exogenous PGE_2, SC-560 (COX-1 inhibitor at 0.01 & 0.10 μM) and Celebrex (COX-2 inhibitor at 0.1 and 1.0 μM) across three loading levels (0, 3 and 12 MPa). Both COX inhibitors decreased PGE_2, strain accumulation, cell death and collagenase while maintaining collagen, sulfated glycosaminoglycan (sGAG), ultimate stress, and energy density, but increasing elastic modulus and stiffness. Exogenous PGE_2 treatment increased strain accumulation and collagenase while reducing stiffness, cell death and showing trends for decreasing collagen and sGAG. These results suggest the pathology of overuse injuries can be accelerated by PGE_2 potentially through the reduction of stiffness, resulting in increased strains, and thus increased cellular responses. Finally, both SC-560 and Celebrex were effective at suppressing many of these changes while initiating no damaging changes, suggesting a potentially positive therapeutic role for COX inhibitors in preventing overused tissue from developing into tendinopathy.

8.2 Introduction:

Overuse injuries account for significant time and productivity losses in both athletic and occupational settings. Tendon overuse injuries comprise nearly 30% of all running injuries and 40% of all tennis injuries (Sharma and Maffulli 2005). Though the exact etiology is not known, overuse injuries are believed to be caused by an accumulation of damage due to repetitive loadings and biochemical expressions (Jozsa and P. 1997; Sharma and Maffulli 2005; Wang *et al.* 2006). In response to external stimuli such as loading, various biochemical factors such as cytokines, growth factors and inflammatory mediators are released. One such load-inducible biochemical expression that is thought to play a key role in the development of tendinosis is the inflammatory mediator prostaglandin E_2 (PGE_2) (Almekinders *et al.* 1993; Almekinders *et al.* 1995; Li *et al.* 2004; Wang *et al.* 2006).

Blocking PGE_2 is one of the main effects of non-steroidal anti-inflammatory drugs (NSAID), a common treatment for tendinopathies (Radi and Khan 2005; Mehallo *et al.* 2006). NSAIDs inhibit cyclooxygenase (COX), a catalytic enzyme responsible for the formation of all prostaglandins and thrombaxanes. COX has two main isoforms, COX-1 and COX-2, the constitutional and inducible isoforms, respectively.

Non-specific NSAIDs, those that block both isoforms of COX, are the oldest class of synthetic drugs and have been used for over 100 years. In tendon, these drugs have both positive and negative effects. Indomethacin has been shown to have both positive (Forslund *et al.* 2003) and negative (Cohen *et al.* 2006) effects on healing tendon through the increase/decrease of failure strength. Negative effects have also been shown, such as decreased cellular proliferation in a fibroblast model (Almekinders *et al.* 1995), and both decreased cellular proliferation and glycosaminoglycan (GAG) synthesis in explant models

(Riley *et al.* 2001). Ibuprofen has also been shown to inhibit both cellular proliferation and migration, effects negative to tendon healing (Tsai *et al.* 2004; Tsai *et al.* 2006). Non-specific NSAIDs been shown to decrease the levels of PGE_2, however it is usually accompanied by a concurrent increase in LTB4 levels (Almekinders *et al.* 1995; Li *et al.* 2004), making its usefulness questionable. Furthermore, negative effects have been seen in vivo as they have been shown to decrease blood flow during exercise (Langberg *et al.* 2003).

NSAIDs that block COX-1 have side effects of causing gastrointestinal bleeding and ulcering. Newer NSAIDs called coxibs have been developed that block predominantly the second isoform of COX. No data exists on the effects of coxibs on overuse tendinopathy, but limited data does exist on ligament injury and acute tendon injury models. In a treating acute ankle sprain, coxibs have been as effective as non-specific NSAIDs (Petrella *et al.* 2004). In transected tendons, coxibs have shown negative effects in the early stages (Elder *et al.* 2001; Virchenko *et al.* 2004; Cohen *et al.* 2006), but beneficial effects later in the healing process (Forslund *et al.* 2003; Virchenko *et al.* 2004). Also, a micro-dialysis study of peri-tendinous space has shown coxibs to decrease the normal increase of blood flow during exercise (Langberg *et al.* 2003).

The current data that exists on the efficacy of NSAIDS is, at best, contradictory but they are still routinely prescribed for tendinopathy treatment. No benefits of COX-1 inhibition from NSAIDs have been seen (Radi and Khan 2005), however they possess great analgesic effects, justifying their use (Jozsa and P. 1997; Mehallo *et al.* 2006). Because little data exists on the effects of NSAIDs on overuse tendinopathy the following study was carried out. The objective of this study was to investigate the ability of COX inhibitors to modulate the biochemical and biomechanical response of tendon explants exposed to cyclical

loading in vitro. We hypothesize that the COX-2 inhibitors will decrease load induced PGE_2, resulting in better mechanical properties and reduced biochemical expressions of tissue injury compared to the COX-1 inhibitor treated and control groups. An additional objective was to examine the influence of elevated levels of exogenous PGE_2 on the biochemical and biomechanical response of tendon explants exposed to cyclical loading. We hypothesized that exogenous PGE_2 exposure would result in inferior mechanical properties and increased biochemical expressions of tissue injury.

8.3 Methods:

8.3.1 Specimen & Treatment: Avian flexor digitorum profundus tendons were harvested from the middle toes of freshly sacrificed 50 – 60 day old commercial, Rock Cornish hen. Three loading levels (unloaded, 3 MPa and 12 MPa) and six treatments comprised 18 total groups with n = 12 per group. Treatments consisted of high and low dosages of SC-560, a COX-1 inhibitor at 0.01 & 0.10 μM, high and low dosages of Celebrex, a COX-2 inhibitor at 0.10 & 1.00 μM, an exogenous PGE_2 group (100 ng/ml) and an untreated group.

8.3.2 Loading Design: Following isolation, tendons were placed on a load-controlled tissue loading device (TLD) (Devkota and Weinhold 2005). An equilibration period of 72 hours followed with preconditioning (3 MPa, 0.50 Hz haver-sine wave) for two hours at 24 and 48 hrs to minimize atrophic changes. Then tendons were loaded 4 hours and allowed to rest for 20 hours the subsequent six days. Loadings were 1.0 Hz, haver-sine waveforms with peaks of 12 MPa, 3 MPa or 0 MPa.

8.3.3 Loading Strain Analysis: Sutures were stitched across the tendon midsubstance for video strain analysis (VSA) (Devkota and Weinhold 2005). Distance

between the sutures was measured just prior to the first, third and after the sixth loading days at 3MPa of tension for static strain. Dynamic strain was measured during the first, third and after the sixth loading days. Static measurements are absolute strain values while dynamic strain values are (peak – trough)/ trough length. Strain measurements were not performed on the unloaded groups.

8.3.4 Media Analyses: Media was replaced just prior to loading, and every 72 hours thereafter. Furthermore, 24 hours before loading started (48 hours into equilibration period), the media was replaced to remove biochemical expressions released during surgery and setup. All media was stored frozen (-80ºC) and analyzed at a later time.

PGE_2 levels in the media were quantified by a commercial immunoassay kit (PGE2 EIA – monoclonal, Cayman Chemical, Ann Arbor, MI). Cellular death was quantified by measuring the levels of lactate dehydrogenase (LDH), a membrane protein released upon cell lysis by a commercial colormetric assay (Cytotox 96, Promega Corp, Madison, WI). Total collagenase protein levels were measured by quantifying the amount of dye released from a mixture of sampled media and Azocoll, a dye impregnated collagen (Chavira *et al.* 1984). Aminophelylmercuricacetate (APMA), a collagenase activating agent was added to half of the samples to distinguish active from total collagenase.

8.3.5 Compositional Analyses: Following loading, 3 of the tendons were sectioned for compositional analysis and viability. Viability was evaluated by the modification of an MTT assay (Klein *et al.* 1996). For compositional analysis, the remaining sections were digested with Papain (0.5mg/ml) and stored at -80ºC for collagen and proteoglycan analysis.

Modified hydroxyproline (Bergman and Loxley 1963) and sulfated glycosaminoglycan (sGAG) (Farndale *et al.* 1986) content assays were performed on the

digest to measure hydroxyproline, a collagen constituent of known proportions and sGAG, the covalently attached glycoprotein chains of proteoglycans. Hydroxyproline and chondroitin sulfate standards were performed to determine absolute levels and were normalized by the dry-weight of the tissue.

8.3.6 Failure Testing: Following loading, 9 of the tendons were wrapped in saline soaked gauge and stored at -20ºC for failure testing. An area micrometer (Butler *et al.* 1984) was utilized for two measurements on the midsubstance prior to failure testing. Tendons were cryo-gripped (Riemersa and Schamhardt 1982) and pre-conditioned with ten cycles of a 1% strain, 0.5 Hz haver-sine waveform prior to failure testing with a 1% strain/sec linear ramp. Load, grip strain and midsubstance strain data were gathered during the failure loading for structural and material property analysis.

8.3.7 Statistical Method: The exogenous PGE_2 and COX treatment groups were analyzed individually (vs. control) because COX inhibition has wide ranging effects beyond reducing PGE_2 production. A two-way (Load x Treatment Concentration) repeated measures analysis of variance (2-way RM ANOVA) was performed for both COX treatments at each measure to determine if dosage effects existed. Within each measure, if a main effect of treatment concentration was not observed, the specimens at each dosage were combined. Media and strain analyses were performed by a 2-way (Load x Treatment) ANOVA at each day (0, 3, 6) and a 2-way (Treatment x Day) RM-ANOVA at each load level (0, 3, 12 MPa). Failure testing and composition data were analyzed by a 2-way (Load x Treatment) ANOVA for each measure. Interactions were assessed by the Holm-Sidak multiple comparisons method with significant set at $P<0.05$.

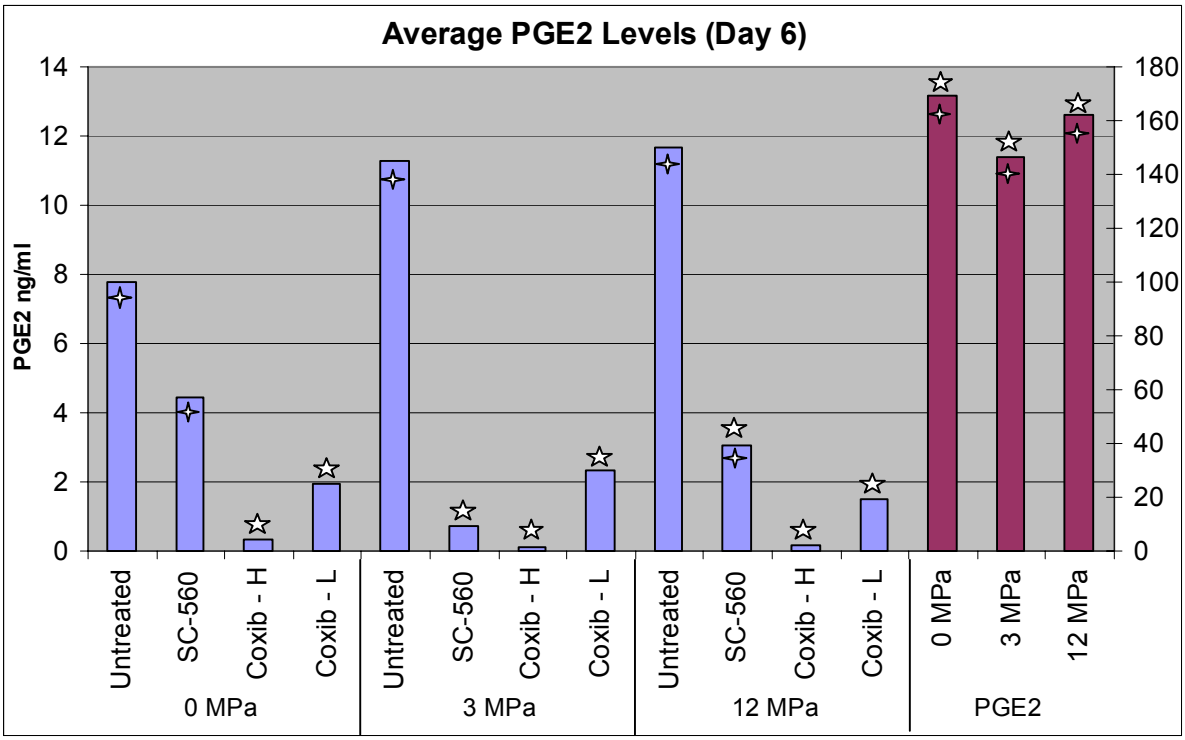

Figure 21: Media PGE_2 levels (ng/ml) in cyclically loaded tendon explants. Five point stars represent difference from control at that load level, while four point stars represent difference from basal values within group ($P<0.05$). PGE_2 levels were greater after six days in untreated and PGE_2 treated groups relative to their pre-loading levels. All treatments of the Coxib (Celebrex) and the 3 MPa treatment of the COX-1 inhibitor (SC-560) eliminated this increase. Note different scale on right for PGE_2 treatments (Dark bars).

8.4 Results:

8.4.1 PGE_2 Analysis: Levels of PGE_2 in the media were found significantly increase in all untreated and PGE_2 treated groups, relative to basal levels (Figure 21). The increase in the exogenous PGE_2 groups was an order of magnitude greater than the untreated groups. COX inhibitor analysis showed a significant dosage difference in the Celebrex group, but not the SC-560 group. All Celebrex and the SC-560 treatments significantly reduced PGE_2 at all load levels relative to the 12 MPa and 3 MPa untreated groups. The untreated, unloaded group was significantly greater than all Celebrex-High, 12 MPa Celebrex-Low and 3 MPa SC-560 Low groups. Within the COX inhibitor groups, a main treatment effect of increased

PGE_2 was observed (SC-560 > Celebrex), independent of load level. All COX inhibitor treatments were able to abolish PGE_2 levels (relative to basal levels) except for the 0 and 12 MPa SC-560 groups. A strong trend for a decrease in PGE_2 levels, relative to basal levels, was observed in the 3 and 12 MPa Celebrex-High groups (P = 0.056 and 0.055, respectively).

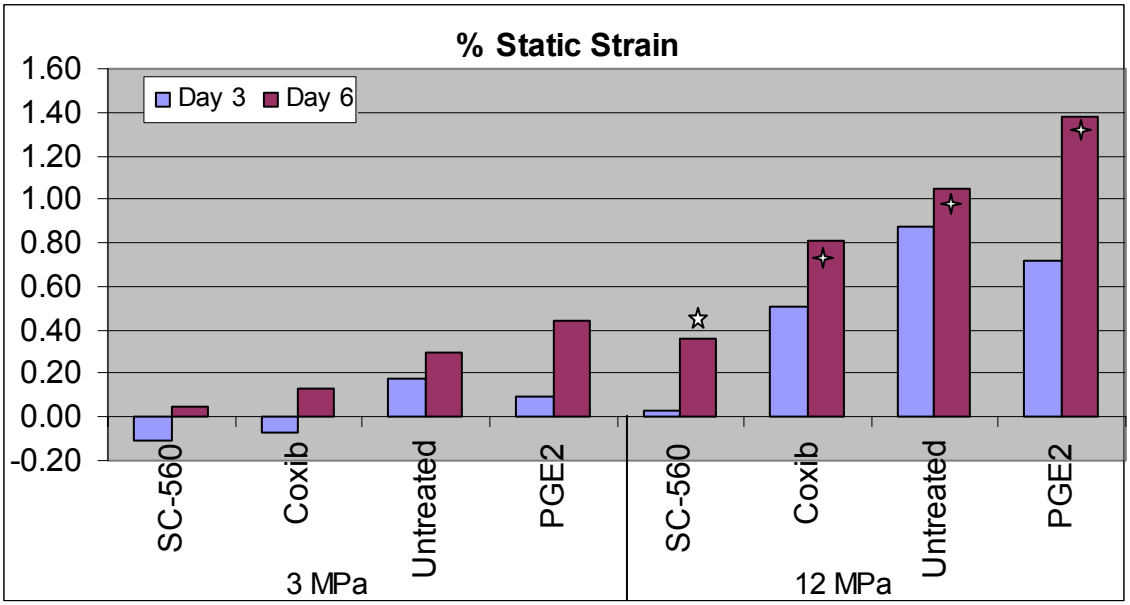

Figure 22: Static strain measured during cyclic loading. Five point stars represent difference from control at that load level, while four point stars represent difference from basal values within group ($P<0.05$). Significant strain accumulated by day six in the highly loaded untreated as well as the PGE_2 and COX-2 treated groups. Day three values are represented for each group as light shaded bars.

8.4.2 Loading Strain Analysis: Static strain analysis showed untreated and PGE_2 treated groups to significantly accumulate more strain than the SC-560 group, which did not accumulate strain, with 12 MPa loading (Figure 22). The Celebrex group also accumulated strain at this loading level, but it was not different from any other treatments. At the 3 MPa loading level, only the PGE_2 treated group accumulated strain, but no inter-treatment differences were seen. A trend for strain accumulation in the untreated group at 3 MPa was

also observed (P = 0.092). An overall duration dependant strain accumulation was observed (6 > 3 > 0), and at day six, a load-magnitude dependant strain increase was also noted (12 MPa > 3 MPa).

Dynamic strain measurements ((peak-trough)/trough) demonstrated no treatment effects of SC-560, Celebrex and PGE_2 groups relative to the untreated groups. An overall significant magnitude (12 > 3 MPa) dependant increase was noted, however a duration dependant increase (6 > 3 > 0) was only noted for all 12 MPa groups (data not shown).

8.4.3 Failure Analysis: Midsubstance tissue strains at failure were found to be significantly increased in the PGE_2 group, relative to the untreated group at 0 MPa (Figure 22). A main treatment effect of decreased midsubstance stiffness was also seen in the PGE_2 groups, compared to the untreated groups, independent of load level. Conversely, a main treatment effect of increased midsubstance stiffness was observed in the Celebrex groups, compared to the untreated groups, independent of load level. A main treatment effect of increased midsubstance elastic modulus, independent of load level, was noted for the Celebrex group relative to the SC-560 and untreated groups. Main effects of load magnitude were demonstrated by the decreased max stress, energy and energy density of the 12 MPa groups relative to the 0 and 3 MPa groups, independent of treatment. No other treatment effects were noted for any analysis parameter.

Table 5: Biomechanics data from failure testing. Note COX inhibitors (A, B; Ω, Λ) and PGE_2 (a, b; λ, ω) were each analyzed separately against the untreated group (PGE_2 and COX inhibitors cannot be directly compared). Latin letters denote load-magnitude difference, while Greek letters denote treatment differences. Groups that do not share a common letter are significantly different.

		COX-1		COX-2		Untreated		PGE$_2$	
Max Stress (MPa)	**0 MPa**	133.0	A	137.7	A	131.3	A a	132.8	a
	3 MPa	128.4	A	140.2	A	132.1	A a	141.4	a
	12 MPa	117.9	B	110.7	B	105.8	B b	106.7	b
Max Load (N)	**0 MPa**	282.4	B	269.4	B	249.9	B	252.9	
	3 MPa	277.5	A	307.0	A	297.6	A	257.4	
	12 MPa	252.4	B	252.0	B	243.0	B	233.9	
Area (mm2)	**0 MPa**	2.121	B	1.970	B	1.899	B b	1.942	a,b
	3 MPa	2.165	A	2.198	A	2.255	A a,ω	1.814	b,λ
	12 MPa	2.139	A	2.275	A	2.295	A a	2.202	a
Grip Strain (mm/mm)	**0 MPa**	0.132		0.137		0.130		0.122	
	3 MPa	0.134		0.132		0.130		0.135	
	12 MPa	0.131		0.119		0.113		0.122	
VSA Strain (mm/mm)	**0 MPa**	0.115		0.163		0.107	a, λ	0.250	a, ω
	3 MPa	0.152		0.120		0.152	a	0.126	b
	12 MPa	0.128		0.115		0.103	a	0.131	b
LVDT Stiffness (N/mm)	**0 MPa**	71.47	C	71.95	C	68.30	C b	69.47	b
	3 MPa	89.65	A	91.27	A	88.19	A a	75.77	a
	12 MPa	77.82	B	81.20	B	86.21	B a,b	73.46	a,b
LVDT Elastic Modulus (MPa)	**0 MPa**	1129.9		1164.8		1130.4		1199.6	
	3 MPa	1106.8		1206.8		1160.9		1231.8	
	12 MPa	1060.1		1116.4		1141.1		1079.4	
VSA Stiffness (N/mm)	**0 MPa**	223.4	Ω, Λ	265.9	Ω	198.9	Λ ω	161.9	λ
	3 MPa	227.1	Ω, Λ	243.4	Ω	181.9	Λ ω	153.6	λ
	12 MPa	184.9	Ω, Λ	212.0	Ω	186.8	Λ ω	149.0	λ
VSA Elastic Modulus (MPa)	**0 MPa**	1479.5	Λ	1661.1	Ω	1362.5	Λ	1080.7	
	3 MPa	1324.8	Λ	1661.4	Ω	1287.3	Λ	1498.2	
	12 MPa	1348.2	Λ	1460.2	Ω	1376.4	Λ	1227.4	
Energy (mJ)	**0 MPa**	740.9	A	684.0	A	601.0	A	591.8	
	3 MPa	641.0	A	700.5	A	677.6	A	626.6	
	12 MPa	574.2	B	534.4	B	471.7	B	521.5	
Energy Density (mJ/mm3)	**0 MPa**	10.14	A	10.93	A	9.80	A a	9.18	a
	3 MPa	10.44	A	10.92	A	10.14	A a	11.26	a
	12 MPa	9.05	B	7.58	B	6.76	B a	7.45	b

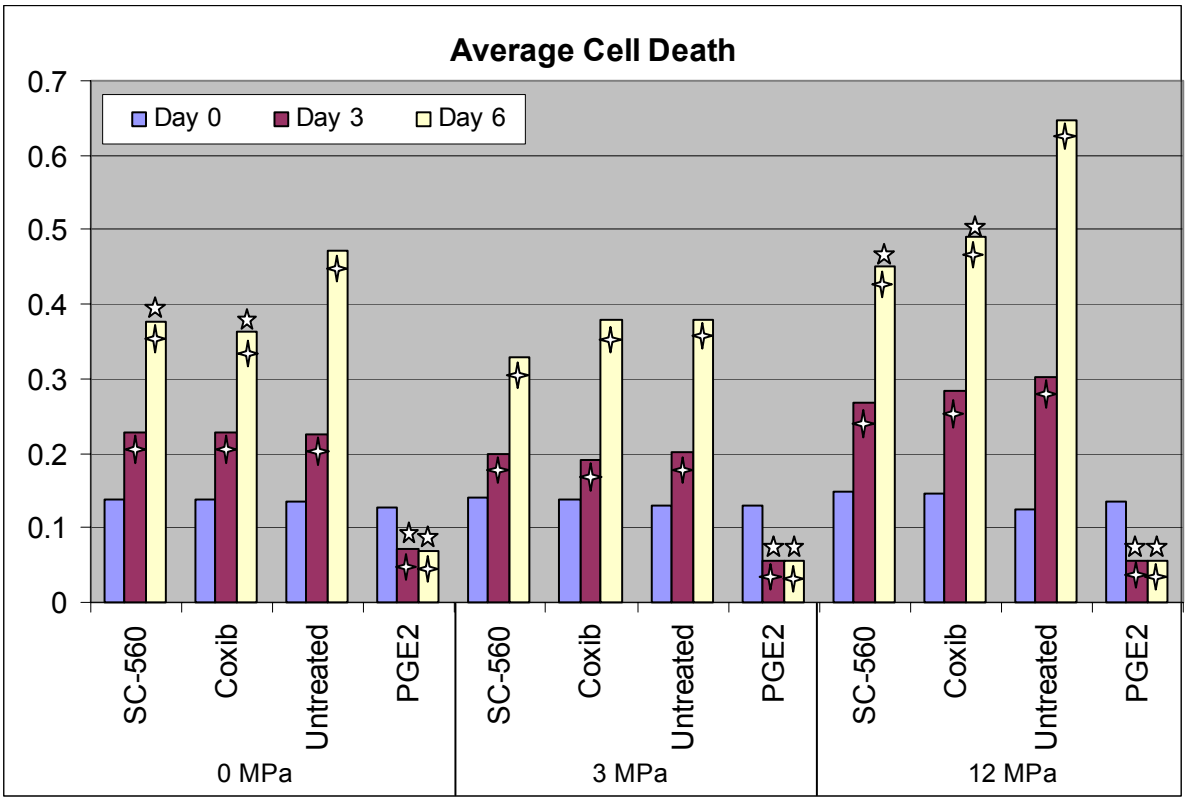

Figure 23: Cytotoxicity, as measured by LDH levels in media. Five point stars represent difference from control at that load level, while four point stars represent difference from basal values within group ($P<0.05$). Celebrex and SC-560 tended to decrease the load based cytoxicity increase, while exogenous PGE_2 eliminated it to sub-basal levels.

8.4.4 Cell Death and Viability Analysis: Exogenous PGE_2 significantly reduced cell death at all loading levels, while all untreated groups displayed a significant increase relative to basal levels (Figure 23). Celebrex and SC-560 significantly reduced cell death relative to untreated groups at 0 and 12 MPa. No differences were seen between the COX inhibitors and untreated groups at 3 MPa. Across days, all COX inhibitor and untreated groups increased significantly ($6 > 3 > 0$) while PGE_2 decreased ($0 > 3, 6$). A load-magnitude effect was also present as the 12 MPa groups were significantly greater than their

3 MPa counterparts. No such load-magnitude effects were seen in the exogenous PGE_2 treated groups.

Cell viability analysis demonstrated no significant difference between treatments, but a trend was observed (P = .091) for the Celebrex group to have more live cells than the untreated group (Table 6). A load-magnitude based response was seen, as the 12 MPa and 3 MPa groups displayed significantly less live cells than the unloaded groups.

Table 6: Composition data from tissue segments. Note COX inhibitors (A, B; Ω, Λ) and PGE_2 (a, b; λ, ω) were each analyzed separately against the untreated group (PGE_2 and COX inhibitors cannot be directly compared). Latin letters denote load-magnitude difference, while Greek letters denote treatment differences. Groups that do not share a common letter are significantly different.

Cell Viability (absorbance/mg)			
Treatment	**0 MPa**	**3 MPa**	**12 MPa**
SC-560	137.1 ± 9.50 ^{A}	120.4 ± 42.4 ^{B}	122.0 ± 26.0 ^{B}
Celebrex	157.2 ± 12.5 ^{A}	140.2 ± 33.2 ^{B}	127.2 ± 32.6 ^{B}
Untreated	141.6 ± 6.66 ^{A}	110.7 ± 34.8 ^{B}	107.7 ± 25.4 ^{B}
PGE_2	139.5 ± 11.4	146.4 ± 21.1	123.7 ± 15.6
Collagen Content (ug/mg)			
Treatment	**0 MPa**	**3 MPa**	**12 MPa**
SC-560	45.96 ± 2.71	42.64 ± 4.00	51.35 ± 3.79
Celebrex	40.38 ± 13.2	47.95 ± 4.18	49.23 ± 6.38
Untreated	53.04 ± 5.59 $^{a^*\lambda^*}$	45.29 ± 5.86 $^{b^*\lambda^*}$	49.13 ± 5.46 $^{b^*\lambda^*}$
PGE_2	49.11 ± 3.25 $^{a^*\omega^*}$	42.49 ± 2.94 $^{b^*\omega^*}$	43.46 ± 4.12 $^{b^*\omega^*}$
sGAG Content (ug/mg)			
Treatment	**0 MPa**	**3 MPa**	**12 MPa**
SC-560	2.42 ± 0.89 ^{B}	2.74 ± 0.79 ^{A,B}	3.02 ± 0.66 ^{A}
Celebrex	1.81 ± 0.86 ^{B}	2.14 ± 0.80 ^{A,B}	3.13 ± 0.74 ^{A}
Untreated	2.55 ± 0.45 $^{B;b,\lambda}$	2.88 ± 0.37 $^{A,B;a,b,\lambda}$	2.88 ± 0.47 $^{A;a,\lambda}$
PGE_2	1.26 ± 0.61 $^{b,\omega}$	2.22 ± 0.70 $^{b,\omega}$	3.20 ± 0.45 $^{b,\omega}$

8.4.5 Collagen and Collagenase Analysis: Total collagenase levels increased nearly 3 fold in the exogenous PGE_2 treated group compared to the untreated group at 0 MPa (Figure 24). A slight, but significant increase was also noted in the PGE_2 group relative to the untreated at 3 MPa, while a slight significant decrease was observed at 12 MPa relative to untreated. Total collagenase levels were not affected by COX inhibitor treatment at any load level. For total collagenase, a main effect of loading duration was present as levels increased from day zero to six, independent of load level. At day six a main effect due to loading magnitude, independent of treatment, was present as the 0 MPa groups were significantly greater than the 3 and 12 MPa groups.

For active collagenase, a main effect due to load level was also seen, but in this case the 12 MPa groups produced more collagenase than the 0 MPa groups (Figure 24). Active collagenase increased significantly in the 12 MPa untreated and PGE_2 treated groups relative to basal levels. This increase was significantly reduced by both COX inhibitor groups. Hydroxyproline content analysis showed no treatment or loading effects in collagen content for COX inhibitors (Table 6). PGE_2 treated groups displayed a trend ($P=0.086$) for a main effect of a decrease in collagen content, independent of loading level.

8.4.6 sGAG Analysis: PGE_2 treated groups displayed a main treatment effect for a reduction in sGAG content relative to the untreated groups, independent of loading level (Table 6). A main effect of load level to increase sGAG content was also noted (12 > 0 MPa) for COX inhibitors, exogenous PGE_2 and untreated groups.

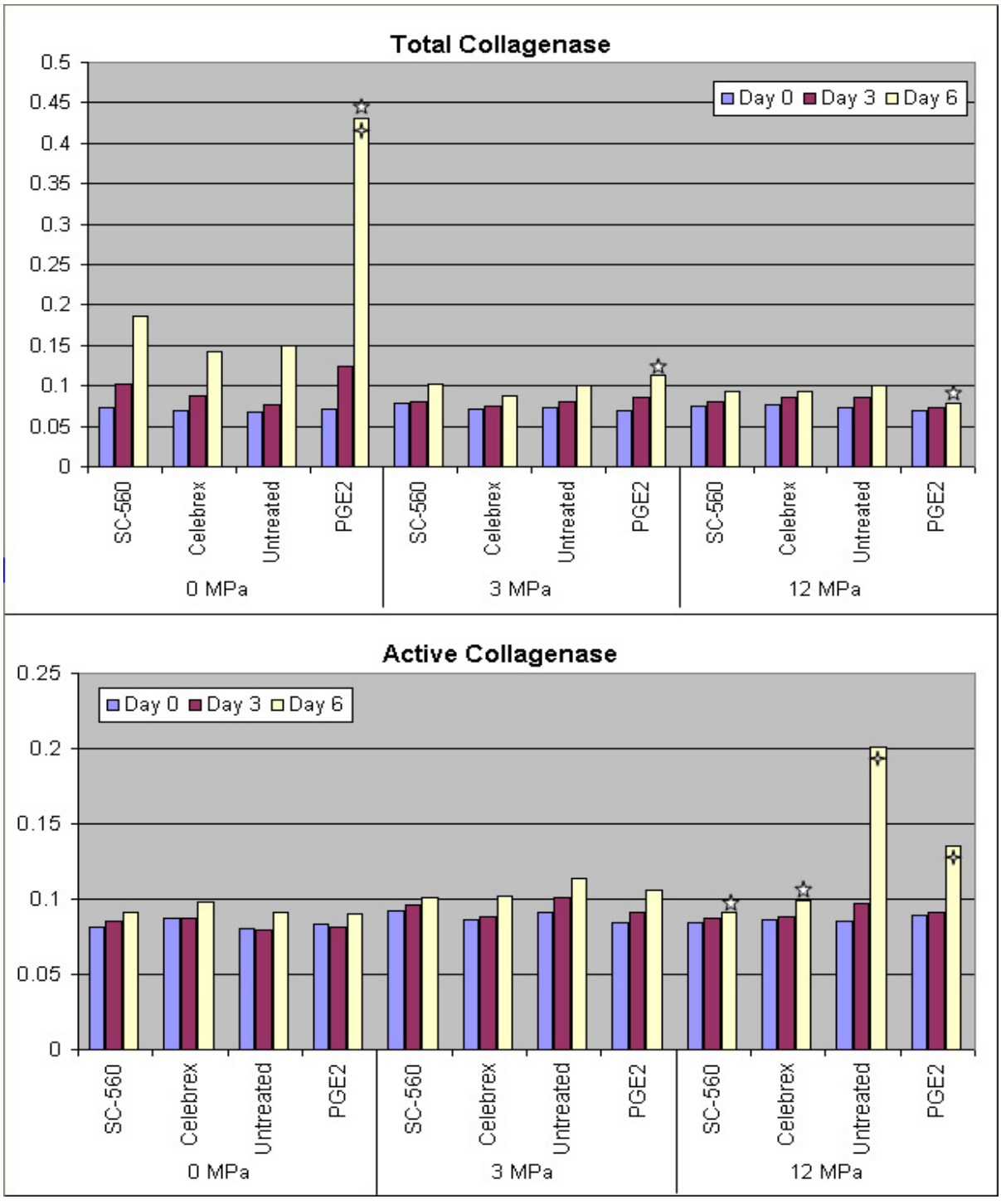

Figure 24: Total collagenase levels measured from media samples treated with AMPA show an increase in unloaded groups and a significant PGE_2 effect. Active collagenase measured from media samples without APMA show highly loaded groups to increase in active collagenase.

8.5 Discussion:

Overuse injuries comprise a significant portion of all orthopaedic injuries. Though the etiology is unknown, a common treatment modality remains pharmaceuticals such as NSAIDs. NSAIDs inhibition of prostaglandin synthesis is believed to be the method of action and is thought to be beneficial to the healing tendon. Because prostaglandins are produced in response to loading, repetitive loading has been hypothesized to causes chronic overproduction that could lead to overuse injury. Selective COX-1 and COX-2 inhibitors (as well as positive and negative controls of exogenous PGE_2 and untreated groups) were administered with our overuse tendinopathy model to examine the influence of prostaglandins on the biochemical and biomechanical response of tendon. The loading levels selected (0 MPa, 3 MPa and 12 MPa) correspond to atrophic, normal loading and fatigue inducing levels of loading, respectively.

Cyclically loaded tendons exhibited a load-induced increase in PGE_2 that was selectively inhibited by both COX-1 and COX-2 specific NSAIDs. Exogenous PGE_2 levels measured 10x that of endogenously created PGE_2 in the untreated groups. This PGE_2 concentration was chosen to balance our culture system, which maintains a 1/500 tissue to media volume ratio, while most cell culture studies maintain ratios near 1/50. A trend for an increase of PGE_2 with load-magnitude was observed in untreated groups. This response has not been seen with tendon tissue, but is consistent with past cell culture studies of tendon and ligament fibroblasts (Yamaguchi *et al.* 1994; Wang *et al.* 2003). The COX-1 inhibitor SC-560, was able to reduce the PGE_2 increase at the 3 and 12 MPa loading levels relative to the untreated groups, but not in the unloaded condition. Because the unloaded, untreated group exhibited a PGE_2 increase, and the SC-560 could not inhibit this, COX-2 would seem be the

dominant isoform for PGE_2 production in this scenario, the atrophic condition. Whether this inducible COX-2 pathway is driven by the lack of load or some other signal is not determinable from this study. However, as hypothesized, Celebrex exhibited inhibitory effects for load induced PGE_2 increase, as well as for the unloaded condition. Celebrex was also found to be dose dependant with the higher concentration showing a strong trend to decrease PGE_2 levels below initial basal levels in the proposed damaging (12 MPa) and non-damaging (3 MPa) groups.

The hypothesized role of PGE_2 treatment to cause the greatest decline in property changes was confirmed by failure analysis. PGE_2 treatment, in the 0 MPa group, caused a significant increase in the tissue strain at failure. This increase in strain will result in the reduction of the tendon's force generation capability, potentially leading to further atrophy and remodeling. This lengthening also caused PGE_2 treatment to make tendons less stiff. Stiffness loss alters a tendon's ability to handle load, and is often used as an indicator of tissue injury. Conversely, the Celebrex treated groups caused an increase in midsubstance stiffness and elastic modulus, compared to the unloaded controls independent of load level. This effect has potential therapeutic benefit in clinically strained tendons, as PGE_2 is strain inducible and known for causing soft tissue laxity (Shi *et al.* 2000), coxib treatment may directly alleviate this.

Though failure stiffness, elastic modulus and strain were affected by treatment, maximum stress was not. The proposed damaging group (12 MPa) exhibited significant decreases in maximum stress, energy and energy density. Statistical analysis showed this to be dependant on loading magnitude level and independent of treatment, implying the damage is a result of mainly material fatigue due to accumulated fibril damage.

Material fatigue was also evident in the strain accumulation during loading. The dominant parameter was load-magnitude as a main effect for load existed. Significant strain accumulated at 12 MPa, and this was reducible by SC-560 treatment unlike in the failure material properties where Celebrex reduced this increase. Strain accumulation at 12 MPa was greatest with PGE_2 treatment, and at 3 MPa, the only group with strain accumulation was the PGE_2 treated group. PGE_2 has been shown to increase the extensibity of the cervix (Shi *et al.* 2000) and the failure testing showed consistent findings of increased strain and decreased stiffness.

PGE_2 treatment also increased total collagenase levels significantly (3x) versus the untreated levels within the unloaded groups. Increase in MMP-1 expression of unloaded tendons (Lavagnino *et al.* 2003) has been previously observed and could partly explain the onset of tendinosis in sedentary people. Total collagenase also exhibited a tendency for a load-magnitude based reduction, which has been seen in past studies (Lavagnino *et al.* 2003). This stocking of collagenase is likely a remodeling response, however, because no differences were noted in active collagenase in the unloaded condition, further signals or factors are likely required before changes that may lead to tendinopathy occur. The opposite effect, a load dependant increase was seen for active collagenase as the 12 MPa PGE_2 and untreated groups were significantly greater than their 3 and 0 MPa counterparts. This increase in active collagenase was inhibitable by both COX inhibitors. Whether this inhibition is positive or negative is debatable because though the collagenase can damage the matrix, they are required to remodel damaged matrix. With tendinosis, before or during the onset of injury it is likely beneficial to inhibit collagenase, but later in the remodeling and repair phase it is likely detrimental. This is supported by collagen content data which

exhibited a trend for a load based collagen content decrease, with a trend for the treatment effect of PGE_2 to decrease collagen content. This likely contributed to the increased strain and decreased stiffness in the PGE_2 groups.

Consistent with clinical tendinopathy, a load magnitude based sGAG increase was observed. This increase is thought to be indicative of remodeling associated with tendinopathy. However, PGE_2 treatment decreased sGAG content (independent of load level) relative to the untreated group. This is contradictory to the clinical situation as well as previous studies of cox inhibitors (Riley *et al.* 2001). GAG loss was likely a physical result of the collagen decrease (trend) with PGE_2 treatment.

Cyclically loaded tendons exhibited a high magnitude and time dependant increase in cell death, which is consistent with past studies (Scott *et al.* 2005). Exogenous PGE_2 treatment completely eliminated this response. This reduction of cell death by PGE_2 is seen in lung, colon and various other cancers and is thought to be through the cessation of apoptosis (Cui *et al.* 2005; Grosch *et al.* 2006). Apoptosis has been suggested to play a role in the hypocellularity noted in tendinosis (Yuan *et al.* 2003). While apoptosis is positive in cancer where there is an over-abundance of cells, it is negative in tendinosis where there is decreased cellularity, implying a positive effect of PGE_2. Paradoxically, COX inhibitor treatment did not have the opposite effect of increased apoptosis. Both SC-560 and Celebrex also reduced cell death (relative to untreated), but since they were effective in reducing PGE_2 production, the COX inhibitors decreased cell death via a different pathway than PGE_2. Past studies have also noted exogenous PGE_2 and COX inhibitor treatments to have the same effect, decreased proliferation (Almekinders *et al.* 1995; Riley *et al.* 2001; Cilli *et al.* 2004; Tsai *et al.* 2004). Though cell death was decreased, an increase in the live cell count (relative

to untreated) was not noted with any treatment, implying a reduction in cellular turnover by these treatments.

In conclusion, our study indicates potentially significant roles for PGE_2 and COX inhibitors in the development of overuse tendinopathy. COX inhibitors maintained tissue composition and properties as evident by a lack of change in collagen, sGAG, ultimate stress and energy density. This was likely accomplished through reduced cellular responses such as collagenase and cell death. Cellular responses are strain dependant, and the COX inhibitor effect of increased stiffness and modulus resulted in decreased strains, and thus decreased responses. Both COX inhibitors had the same trends, but Celebrex was generally more effective. Conversely, the PGE_2 treatment resulted in decreased stiffness, and trends for decreased collagen and sGAG content, likely due to increased strains and collagenase. The underlying pathology leading to the stiffness change could be due to increased/decreased cross-linking, and warrants further investigation. However, the changes noted in this study could lead a healthy tendon to overuse tendinopathy if allowed to progress. These changes are accelerated by PGE_2, but reducible by COX inhibitors, mainly COX-2, suggesting a potentially therapeutic role against the development of overuse tendinopathy.

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CHAPTER 9

SUMMATION

Overuse tendinopathy remains a significant clinical concern, yet the etiology remains unclear. Most research on tendinopathy continues to focus on the healing response of tendon, usually following acute injury. While clinically relevant and potentially able to offer treatment options, the pathology of the condition cannot be fully addressed without better understanding the basic tendon material properties, then systematically examining the effects of loading, then repetitive loading on tendon to isolate the causative factors. The following studies took such an approach by first determining the absence of an elastic strain limit, then creating a tendon explant model which allows for short term overuse studies. Several load induced biochemical expressions that reduce composition and mechanical properties were isolated. A factor of particular interest proved to be PGE_2, which appears linked to several factors that combine to cause property losses resembling overuse tendinopathy. COX inhibitors proved effective at reducing the PGE_2 linked property loss, suggesting COX inhibitors as potential therapeutic measures to impede PGE_2 from driving repetitively loaded tendons into the tendinopathy state.

In an effort to reexamine a basic material property of tendon, the elastic strain limit, tendons were loaded to various subfailure strains, and then loaded to failure. Surprisingly, even the highest subfailure strains tested (90% of failure) did not differ from any other subfailure strain level (1%) in terms of material properties (Figure 4, Figure 5, Table 1).

These results are likely due to methodology of cryo-grips which reduce stress concentration at edges of grips that employ only pressure for clamping. Cryo-gripping resulted in failure within the midsubstance for over 98% of tendons tested, while previous studies in this lab saw 95% failure at grip edges. These findings are supported by the study of bone-ligament-bone complexes (which do not require mechanical gripping of the soft tissue) which saw no effects of subfailure loading up to 80% of failure strain (Panjabi *et al.* 1996).

These findings suggest tendons are not directly damaged by a single subfailure tensile loading. Because tendons are rarely loaded near failure levels, an alternate pathway, mediated by cellular responses must be responsible for a significant portion of the damage. Furthermore, because minimal fibrillar damage occurred, cellular responses are likely predominately load-induced and not damage induced. Therefore, if these damage inducing cellular responses are identified, pharmaceutical treatments may be effective in preventing cyclically loaded tendons from developing into tendinopathy.

To determine the cellular responses directly responsible for driving an otherwise healthy tendon into a degenerative state, overuse studies are required. However, before true overuse studies are conducted, studies of the basic response of tendon to cyclic load must be examined. For this reason, an explant model was developed and its performance was characterized. The explant model consists of two hexagonal, load controlled tissue loading devices and a video strain analysis system. The loading device is superior to existing devices in its ability to maintain tissue viability, cause fatigue damage and operate in load control. The video strain analysis component of the model is superior to existing systems in its high speed of operation, reduced cost and programmability.

The VSA system is able to quantify the fatigue damage produced by the loading device in terms of strain accumulation. The necessity of this feature cannot be overstated, especially considering the method of loading. Our tests confirmed significantly increased strain accumulation across the grips, versus across just the midsubstance. This increase is a result of slippage and stress concentrating effects from clamping, the same effects that caused the elastic limit of tendons to be initially reported at 4%. Furthermore, were the tissues loaded in strain control, as many existing devices do, this damage and slippage would result in decreased loads across the tissue. However, because compressive damage is occurring at the grip edge, cellular responses initiated by damaged cells must be considered. Tests of control tendons (unloaded) have been performed with and without gripping, and shown no significant differences in material properties, composition or biochemical response.

This damaging explant system nicely compliments in vivo animal models as well as cell culture models. Cell culture studies lack the extracellular matrix and thus cannot determine the effects of the cellular responses while in vivo models are inherently difficult to control. In order to directly, and independently assess the effects of the various parameters of loading on tendon tissue, explant models are required. The current model, being able to cause and monitor fatigue damage while maintaining tissue viability, seemed capable of producing the beginnings of overuse tendinopathy, but needed confirmation.

Overuse tendinopathy is a result of damage induced by cyclic loading and fibroblast response. However, how the cellular response to this cyclical load contributes to or lessens the fatigue-based decline in tissue properties, that is, their interaction is less clear. To establish the usefulness of our system in overuse studies, both of these factors, and their interaction needed to be established. To assess this, a loading design consisting of a set

number of cycles of load, distributed across varying periods was undertaken. The numbers of cycles selected (86,400) corresponds to the number of seconds in one day. These cycles were either administered consecutively for 24 hours, or divided into twelve equal portions (2 hours each) and distributed over twelve days. The twelve days was arbitrarily selected to allow sufficient time for cellular response to be induced.

The model was successful in inducing material fatigue, as evident by the decreased material properties (failure stress, failure strain, elastic modulus). Fibroblasts also expressed many responses with these loadings, among them collagenase, PGE_2, sGAG and cell death. In general, these responses were magnified when applied across the longer duration, and their interaction was statistically significant. When changes are observed across time, it is important to establish that they were not solely a function of time in tissue culture. This was evident in most responses which, though increased over time, also increased by loading magnitude. Though many cellular responses were observed, the compositional changes were minimal, reiterating overuse tendinopathy as a long term degenerative condition. However, load based changes in sGAG was observed indicating that changes were likely imminent, if not already in progress. The proteoglycans that GAGs attach to are involved in, among other thing, matrix reorganization.

Overall, fibroblast and load based changes were observed in cyclically loaded tendon explants with our new model. However, the precise pathway by which the damage occurs is not clear. Whether load induces cellular responses which then damage the tissue, or load causes damage which then induces cellular responses needed further clarification. A great effect for the cellular responses to cause damage was seen as the longer duration of the normal loading level caused marked cellular responses and material property damage relative

to the same loading applied across one day. Also load induced damage was noted as the aggressively loaded groups caused the same amount of mechanical damage, regardless of the duration across which the loadings were applied. Though clinical tendinopathy wasn't developed, changes that would likely lead to tendinopathy if allowed to progress are present.

A cellular response that likely plays a significant role in driving a cyclically loaded tendon into overuse tendinopathy is PGE_2. PGE_2 is seen in bone to play a significant role in the modulation of osteoblasts and osteoclasts formation, thereby affecting bone formation and resorption. Though inflammatory cells are not found in tendinopathy, inflammatory mediators such as PGE_2 are frequently observed. PGE_2 is load inducible in vivo through microdialysis and animal exercise, in seeded fibroblasts, and now in animal explants. Though it is not directly ascertainable from our studies what cells caused this response, studies of tendon fibroblasts in culture suggest their role. PGE_2 has also been shown to be load inducible in a dose dependant manner, cause localized degeneration as well as affect cellular proliferation and collagen synthesis, implicating its likely role in tendinopathy. In other tissue such as bone, these inflammatory mediators play a key role in general remodeling, as well as remodeling after damage however, their role in tendon has not been established.

We found tendon explants to exhibit a dose and duration dependant increase in PGE_2. Though not directly comparable to the results of cell culture studies, the levels of PGE_2 measured due to loading in our model are consistent with in vivo micro-dialysis studies of human tissue. Also, PGE_2 is load-magnitude dependant, as the higher loading levels produce more PGE_2 than the low and unloaded groups. PGE_2 also may play a role in modulating other responses seen as, collagenase, cell death and collagen content have all been previously

linked to PGE_2 or its inhibition. To establish these links directly, and simultaneously, in our model would help confirm PGE_2's role in tendinopathy.

To help determine PGE_2's role in tendinopathy, treatments of COX inhibitors and PGE_2 were administered to our existing model. The model consisted of three loading levels (0 MPa, 3 MPa and 12 MPa) which correspond to unloaded/atrophic condition, normal use condition and overuse condition, respectively. The findings suggest that tendon may become damaged in the unloaded and overuse condition, but not the regular use condition.

After six days of loading, as expected, load inducible PGE_2 as well as various other factors were expressed in high quantities. The COX inhibitors were able to reduce this PGE_2 increase in most cases and the exogenous PGE_2 produced the greatest negative changes in the tendon. COX inhibitors maintained tissue composition and properties as evident by a lack of change in collagen, sGAG, ultimate stress and energy density. This was likely accomplished through reduced cellular responses such as collagenase and cell death. Cellular responses are strain dependant, and the COX inhibitor effect of increased stiffness and modulus resulted in decreased strains, and thus decreased responses. Both COX inhibitors had the same trends, but Celebrex was generally more effective. Conversely, the PGE_2 treatment resulted in decreased stiffness, and trends for decreased collagen and sGAG content, likely due to increased strains and collagenase. The underlying pathology leading to the stiffness change could be due to increased/decreased cross-linking, and warrants further investigation. However, the changes noted in this study could lead a healthy tendon to overuse tendinopathy if allowed to progress.

The significant role of PGE_2 in tendinopathy is further highlighted when correlated to the other responses. Our earlier examinations saw the response to PGE_2 to be identical to the

collagenase and cell death response, and similar to the sGAG response. This does not provide a direct link between the responses because they could all be load inducible in the same matter, however, their reduction with COX inhibitors and increase with exogenous PGE_2 suggests that PGE_2 plays a significant role in their expression. However, not all PGE_2 and COX responses were antagonistic, which suggests that COX inhibitors and PGE_2 may act by different pathways to perform the same task (such as reducing cell death).

The importance of our load controlled device is evident here as these response to PGE_2 would not be seen in other position controlled devices. With the decreased stiffness, if in strain control, the loading would decrease and thus the responses would be different. With our model, where the loading was constant, these changes were accelerated by PGE_2, but reducible by COX inhibitors, mainly COX-2, suggesting a potentially therapeutic role against the development of overuse tendinopathy.

The findings and conclusions provided are applicable under the assumption that the changes will continue to progress long term, under similar conditions. However, if they are not found to continue, then the actions of PGE_2 can be observed as benificial. Mechanical properties were expected to diminish significantly in the PGE_2 treated groups, especially the ultimate strength. However, only certain properties decreased (strain, stiffness, modulus) while failure strength and energy density did not. This translated into lengthening of the tissue, not property loss and was supported by the lack of collagen content change. This is beneficial to tendon as it reduces overall load and thus reduces the possibility of failure. Decreases in GAG content of the PGE_2 group demonstrate that this is likely not tendinosis, where GAGs increase. Also, PGE_2 reduces apoptosis rates which would be helpful in the tendinosis state where reduced cell numbers are seen. Also, with no change in total cell

count, reduction of cellular turnover would be quite beneficial if tendon behaves like bone. These findings could suggest that PGE_2 is actually beneficial in tendinopathy. PGE_2 did modulate collagenase, but again only for total collagenase and not active collagenase. Finally both existing studies of biomechanical properties for PGE_2 in whole tissue have shown positive roles for PGE_2 as an injection study found increased structural properties (Ferry, et al unpublished) and a second study saw no decrease in strength, but a trend (P=.06) for increased strain in with an internal pump of PGE_2 (Wilkes, el al unpublished). Overall, if PGE_2 only lengthened tendons as these data might suggest, PGE_2 would be beneficial in preventing damage.

From this work many future studies are possible. The device can be utilized to perform basic material properties of tendon that have not been done in such a model. They include basic creep, dynamic creep, fatigue as well as similar tests to determine the effects of frequency. Expansions of the existing tests could also prove beneficial as histology and specific analysis of MMPs and GAG were not performed. To induce tendinopathy however, more damage must be created. This could be achieved by either introducing more factors like PGE_2 that could drive tendon to tendinopathy (collagenase, cytokines, etc) or by artificially creating injury. This injury could be created by compression, hypo-thermally or through an incision. Once clinical tendinopathy type injury in created, a series of rehabilitation studies can be performed that test all the mechanical characteristics of loading (magnitude, frequency, duration, rest period, etc.) with and without therapeutic intervention (COX inhibitors, PGE_2, etc.) to determine the best course of action for recovery.

APPENDICES

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APPENDIX 1

Loading Control Program

The tissue loading device is controlled by the following waveform generation program (code not shown) written in LavView (Figure 25). Six individual regimens (three per channel) can be programmed manually or selected from preset waveforms. The regimens can be repeated an indefinite number of times at a set interval. All inputs are in MPa, calculated from a mean cross sectional area (default 1.713).

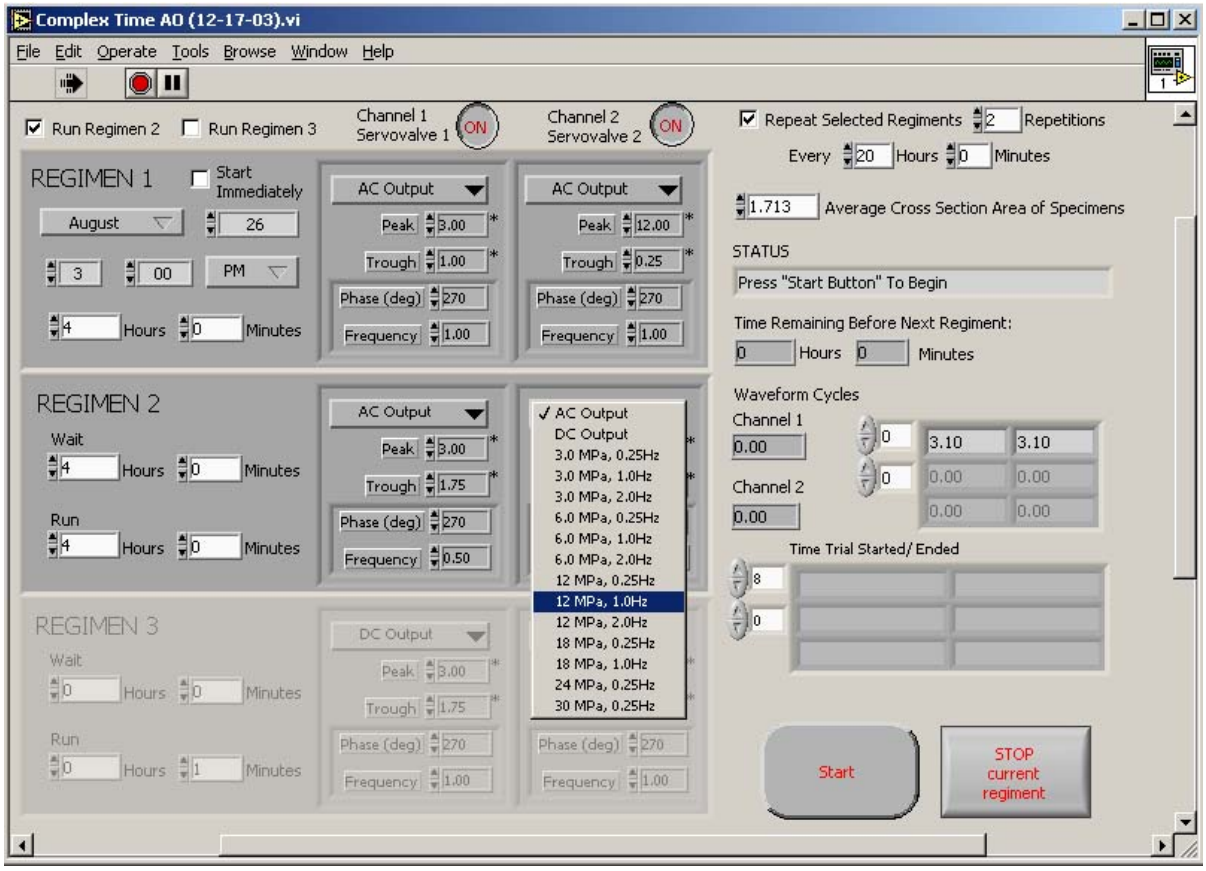

Figure 25: Sample window of waveform generation program.

APPENDIX 2

Video Strain Analysis Program

The real-time video strain analysis (VSA) is performed with the following program (code not shown) written in LabView. The procedure is outlined below.

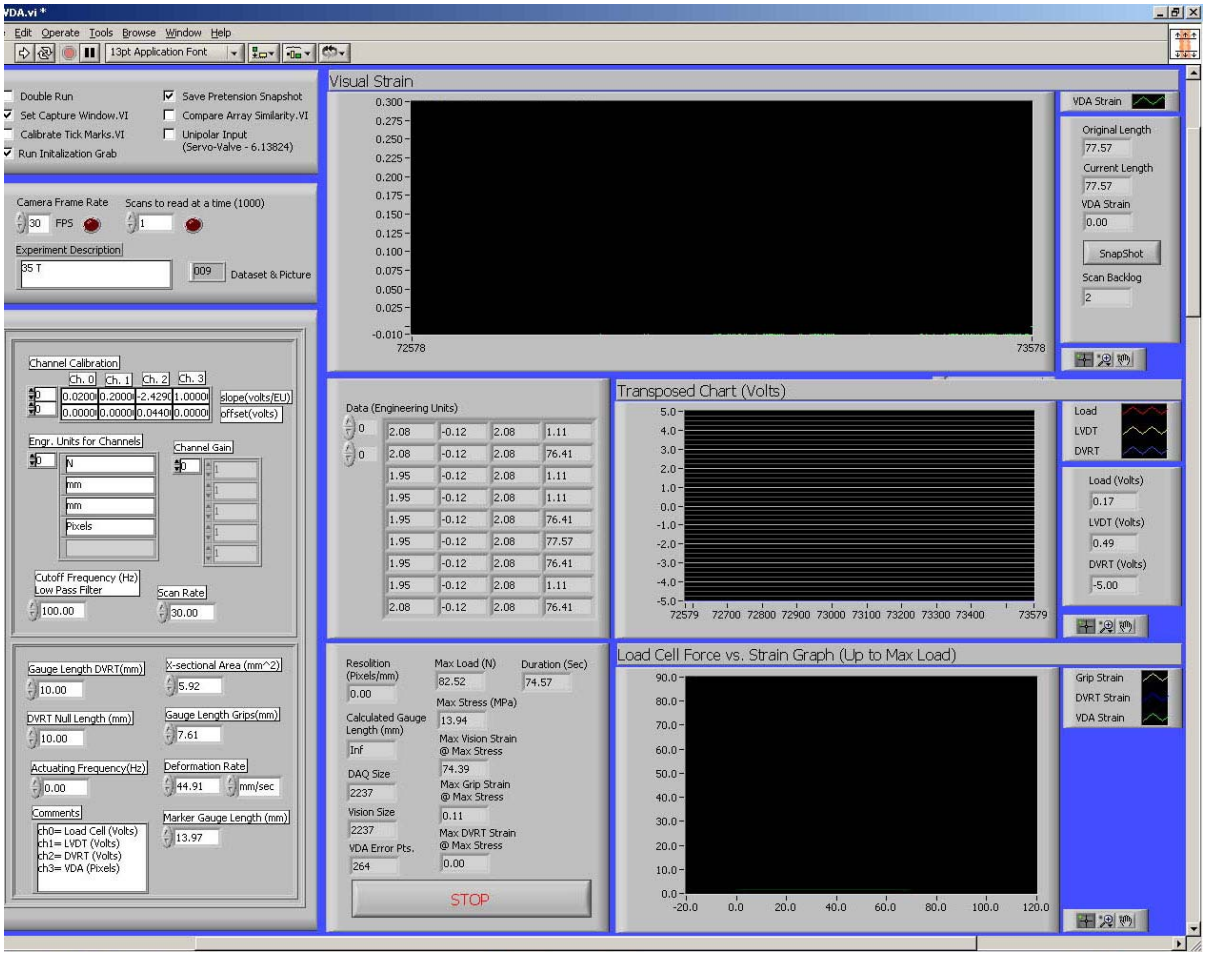

Figure 26: VSA Step 1 - Define channels and input specimen information.

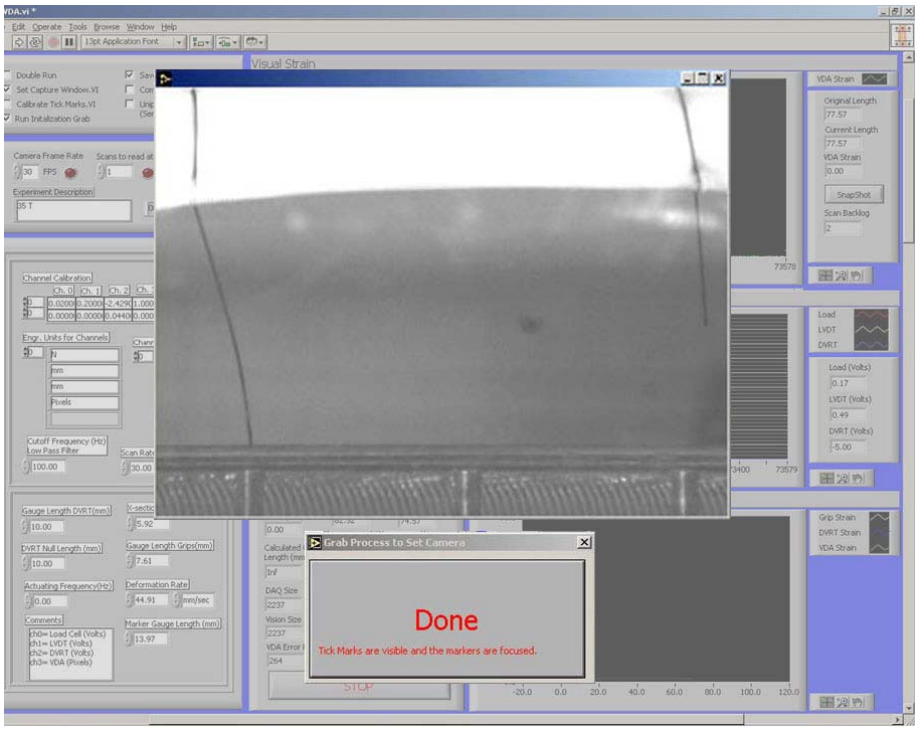

Figure 27: VSA Step 2 – Position camera orthogonally such that both markers (including their intended path) and calibration marks are visible.

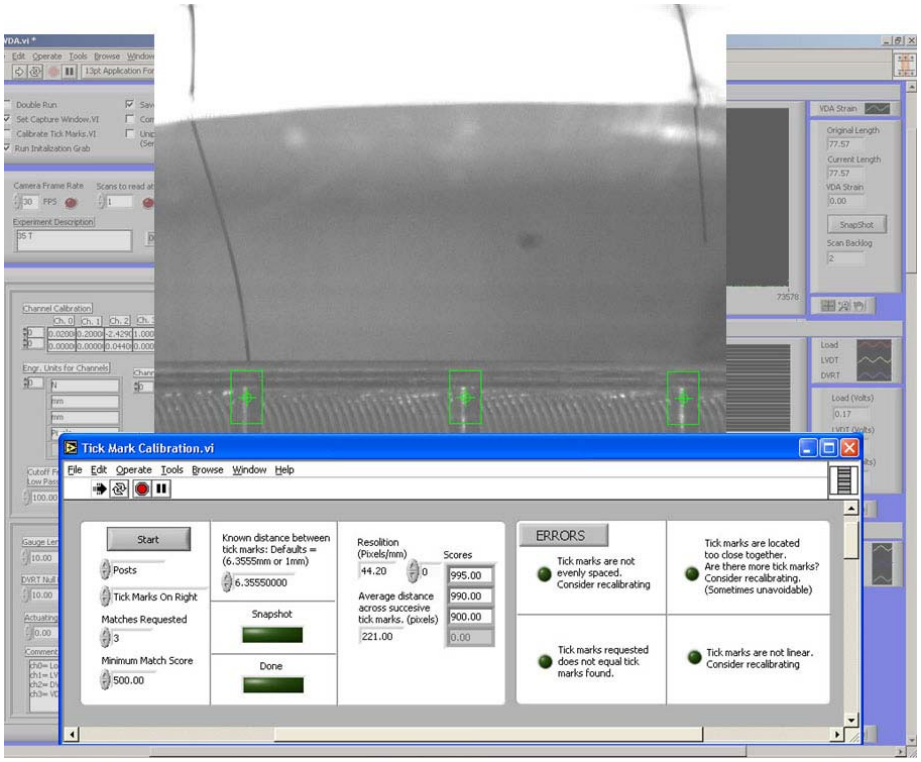

Figure 28: VSA Step 3 – Select number of tick marks on the screen of a known distance and allow program to locate them to calibrate the system.

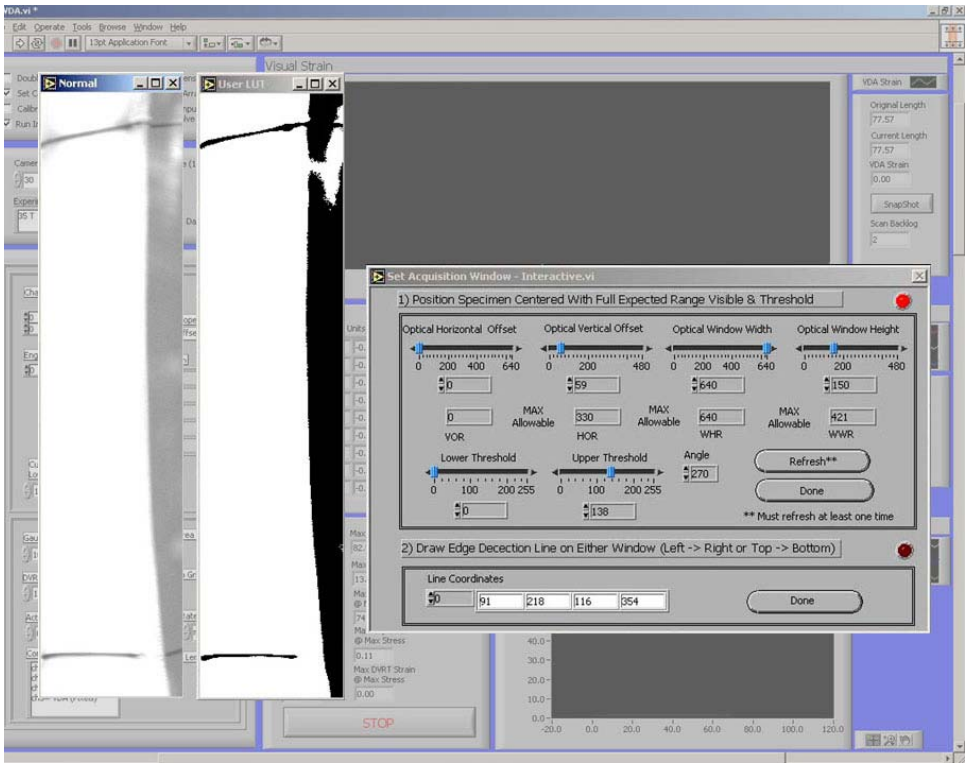

Figure 29: VSA Step 4 – Select threshold parameter such that the original image (on left) is converted to binary image (on right) with markers clearly visible and no extra noise.

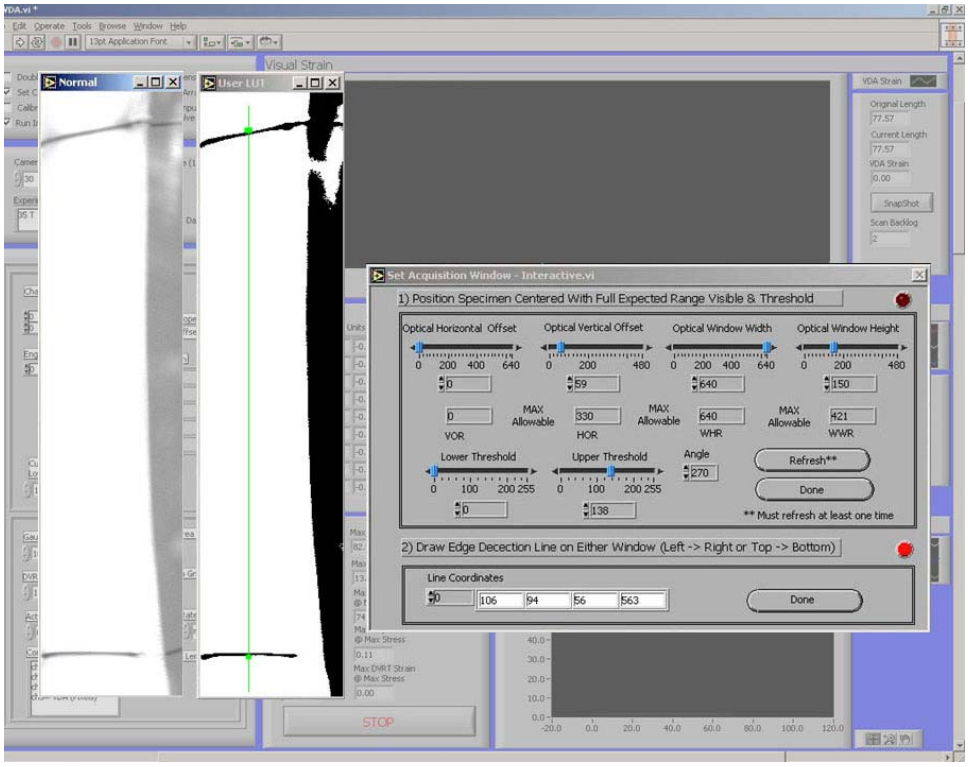

Figure 30: VSA Step 5 – Draw line of action across which to measure strain (first and last marks). Make sure to include full range of motion of markers.

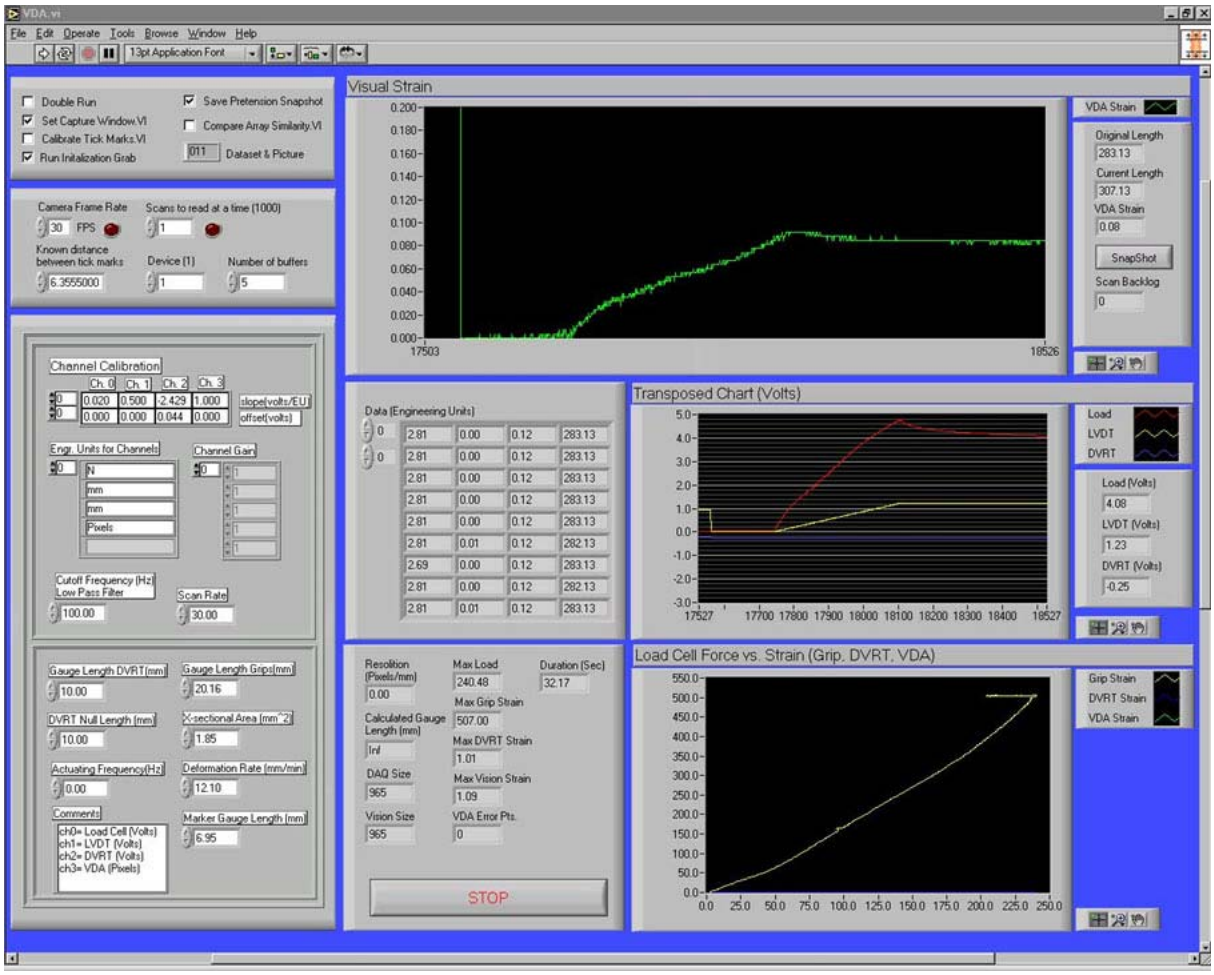

Figure 31: Run Test – Shown is a typical result after failure testing.

APPENDIX 3

Biochemistry

The biochemistry portion of this project consisted of the analysis of the media by two commercial analysis kits, as well as a third analysis procedure derived from literature. Furthermore, select tendons were cut with a special tool to gather sections used for either digestion or MTT analysis. This tool provides an average of 7.11mm sections when cut with a slight bit of pretension. For the future, it is suggested to shorten this section width by decreasing the size of the aluminum block between the scalpel blades so more sections only from the midsubstance may be utilized.

APPENDIX 4

MTT Cell Viability Assay

Immediately following loading, select tendons were sectioned (Appendix 6.0). One of these sections was analyzed for viability by a (MTT) protocol. The protocol used (Flick, 2005) was developed by a former student Jason Flick, and is a modification of a common viability assay (Klein *et al.* 1996). The chemicals used, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide) and 2-methoxyethanol were purchased from Sigma (St Louis, MO), while the PBS (Phosphate Buffered Saline) and DMEM-H (Dulbecco's Modified Eagle Medium, High Glucose) was manufactured by Invitrogen (Carlsbad, California) and purchased from the local tissue culture facility. The following is the protocol used:

MTT Protocol

Preparation:

1. Weigh 50mg of MTT wrapped in aluminum foil.
2. The 50mg of MTT will be transferred to the Tissue Culture hood where it will be dissolved in 10ml of sterile PBS in a sterile 15ml centrifuge tube.
3. 1ml aliquots of the 10ml MTT solution will be sterilely transferred to 1.5 ml micro-centrifugation vials and labeled with the concentration (5mg/ml), medium, date, and stored frozen at –20C.

Protocol:

1. Aliquot 900µl of DMEM-H media (37ºC) with no additives into micro-centrifuge tube (1 tube/sample to be analyzed).
2. Add 100µl of MTT stock solution (5mg/ml), for final concentration of 1mg/ml
3. Transfer sectioned sample into tube with MTT solution.
4. Incubate tube with tendon for 4hrs in incubator (37ºC, 5% CO_2)
5. Transfer tendons into tubes with 1ml, 2-methoxyethanol (Keep one extra tube with 2-methoxyethanol only for background)
6. On large orbital shaker, shake tubes (sealed) for 12 hrs at 120rpm
7. Aliquot 200µl x 4 from each tube into 96-well plate
8. Record absorbance in dual mode. 570nm with background (690nm) subtracted.
9. Dry tendon segments in speed-vac (100mTorr) for 2hrs.
10. Final value is average absorbance / dry weight (obs/mg)

APPENDIX 5

Papain Digest

Immediately following loading, select tendons were sectioned (Appendix 6.0). One of these sections was stored frozen (-80ºC). At a later date, the section was digested with Papain from Papaya Latex for compositional analysis. All chemicals are listed below and were purchased from Sigma. The protocol was based on a modified procedure performed previously (Riley *et al.* 1994). The following is the protocol used:

PAPAIN DIGESTION

(Ref.: Riley et al., 1994)

Make phosphate buffer and store at 4 ºC. Each sample requires 250µl, i.e. you can do four samples/ml of buffer. You don't want to store buffer too long b/c of degradation issues. Buffer is 0.1 M phosphate buffer (pH 7) with 10mM Cystine HCL & 2 mM EDTA. It's sampled and brought to 0.5 mg/ml papain, this is the final working buffer which will digest the samples.

Reagents:

1.	Monobasic	$NaH_2PO_4 \cdot H_20$	137.99 g/mol	Powder (Chemical Room)
2.	Dibasic	$NaH_2PO_4 \cdot 7H_20$	286.07 g/mol	Powder (Chemical Room)
3.	L-Cysteine-HCl	$C_3H_7NO_2S \cdot HCl \cdot H_20$	175.63 g/mol	Powder (Chemical Room)
4.	EDTA	$C_{10}H_{14}N_2Na_2O_8$	336.21 g/mol	0.5M Stock (Cold Room)
5.	Papain (Papaya Latex)		21 kDa	Powder (Freezer)

Stock Buffer Preparation:

1. Make (100ml) 0.1M phosphate solutions
    a. 100ml ddH_20 + 1.3799g (Monobasic)
    b. 100ml ddH_20 + 2.8607g (Dibasic)
2. Make buffer base – Final ratio of dibasic/monobasic will be close to 65:35. Add 70ml of dibasic solution and slowly add monobasic solution until pH is 7.0. (Total volume will be slightly > 100. This is not an error.)
3. Add Cysteine HCL directly to make final solution 10mM (0.17564g for 100ml).
4. Add EDTA to make final solution 2mM (400µl (.5M stock) for 100ml).
5. This does not store well (precipitates). Store at 25C < 2 weeks. It's the additives (Cysteine, or EDTA) that precipitates out, so don't add these until ready to use.

Working Buffer Preparation:

1. Prepare 0.5 mg/ml working buffer
2. Vortex well and warm to 37 ºC to completely dissolve Papain
3. Use warm solution when assaying

Protocol:

1. Record dry weight of individual tendons (SpeedVac 4-hrs).
2. Add 250 µl of Papain/Buffer Solution. (Ensure solution is warm before adding).
3. Seal lids of microtubes with Teflon tape and the plastic snaps designed for microtubes. This is to prevent evaporation. (not required if o-ring capped microtubes are used)

4. Digest for 3 hrs. at 65 °C. Use oven under bench in 2332 with test tube shaker/rotator to ensure complete digestion.
5. Periodically (60min), flick the tubes to ensure mixing, as well as to loosen any pellet particle that may be sticking to the tube.
6. Double check for evaporation (monitor volume of digest when finished).
7. May have to add another 250 µl of Papain/Buffer solution (if not digested within 6hrs).
    a. Note calculation changes will be required
8. Aliquot samples into three more tubes (µ-centrifuge w/O-ring cap). Separate aliquots in different boxes and store at -80C.
    a. Hydroxyproline – 40µL in 2.0ml clear o-ring tube
    b. GAG - 20µL in fliptop tube A
       20µL in fliptop tube B
    c. DNA - 40µL in 2.0ml clear o-ring tube
       40µL in 2.0ml clear o-ring tube

APPENDIX 6

sGAG Content Assay

One set of aliquots from the papain digest (stored at -80ºC) was used for sulfated glycosaminioglycan (sGAG) assay. All chemical used are listed below and were purchased from Sigma. The protocol was based on a modified procedure performed previously (Riley *et al.* 1994) based on the original procedure of (Farndale *et al.* 1986).. The following is the protocol used

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Glycosaminoglycan Assay

(Ref.: Riley et al, 1994 from Farndale et al, 1986)

The working solution is a DMMB reagent with the constituents listed below, brought to 1 L total volume with ddH_20. Storage is recommended in a brown (or Al Foil covered) bottle at room temperature. Standards/samples must be assayed immediately after combining with the reagent buffer, as the combination is very unstable.

Reagents:

	Usage	Required (per sample)	Stability
Color Reagent	Make stock	200µl	Few Months (25ºC In dark bottle)
Chondroitin Sulfate	Make fresh Daily	2 ml (per plate)	Few Weeks (4ºC)

Notes:

1. Will do 8 standards (100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0 µg/ml)
2. Will be doing two dilutions of each sample (in duplicate)
3. Can accommodate up to 20 samples per plate (Do not do much more than 10/plate)
4. Timing is very important, but multi-pipetting consistency cannot be compromised
5. Must use multi-pipettor to disperse buffer

Working Solution:

Color Reagent:

a) 16 mg Dimethylmethylene Blue (DMMB)

b) 3.04 g Glycine

c) 2.34 g NaCl

d) 95.0 ml HCl, 0.1 M

* Total Volume = 1.0 L ddH_20

Standard:

1) Stock Solution (0.5 mg / ml)
   - 5mg of Chondroitin Sulfate in 10ml of ddH_20
   - This is to reduce weighing errors
   - Store this at 4ºC
2) Bring stock solution to room temperature
3) Temp Solution (100µg / ml)
   - 2ml of stock solution (vortex first) + 8ml ddH_20

4) Make Standard Dilutions (Serially)

- Fill 1 flip-top µ-centrifuge tube with 1000µl of ddH_20 (1)
- Fill 6 flip-top µ-centrifuge tube with 500µl of ddH_20 (2-7)
- Fill 1 flip-top µ-centrifuge tube with 1000µl of Stock (8)
- Transfer 500µl from stock (8) to next tube (7)
- Shake tube (7) and transfer 500µl to next tube (6)
- Continue until 1000µl is in tube (2)

Standard #	**Concentration**
1	0.00 µg/ml
2	1.5625 µg/ml
3	3.125 µg/ml
4	6.250 µg/ml
5	12.50 µg/ml
6	25.00 µg/ml
7	50.00 µg/ml
8	100.0 µg/ml

Protocol:

1) Set up Plate-reader and make it ready to go
    - Prepare template
    - Prepare protocol (Kinetic 2 reads/min, 30min)
2) Prepare samples
    - Aliquot 20ul of digest samples into each (of 2) flip-top tubes
    - Dilute first samples 1/3 by adding 40ul ddH_20
    - Dilute second samples 1/4 by adding 60ul ddH_20
3) Prepare standards serially as described above
4) Prepare 96-well plate
    - Aliquot (double) 8µl of standards
        i. Start with 0 µg/ml, and end with 100 µg/ml
        ii. Use same pipet-tip
    - Aliquot 8µl of each sample to wells (in duplicate)
5) Add 20ml of DMMB Reagent to multipipetting tray
    - Add 200µl of buffer to each well using multi-pipettor*
6) Take immediately to Plate Reader
7) Read absorbance at (525), but actually 540 b/c that's what we got
    - Will kinetically read 2 reads/minute for 30 minutes

*Timing important, But multipipetting consistency is more important…

So, **MULTIPIPET CAREFULLY AND CONSISTENTLY!!!!!!!**

APPENDIX 7

Hydroxyproline (Collagen Content Assay)

One set of aliquots from the papain digest (stored at -80ºC) was used for the collagen content assay (measuring hydroxyproline). Hydroxyproline is an amino acid that is found primarily in collagen at known proportions. All chemical used are listed below and were purchased from Sigma. The protocol was based on a modified procedure performed previously (Riley *et al.* 1994) based on the original procedure of (Bergman and Loxley 1963). The following is the protocol used

---

Hydroxyproline Assay

(Ref.: Riley et al, 1994 from Bergman & Loxley, 1961)

Reagents:	Usage	Stability
Chloramine T	Make fresh as needed	Few weeks
Na Buffer	Make stock	Indefinite
DAB	Make fresh as needed	Few weeks (in dark bottle)
Isopropanol	Straight from bottle	Indefinite
Hydroxyproline	Make fresh as needed	Few weeks (at 4ºC)

Notes:

1. Will do 8 standards (100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0 µg/ml)
2. Will be doing two dilutions of each sample (in duplicate)
3. Can accommodate up to 20 samples per plate
4. Do not make working solutions in advance

Working Solutions:

1. 1:4 (20/80%) of Chloramine T and Buffer — **Amount Required**

    a) Chloramine T — 0.4 ml + 0.1 ml / sample

        - 7 % w/v Chloramine T in H_20 — 2.4ml max (Per Plate)
        - 7g Chloramine T per 100ml water

    b) Na Buffer

        - 57g Sodium acetate trihydrate
        - 37.5g Trisodium citrate dehydrate
        - 5.5g Citric Acid monohydrate
        - 385ml Isopropanol
        - ≈615ml Water (Bring to 1L Total)

2. 1:3 (25/75%) of DAB and Isopropanol — **Amount Required**

    a) DAB (p-demethylamino-benzaldehyde) — 2.4 ml + 0.6 ml / sample

        - 66.6% w/v DAB in 60% perchloric acid — 14.4ml max (Per Plate)
        - 20g DAB per 30ml of 60% perchloric acid

    b) Isopropanol (Propan-2-ol)

Standard:

1) Temporary Solution (0.5 mg / ml)

    - 5mg of Hydroxyproline in 10ml of ddH_20
    - This is to reduce weighing errors

2) Stock Solution (100µg / ml)

    - 2ml of temporary solution + 8ml ddH_20

3) Make Standard Dilutions (Serially)

- Fill 1 flip-top µ-centrifuge tube with 1000µl of ddH_20 (1)
- Fill 6 flip-top µ-centrifuge tube with 500µl of ddH_20 (2-7)
- Fill 1 flip-top µ-centrifuge tube with 1000µl of Stock (8)
- Transfer 500µl from stock (8) to next tube (7)
- Shake tube and transfer 500µl (7) to next tube (6)
- Continue until 1000µl is in tube (2)

Standard #	**Concentration**
1	0.00 µg/ml
2	1.5625 µg/ml
3	3.125 µg/ml
4	6.25 µg/ml
5	12.5 µg/ml
6	25.0 µg/ml
7	50.0 µg/ml
8	100 µg/ml

Protocol:

1) Aliquot 40µl of digested sample into **Clear 2.0ml tubes** (screw cap) w/**Black O-ring**
2) Hydrolyze sample
   - Add 200µl of 7.2 M HCl (Final conc. = 6.0 M)
   - Screw tightly & Incubate (105ºC) in oven overnight (20hrs)
3) Dry down in Speed-Vac (6 hrs)

4) Reconstitute with 1.0ml H_20 and dry down in Speed-Vac (20 hrs)
    - (This is to reduce acidity of pellet which has liquid stuck under it)
5) Make working solutions (4ml and 16ml max)
6) Reconstitute sample with 1000µl ddH_20 (Stock)
    - Dilute 1/8 ≈ 10 µg/ml 125µl sample + 875 µl ddH_20
    - Dilute 1/4 ≈ 20 µg/ml 125µl sample + 375 µl ddH_20
7) Store Stock at -80ºC
8) Prepare 96-well plate
    - Aliquot (double) 40µl of standards
        i. Start with 0 µg/ml, and end with 100 µg/ml
        ii. Use same pipet-tip
    - Aliquot (double) 40µl of sample (both dilutions)
    - Add 25µl of Working Solution 1
    - Wait 4 min
    - Add 150µl of Working Solution 2
9) Apply parafilm seal and cover plate,
    - Incubate 60ºC for 40min
    - Cool 5 minutes to room temperature
10) Scan at 540nm & 575nm (will use the 540)

APPENDIX 8

PGE_2

One set of media aliquots were analyzed for PGE_2 content by a commercial, competitive enzyme immunoassay kit from Cayman Chemical (Prostaglandin E2 EIA Kit – Monoclonal, Catalog #514010). The manufacturer recommended protocol was utilized with minimal changes. The suggested plate layout was used with each sample measured in two dilutions, each in duplicate. A summary of the procedure is outlined below.

PGE_2 Assay

Cayman Chemical Part # 514010

Pre assay:

1) Make EIA buffer (Ultra-pure water source in MBRB 23)
2) Make wash buffer

Assay:

1) Make two dilutions/samples (Chose from 1/10, 1/30, 1/60, 1/100, 1/200, 1/400)
2) Prepare PGE_2 standard, tracer and antibody
3) Prepare Plate
    a) EIA Buffer – 100µl in NSB wells, 50µl in Bo wells
    b) PGE_2 Standard - 50µl of each standard (8 concentrations, 0 – 1ng/ml)
    c) Samples – 50µl

d) Tracer – 50µl to all well except TA

e) Antibody – 50µl to standards, samples, and Bo

4) Incubate overnight (18 hrs) at 4ºC

5) Reconstitute Ellman's reagent

6) Wash plate 5 times

7) Add Ellman's reagent (200µl) to every well

8) Add 5µl tracer to TA well

9) Develop (in shaker, protected from light) 60-90 min

10) Read plate (405-410nm)

APPENDIX 9

LDH

One set of media aliquots were analyzed for cellular toxicity by measuring LDH content by a commercial, competitive enzyme immunoassay kit from. The manufacturer recommended protocol was utilized with minimal changes. The suggested plate layout was used with each sample measured in two dilutions, each in duplicate. A summary of the procedure is outlined below.

LDH Assay

Promega Part Number TB163

Pre assay:

1) N/A

Assay:

1) Aliquot 50µl of sample (in triplicate) to 96-well plate
2) Make substrate mix
3) Add 50µl substrate mix to each well
4) Cover plate and incubate 30 min at room temp
5) Add 50µl Stop solution to every well
6) Read absorbance at 490nm

APPENDIX 10

Collagenase

During the loading portion of the experiment, the media was collected at various periods and stored. During Aim 3, the media was stored frozen at -20ºC, while it was stored at -80ºC for Aims 4 and 5. Of set of the media was analyzed for collagenase activity by the modification of an Azocoll procedure. This was done originally by Dr. Mari Tsuzaki in this lab, based on a previously described method (Chavira *et al.* 1984). Azocoll was purchased from EMD Biosciences (San Diego, CA). All other reagents were purchased from Sigma. The following is the procedure used:

Collagenase Activity Assay (Azocoll)

(Chavira et al 1984)

Media from the fixed cycles experiment will be analyzed for collagenase activity. The basic procedure of Chavira et al 1984 will be used as interpreted by Mari (her protocol also in directory). APMA will be added to all samples and controls. Media from each day, plus background and controls will be run. Final concentrations of Azocoll and sample are 1:1.

Pre Assay Preparation

1) Make reaction buffer stock
    - 50 mM Tris-HCL
    - 1 mM $CaCl_2$
    - pH 7.8

Samples for Assay

1) Samples
2) Background (Buffer, Azocoll)
3) Controls (LDH +)

Procedure

1) Make Azocoll buffer
    a. Suspend Azocoll in buffer (5mg/ml)
    b. Incubate (25C) for 90min
    c. Aspirate supernatant and replace buffer
2) Add AMPA, final conc. 1mM
    a. Already in warm buffer to help dissolve
3) Prewarm tube at 37C for 15min
4) Distribute 500µl sample to flip-top tubes
5) Vortex azocoll buffer and add 500µl to samples
    a. Vortex again after every 2-3 tubes
6) Incubate 24hrs, 37C while rotating slowly to reduce sedimentation
7) Stop reaction by immersing in ice-water bath (10 min)
8) Centrifuge tube and sample supernatant to 96-well plate (double aliquot)
    a. 30sec, 1000 RPM: 200ul
9) Record absorbance at 520nm (subtract background)

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