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Characterization Of Inflammation In Tendinitis And Application Of Electromagnetic Fields

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CHARACTERIZATION OF INFLAMMATION IN TENDINITIS AND APPLICATION OF ELECTROMAGNETIC FIELDS

A Dissertation

Presented to

The School of Graduate Studies

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by

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ABSTRACT

Tendinitis is a disorder caused by inflammation of the tendon fibers, characterized by collagen fiber disruption and cellular infiltration. Current treatments are lacking, in that they often are associated with unwanted side effects or can even cause an increased amount of damage via scar tissue formation. Pulsed electromagnetic fields (PEMFs) have been shown to be useful in treating wounds and healing non-union fractures. The purpose of the current study was to develop a quantitative rat model of mild tendinitis and subsequently to examine the efficacy of therapeutic electromagnetic fields (EMFs) on this injury. The EMFs used in the current study were: a pulsed field generator obtained from Electro Biology, Inc. (EBI coil) and a field generator obtained from EMF Therapeutics that is capable of delivering two signals (TEMF coil).

Tendinitis was induced in Harlan Sprague Dawley rats by collagenase injections into the Achilles tendon, and animals were then exposed to one of the above EMFs. Tendons were collected at 24, 48, and 72 hours post-injury, as well as at 1 and 2 weeks post-injury for the acute phase analysis. Chronic phase analysis included tendon collection at 4 weeks post-injury. Methods used to assess edema revealed swelling to be maximal at 24 and 72 hours post-injury and to return to pre-injury levels by 1-2 weeks post-injury. The Achilles Functional Index was used to determine function. This method revealed a drop in function at 72 hours post-injury, as well as a return to normal by 1-2 weeks post-injury. A materials testing system (MTS) was used to determine the force required to rupture each tendon at the various time points. This data revealed a drop in

strength of the tendon at 72 hours post-injury, with a return to higher levels by 2 weeks. No significant effects were seen with EMF exposure using the EBI coil. In contrast, differences in healing were observed using one of two signals from the TEMF coil.

The work presented here was part of a comprehensive study which examined protein expression, blood parameters, and immune modulators to thoroughly characterize tendon injury and the possible role of EMF therapy in decreasing healing time.

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I wish to dedicate this paper to all of the people who have helped me reach this point in my life: my parents, family, and friends. A special thanks to Mom and Dad for encouraging words given at just the right times. Thank you to my family for always telling me how proud you are - what an honor to be thought of so highly by so many wonderful people. Thank you to my friends who have continued to support me in spite of the fact I might not return calls or see them as often as I should. I could not have come this far without those blessings of friends and family. This paper is also dedicated to deceased friends and family; we shall meet again.

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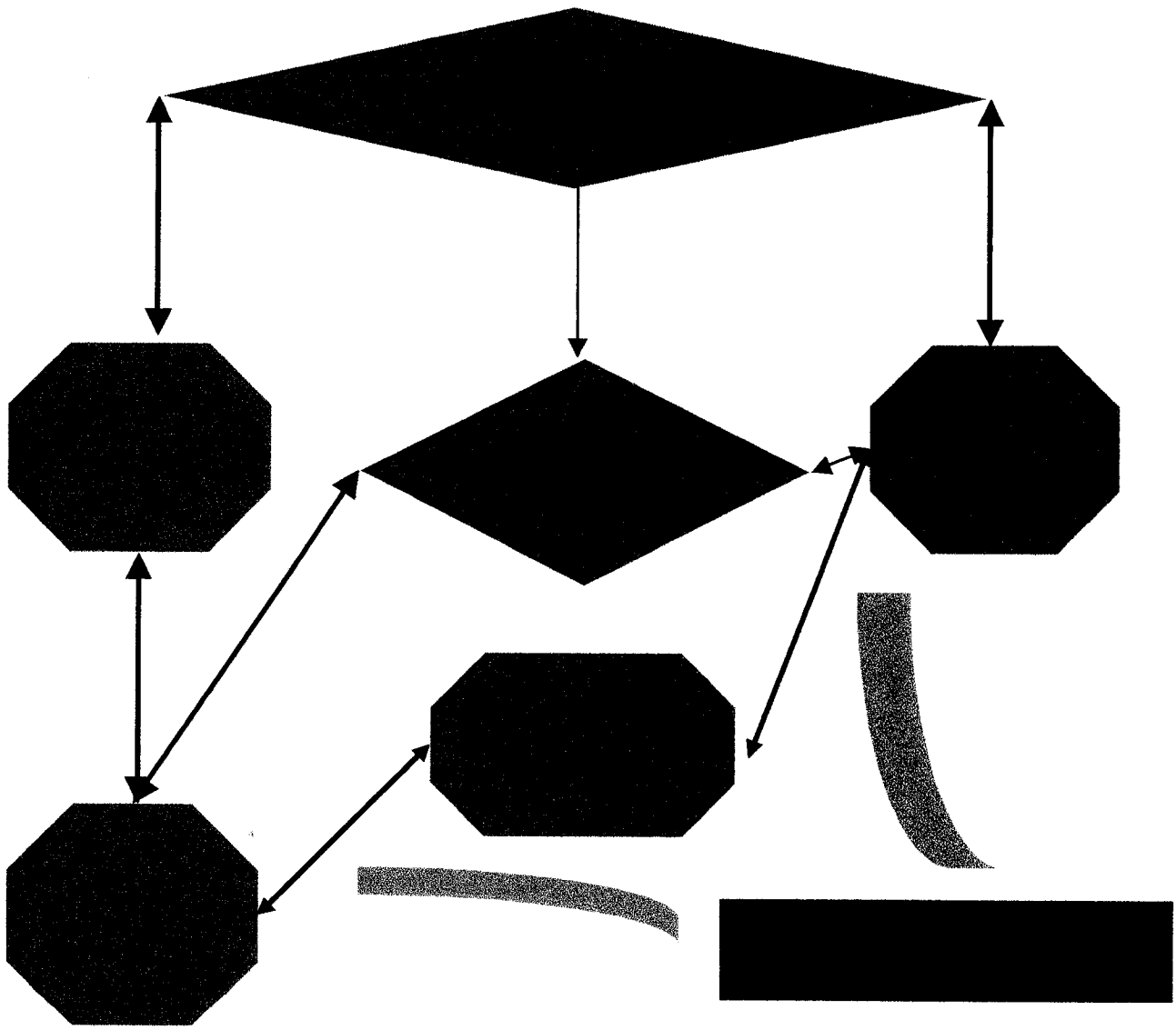
INTRODUCTION

Tendinitis Background

The focus of this study is on a specific inflammatory pathology known as tendinitis. Achilles tendinopathy is a general term used to describe the pathological disorders that occur in the Achilles tendon (tendinitis) or the surrounding sheath or paratenon (paratenonitis). Tendinitis is most often caused by overuse or repeated motion that eventually leads to development of pain, such as repetitive running or jumping in athletes (Benazzo et al., 2001). The number of work-related tendon injuries is increasing in spite of implementation of ergonomically-correct workstations in many businesses and industrial settings. As of 2000, overuse injuries accounted for sixty seven percent of total injuries in the workplace (Bureau of Labor Statistics, 2001). This in turn translates to lost work time and treatment costs for the patient. Current interventions available for the treatment of tendinitis are effective only in reducing pain temporarily, not diminishing the causative agents of the inflammation.

The goal of the current study was to develop a quantitative, collagenase-induced, small animal model of rat Achilles tendinitis. Applying measures at several levels of analysis including gross anatomical, cellular, biomechanical, and functional, we assessed the natural healing time course in a rat model of tendon injury. Once we had determined this normal time course of healing, we then applied electromagnetic fields (EMFs) to examine their potential ability to decrease healing time in this highly developed model (Fig. 1). Understanding the anatomy and function of the Achilles tendon, as well as

Figure 1. Overall Study Goal. The flow chart shows the various aspects that were involved in characterization of a rat model of tendon injury (green octagons). Italicized text within octagons represents level of analysis used, while method used to achieve this analysis follows in regular font. Acute and chronic phase assessments contributed to the overall characterization and subsequent ability to apply pulsed electromagnetic fields as a potential therapeutic modality (purple rectangle). Please note the interaction of all levels of analysis indicated by interlocking arrows between groups.



reviewing the current literature and state of knowledge of tendinitis and its therapies is important before full appreciation of the developed model can be achieved.

Tendinopathy can encompass both degenerative and inflammatory processes. Tendinitis implies an inflammatory process, whereas tendinosis implies a process related to a type of degeneration (Speed, 2001). Some disagreement about the presence of inflammatory cells in tendinosis exists in the literature. The majority of publications conclude tendinosis is characterized by a lack of inflammatory cells (Jarvinen et al., 1997). However, a 2001 study by Rolf et al. concluded that increased cellularity is seen in cases of patellar tendinosis. More studies to quantitatively characterize the cells present are needed to more clearly delineate this difference.

The general consensus is that tendinitis is characterized by cellular infiltration into the tendon substance, as well as disruption of the collagen fibers of the tendon. Under normal conditions, tendon is composed of a greater amount of extracellular matrix than cells, (Fig.2 A), with sixty five to seventy five percent of the dry weight of the tendon being contributed by collagen (Josza and Kannus, 1997). Upon injury, the tendon becomes inflamed and cellular infiltration occurs, along with collagen fiber misalignment (Fig. 2 B). Inflammation is a necessary step in the progression of healing in any tissue pathology. Although a prerequisite to healing, inflammation can often lead to debilitating effects such as pain and loss or diminution of function. Cell types that infiltrate the tendon immediately following damage include polymorphonuclear (PMN) cells and macrophages. In later healing stages, those cells are replaced by lymphocytes that regulate the healing process and fibroblast cells that synthesize new fibers to replace damaged collagen.

Figure 2. Healthy and Injured Rat Achilles Tendon. Achilles tendon of healthy, uninjured rat (A) and tendon of injured rat (collagenase-injected) (B). Magnification 200X. Tendon sections were stained with hematoxylin and eosin (H & E) as described on page 50. Note the wavy appearance of the collagen fibers in the healthy tendon and the fiber disruption that has occurred in the injured tendon. Also note the predominance of extracellular matrix with few cells present in the healthy tendon as compared to the injured tendon where cellular infiltration and collagen fiber disruption has occurred.

A

B

To date, there have been few papers published investigating specific cell types that infiltrate a tendon upon injury. In a 1998 study by Iwuagwu et al., the cellular response of Fischer rat tendon to loading was examined. In this paper, a partial tenotomy was performed, forming what was described as a "window" in the tendon midsubstance. Tendon normally contains very few cells in comparison to the amount of extracellular matrix present, therefore one aim of the study was to determine a time course for cellular infiltration by examining the cells that penetrated the injured area over the post-injury healing period. Using a light microscope and a square graticule, cells were counted within this window. The cell types were grouped into inflammatory cells, fibroblasts, and "other" cells. Sixteen randomly chosen small squares within the graticule were counted. Cells were identified by appearance and a sample of immunohistochemical stains to confirm the specifically acknowledged types. At twenty-four hours post-injury, abundant mononuclear cells and macrophages were observed. By seventy-two hours, there were fewer cells present in the window, with fibroblasts emerging, and consequently, new collagen fibers also appearing. At one week post-injury, the window area of the tendons was very cellular, with fibroblasts along the longitudinal axis of the tendon. This study observed the tendons through one week post-injury, while the current study quantitated cell numbers throughout the entire tendon over a longer, four week time course.

It is unclear whether inflammation that results from traumatic injury to a tendon is the result of recruitment of new cells from the circulation to the injured area or is a result of cellular activation of inflammatory cells that were already resident in the injured

tendon (Marsolais et al., 2001). From research conducted to date, it appears most inflammatory cells are brought into the area of injury via the surrounding circulation. This fact is supported by the current study's findings that CD3+ T cells at forty-eight and seventy-two hours post-injury accumulate around the tendon substance, although at these two time points, they have not yet penetrated into the area. These cells, along with others such as PMNs and macrophages contribute to the overall process of inflammation in the tendon.

Stages of Inflammation

The process of inflammation can be divided into three stages: inflammatory, proliferative, and maturation (Humble and Nugent, 2001). Each of these stages is essential to the ultimate objective of healing an injured tissue.

The inflammatory stage of inflammation involves the initial tissue response to injury. This stage normally comprises the first day of injury through day four to day seven post-injury. Specifically, in the injured tendon, fibrin and fibronectin cross-link to collagen in the tendon substance to form a type of temporary "plug." Changes in the vasculature lead to infiltration of inflammatory cells, such as PMNs. Scavenger cells such as macrophages work to clear debris present due to the injury.

Changes in the vasculature that occur subsequent to injury and allow infiltration of inflammatory cells are known as extravasation (Parham, 2000). Extravasation is the process by which cells from the blood maneuver themselves between the gaps among endothelial cells in the injured tissue. The first step in the extravasation process involves an interaction between circulating leukocytes and the endothelial cells of blood vessel

walls, which recognize signals present on the surface of invading cells. Vascular adhesion molecules called selectins, which are expressed by endothelial cells, bind to and slow down circulating leukocytes, causing them to adhere to the blood vessel walls. These selectins present on the endothelial cell surface allow interaction with the integrins found on leukocyte surfaces in a process known as "rolling." Contact with other adhesion molecules on endothelial cells leads to a subsequent increase in the adhesive properties of the leukocytes. This change leads to the cessation of the rolling process and the strong attachment of the leukocytes to the endothelial cells of the tissue.

Diapedesis is the last step in the extravasation process. It is the means by which inflammatory cells enter an injured area. Diapedesis is the specific process by which leukocytes cross the blood vessel wall between endothelial cells (Imhof and Dunon, 1997). Once a cell has entered into the injured area and reaches the basement membrane, it begins secreting proteases that break down this extracellular matrix. From this point on, chemoattractants take over the process, guiding the leukocytes toward the site of inflammation (Imhof and Dunon, 1997). Chemoattractants are signaling molecules released by a number of cells. The signal is usually one that recruits other cells to a site of injury, so that mediators of inflammation can be released to perform their duties and subsequently resolve the injury.

As with most forms of inflammation, phagocytic PMNs are the first warriors on the scene after tendon injury. These cells are recruited due to the presence of chemotactic factors from the surrounding tissues that are released when injury occurs. PMNs release cytokines that are responsible for many processes, especially that of recruiting macrophages. Two of the cytokines released by PMNs are interleukin-1 (IL-1) and tumor

necrosis factor-α (TNF- α). These cytokines act in either and autocrine or paracrine manner, either acting to increase PMN activity, or to activate and recruit macrophages.

Over time, macrophages become the predominant cell type, phagocytosing the PMNs that were the first responders. Throughout the remainder of the inflammatory process, macrophages are the principal cell type present. Macrophages are capable of releasing numerous cytokines and growth factors (Parham, 2000), and each contributes to the overall goal of resolution of inflammation. The main function of macrophages in the injured tendon is to release factors capable of promoting extracellular matrix synthesis through increasing fibroblast activation. Two major growth factors produced by macrophages are fibroblast growth factor (FGF) and tumor growth factor (TGF-β). These growth factors have multiple roles including fibroblast chemotaxis and stimulation of the synthesis of collagen and noncollagenous proteins (Murphy et al., 1993).

The infiltration of cells from the surrounding vasculature into the injured area also brings with it some fluid accumulation. This fluid accumulation is due to changes in the adhesive properties of the endothelium as well as changes in the permeability of blood vessels that lead to fluid leakage into tissues. The swelling that is the result of this fluid is known as edema (Imhof and Dunon, 1997) and can be used as an indicator of the level of tissue injury. The current study utilized the evaluation of edema formation as one method to determine the natural time course of tendon inflammation and tissue healing.

The second stage of inflammation is known as the proliferative stage. This stage comprises the period from one week post-injury to about two to three weeks post-injury in humans. As its name implies, this phase of inflammation is vital to the healing of the injured tissue. The main hallmark of this phase of inflammation is the infiltration of

fibroblasts. Fibroblast cells enter the injured area and begin secreting fibronectin. Fibronectin acts as an adhesive for attachment of cells and the laying down of new fibrils of collagen type III (Kannus et al., 1998). The newly synthesized collagen acts to replace the old, damaged collagen type I. It is important to note that collagen type III is weaker and thinner, as well as lower in tensile strength due to decreased cross-linking as compared to that of collagen type I (Humble and Nugent, 2001). These smaller diameter collagen fibrils are characteristic of injured tendon tissue and collagen type III (Yamamoto et al., 2002). This lack of collagen strength leads to decreased tensile properties in the tendon substance. Tensile properties can be classified into two areas: stress and strain. Stress is the internal resistance to load that is common to all materials, while strain describes the shape change induced in a material upon loading (Whiting and Zernicke, 1998). The current study determined a force to rupture or breaking strength for the tendons examined, without regard for positioning to delineate stress vs. strain.

The last phase of the inflammatory process is known as the maturation stage. Again, the name is descriptive of the process involved. This stage begins at the end of the proliferative stage and can continue for up to one year post-injury. This is the period where mature collagen fibers are formed. This is also the stage where scar tissue is formed. The organization of the collagen fibers into neatly arranged fibrils occurs in this stage. By applying tensile stress during fibroplasia, the alignment of the collagen fibers is optimized to provide the greatest tensile strength (Enemweka, 1989). Many studies, discussed in the treatment section of this paper, have examined the use of applied stress or motion and its effects on healing in the injured tendon.

It is important to note that there are also two overall stages of generalized inflammation. These two stages are classified as the acute and the chronic stages. The acute stage of inflammation includes the immediate post-injury period, but the exact transition time from the acute stage to the chronic stage has not yet been well defined. A few studies have aimed to further elucidate the time point at which the acute phase ceases and the chronic phase begins (Iwuagwu 1998, Enemweka, 1989). The acute phase normally lasts up to two weeks post-injury in humans and the chronic phase begins after about 6 weeks post-injury (El Hawary et al., 1997).

The tendon degeneration theory describes a pre-existing contributing factor that ultimately leads to the rupture or injury of the tendon upon mechanical overstimulation of that particular tendon. Some of the factors that cause a tendon to be more susceptible to injury are age, hypovascularity, or even lack of use. It seems that age is more a factor in chronic tendon injuries (Yinger et al., 2002). Animals used in the current study were from a cohort in the middle age group in terms of rat lifetime, to eliminate the possibility of older age being a factor in the results.

There also has been some controversy in the literature as to what processes occur within which inflammation stage. Acute inflammation includes three main events: changes in the vasculature leading to increased blood flow, extravasation as discussed previously, and accumulation of cells at the site of injury (Enemweka, 1989). Enemweka also gave insight into the end of the acute stage of inflammation via histological appearance of injured tendons. It was shown that an abundance of cells and inflamed vessels were present in the five day post-injury samples, but not in the seven day post-injury samples. Defining the acute stage of inflammation as a time of increased

cellularity and neovascularization, the study concluded that a decrease in these two factors would indicate a crossover to the chronic stage. Another observation of the Enemweka study was that after the initial acute phase ended, there appeared to be rapid fibrillogenesis. The results of the current study support the findings of the Enemweka group, although increased cellularity was observed as early as twenty four hours post-injury.

Understanding the structure and anatomy of tendons is important for correlating the process of inflammation to injuries within the tendon. Knowledge of normal, healthy tendon, its components and structure, are important when defining various levels of analysis that can be examined to determine healing time and effectiveness of potential therapeutic modalities.

Tendon Structure and Anatomy

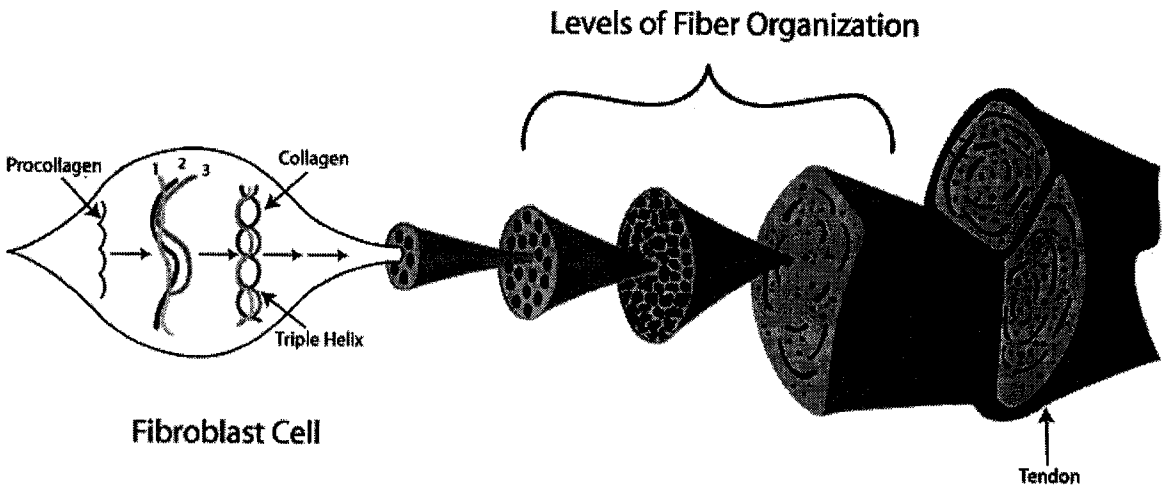
Normal, healthy tendon is composed of approximately seventy percent collagen type I and two percent elastin in an extracellular matrix. The function of elastin in the tendon is not clear, but it is thought that it contributes to the recovery of the wavy collagen fiber structure after muscle contraction and tendon stretch. The collagen in the tendon provides strength under pressure, and the elastin provides the flexibility needed with movement (Renstrom and Leadbetter, 1992). The ground substance, or extracellular matrix, is a gel-like material surrounding the fibrils of collagen within the tendon. It serves as a means of structural support and also a means for diffusion of nutrients throughout the tendon. The main components of the ground substance are proteoglycans and water, along with fibronectin. Upon injury, types of collagen within the tendon

change. Injured tendons display the presence of some collagen type III, as well as the collagen type I that is present in normal tendon (Coombs et al., 1980).

Collagen molecules are formed via a complex process. Amino acids form structures called alpha (α) chains. Three of these α chains interact to form a triple helix collagen molecule. These collagen molecules then orient themselves in an overlapping manner to form tropocollagen molecules. Originally formed in the fibroblast cell as procollagen (Fig. 3, left side), tropocollagen consists of three polypeptide chains. These soluble molecules cross link to form insoluble collagens, which then aggregate to form units that can be seen under the light microscope, called collagen fibrils. A bundle of fibrils form a collagen fiber, which is the basic unit of a tendon (Fig. 3, right side). An endotenon surrounds each collagen fiber, and also binds the fibers to one another. Fibers within a tendon are organized into a hierarchal scheme (Fig. 3, right side). A group of fibers is called a primary fiber bundle (subfascicle). Subsequently, a group of subfascicles forms a fascicle or secondary fiber bundle. Further, these fascicles are bundled to form a tertiary fiber bundle, which comprises the main substance of the tendon (Josza and Kannus, 1997).

Of the twenty types of collagens that have been identified in the human body (Whiting and Zernicke, 1998), types I, II, III, and IV are the most common. The relative distribution of each collagen type is specific. Collagen type II is found in articular cartilage and intervertebral discs as well as in the osteotendinous junction and type IV is found in the basement membranes of capillaries. Collagen types are chemically different, based on the types of α chains present. Collagens contain either one type of chain or two

Figure 3. Hierarchy of Tendon Fiber Organization. Tendon is composed of many different fiber bundles, each contained within the next larger entity. The fibroblast cell (left side of figure) is the site of collagen synthesis. Collagen begins as a procollagen molecule and through a series of modifications, becomes a triple helix structure. This is the structure of mature collagen, which is then incorporated into the first level of fiber organization in the tendon substance. Subsequent levels of organization are then formed, ultimately resulting in the tendon (right side of figure).



genetically different chains. Collagen type I contains two α_1 (I) chains and one α_2 (I) chain and is found in highest abundance in tendon, ligament, bone, and skin. Collagen type III on the other hand, contains three α_1 (III) chains and is predominantly found in the paratenon and vascular walls. α_1 chains differ from α_2 chains in their amino acid sequence. All α chains consist of a repeating triplet (glycine-X-Y) of amino acids. The presence of glycine at every third residue makes it possible for the α chains to coil around one another in the process of the triple helix formation. Proline and hydroxyproline often occupy the 'X' and 'Y' positions in the amino acid triplet, promoting stability of the triple helix structure (Jozsa and Kannus, 1997).

In the resting state, collagen appears as a wavy fiber upon histological staining. Under stress, the fibers become straightened. The size and number of fibers present in a tendon will determine the amount of strain or stress a tendon can withstand. Collagen, being one of the main components of normal, healthy tendon, can be used as a means of assessing healing in an injured tendon. Healthy tendon is comprised mainly of collagen Type I. Upon injury, collagen Type I is replaced by newly synthesized collagen Type III (Lehto et al., 1990). Collagen Type III is found in injured tissue and is known to be the less stable form and exhibits decreased tensile strength when present in tendon, as it forms thinner fibers than that of type I (Williams et al., 1980).

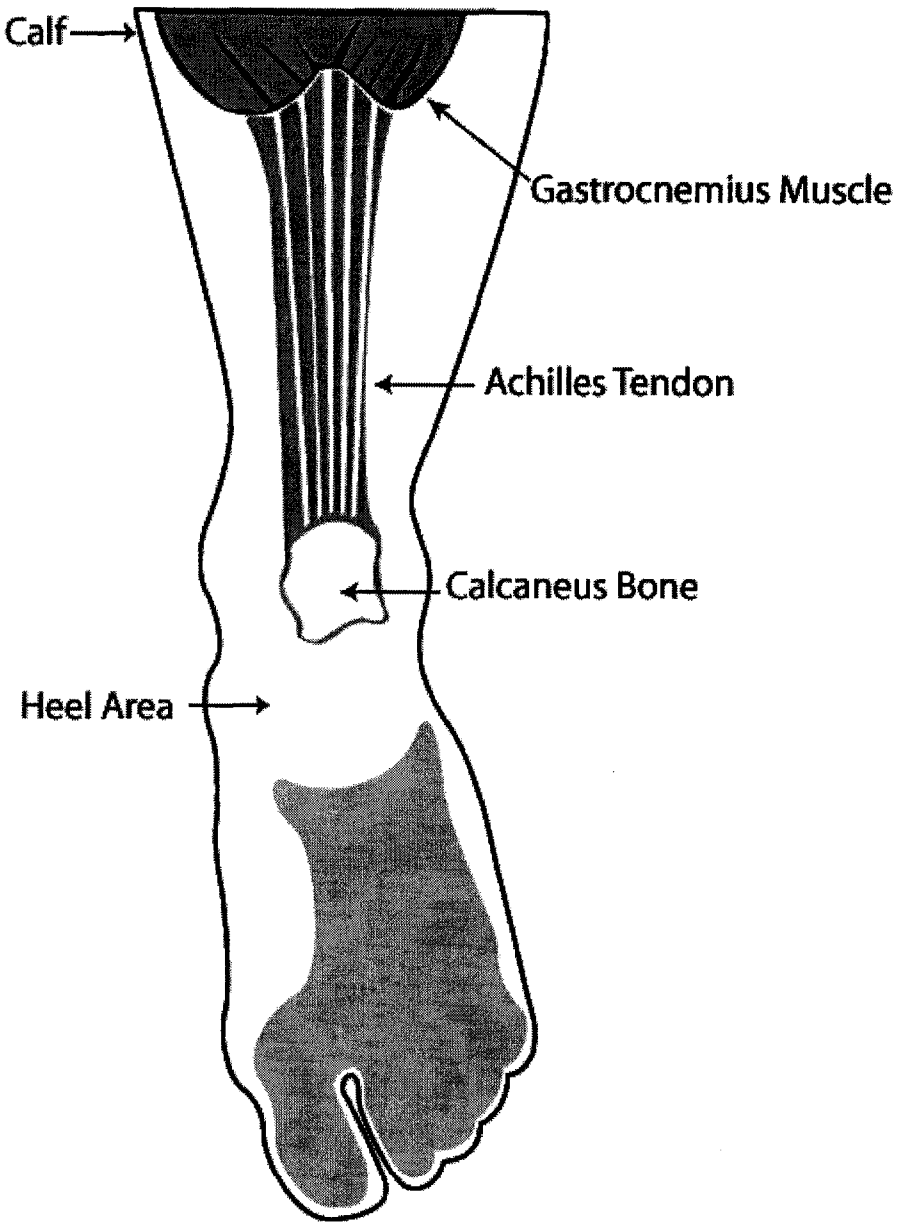
Noncollagenous matrix proteins within the tendon include fibronectin and tenascin-C. High concentrations of fibronectin exist at the myotendinous junction as well as between collagen fibers. This protein is known to serve a binding function. Tenascin-C is found in the extracellular matrix in areas where forces are highest, i.e., at the myotendinous and osteotendinous junctions (Kannus et al., 1998).

A tendon acts to transfer the force from a muscle to a bone, which requires the tendon to have great tensile strength. The current study focuses specifically on the Achilles tendon. The Achilles tendon connects the gastrocnemius muscle of the leg to the calcaneus bone in the foot (Fig. 4). It acts to transfer the force of the gastrocnemius muscle contraction to the calcaneus bone. As mentioned in the previous section, tenascin-C is a non-collagenous protein found in the extracellular matrix, which plays a vital role in the transmission of force within the muscle-tendon-bone unit. Non-collagenous proteins such as tenascin-C are expressed during fetal development, in normal adult tissues, and also in damaged or injured tissue (Jarvinen et al., 1999).

In order that the tendon proper senses the muscle contraction, tendon innervation is of utmost importance. This innervation is mainly afferent. The afferent receptors are the organs of Golgi, found near the neuromuscular junction. The Golgi tendon organs respond to mechanical changes and therefore are classified as mechanoreceptors. The blood supply to the tendon can be divided into three different regions: the myotendinous junction, the paratenon, and the osteotendinous junction (Ahmed et al., 1998). A true synovial sheath is not present in the Achilles tendon. Instead, the paratenon structure is present. The paratenon provides the tendon with the ability to glide smoothly, due to the presence of synovial fluid (Saltzman and Tearse, 1998). The blood vessels originate from the paratenon, which provides the main blood supply to the central portion of the tendon. These vessels then branch into the tendon proper, where they incorporate into the spaces between collagen fibers.

In the Achilles tendon, the arteries are mainly longitudinal and course the length of the tendon. The area of least vascularity in the Achilles tendon is just above the

Figure 4. Achilles Tendon Anatomy. The Achilles tendon is located between the gastrocnemius muscle in the calf and the calcaneus bone of the foot. The Achilles tendon acts to transmit force from muscle to bone.



calcaneal insertion point, and this is commonly a site of tendon injury (Curwin and Stanish, 1984). Altered blood flow is often a factor in tendon injuries. Tendons are at a disadvantage in that they are not well vascularized in the normal state. Due to this, healing occurs more slowly in tendon tissue than it would in a highly vascularized tissue. This fact has contributed to studies that focused on altering the tendon's vasculature to promote healing.

Evaluation of vascular endothelial growth factor (VEGF) is one way to quantitate the amount of neovascularization occurring within an injured tissue (Pufe et al., 2001). VEGF is an angiogenic growth factor that aids in extracellular matrix rearrangement to promote healing (Ferrara and Davis-Smyth, 1997). VEGF appears to peak in concentration at seven days post-injury in a canine model of flexor tendon injury (Boyer et al., 2001). This result supports the idea that maximal ingrowth of vessels occurs in the injured tendon at one week post-injury. Angiogenesis is important for resolution of wounds and generally promotes healing. Transmission of angiogenic factors between cells of the tendon is important if they are to mount a stronger response to injury.

In vivo, tendon cells form a complex system via gap junctions (McNeilly et al., 1996). Due to the presence of these gap junctions, it is possible for individual tendon cells to respond to the injury or insult with increased amplitude, and throughout the length of the tendon. A 1999 study demonstrated that tendon cells need gap junction communication to sustain growth and integrity of the extracellular matrix (Banes et al., 1999). In this study, mechanical load was examined in regard to its effects on intercellular communication in flexor tendons from chickens. Results indicated increased DNA and collagen synthesis with load, as measured by ^{3}H-thymidine and ^{3}H-proline,

respectively. Further, this study demonstrated that addition of a gap junction blocking agent, octanol, blocked this increased synthesis.

Although many of the aforementioned factors might be in place and ready for action upon injury, tendon healing is a slow process. Many current therapies are ineffective and can lead to further debilitation or scar tissue formation. A review of the current treatments for tendon injury is in order to understand the need for an alternative therapeutic modality.

Therapeutic Interventions

NSAIDs

Inflammatory diseases are widespread, costly, and painful. Current treatments for inflammatory diseases such as non-steroidal anti-inflammatory drugs (NSAIDs), often have side effects such as gastrointestinal irritation. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for inflammatory disease to counteract the pain associated with injury. The mechanism of action of NSAIDs is to block the cyclooxygenase enzymes (COX-1 and COX-2) that are involved in prostaglandin production. The COX-1 enzyme is expressed consitutively throughout the body, while COX-2 is inducible and by inflammatory stimuli (Vane and Botting, 1998). Prostaglandins are, in turn, responsible for the production of mediators of the inflammatory process. NSAIDs are normally only recommended for use early in the post-injury period. Their use is recommended to be discontinued by the peak period of the acute inflammatory reaction (Almekinders and Deol, 1999).

In a review by Almekinders and Temple (1998), the literature on chronic tendon problems was evaluated. The authors investigated the literature on human tendinitis in regard to etiology, diagnosis, and treatment. Based on the studies reviewed in this paper, it became apparent that NSAIDs are commonly prescribed for treatment of tendinitis. However, only about fifty percent of the studies which examined NSAID effectiveness showed improvement in follow-up analysis. Clearly, other therapies with a higher degree of efficacy are important.

Corticosteroids

Another form of treatment for tendon injuries is corticosteroid injections (Speed, 2001). The mechanism of action of corticosteroids involves inhibition of proinflammatory mediators such as nitric oxide, prostaglandins, and cellular transcription factors. One particular example involves the nuclear transcription factor factor- κB (NF- κB). Upon binding of an immune stimulator to the cell's receptor, NF- κB is released from the inhibitory factor that maintains it in an inactive form (I- κB). Once NF- κB has been released, it is free to go to the nucleus and activate proinflammatory cytokine genes. Corticosteroids act to increase levels of I- κB, thereby keeping NF- κB in its inactive state (Paavola et al., 2002). A major problem with this form of treatment is that the injection itself can lead to decreased tensile strength, as well as decreased tendon mass and a decline in the biomechanical integrity of the tendon. It has been recommended that this form of treatment should be used only when all other conservative modalities have failed (Fredberg, 1997).

Surgery

In extreme cases of tendon rupture, surgical repair or immobilization are the available options. The most frequently used method of surgical repair in Achilles tendon injuries involves stripping of the paratenon and freeing the tendon from adhesions it has formed with the surrounding tissue (Kvist and Kvist, 1980). After stripping of the paratenon, multiple lengthwise incisions are made along the main axis of the tendon. This is to induce formation of new blood vessels and new extracellular matrix, via microinjuries within the main substance of the tendon. In a 2002 review of the efficacy of surgical intervention in the case of acute Achilles tendon ruptures in humans, Bhandari et al. found that surgery led to a significant reduction (29%-86%) in the risk of rerupture when compared to conservative treatment. However, a downside to surgical intervention is an increased risk of infection. A recent study examined the effectiveness of ultrasound-guided percutaneous tenotomy as an alternative to the conventional method of surgery. This method requires only one incision, as compared to four or five required by traditional methods of surgical intervention for tendon injuries (Testa et al., 2002). Immobilization has a higher rerupture rate than surgery and is also characterized by a longer time between injury and full participation in sports-related activities (Lo et al., 1997).

Shock Wave Therapy

One form of alternative treatment for tendinitis is known as shock wave therapy. Shock waves are a low intensity form of energy produced by delivering a strong electric current through a flat coil. This current creates a magnetic field in an overlying metal

sheet. The magnetic fields in the sheet and the coil repel one another and lead to movements called shock waves (Rompe et al., 1998). In a recent study, the effect of shock wave therapy on neovascularization at the tendon-bone junction in dogs was examined (Wang et al., 2002). It is thought that shock wave therapy induces microtrauma within the injured tissue, which in turn stimulates neovascularization. The study by Wang et al. showed an increase in new capillaries for all samples that had undergone shock wave application, as early as four weeks post-treatment. Also observed was an increase in muscularized blood vessels. The summation of the stimulation of these shock waves is thought to provoke analgesia at the site of injury, thereby relieving pain (Rompe et al., 1998).

Relieving pain and directly affecting inflammatory mediators are two different outcomes of therapy. NSAIDs and shock wave therapy appear to promote analgesia, yet not directly decrease the pro-inflammatory intermediaries that are the basis for the injury itself.

Ultrasound and Laser Photostimulation

Another controversial area of research in terms of possible therapies for healing tissues is therapeutic ultrasound. Ultrasound is defined as any sound frequency of 20,000 Hz or higher (Rivenburgh, 1992). It is possible that ultrasound works through decreasing edema present in the early stages of inflammation, as well as increasing neoangiogenesis (Young and Dyson, 1990). Using Wistar rats, Da Cuhna et al., 2001, observed an increase in collagen synthesis at the site of injury and better collagen fiber alignment in the tendons of the animals that were exposed to a 1MHz frequency ultrasound wave for

five minutes each treatment day for a total of twelve treatments. However, a recent review of ultrasound therapy (Robertson and Baker, 2001) found little evidence supporting therapeutic ultrasound as a means of treating people with pain or for promoting soft tissue healing.

Laser photostimulation has also been investigated as a means of healing injured tendons. In a 1998 study (Reddy et al), rabbit Achilles tendons were treated with a He:Ne laser for fourteen days post-injury. Using a method to measure hydroxyproline content in the tendons, collagen concentration was determined for each group. Results showed a 26% increase in collagen concentration in the group exposed to the He:Ne laser, as compared to sham treated animals.

Growth Factor Therapy

Growth factor therapy has also been heavily investigated as a means of treating tendon injuries. Growth factors such as TGF-β contribute to the cessation of the inflammatory process by decreasing macrophage activation (Parham, 2000). A certain amount of macrophage activation is necessary after a tissue is injured, but inevitably, the tissue will suffer further damage if cessation of macrophage activity does not occur at the appropriate time. Tendons are capable of synthesizing TGF-β under homeostatic conditions as well as in response to an injury. TGF-β has been shown to be a potent stimulator of collagen production (Itayem et al., 1997). Tendons also have endogenous stores of insulin-like growth factor-I (IGF-I), which has been investigated for its possible use as a treatment modality for tendinitis.

IGF-I acts as a mitogen and stimulates collagen and extracellular matrix molecule production. IGF-I treatment has been shown to improve healing in a horse, collagenase-induced model of tendon injury (Dahlgren et al., 2002). This study showed that injections of IGF-I after collagenase-induced tendon injury, were beneficial to healing. Improved mechanical function, increased DNA synthesis, and a decrease in swelling of the tendon were all observed in the IGF-I treated samples. It is important to recognize that the horses used in this study were pre-treated with non-steroidal anti-inflammatory drugs prior to collagenase injections. NSAIDs have been administered precisely for the purpose of decreasing the pain associated with tendinitis for many years. In addition, the delivery route for IGF-I was also via injection. The method for tendon injury was by way of collagenase injections. It is highly likely that the volume contributed by the IGF-I suspension contributed in some way to the development of the injury in the tendon. These two confounding factors make the results of this study hard to decipher.

Fibroblast cell proliferation is indicative of healing, and can be measured by DNA synthesis. Combined treatment with platelet derived growth factor (PDGF) and IGF-I has been shown to increase levels of DNA synthesis in avian tendon fibroblasts (Banes et al., 1995). Mechanical load alone can increase DNA synthesis as well. In the Banes study, mechanical load alone was compared to mechanical load plus growth factors to determine if an additive effect for DNA synthesis occurred with the addition of growth factors. It was found that cell division did not occur unless a growth factor was present to costimulate cells. This report correlates well with our idea that therapy for tendon injury must be multifaceted. Proposed therapies that work at both the mechanical or gross level as well as at the cellular level will be more likely to achieve maximum healing. These

various interacting levels of healing have been considered in the model developed in the current study, as can be seen in Fig. 1.

Augmented Soft Tissue Mobilization

Another form of conservative treatment that has been investigated as a possible means for treating tendon injuries is soft tissue mobilization. In a 1997 study by Davidson et al., a procedure known as augmented soft tissue mobilization (ASTM) was employed. The ASTM treated animal tendons were compared to controls and the process was investigated as a possible noninvasive means of decreasing healing time in tendinopathy. The idea behind tissue mobilization is that small microtraumas are induced by massaging the injured tissue. These microtraumas then lead to increased rates of healing through increases in fibroblast activity and increased rates of collagen deposition by these highly active fibroblast cells. The study showed an increased rate of healing via increased numbers of fibroblasts. It is important to note that the samples were examined only at the three weeks post-injury time point and no acute time points were investigated. Also, a thirty microliter dose of collagenase was used to induce injury. This volume alone may produce alterations in collagen fiber alignment and a type of inflammatory reaction. In the current study, volumes no greater than 25 microliters were used during collagenase injections. A comparison of injection volumes and functional indices achieved showed no differences across animals (Data not shown).

The amount of stretching needed to promote healing of the tendon substance post-injury also is debatable. Future studies examining the effectiveness of movement of the tendon after injury are likely to contribute to how the injury is managed by clinicians.

Often, rest is prescribed after injury, but how long the person should remain inactive is unclear. A study in 2001 by Skutek et al., used cytokine measurement to determine the effect of stretching on human tendon fibroblasts *in vitro*. The results of this study showed increased secretion of interleukin-6 (IL-6) by fibroblasts that had been subjected to a physiologically–relevant level of stretching. IL-6 is a proinflammatory cytokine known to be involved in the inflammatory response seen after accelerated rehabilitation in tendon injuries. Using an *in vitro* model, it is difficult to assess if the result would translate directly to the *in vivo* system, where other cell types would be present and contribute to the overall outcome of healing.

Tissue Engineering and Gene Therapy

A more recent and cutting edge type of treatment involves tissue engineering and gene therapy (Kreeger, 2003). A new concept for treating rotator cuff tendinitis involves use of a patch implant that consists of Type I collagen embedded in layers of porcine intestinal material. This patch aids in healing by acting as a scaffold and stimulating tissue regeneration. Using bone marrow cells to replace or enhance biologic function of damaged tissue is an idea currently being heavily investigated. Bone marrow cells are capable of producing fibrous tissue and differentiating into fibroblasts, the cell type responsible for producing collagen (Prockop et al., 1997). Other studies have found tendon tissue can be engineered *in vivo* and is capable of bridging tendon defects (Cao et al., 2002; Hildebrand et al., 2002).

In most cases of tendon injury, the patient does not seek medical assistance until the pathology has reached the chronic stage. Once it has reached the chronic phase,

tendinitis is characterized by scar tissue formation and loss or inhibition of function. Applying therapeutic interventions during the acute phase of injury could lead to decreased healing time and subsequent function being fully regained. Effective therapy used during the time when inflammatory indicators should be returning to pre-injury levels could lead to a more rapid recovery for people suffering from the pain of tendinitis.

Investigation of new alternative therapies for decreasing healing time in tendon injuries is important. Electromagnetic fields are one possible therapeutic alternative to current treatment modalities in current clinical use.

Electromagnetic Fields (EMFs)

EMFs are invisible lines of force that consist of an electric and a magnetic component. Electric fields are produced by a voltage source (field generator) and increased voltage results in a stronger electric field. Electric fields are shielded by conducting objects and therefore create weak induced fields in humans. The magnetic component of the EMF is produced by current and is perpendicular to the current flow (NIEHS, 2002). With an increased current flow, a stronger magnetic component is produced. Magnetic fields are capable of passing readily through the human body and most conducting objects. The intensity of both electric and magnetic fields drops off rapidly with increasing distance from the source of the field. Electric fields extend from positive to negative poles, while magnetic fields form continuous loops around lines of current.

There are different types of electromagnetic fields, which are characterized by frequency, intensity, waveform, and duration. One of the most widely studied types of

EMFs is called pulsed electromagnetic fields (PEMFs). PEMF application, which has been shown to be useful in treating soft tissue injuries, has been widely investigated as a therapeutic modality for many pathologies. Pulsed electromagnetic fields are different from other EMFs in that the field is alternately cycled on and off, whereas EMFs are characterized by a continuous on signal. The underlying mechanism of action of PEMFs is yet to be fully understood. It is thought that PEMFs act to inhibit cell proliferation via increasing apoptosis (Nindl et al., 2000). Apoptosis or "selective cell death" is the process by which damaged or incompetent cells are disposed of by a natural process of programmed cell death. An increase in the apoptosis of pro-inflammatory T cells would correlate with decreased inflammation and would therefore provide a treatment option for tendinitis. It is important to once again note that the increased amount of cellularity (as compared to normal tendon) that occurs with inflammation of the tendon is a prerequisite to healing. Therefore by selectively inhibiting cell proliferation or increasing apoptosis of only certain cell types, one might be capable of modulating the inflammatory response and thereby decreasing healing time.

PEMFs as Therapeutic Modality

Several papers have examined the use of PEMFs for treatment of tissue injuries (Lee et al., 1997; Scardino et al., 1998; Jasti et al., 2001). As discussed below, a number of of these papers have examined the effects of EMFs on collagen, cytokine, and growth factor production.

It has been shown that *in vitro* exposure to PEMFs promotes collagen production in fibroblasts from rabbit bone marrow (Farndale and Murray, 1985). This study

observed changes in collagen production *in vitro* via the conversion of ^{3}H-proline to ^{3}H-hydroxyproline. Increases in hydroxyproline were observed in postconfluent cultures of fibroblasts that had been exposed to the PEMF. Another study further supported the idea that PEMFs can increase collagen production *in vitro* (Murray and Farndale, 1985). A 15 Hz PEMF was applied to cell cultures for twelve hours per day. Results showed again, that only non-dividing, postconfluent cells responded to PEMF treatment. A doubling in the amount of collagen present in the PEMF-exposed cultures occurred after six days of treatment. Another early study examining the effects of different intensities of PEMFs (0.2, 1, and 5 mT) showed a dose-dependent increase in collagen production and tensile strength (Lin et al., 1993). The exposures in this study were also performed for up to six hours daily.

PEMFs also have been investigated for their ability to affect cytokine levels in injured tissue. The inflammatory cells that infiltrate the tendon after injury secrete cytokines that contribute to the inflammatory process, thereby making cytokines a logical entity to examine in terms of modulation by PEMFs. Whole body exposure to a 0.12mT PEMF (Electro-Biology, Inc., Parsippany, NJ), resulted in no changes in cytokine mRNA expression for interferon-γ, interleukin-Iβ, interleukin-6, or tumor necrosis factor–α in rats (Jasti et al., 2001). It is important to note that this study focused on systemic cytokine expression in the spleen. Tendinitis was induced by injection into the Achilles tendon and cytokine levels were examined at the level of the spleen. Also, this study only examined the twenty-four hour post-injury time point.

One reason PEMFs were considered as a possible therapeutic modality was due to their influence on nonunion bone fractures (Bassett et al., 1982). A 2001 study (Guerkov

et al.), demonstrated that a 15 Hz PEMF was capable of increasing levels of TGF-β in cultures of human nonunion bone cells exposed for eight hours per day. TGF-β is a known anti-inflammatory cytokine, therefore increased expression would lead to decreases in inflammation over time. TGF-β has also been shown to be beneficial in tendon healing due to its ability to increase collagen type I and III levels in tendon cell cultures (Klein et al., 1994). The 2001 study by Jasti et al. also observed increased levels of TGF-β in spleen at twenty-four hours post-injury, although there were no significant differences between the PEMF exposed and the control samples.

PEMFs have also been investigated as potential therapies for treatment of chronic wounds. In a 1998 study by Scardino et al., open wounds in dogs were examined after exposure to a gradually increasing frequency PEMF (0.5 – 8.0 Hz). Animals that received the PEMF exposures displayed increased healing compared to controls by day ten post-injury. Another study which used an *in vitro* model of skin, (Binhi et al., 2000) found a frequency of 10 Hz to be most effective at increasing proliferation of dermal fibroblasts. This is an important concept, in that higher frequency fields do not necessarily correlate with more beneficial effects; the result of PEMF exposure depends on the field characteristics, as well as the biological characteristics of the system being examined. Having reviewed the literature in terms of general studies on PEMF application, we now turn to focusing on studies examining PEMF application to improve healing of tendon injuries.

Two studies by Greenough (1996) and Robotti et al. (1999), examined injured rabbit and chicken flexor tendons after PEMF exposure. The Greenough study found no

significant differences between PEMF-exposed (15 Hz, .12mT) and control tendons in terms of load to failure for the examined specimens. The Robotti study incorporated use of a 15 Hz, 0.02 mT clinical PEMF. This study found no benefit in terms of increasing tensile strength in the PEMF-exposed tendons after three weeks post-injury. The field characteristics used in these two studies were chosen due to their effectiveness in treating nonunion fractures. It is possible that these signals were not optimal for tendon or connective tissue healing.

A study conducted by Lee et al., 1997 investigated the potential healing effects of PEMFs on rat Achilles tendon. Using a 15 and a 46 Hz field, the authors found the 15 Hz field was not significantly different from the control group as far as tendon water content by the end of the experiment, four weeks post-injury. The 46 Hz PEMF was found to have no effect by the end of the experiment as well.

One of two fields (EMF Therapeutics, TEMF coil) used in the current study has been shown to decrease angiogenesis and reduce tumor growth (Williams et al., 2001). As mentioned previously, a decrease in angiogenesis could lead to a more rapid resolution to inflammation within the tendon. By decreasing vascularity back to the level present in normal, healthy tendon, reorganization of the extracellular matrix into neatly aligned collagen fibers could take place, leading to increased strength and functional capacity of the tendon. However, increased vasculogenesis could aid in healing if it occurred at the proper time point when cells need to come into the injured area to perform their specific tasks. Therefore, timing and potential modulation of angiogenesis might be of critical importance in the therapeutic application of EMFs. To determine the optimum timing of EMF application in a mild form of tendinitis, development of a fully

characterized model, examining many levels of healing is required and currently does not exist in the literature.

Models of Tendon Injury

The current study has focused on developing a quantitative small animal model of tendon injury. Previously tendinitis/ tendinosis models have been developed, although most are in large animals. Means of inducing tendinitis range from injection of chemicals like collagenase (Williams et al., 1984) and cytokines (Stone et al., 1999) to the physical trauma such as dropping a large mass to induce a contusion type injury in the tendon (Lee et al., 1997). Other investigators have tried to emulate an exercise-induced injury via use of a kicking machine in a rabbit model (Backman et al., 1990) and treadmill running in a rat model (Soslowsky et al., 2000).

In a 2001 study by Archambault et al., a refinement to the 1990 Backman study was made. The Backman study was important, in that it was the first to attempt to induce a true overuse injury in an animal model of tendinopathy. One problem with the study was that the magnitude of the load applied to the tendon during loading sessions was not measured. In the Archambault study, controlled, quantified loads were applied to induce the injury. In contrast to the earlier study by Backman et al., Archambault observed no inflammatory reaction in response to repetitive loading. It is significant to note that the movement frequency of 150 flexions and extensions/ minute used by Backman et al., was not a physiologically relevant one, whereas the frequency used by Archambault et al. was a less intense, more physiologically relevant rate, shown to be equivalent to slow hopping in the rabbit.

Tendon overuse injuries occur in the horse, making it one of the animals investigated in the field of tendon injuries (Archambault et al., 1995). The 1984 Williams study was an enzyme induced horse model of tendon injury. This model is not practical for many laboratories, as most would not have the facilities or the funding to maintain these large animals. And of course, animal number per treatment group would be much smaller, due to high cost of purchase and maintenance of the animals.

Another aspect that must be considered with an enzyme-induced model of tendon injury is the enzyme's activity. In the current study, we measured the collagenase specific activity using a continuous spectrophotometric method (Van Wart and Steinbrink, 1981). This assay utilizes a synthetic collagenase peptide substrate known as 2-furanacryloyl-L-prolyly-L-alanine (FALGPA). Upon hydrolysis, a blue shift in the near ultraviolet absorption band occurs. This can then be seen as a decrease in absorbance over time. The enzymatic activity of the three different lots of collagenase used in the current study was equivalent (approximately 0.125 U change in absorbance).

In the 1999 study by Stone et al., a comparison was made between a cytokine-induced tendon injury and the collagenase-induced model. In this study, rabbit patellar tendons were injected either with a cytokine preparation or collagenase. Results showed no matrix damage with cytokine injury in contrast to collagenase injury in rabbit tendon. Matrix damage and disruption is a hallmark of tendon injury, therefore the cytokine injury model does not accurately reflect what occurs in the initial stages of tendinitis. Collagenase injection as a means to induce injury, and used in the present study, provides the two hallmarks of tendinitis; collagen fiber disruption and cellular infiltration (Fig. 2 B).

The 1997 study by Lee et al. used a falling weight to induce tendon injury in rats. This model appears to induce the same type of injury both histologically and biochemically that is seen in human overuse induced tendinitis. Infiltration of inflammatory cells as well as disruption of collagen fibers was observed as early as two hours post-injury. A major confound of this model of tendon injury is the issue of precisely repeating the injury each time the weight is dropped.

The exercise-induced model of tendon injury by Backman, specifically focused on paratenonitis. Paratenonitis is an inflammation of the paratenon that surrounds the tendon proper. Rabbits were placed into a kicking machine for two hours per day three times a week for five to six weeks. A thickening of the exercised tendon could be felt upon palpation by four weeks into the exercise regimen. Histological observation of the tendons post-exercise revealed edema and infiltration of inflammatory cells, both of which indicate tendon injury. The method of analysis used was semiquantitative, providing a numbered assessment on a scale of zero to three, with zero being equivalent to no change and three representing marked change in histological appearance. Induction of injury using this type of model is also a concern, as it takes up to six weeks of repetitive motion in the machine to achieve a noticeable injury.

As can be seen from the aforementioned models of tendinopathy, there is a need for a reliable, affordable, fully-characterized small animal model of tendon injury. This is the basis for the model we have developed and the data that will be presented in the current paper was collected using the model as a tool.

Most of the research done in this area has focused on only one or two aspects of tendon healing. The goal of this paper is to quanitate and examine a rat model of

tendinitis at several levels of analysis. This examination will include analyses at the following levels: gross anatomical, cellular, functional, and biomechanical (Fig. 1).

Overview of Current Study

A tendinitis model as described in this study (Fig. 1) can be used as a baseline to determine the efficacy of various tendinitis treatments. Many studies to date have been conducted to characterize this disorder. Such studies used histological examination of tendon sections and provided visual analyses and qualitative descriptions of cell types present (Davidson et al., 1997 and Messner et al., 1999). The current study is the first to demonstrate a model of rat tendon injury over a prolonged time period characterized at several levels of analysis. This model was then employed to assess the impact of two EMFs on tendon healing.

At the gross anatomical level, we have examined hindlimb edema via caliper measures (p. 52). This measurement is indicative of the amount of leg swelling that has occurred with injury in our model. A refinement of the caliper method is the lyophilizer method, which takes a direct measure of the amount of water within the tendon itself (p.52). The cellular level analysis includes quantitation of inflammatory cells over a time course of four weeks post-injury throughout the volume of the tendon. In addition, lymphocytes were specifically identified in some samples with an anti-CD3 antibody that recognizes the CD3 portion of the T cell receptor on rat T cells (p.48). The functional level analysis was also a quantitative one. Using the Achilles Functional Index (Murrell et al., 1992), we were able to evaluate function in the injured animals over time (p. 52). Biomechanical analysis included measuring the force required to rupture each sample (p.

58). After development of this model, the normal (control) and EMF exposed samples were compared at each of the analysis levels to determine if differences existed.

One of the goals of the present study was to define a time course for normal healing, concurrently establishing a quantitative portrait of cells and their time of appearance as well as the corresponding amount of edema that occurs as a result of the damage to the tissue. Additionally, return to function and force to rupture for each tendon was determined as a correlation to the clinical aspects of the injury.

This study was designed to delineate the natural healing time course in a chemically-induced model of tendinitis. To our knowledge, no quantitative analysis of total cell numbers in whole tendon samples over the extended time course of healing has been published to date. Previous studies have examined histological sections of rat tendon and outlined a time course of appearance of cell types without estimating total cell number in whole tendons over a four week post-injury time period. By understanding the cellular dynamics over the progression of the injury, we can better focus possible therapies such as EMFs on certain time points before chronic injury develops and leads to a loss or limitation of normal function.

Cumulative analysis at all levels of healing, including gross anatomical, cellular, functional, and biomechanical will contribute to a developing picture of tendon injury and the potential therapies that show the optimum benefits.

EXPERIMENTAL METHODS

Animals. Sprague Dawley rats aged 4-6 months old, (Harlan Laboratories, Indianapolis, IN) weighing between 250 and 500 g were used in this study. Animals were quarantined for two weeks to guard against potential pathogens before being housed with in-house animals or used in any experimental studies. Animals were then housed two per cage in plastic cages at the vivarium. The environment in the vivarium is maintained at 21-22^0 C with a dark/light cycle of 12/12 hours, and the animals are fed rat chow and water ad libitum. All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals, under an institutionally approved protocol (02-14:MJ).

Collagenase Injections. All animals were weighed pre-injection and a ten percent value was obtained from this starting weight for each animal. Daily weight measures were obtained and used as a means of monitoring animal health and well-being (Sandrey et al., 2002). Any animal whose weight dropped below the ten percent level was removed from the study, although this did not occur in any of the trials reported here. Animals were also monitored daily for signs of discomfort, including chewing and/or biting at the injection site, as well as any major changes in gait, such as limping.

Once the pre-injection weight was obtained and recorded, each animal was anesthetized with 3% Enflurane (Ohmeda, Liberty Corner, NJ) or 2-3% Isoflurane (Halocarbon Labs, River Edge, NJ). Collagenase-XI from *Clostridum histolyticum*

(Sigma Chemical Co., St. Louis, MO) [150 IU/ kg body weight for the model development and EBI exposure experiments and 900 IU/ kg body weight for the TEMF exposure experiments] was injected into the mid-portion of the Achilles tendon of each animal. The hindleg was paplpated between the thumb and forefinger to find the Achilles tendon previous to injection. For studies involving gait analysis, animals were only injected in the right Achilles tendon. All other studies utilized injections into both left and right tendons. Animals were allowed a short recovery period and were then placed back into their respective home cages. At each of the following time points post-injury animals were euthanized by CO_2 narcosis followed by bilateral pneumothorax to ensure death: 24 hours, 48 hours, 72 hours, 1 week, 2 weeks, and in some experiments, 4 weeks. In some of the experiments, death was ensured by decapitation to obtain blood samples and brains. For histological analysis, Achilles tendons were excised and placed in Bouin's fixative (saturated picric acid containing 23.8% formaldehyde(v/v) and 4.76% glacial acetic acid (v/v)) for four hours, then put into 70% ethanol until processed for embedment. Briefly, the embedment procedure consisted of one hour exposures to a graded alcohol series, followed by clearing and paraffin embedment (Presnell and Schreibman, 1997).

Collagenase Activity Assay. Following the method of Van Wart et al., (1981), we measured the activity of the collagenase enzyme used in the experiments reported in this paper. The assay was developed based on the fact that collagenase is capable of hydrolyzing a synthetic peptide known as FALGPA (Yu and Lee, 1999). Briefly, a FALGPA concentration of 0.05 mM in 50 mM Tricine, 0.4 M NaCl, 10 mM $CaCl_2$, pH

7.5 was used. Hydrolysis at 37°C of the substrate was monitored using a Perkin Elmer Lambda 20 UV/ Vis spectrophotometer. Samples were placed in a one cm x one cm cuvette at 324 nm wavelength after addition of 10 ul of collagenase to give a final enzyme concentration of 4ug/ml. Hydrolysis was measured over a time course of eight hours at one minute intervals.

Electromagnetic Field Exposures. To determine if EMFs might alter the normal timecourse of healing in our model of rat tendinitis, we tested two clinically employed EMFs. Results from the current study will be representative of these two different EMF exposure groups. The two fields used to obtain data for this paper were as follows:

Electro-Biology Incorporated (EBI) field

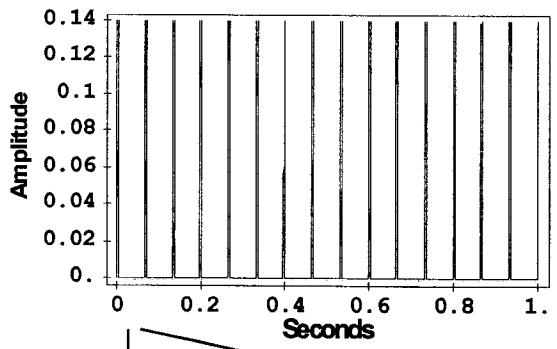
EMF Therapeutics (TEMF) field

Exposures for both fields were carried out by placing animals into Plexiglas cages covered with Plexiglas lids, which provided gaps for air exchange. These Plexiglass cages were then placed into the uniform magnetic field exposure area within the appropriate coil. Each chamber could house two adult rats without heat buildup (determined to be +/- 1.0°C; data not shown). Bedding used in the chamber was identical to that used in the home cages.

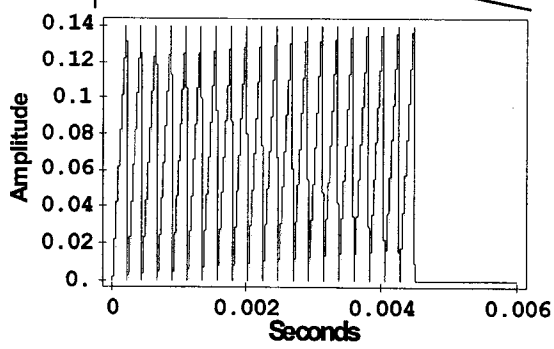
EBI field. Animals were exposed to the field at the same time each day in cages placed within the center of a 4-coil Merritt coil system large enough to guarantee simultaneous exposure to a homogenous magnetic field for all rats in the center of the coils for four hours each day. The coil was powered with a proprietary, complex 0.12 mT pulsed magnetic field (Fig. 5),

Figure 5. Characteristics of the Electro-Biology, Inc. (EBI) Field. This field is characterized by 15 pulses per second with a 200 ms rise time. Each pulse consists of 20 sawtooth waves.

15 pulses per second



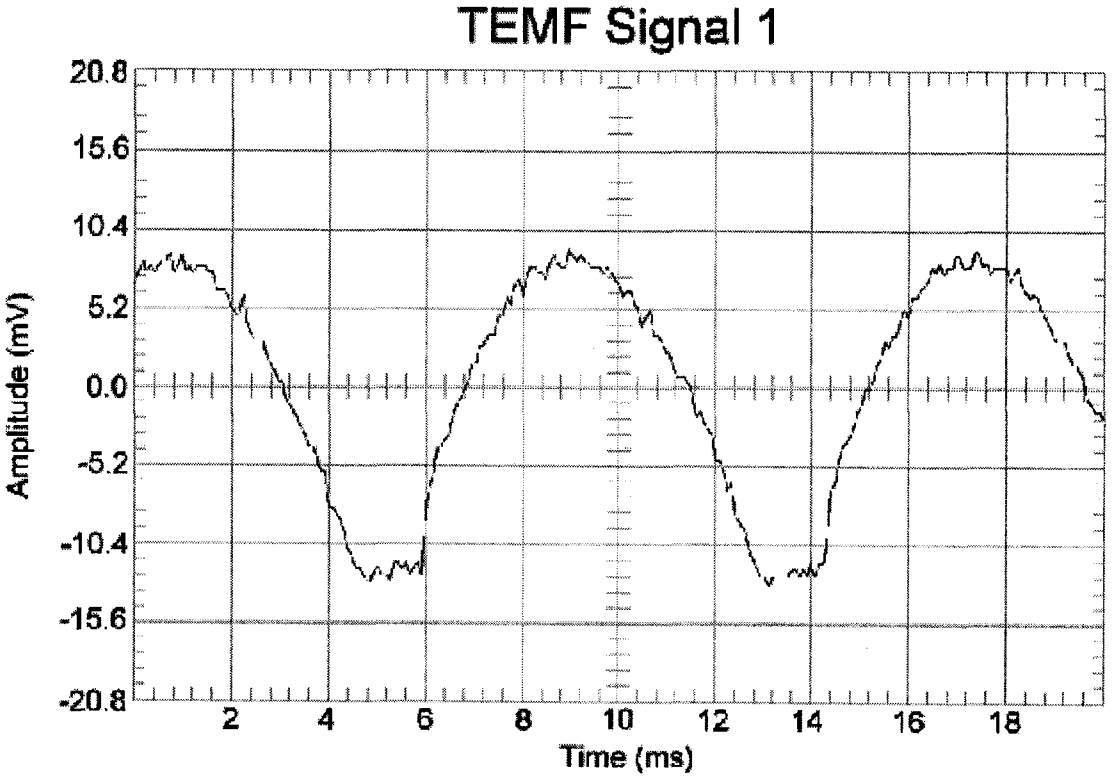
Each pulse is 20 sawtooth 0.12mT waves with a 200 ms rise time



which is used in therapeutic settings and was kindly provided by Electro-Biology, Inc. (EBI, Parsippany, NJ). The field characteristics include a 200 ms rise time with each pulse consisting of 20 sawtooth 0.12 mT waves. In all the EBI experiments, the coils were oriented so that the horizontal magnetic field generated was parallel to the 0.04 mT horizontal component of the earth's geofield. The control for this set of experiments was the Earth's geomagnetic field ("GEO").

TEMF field. Field was located in a dedicated room at the vivarium. Animals exposed to the TEMF field were placed into cages, which were then placed within the center of the exposure coil for thirty minutes each day, at the same time each exposure day. The field generating device was kindly provided by EMF Therapeutics (Chattanooga, TN). The generator produces a pulsating, half sine-wave magnetic field, 120 pulses per second, as described elsewhere (Williams et al., 2001; Nindl et al., 2002). The generator is capable of producing fields with intensities of 10, 15, and 20 mT. For the experiments discussed here, the 15 mT signal was used in all instances (Fig. 6). The 15 mT signal will then be further subdivided in the results and discussion section into "SIGNAL 1" and "SIGNAL 2". SIGNAL 1 and SIGNAL 2 differ in their off times in the following manner: SIGNAL 1 is characterized by an 8 ms off time and SIGNAL 2 by a 4 ms off time (EMF Therapeutics, Inc.). In our experience, the signals are complex and contain high frequency components. The control for this set of experiments will be called "SHAM". This SHAM setting was achieved by turning the field generator switch to the standby position and placing the animals into exposure cages within the

Figure 6. Characteristics of the EMF Therapeutics (TEMF) Field. This field is characterized by a 60 Hz half sinewave signal. The levels of intensity possible with this field include 10, 15, and 20 mT. The signal shown is a 15mT signal from the SIGNAL 1 setting for this field.



coil. It is important to note this field condition is not a true sham, because in a true sham the coils are double wound such that the current cancels out and no magnetic field is produced. Vibration was minimized by placing the exposure chambers on a suspended platform within the uniform coil space. Table 1 is a representation of the number of exposures received per animal, for both the EBI field and the TEMF field experiments.

Histology. Tendon samples were dehydrated through a graded series of ethanol solutions ending in xylene and embedded in paraffin (Paraplast, Fisher Scientific) (Presnell and Schreibman, 1997). Once embedded in paraffin, 6 μm longitudinal sections were cut using a rotary microtome and placed onto pre-coated Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) or poly-L-lysine (Sigma Chemical Co., St. Louis, MO) coated slides. For cell enumeration, slides were stained with H & E to allow for cellular visualization (Presnell and Schreibman, 1997).

Immunohistochemistry. For anti-CD3 visualization, paraffin embedded sections were placed in a 60^0C oven for 1.5 hours to eliminate excess paraffin before beginning the staining process, as described in Presnell, 1997 (pp.367-369). Slides were then washed three times in histological grade xylene (Fisher Scientific, Hanover Park, IL) for ten minutes each. Rehydration was then carried out by immersing the slides in an ethanol series (100%, 95%, and 70%) two times each. Slides were then allowed to air dry. Endogenous peroxidase was blocked using a 3% H_2O_2 solution in methanol. Nonspecific binding was blocked using normal donkey serum (Jackson ImmunoResearch, West

Table 1: Number of EMF Exposures Per Animal. The number of EMF exposures received per animal varied depending on which group they belonged to. Increasing numbers of exposures were given to the animals in later time period groups.

TIME POINT	24 hour	48 hour	72 hour	1 week	2 week
NUMBER of EXPOSURES	1	2	3	7	10

Grove, PA). To block nonspecific binding, avidin and biotin sites were pre-blocked using an avidin biotin blocking kit (Vector Laboratories, Burlingame, CA) and primary antibody (mouse anti-rat CD3; Accurate Chemical Co., Westbury, NY) diluted into 0.1% BSA/PBS (Accurate Chemical Co., Westbury, NY) was applied at a concentration of 10 μg/ml overnight at 4°C. After incubation in primary antibody, secondary antibody (biotin-SP-conjugated anti-mouse IgM; Jackson ImmunoResearch) was applied at a 1:500 dilution into 0.1% BSA/PBS. The Avidin Biotin Complex kit (ABC kit; Vector Laboratories, Burlingame, CA) was then used, in addition to 3, 3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) for visualization. Slides were counterstained with hematoxylin and mounted using Vecta Mount mounting medium (Vector Laboratories, Burlingame, CA). The slides were then examined for anti-CD3 positive staining. As an isotype control, mouse IgM (Accurate Chemical Co., Westbury, NY) was utilized. As a negative control, sections with no primary antibody were used. Also, as a positive control tissue, rat thymus was used.

Stereology. Stereo Investigator software (MicroBright Field, Inc., Colchester, NY) was employed for obtaining statistically unbiased inflammatory cell numbers in H & E stained sections. Sections of 6 μm thickness were analyzed using the optical fractionator method (Gundersen et al., 1988), which provides an unbiased estimate of the number of cell nuclei/ μm^3. Sections to be counted within each tendon sample were selected using a random numbers generator available in the Stereo Investigator software package. The

stereological method used in the current study was previously published (Wetzel et al., 2001).

Caliper Measurements. As a crude estimate of inflammation, ankle width was obtained from each animal both pre-injection and pre-harvest. A caliper (Forestry Suppliers, Jackson, MS) capable of determining very small widths (+/- 0.02 mm) was used to determine ankle thickness in the area immediately above the calcaneus bone and below the gastrocnemius muscle of the leg. The difference of the measures between pre-injection and pre-harvest is representative of inflammation occurring in each tendon and reflects the changes in vascular permeability that accompany acute inflammation (Lee et al., 1997).

Lyophilizer Measurements. At harvest, tendons were excised from the hindlimb and placed into pre-weighed, coded tubes and wet tendon weight was obtained. Each sample was weighed in triplicate and an average of the three measurements was used as the "wet weight" determination. Samples were then placed into a Unicool unit lyophilizer (Vestis Co., Gardiner, NY) for 24 hours to remove all water in the tendon substance. A dry tendon weight was then obtained, again being measured in triplicate, with the average being the "dry weight" measurement. The difference between the wet and dry weight was used to reflect the amount of water present in the tendon substance at each of the evaluated time points.

Functional Analysis. To monitor function, the Achilles Functional Index (Murrell et al., 1992) was utilized. Animals were tested in a confined Plexiglass walkway, 6.0 cm wide x 60 cm long (Fig. 7). A bright light was placed over the starting area to promote movement toward the other, unlit end of the walkway. A 6 x 60-cm piece of register roll paper was placed on the floor of the walkway. Held by their tail, a light cooking spray was applied to the animals' back paws before placement into the starting area. The cooking spray provided the oil for visualization of footprints by fingerprint powder used in the later steps of the functional analysis. The animals' footprints were then deposited onto the register paper as it traversed the gait walkway. The register papers were coded with animal number and then dusted with fingerprint powder (Law Enforcement Co., Kansas City, MO) to allow for print visualization and sprayed with artists' fixative to prevent smearing or loss of print integrity. Prints were then analyzed according to the method of Murrell et al., 1992. When an animal is injured, the print length should increase and the distance between toes should decrease, thereby resulting in a negative AFI. Correspondingly, a zero or positive AFI value correlates with a healthy or uninjured animal. Briefly, print length (PL), toe spread (TS), and intermediary toe spread (IT) were measured for the back footprint from both the control and injured sides for each animal (Fig. 8). PL is the distance from the third toe of the foot to the heel. TS is the distance between toes 1 and 5, and IT is the distance between toes 2 and 4. We recorded the measurements of all footprints on each strip of paper (range 2-6 prints per paper) and calculated the mean for each variable. These values were then used in the AFI formula:

$$\mathbf{AFI = 74(PL) + 161\ (TS) + 48\ (IT) - 5}$$

Figure 7. Gait Analysis Walkway. Diagram of the Plexiglass gait walkway used to assess level of function in injured animals at different time points post-injury. The Achilles Functional Index was used as a means of determining return to function, as described on page 46. Dimensions for the gait analysis walkway are 60cm x 6cm with plastic cages on each end.

Top View

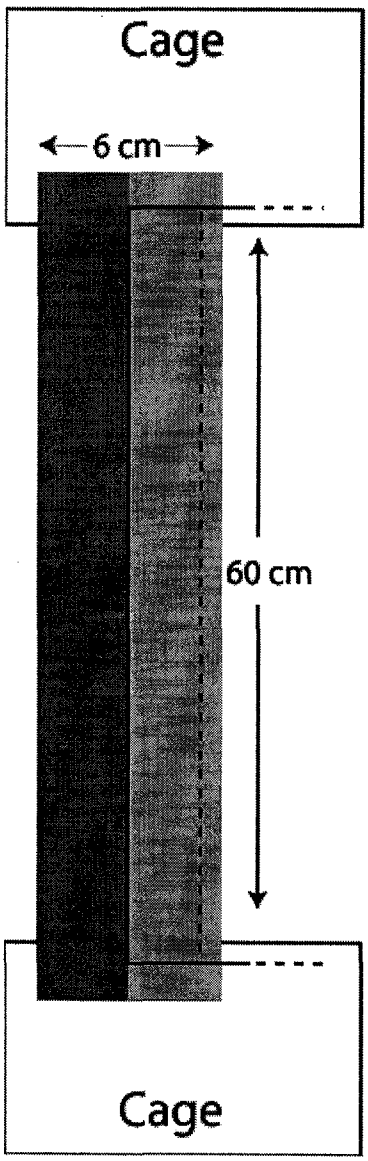
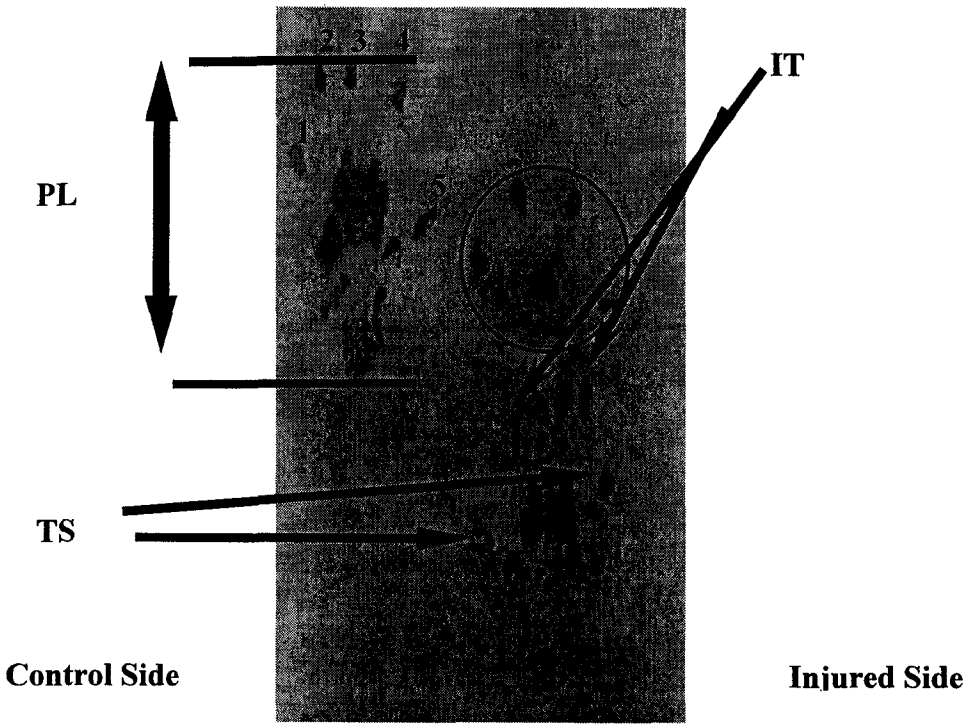


Figure 8. Example Rat Footprint Used in Gait Analysis. Example of an uninjured (left side) and an injured (right side) rat footprint. Animals received collagenase injections into the right Achilles tendon only. The left side was uninjected. The back paws of each animal were sprayed with cooking spray to provide oil to deposit onto the paper in the walkway. Animals traversed the gait walkway and deposited their footprints onto the underlying paper. The paper was then dusted with fingerprint powder to allow print visualization, described in detail on page 55. Both injured and uninjured sides were analyzed, measuring the following parameters, which are labeled in the figure: PL = Print length; TS = toe spread; IT = intermediary toe spread. Note the circled area in the figure represents a front paw print, which is not considered in the AFI analysis.



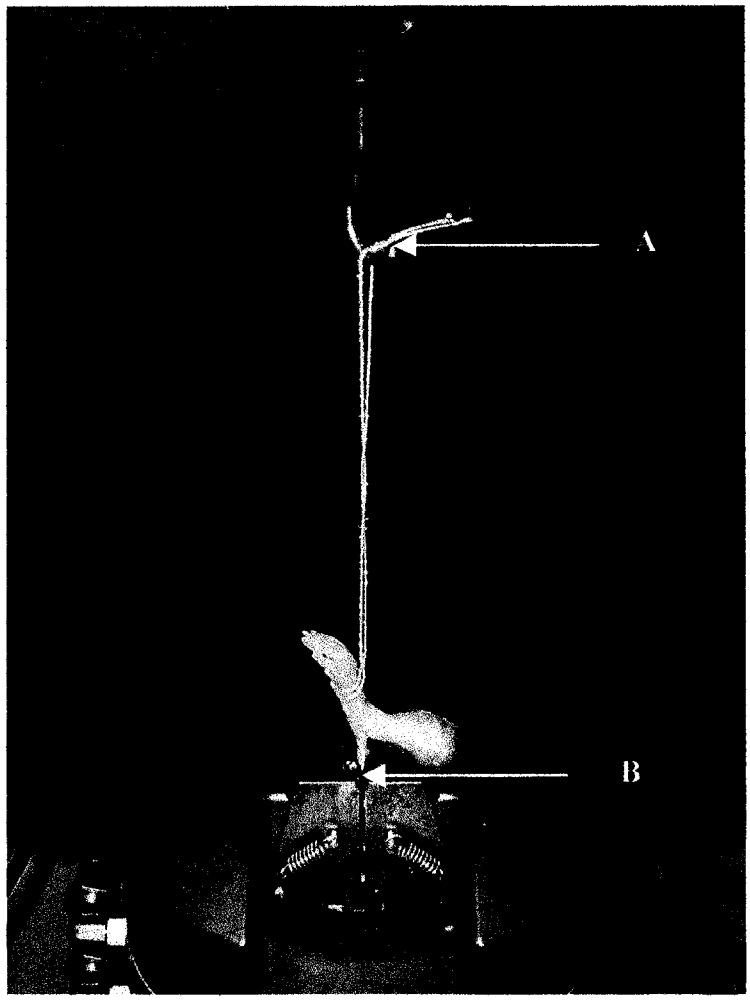
Due to observing differences in print length from print to print within one animal's prints for a single day, we made a modification to the above formula, whereby we used the actual foot length (FL) for each animal to determine how accurate the print length measurement had been:

$$\mathbf{AFI = 74(FL) + 161\ (TS) + 48\ (IT) - 5}$$

Foot length was measured by placing the paw on a flat surface and measuring from the tip of the longest toe to the back of the heel. We used this measurement in one experiment to determine if this significantly changed the AFI results. Unless stated otherwise in the results section, the formula used was the original one with print length rather than foot length.

Materials Testing System (MTS). After collecting intact left (normal) and right (injected) limbs, the samples were placed into 50 ml centrifuge tubes containing PBS (Phosphate Buffered Saline, Gibco BRL, Gaithersburg, MD) plus 0.1% sodium azide. Samples were kept at 4^0C for up to four days before analysis. Immediately prior to analysis, the tendon was freed from the muscle junction by cutting with scissors. The tendon remained attached to the calcaneus bone of the foot. Tissues that were not part of the tendon substance were dissected away. The intramuscular tendinous fibers were secured between two pieces of Skid Guard Safety Tape (Sure Foot Industries, Cleveland, OH) with Super Glue Gel (Pacer Technology, Rancho Cucamonga, CA) (Fig. 9A). The foot was tied with a noose-like apparatus, made from string, with a loop at one end for attachment to a hook on the load cell of the MTS machine (Fig. 9B). To eliminate the

Figure 9. Diagram of Set Up for Materials Testing System. The foot is attached with a noose-like string to the top hook in the machine (A). Tendon is glued between two pieces of no slip tape and this tape is placed between grips on the opposite end (B) before pulling at a constant rate of 1cm/ sec begins.



potential for the Super Glue affecting the tensile properties of the tendon, throughout the procedure care was taken to ensure that the Super Glue did not contaminate the tendon substance. Also, to prevent dehydration of the tendon during testing, PBS was applied using a squirt bottle when needed. To monitor the maximum force required to rupture each tendon (force to rupture), we utilized an MTS 858 Material Strength Testing Machine (MTS, Eden Prairie, MN) connected to a personal computer, kindly provided by Rose Hulman Institute of Technology. The load cell used was 500 N with a range of –0.05% of full scale in tension and +0.02% of full scale in compression. Failure was defined as complete breaking of the tendon sample between the two grips attached to the load cell. All tendons underwent a test of constant velocity ramp to failure at 1 cm/s. Force, time, and displacement were recorded and used for development of failure profiles during the subsequent analysis phase. Upon conclusion of testing, each tendon sample was observed for location of failure. These failure locations were recorded in the following manner:

NMP: Near muscle partial; tendon fibers break away partially from near the myotendinous junction

NC: Near calcaneus; tendon breaks near the calcaneal insertion point

MTS: Mid tendon substance; tendon breaks perpendicular to the fiber alignment, near the center of the tendon substance

Statistical Analysis and Power. Sample size was determined using power calculations. Assuming a 25% standard deviation from the mean, power analysis estimates an $n = 8$ animals (4 tendons) per condition and $\alpha = 0.05$. The uninjured limb served as a control

for each animal and a total of four to six animals per condition were used. Data was analyzed using Windows Excel. A two way analysis of variance (ANOVA) design was employed where noted in figure legends. The factors studied included treatment received (GEO, EBI, SHAM, SIGNAL 1, or SIGNAL 2), the time between injury and harvest (time 0, 24 hr., 48 hr., 72 hr., 1 week, and 2 week), and the possible interaction between treatment and harvest time. Significant differences were determined using ANOVA, followed by a Student's two-tailed T test. For experiments with an unequal sample size, the F test for variance was employed. The confidence limit was set at a level of $\alpha = 0.05$. For GEO versus SHAM comparisons, the Bonferroni correction factor was used to determine the significance level. Based on this calculation, a $p < 0.017$ (0.05/ 3 tests) level was established for significance in these data sets. Results are expressed as means +/- SEM.

RESULTS

Normal Healing Model

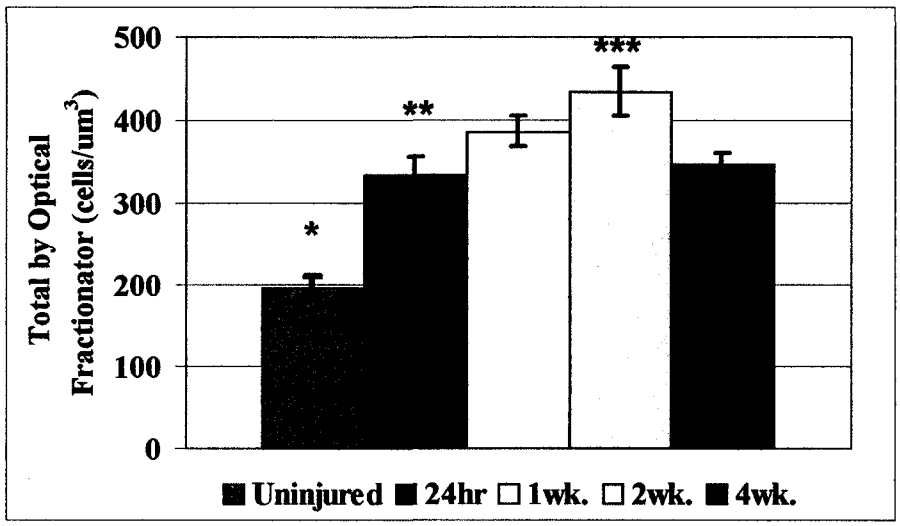
As mentioned previously, quantitative small animal models of tendon injury in the literature are lacking. Many of the existing small animal models have investigated only one of the various levels possible (gross anatomical, functional, cellular, and biomechanical) for evaluating injury and subsequently, healing (Iwuagwu et al., 1998; Jarvinen et al., 1999). Accordingly, we set out to develop a comprehensive, quantitative, model of tendinitis in a rat.

Based on the definition of tendinitis as an inflammation of tendon fibers associated with collagen fiber disruption and infiltration of cells from the surrounding vasculature, we first focused on examination of total cell numbers in normal, uninjured tendon. Staining with H & E, we first examined normal, uninjected tendons under a light microscope. As can be seen in Fig.2 A, in normal tendon, the placement of the collagen fibers is regular and the collagen displays a wavy appearance. In addition, very few cells are present, based on few dark purple stained areas that represent cell nuclei.

Once we had established the morphological characteristics for normal, uninjured rat Achilles tendon, we then sampled five 6 μm sections per tendon, along the longitudinal axis. The five sections chosen were selected to accurately characterize the entire tendon and included the top layer of the sample and four deeper lying sections.

As can be seen from Fig. 10, a timeline for normal tendon healing based on cellular infiltration was established. Based on this graph, it can be seen that normal

Figure 10: Inflammatory Cell Profile in Rat Tendinitis Over a Four Week Time Course. Animals received collagenase injections into the Achilles tendon and tendons were harvested at the noted time points (24 hours, 1, 2, and 4 weeks post-injury). Cell nuclei counts were enumerated by optical fractionator as described on page 51. Bars are representative of number of cells/ um^3 from a total of 23-30 sections/ time point. Error bars are representative of SEM of n = 6 animals/ time point. Significant differences are indicated, calculated using Student's T-test, $p < 0.05$. * Normal significantly different from all other time points. ** 24 hr. significantly different from 2 wk $p = 0.032$ and *** 2 wk significantly different from 4 wk, $p = 0.022$.

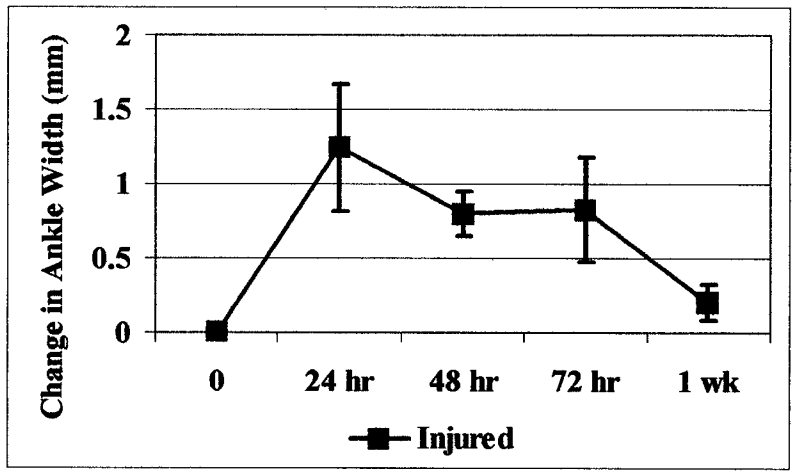


tendon, on average, contains two hundred cells/μm^3. In injected tendons, as early as the twenty-four hour post-injury time point, the cell number increases to almost three hundred and fifty cells/μm^3 and by one-week post-injury, cell number is almost 100% greater than uninjured controls. The two-week post-injury time point is also the time at which inflammatory cell numbers reach their peak in this model (> 400 cells/ μm^3). By two weeks post-injury, lymphocyte and fibroblast cells are beginning to be replaced mainly by highly proliferative fibroblasts (Iwuagwu et al., 1998). These are the cells responsible for laying down new collagen to replace that damaged by injury.

At the four week post-injury time point, it is clear that cell numbers have begun a trend back toward that of normal, uninjured tendon samples and display cell numbers in the range of three hundred cells/μm^3. This decrease in cell numbers may be attributed to proliferative fibroblasts exiting the tendon after deposition of new collagen to replace that which had been damaged upon injury. Ours is the first study to quantitate cell numbers over this prolonged time period in whole tendon samples, using an unbiased method.

As another means of assessing natural tendon healing, we injected tendons with collagenase and determined the change in ankle width via caliper measures. Ankle widths were determined both pre-injection and pre-harvest for each animal. Results are expressed as the difference between the pre-harvest and the pre-injection ankle width at each of the time points considered. Figure 11 shows the resultant time course for natural healing of tendon injury via measurement of swelling in the ankle. Note that although the ankle width has increased as much as 1.25 mm by twenty-four hours post-injury, by one week the change in ankle width has returned to the baseline level.

Figure 11. Caliper Measurements of Rat Ankles in Normal Tendon Healing. Animals received collagenase injections into the Achilles tendon and were exposed to the Earth's magnetic field until tendons were harvested at the noted time points (24, 48, 72 hours, and 1 and 2 weeks post-injury). The change in ankle width from pre-injection to pre-harvest was determined through use of calipers, as described on page 52. Results are expressed as the difference between pre-injection and pre-harvest ankle widths for each of the time points indicated. n = 5 animals per time point. Error bars are representative of SEM.



In addition to analyzing normal healing in rat tendinitis via cell enumeration and caliper measures, we also examined how quickly function was regained without intervention after injury. Figure 12 is a representation of normal healing in regard to function, using the Achilles Functional Index. It is clear that by one week post-injury, animals have regained complete function, as indicated by the increase in AFI to pre-injury levels of zero or above.

EBI Field

From the characterization of normal healing in the rat, we determined an approximate time for changeover from the acute to chronic phase. Based on cell numbers (Fig. 10), we determined this crossover point between acute and chronic phases occurred around 2 weeks post-injury. Chronic inflammation is characterized by a lack of inflammatory cells in parts of the tendon (Jarvinen et al., 1997). We then turned our focus to the acute phase time points. In addition to investigating healing without application of PEMFs, as in the model development experiments, we also applied PEMFs to determine if they had any effect on the normal acute phase time course.

Beginning at the cellular level of analysis, we injected 150 IU/ kg rat body weight into the Achilles tendon of the animals in two exposure groups: GEO (control) and EBI. Tendons were harvested at the following time points: twenty-four, forty-eight, and seventy-two hours, as well as one week post-injury. By twenty-four hours post-injury, the normal tendon number again had increased, as in the model, to almost four hundred cells/μm^3 (Fig. 13). This type of increase is expected, due to rapid infiltration by PMNs

Figure 12. Achilles Functional Index in Normal Tendon Healing. Animals received collagenase injections into the right Achilles tendon and were exposed to the Earth's magnetic field until harvest at the noted time points (24, 48, 72 hours and 1 and 2 weeks post-injury). Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest, as described on page 52. Results are expressed as Achilles Functional Index (AFI) units. n = 4 per time point. Error bars represent SEM.

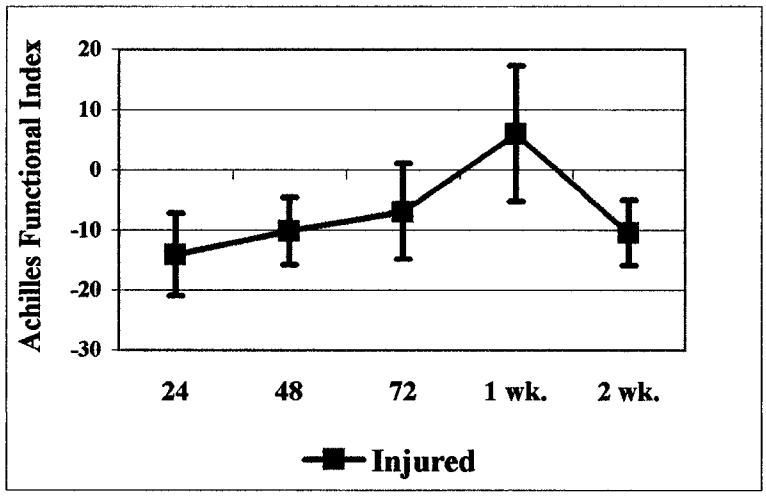
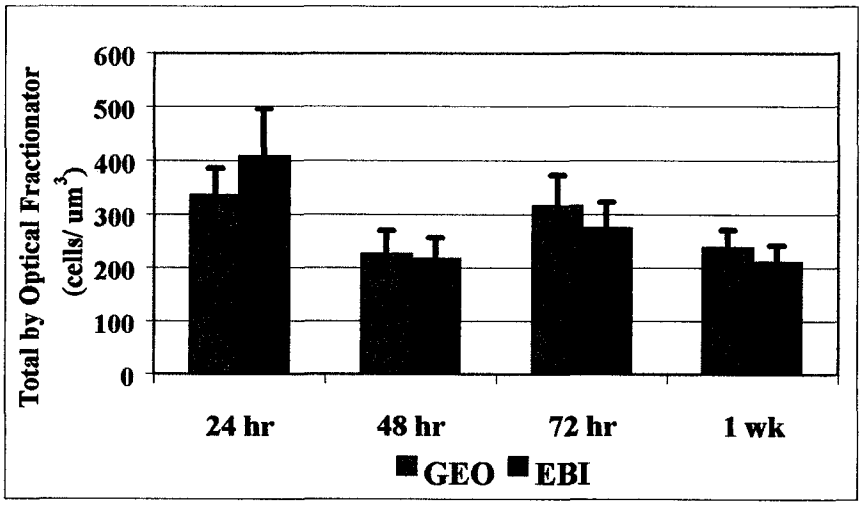


Figure 13. Inflammatory Cell Profile in EBI-Exposed vs. GEO-Exposed Rat Tendon. Animals received collagenase injections into the Achilles tendon and were exposed to the EBI or the GEO field for 4 hrs./ day until tendons were harvested at the noted time points (24, 48, 72 hours and 1 and 2 weeks post-injury). Cell nuclei counts were enumerated by optical fractionator as described on page 51. Bars are representative of number of cells/ um^3 from a total of 23 sections/ time point. n = 5 animals/ time point. Error bars are representative of animal number (n = 5) SEM. Using ANOVA, F for time points (3, 176) = 21.4; $p << 0.00001$; F for field (1, 176) = 0.013; p = 0.901; F for interaction (3, 176) = 3.47; p = 0.017.

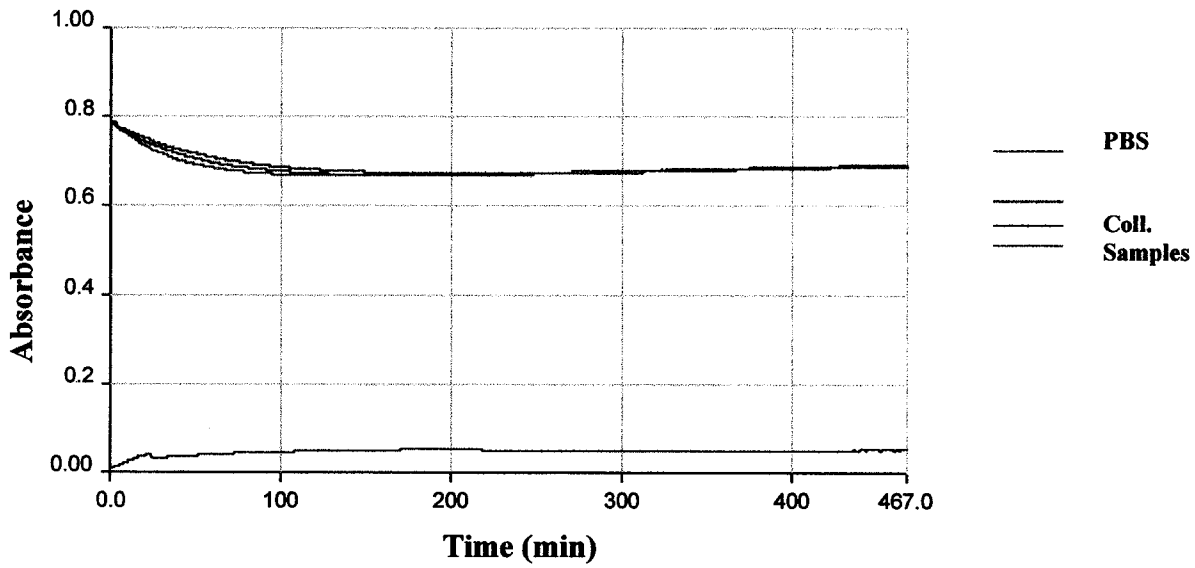


upon injury. A significant difference between time points was noted, as can clearly be seen from the graph. At forty-eight hours post-injury, we observed a decrease in inflammatory cell nuclei, back to the level of the normal tendons. This decrease could be related to a drop in the presence of PMNs, prior to fibroblasts entering to repair damage. By seventy-two hours post-injury, the inflammatory cell number had begun to again increase. At this time point, cell numbers reached about three hundred/μm^3. At one week post injection, the cell numbers had dropped off nearly to the level of normal tendon.

Due to differences seen between experiments in regard to cell number (Figure 10 and Figure 13) at the one week post-injury time point, we investigated the possibility that the different lots of collagenase used had different enzymatic activities associated with them. To do so, we conducted a collagenase activity (FALGPA) assay. As can be seen from Figure 14, the FALGPA assay shows there was no marked difference in activity between any of the lots used for any of the experiments described within this paper. All enzymes hydrolyzed FALGPA at approximately the same rate over time and complete hydrolysis had occurred by two hours after addition of the collagenase enzyme to the buffer containing the FALGPA synthetic substrate. Therefore, the possibility of differences in collagenase enzyme activity contributing to the discrepancy in cell numbers was ruled out. As yet uncontrolled variables may have contributed to this difference.

In addition to enumerating inflammatory cell numbers over the acute time course with EBI exposure, we also measured hind leg edema via calipers (Wetzel et al., 2002). The ankle width was measured both pre-injection and pre-harvest for each animal.

Figure 14. Collagenase Enzyme Activity Assay. The synthetic collagenase substrate, 2-furanacryloyl-L-prolyly-L-alanine (FALGPA) was used to assess the activity of the three collagenase enzyme lots used in the current study as described on page 41. Decreasing absorbance over time indicates activity of the collagenase enzyme is intact. PBS was used as a control (see pink line near bottom of graph).



Results are expressed as the difference between the pre-harvest and the pre-injection ankle width at each of the time points considered. It is apparent that with the EBI field exposure setup, there were no significant differences between the experimental and the GEO (control) groups at any of the time points (Figure 15). From Figure 15, it is also apparent that the change in ankle width by twenty-four hours post-injury is 0.5 mm. This level is maintained until seventy-two hours post-injury, when the ankle width increases to almost 1.0 mm greater than the overall pre-injection value. This seventy-two hour time point is the peak in terms of hindleg edema, as measured by the caliper method. By one week post-injury, the change in ankle width (pre harvest minus pre injection) has dropped to zero, which is indicative of a normal, or uninjured hind limb. The caliper method of measuring hind leg edema gives a gross measurement of the amount of swelling that has occurred in each animal over the post-injury time course. To determine if hindlimb swelling was related to the exact amount of edema that occurred within the tendon substance itself, as compared to its pre injury water content, we measured the tendon water content via a lyophilizer method.

Figure 16 shows there was no significant difference between EBI-exposed and the GEO (control) tendons in regard to water content of the tendon substance at any of the time points measured. By twenty-four hours post-injury, both the EBI exposed and the control groups have begun an upward trend in terms of water content. This trend increases toward an ultimate peak that is reached for both groups between forty-eight and seventy-two hours post-injury. By one week post-injury, a downward trend has begun in both groups. By two weeks post-injury, the level of water content in all tendons has reached that of the normal or injured tendons. The lyophilizer results show tendon water

Figure 15. Caliper Measurements of Rat Ankles in EBI-Exposed vs. GEO-Exposed Animals. Animals received collagenase injections into the Achilles tendon and were exposed to the EBI or the GEO field for 4 hrs./ day until tendons were harvested at the noted time points (24, 48, 72 hours, and 1 and 2 weeks post-injury). The change in ankle width from pre-injection to pre-harvest was determined using calipers, as described on page 52. Results are expressed as the difference between pre-injection and pre-harvest ankle widths for each of the time points indicated. $n = 5$ animals per exposure group. Error bars are representative of SEM. There were no significant differences between EBI-exposed and GEO-exposed animals for any of the time points. Using ANOVA, F for time points $(5, 48) = 10.7$; $p << 0.00001$; F for field $(1, 48) = 0.197$; $p = 0.659$; and F for interaction $(5, 48) = .572$; $p = 0.721$.

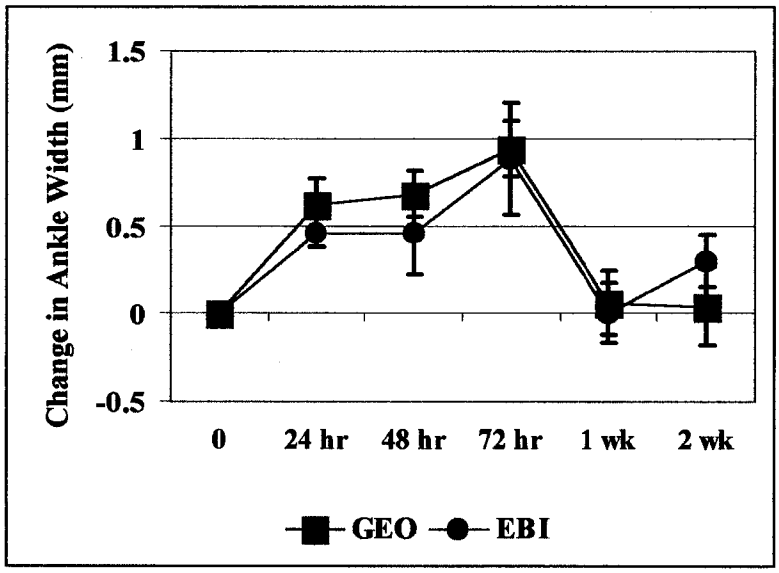
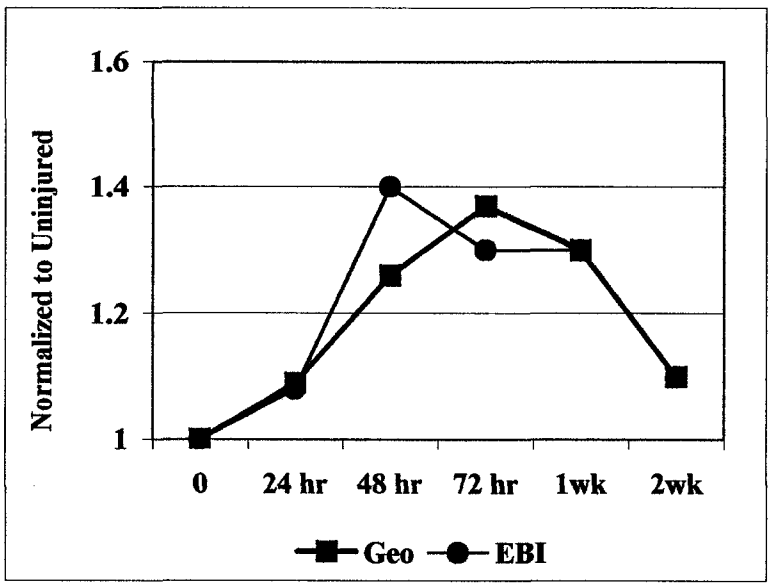


Figure 16. Water Content as Measured by Lyophilizer in EBI-Exposed vs. GEO-Exposed Rat Tendon. Animals received collagenase injections into the Achilles tendon and were exposed to the EBI or the GEO field for 4 hrs./ day. Tendons were harvested at the noted time points and wet tendon weights were taken for each sample. All samples were then dried in a lyophilizer for 24 hours, as described on page 52. A dry tendon weight was then taken for each sample. Tendon water content is expressed as the percent wet weight (wet-dry weight/ wet weight) normalized to an uninjured sample. $n = 5$ per exposure group. There were no significant differences between EBI and GEO exposed samples. Using ANOVA, F for time points $(5, 48) = 10.1$; $p < 0.00001$; F for field $(1, 48) = 0.001$; $p = 0.973$ and F for interaction $(5, 48) = 0.742$; $p = 0.596$.



content remains above normal past the one week time point while hindleg swelling (via caliper) had returned to normal. This difference can be explained by the fact that the caliper method is measuring the entire hindleg, while the lyophilizer method is taking a direct measure of tendon water content.

We used the Achilles Functional Index (AFI) to determine the effects of injury on hindleg function and to correlate this function to the amount of tissue damage. From Figure 17 it is apparent that the only significant difference in function at any of the measured time points between the EBI-exposed and the GEO animals occurred at one week post-injury. The EBI-exposed group displays decreased function as compared to the GEO-exposed group at this time point. This decrease in function continues through, and peaks at seventy-two hours post-injury. Following the seventy-two hour time point, an upward trend has begun. By one week post-injury both groups have nearly regained their original function. The two week time point shows the return to normal for both the EBI exposed and the GEO animals. This return to normal follows nicely with the return seen in the caliper and lyophilizer methods.

The AFI formula incorporates the measurement of three different parameters from each footprint. To determine if any one of these three parameters contributed more to the overall AFI formula, we examined each of the parameters alone. The first of these parameters is print length. Print length is the distance from the third toe of the foot to the back or end of the foot (Figure 8). From Figure 18 it can be seen that print length definitely contributes to the AFI at the twenty four and seventy-two hour post-injury time

Figure 17. Achilles Functional Index in EBI-Exposed vs. GEO-Exposed Animals. Animals received collagenase injections into the right Achilles tendon and were exposed to either the EBI or GEO field for 4 hrs./ day. Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest, as described on page 52. Results are expressed as Achilles Functional Index (AFI) units. $n = 5$ per exposure group. Error bars represent SEM. Using ANOVA, F for time points $(5, 48) = 2.36$; $p = 0.054$; F for field $(1, 48) = 0.117$; $p = 0.734$. and F for interaction $(5, 48) = 1.09$; $p = 0.380$. * EBI-exposed significantly different than GEO-exposed, $p = 0.011$.

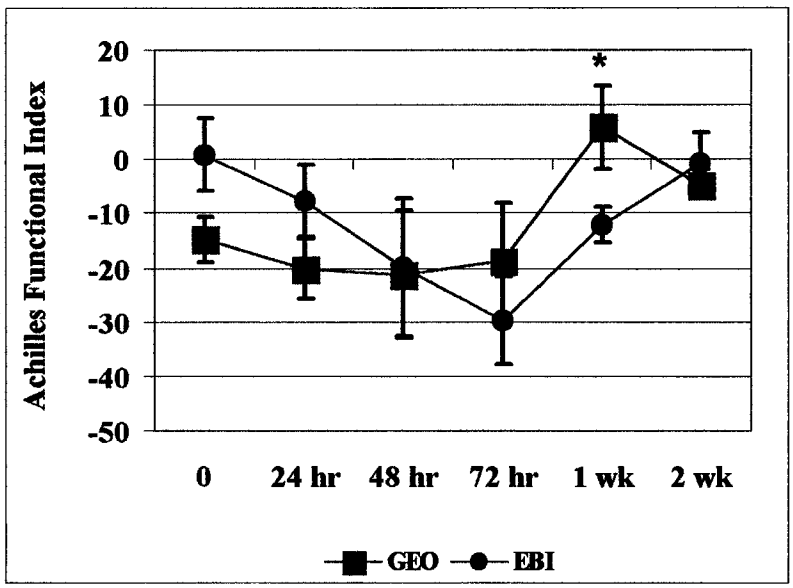
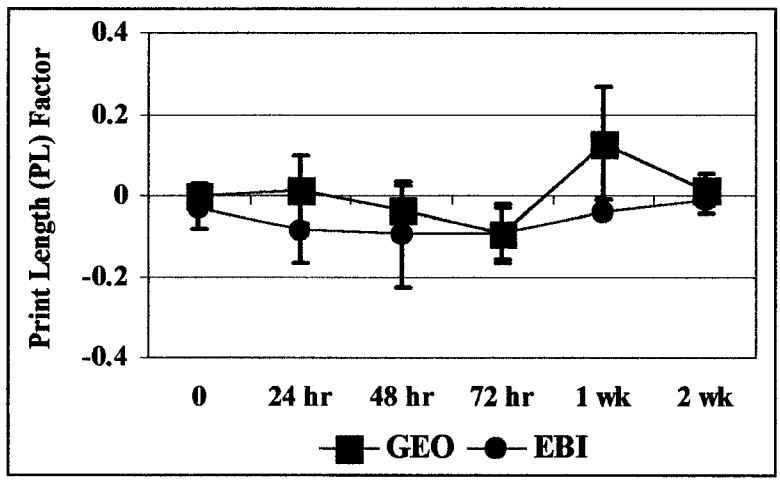


Figure 18. Print Length in EBI-Exposed vs. GEO-Exposed Animals. Results are expressed as print length factor for each time point. Print length factors were obtained by measuring the print length of the uninjured and the injured side. The difference between the two sides was divided by the print length of the uninjured side to obtain the print length parameter, as described on page 53 and 57. n = 5 per exposure group. There were no significant differences between the EBI-exposed and the GEO-exposed animals in regard to print length for any of the measured time points. Using ANOVA, F for time points (5, 48) = 1.15; p = 0.349; F for field (1, 48) = 2.90; p = 0.095; and F for interaction (5, 48) = 0.414; p = 0.837.



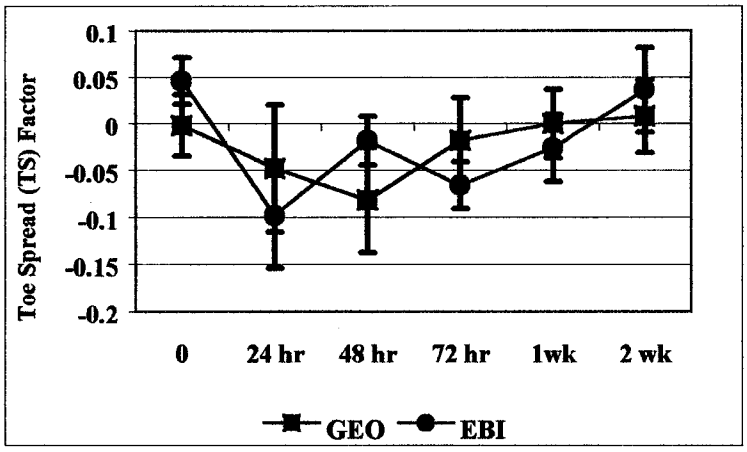
points. This is clear from the negative PL values at these two time points. The second parameter that is included in the AFI formula is toe spread. Toe spread is the distance between the first and the fifth toes of the foot (Figure 8). As can be seen from the graph in Figure 19, there were no significant differences between EBI exposed and control (GEO) prints in regard to toe spread. Additionally, the toe spread factor seems to decrease most at the seventy-two hour post-injury time point and this contributes to the overall decrease in function. Toe spread, as well as print length plots returned to the normal or zero level by the one week post-injury point on the graphs.

The last factor that is included in the overall functional analysis using the AFI is the intermediary toe spread. This factor is taken as the distance between the second and fourth toes of the foot. The intermediary toe factor again showed no significant difference in terms of the EBI exposed vs. the control prints (results not shown).

The profile of intermediary toe spread also shows a decrease at seventy-two hours and a corresponding increase toward normal levels by two weeks post-injury. Taken as a whole, the toe spread and the intermediary toe spread factor correlate nicely with the overall AFI formula results and consistency within the same animal was fairly constant. Print length results displayed some variability within the same animals, and this fact contributed to the use of actual foot length in later experiments.

Cumulative results from the EBI experiments show no significant effect of the field on tendon healing. The two statistically significant differences observed were an increase in cell number at twenty four hours in the EBI animals, as well as a decrease in function at the one week time point, as compared to the GEO (control) animals. Based

Figure 19. Toe Spread in EBI-Exposed vs. GEO-Exposed Animals. Results are expressed as toe spread factor for each time point. Toe spread factors were obtained by measuring the toe spread of the uninjured and the injured side. The difference between the two sides was divided by the toe spread of the injured side to obtain the toe spread parameter, as described on pages 52 and 57. n = 5 animals per exposure group. There were no significant differences between the EBI-exposed and the GEO-exposed animals in regard to toe spread for any of the measured time points. Using ANOVA, F for time points (5, 48) = 2.19; p = 0.071; F for field (1, 48) = 0.015; p = 0.902; and F for interaction (5, 48) = 0.894; p = 0.493.



on this result, we investigated the possibility of increasing the collagenase dose and its effects on function.

Collagenase Dosage Trial

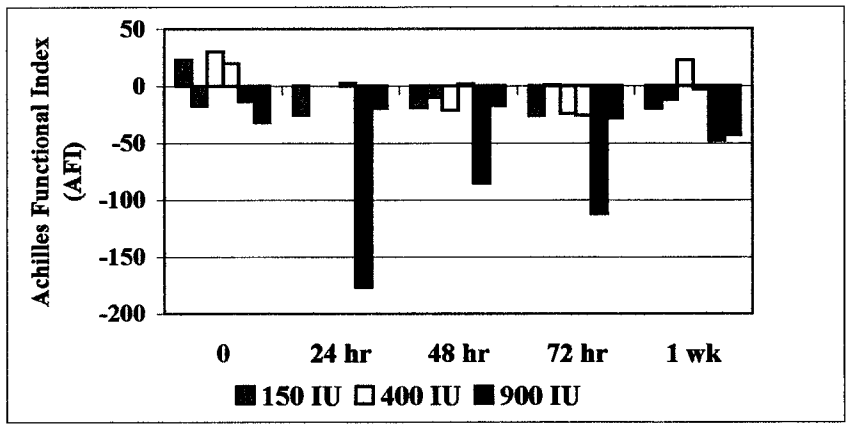
Three different doses of collagenase were chosen for a pilot experiment to examine the effect of increasing enzyme concentration on the various parameters we were using to investigate injury. All dosages were IU/ kg rat body weight and injected into the Achilles tendon. The doses used were 150, 400, and 900 IU.

The AFI parameter was used to identify any differences in function that could be observed from increasing enzyme concentration. As can be seen from Fig. 20, the 900 IU dose had a greater effect on the AFI than did the 150 and 400 IU doses. The AFI for all three groups had returned to normal levels by two weeks post-injury. From this small (n = 2) pilot experiment, we learned that increasing the dose of collagenase to 900 IU/ kg rat body weight would induce an injury that would provide a greater range of values for the AFI. Therefore the dosage used in the remaining results (using the TEMF field) was 900 IU/ kg rat body weight.

TEMF Field

After determining the timing for edema formation and functional capability through the EBI exposure experiments and the dosage trial, we focused on utilizing a different pulsed EMF. The field used for this set of studies was the EMF Therapeutics field (TEMF). This field has been shown to reduce tumor growth (Williams et al., 2001) and also is undergoing clinical trials for use with humans suffering from lower back pain

Figure 20. Collagenase Dosage Trial- Analysis Using the Achilles Functional Index. A comparison of injections of 150, 400, and 900 IU of collagenase/ kg rat body weight. Animals were injected into the Achilles tendon with one of the three doses mentioned above and functional analyses were conducted at the time points shown on the graph (pre-injection or time 0, 24, 48, 72 hours, and 1 week post-injury). Note the 900 IU/ kg body weight dose appears to induce the greatest loss of function at all measured time points. All three groups have reached normal, pre-injury levels by one week post-injury. All data points shown; n = 2 animals per dosage group.



(Harden et al., 2002). The TEMF field delivers two signals, the difference lying in a slight variation in the pulse off time, as presented in the methods section of this paper.

Results from this set of experiments are shown in terms of animals exposed to either SIGNAL 1 or SIGNAL 2 or the SHAM (control) configuration.

To determine specific cell types that infiltrated the tendon after injury, we used an antibody specific to the CD3 portion of the T cell receptor. Figure 21 displays the results of the cell counts for CD3-positive cells in both the SIGNAL 2 and SHAM exposed groups for the forty-eight and seventy-two hour time points. There were no significant differences between the numbers of $CD3^+$ cells in the SHAM vs. the SIGNAL 2 groups. Figure 22 is a representation of this data which incorporates total inflammatory cell numbers at both the forty-eight and seventy-two hour time points. Results are expressed as the percentage of $CD3^+$ T cells in the total inflammatory T cell population. It is possible that T cells have not received signals to enter the tendon at these early time points post-injury. The amount of hind limb swelling in the TEMF exposed animals was compared to that of the SHAM animals. Again, the results are expressed as the difference between the pre harvest and the pre injection ankle widths at each of the measured time points.

From Figure 23 it is evident that there exists no significant difference between either of the signal-exposed groups vs. the SHAM at any of the time points measured. Also, it is clear that there were two peaks in hind leg edema in this experiment: one at twenty four hours and one at seventy-two hours post-injury. Hind leg edema has dropped to normal levels by one week post-injury, and this normal level is maintained at the two

Figure 21. $CD3^+$ Cells in TEMF (SIGNAL 2)-Exposed vs. SHAM-Exposed Rat Tendon. Animals were injected with collagenase into the Achilles tendon and exposed to either the TEMF (SIGNAL 2) or the SHAM field for 30 mins./ day and tendons were harvested at forty-eight and seventy-two hours post-injury, then embedded in paraffin. Sections were cut and a monoclonal antibody to the CD3 portion of the T cell receptor was used to determine the number of $CD3^+$ T cells at each of the time points, as described on page 50. Results are expressed as the total number of $CD3^+$ cells at each time point and condition of exposure. $n = 2$ animals per exposure group. Using a Student T test, there were no significant differences between the TEMF-exposed and the SHAM-exposed tendons in regard to numbers of $CD3^+$ T cells at the forty-eight ($p = 0.967$) and seventy-two hour ($p = 0.233$) time points.

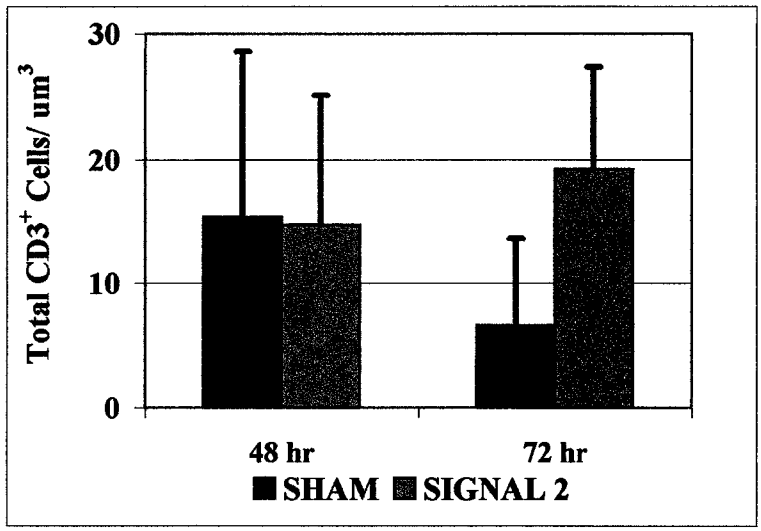


Figure 22. Percentage of $CD3^+$ Cells in TEMF (SIGNAL 2)-Exposed vs. SHAM-Exposed Rat Tendon. Animals were injected with collagenase into the Achilles tendon and exposed to either the TEMF (SIGNAL 2) or the SHAM field for 30 mins./ day and tendons were harvested at forty-eight and seventy-two hours post-injury, then embedded in paraffin. Sections were cut and a monoclonal antibody to the CD3 portion of the T cell receptor was used to determine the number of $CD3^+$ T cells at each of the time points, as described on page 50. Results are expressed as the percentage of $CD3^+$ cells in the total inflammatory cell population for each time point and condition of exposure. n = 2 animals per exposure group.

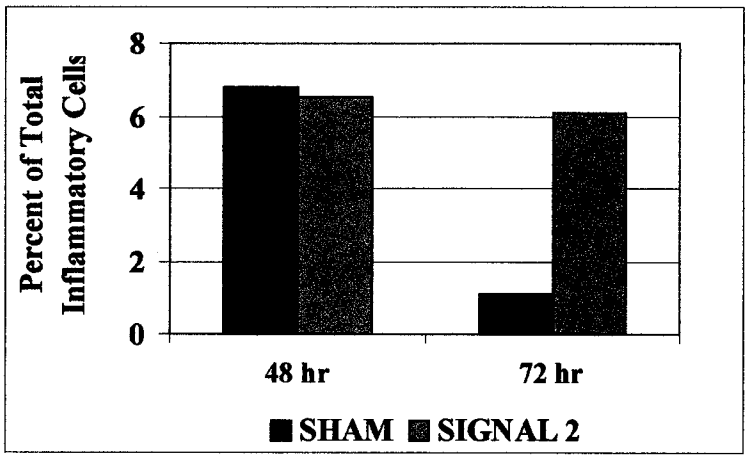
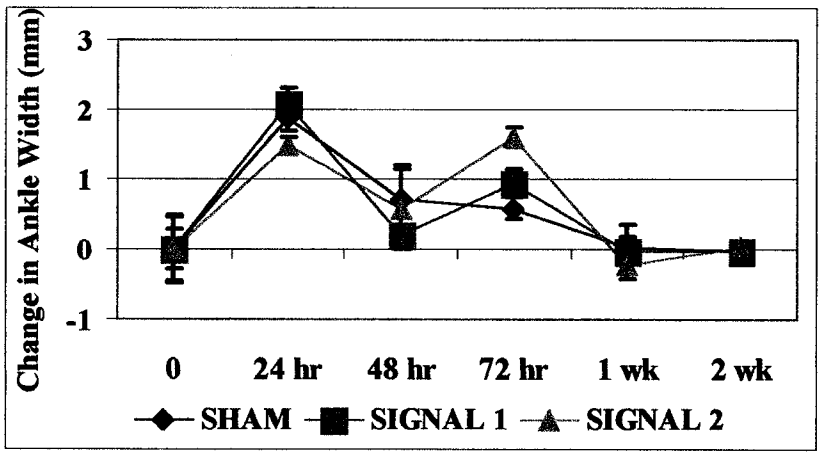


Figure 23. Caliper Measurements of Rat Ankles in TEMF-Exposed vs. SHAM-Exposed Animals. Animals received collagenase injections into the Achilles tendon and were exposed to the TEMF or the SHAM field for 30 mins./ day until tendons were harvested at the noted time points. The change in ankle width from pre-injection to pre-harvest was determined through use of calipers, as described on page 52. Results are expressed as the difference between pre-injection and pre-harvest ankle widths for each of the time points indicated. $n = 4$ animals per exposure group. Error bars are representative of SEM. There were no significant differences between TEMF-exposed and SHAM-exposed animals for any of the time points. Using ANOVA, F for time points $(5, 54) = 31.3$; $p << 0.00001$; F for field $(2, 54) = 0.045$; $p = 0.956$; and F for interaction $(10, 54) = 1.58$; $p = 0.138$.



week time point, indicating that swelling of the ankle has decreased to normal in all observed groups by two weeks after collagenase injection.

To again correlate the hindlimb swelling measured via caliper with actual tendon water content, we used a lyophilizer method. We observed an increase in water content of near 50% in the tendon by twenty-four hours post-injury (Figure 24). By forty-eight hours post-injury, water content in the tendon had drastically declined. Over the remaining time period through two weeks post-injury, the tendons acquired more than 100% of their original water content. SIGNAL 2 appears most effective at reducing water content at the forty-eight hour time point. There appeared to be no difference between the SHAM and SIGNAL 1 exposed samples at any of the measured time points. However, SIGNAL 2 displayed a trend of maintaining a water content closer to that of uninjured tendon over time.

Once we had established the time course for healing with SIGNAL 1 and SIGNAL 2 exposure in terms of inflammatory cell infiltration, hind leg edema, and tendon water content, we focused our energy on examination of functional indices. Using the AFI method, we found that we again observed a maximal decrease in function at seventy-two hours post-injury (Figure 25). This decrease was subsequently followed by a return to normal by one week post-injury. This return to normal was maintained at two weeks post-injury. There were no significant differences among any of the three exposure sets in regard to function, although a clear trend of increased function in the SIGNAL 2 exposed animals can be seen, corresponding to the trend observed with the lyophilizer data (Fig. 24).

Figure 24. Water Content as Measured by Lyophilizer in TEMF-Exposed vs. SHAM-Exposed Rat Tendon. Animals received collagenase injections into the Achilles tendon and were exposed to the TEMF or the SHAM field for 30 minutes/ day. Tendons were harvested at the appropriate time points and wet tendon weights were taken for each sample. All samples were then dried in a lyophilizer for 24 hours, as described on page 52. A dry tendon weight was then taken for each sample. Tendon water content is expressed as the difference between the wet and dry weight, normalized to an uninjured sample. n = 4 per exposure group. There were no significant differences between the TEMF-exposed and the SHAM-exposed tendons in regard to water content at any of the measured time points. Using ANOVA, F for 24 hr (2, 9) = 3.35; p = 0.08; F for 48 hr (2, 9) = 2.29; p = 0.157; F for 72 hr (2, 9) = 0.353; p = 0.712; F for 1 wk (2, 9) = 1.83; p = 0.216; and F for 2 wk (2, 9) = 0.287; p = 0.757.

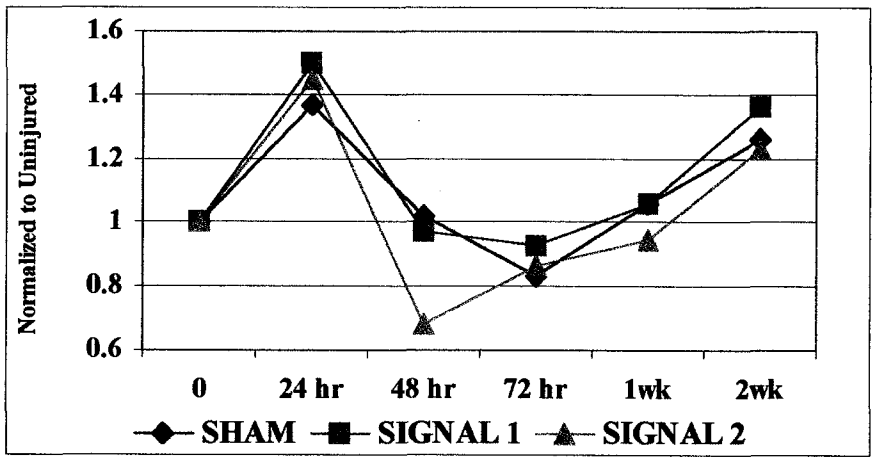
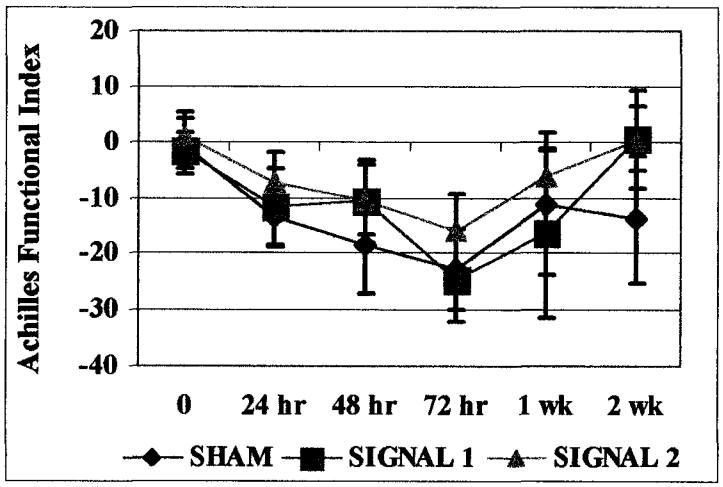


Figure 25. Achilles Functional Index Using Print Length in TEMF-Exposed vs. SHAM-Exposed Animals. Animals received collagenase injections into the right Achilles tendon and were exposed to either the TEMF or SHAM field for 30 minutes/ day. Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest as described on page 52. Results are expressed as Achilles Functional Index (AFI) units. n =4 per exposure group. Error bars represent SEM. There were no significant differences between TEMF-exposed and SHAM-exposed animals at any of the measured time points. Using ANOVA, F for time 0 (2, 48) = 0.310; p = 0.735; F for time 24 hr (2, 54) = 0.300; p = 0.744; F for time 48 hr (2, 42) = 0.358; p = 0.701; F for time 72 hr (2, 30) = 0.284; p = 0.755; F for time 1 wk (2, 21) = 0.202; p = 0.818; and F for time 2 wk (2, 9) = 1.16; p = 0.358.



To observe individual functional differences across animals, we plotted the AFI for each animal from the SIGNAL 1 exposure group individually (Figure 26). It is clear that some variation exists, although the amount of variability is fairly small. Based on the variability in print length observed, we measured individual foot lengths post harvest. This dimension was used to replace the print length factor in the AFI formula. The graph of this result is depicted in Figure 27. It is apparent that use of animal foot length does not markedly alter the outcome of the AFI functional analysis as compared to Figure 25 results, using print length in the formula. This result confirmed the utility of the AFI formula as applied to our model, in spite of the variation in print length that we observed within one animal.

An additional matter of interest was variability in pre-injury AFI. Due to this observed difference in pre-injection AFI , we refined our methodology by pre-selecting a population of animals with a pre-injection AFI of +/- 10, an error range defined by De Medinaceli et al., 1982 as acceptable for normal, uninjured animals when considering a similar functional index, the sciatic functional index or SFI. Animals were also grouped according to weight, to determine if excess weight bearing was a contributing factor to abnormally low pre-injury AFI values. Figure 28 is a representation of the results from the pre-injection analysis. It is clear that all weight groups (including the heaviest animals), were within the pre-injury AFI boundaries of +/ - 10. Therefore, animal weight does not dictate normal function in regards to the AFI methodology.

Figure 26. Individual AFI Results in TEMF (SIGNAL 1)-Exposed Animals. Animals received collagenase injections into the right Achilles tendon and were exposed to the TEMF (SIGNAL 1) field for 30 minutes/ day. Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest as described on page 52. Results are expressed as Achilles functional Index (AFI) units. Plot shows individual AFI results to assess variability among animals.

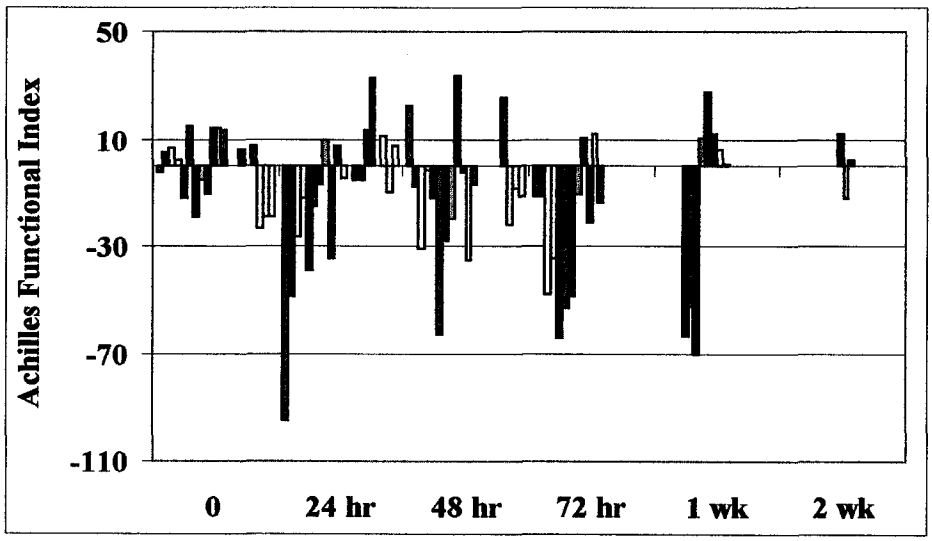


Figure 27. Achilles Functional Index Using Foot Length in TEMF-Exposed vs. SHAM-Exposed Animals. Animals received collagenase injections into the right Achilles tendon and were exposed to either the TEMF or SHAM field for 30 minutes/ day. Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest as described on page 52. Results are expressed as Achilles Functional Index (AFI) units. n = 4 per exposure group. Bars represent SEM. Foot length was used to replace print length in this graph. There were no significant differences between the TEMF-exposed and SHAM-exposed animals in regard to AFI using foot length at any of the measured time points. Using ANOVA, F for time 0 (2, 48) = 0.369; p = 0.693; F for time 24 hr (2, 54) = 0.398; p = 0.674; f for time 48 hr (2, 42) = 0.413; p = 0.664; F for time 72 hr (2, 30) = 0.207; p = 0.814; F for time 1 wk (2, 21) = 1.28; p = 0.299; and F for time 2 wk (2, 9) = 0.481; p = 0.633. Note no marked difference between this result and that in Figure 23 using print length in the AFI formula.

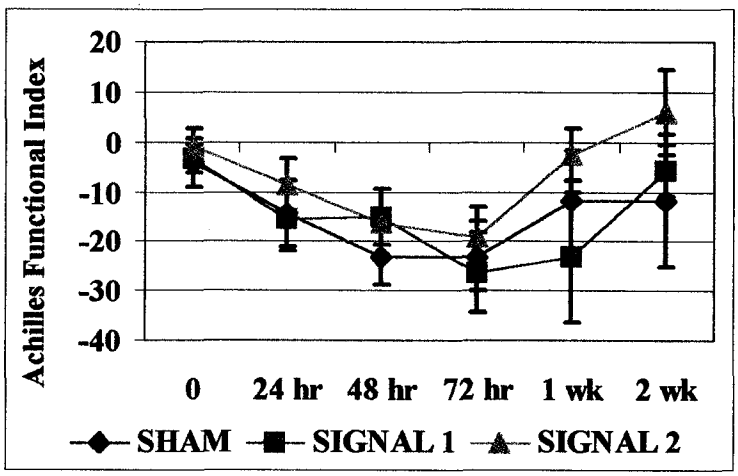
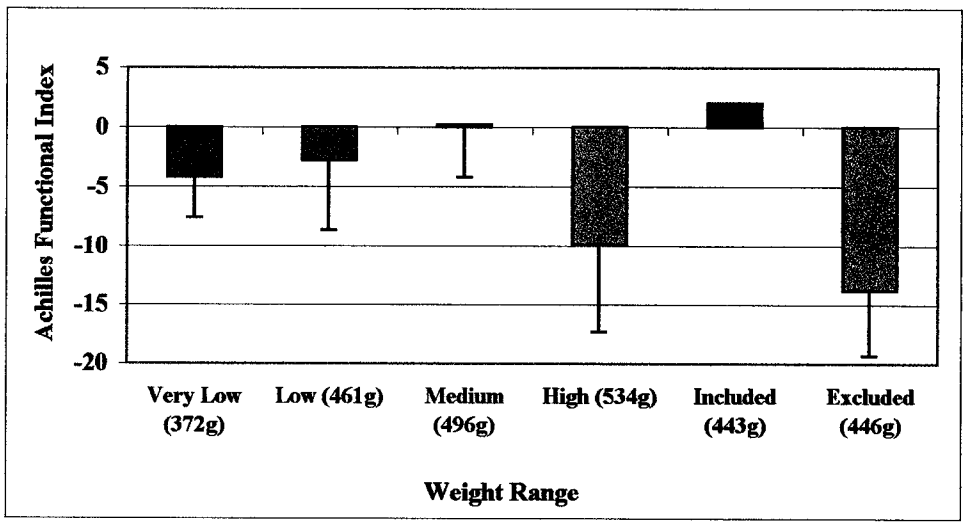


Figure 28. Pre-Injection Achilles Functional Index as Normalized to Animal Weight. Animals received collagenase injections into the Achilles tendon and were exposed to either the TEMF or SHAM field for 30 minutes/ day. Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest as described on page 52. Results are expressed as Achilles functional Index (AFI) units. Error bars represent SEM.



To evaluate tensile properties of the tendons over time, we measured the force required to rupture each sample. As a means of assessing force to rupture, we used an MTS 810 machine, as described in the methods section of this paper. Figure 29 is a representation of this data. From this graph, it is clear there was no significant difference in breaking strength of the tendons between exposure groups except at the seventy-two hour time point. The SHAM tendons displayed a much greater mean force to rupture than did the signal exposed samples at this time point. This observed decrease in tendon strength with field exposure could be due to increased cell proliferation that causes fiber disruption and subsequent loss of integrity of the tendon substance. It is also clear that the mean breaking strength in all three groups reaches a minimum at seventy-two hours post-injury and returns to the twenty-four hour injury level by two weeks. SIGNAL 2 also displays a more rapid return to normal levels than the SHAM or SIGNAL 1 exposed groups.

To rule out the possibility of animal weight being a contributing factor in the tendons' force to rupture, we also normalized the breaking strength to the animals' pre-harvest weight. Figure 30 is a representation of this data. It is clear that normalizing to animal weight does not markedly change the trend for force to rupture over the two week time course examined in this study. Figure 31 is a graph of the normal uninjured tendon vs. the collagenase-injected tendons for the one week time point. This graph shows SIGNAL 2 to again be most effective at increasing the force to rupture as compared to the SHAM exposed and SIGNAL 1 groups. This trend remains consistent by the two week post-injury time point.

Figure 29. Force to Rupture in TEMF-Exposed vs. SHAM-Exposed Rat Tendon. Animals received collagenase injections into the right Achilles tendon and were exposed to either the TEMF or SHAM field for 30 minutes/ day. Results shown are the amount of force required to rupture each tendon while being pulled by a materials testing system at a rate of 1 cm/ s, as described on page 58. n = 4 per exposure group. Error bars represent SEM. Using ANOVA, F for time points (4, 45) = 12.2; $p << 0.00001$; F for field (2, 45) = 3.71; p = 0.032; and F for interaction (8, 45) = 2.3; p = 0.039. *At the seventy-two hour time point, SHAM was significantly different from Signal 1 (p = 0.031) and SHAM was significantly different from SIGNAL 2 (p = 0.025).

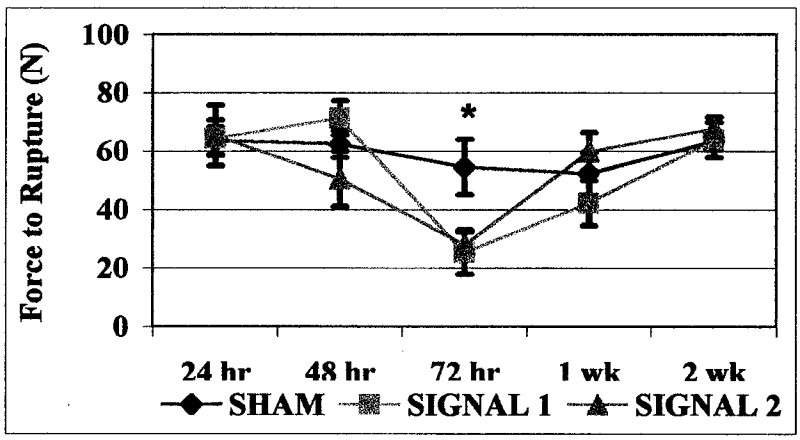


Figure 30. Force to Rupture Rat Tendon as Normalized to Animal Weight. Animals received collagenase injections into the right Achilles tendon and were exposed to either the TEMF or SHAM field for 30 minutes/ day. Results shown are the amount of force required to rupture each tendon while being pulled by a materials testing system at a rate of 1 cm/ s, as described on page 58, then normalized to animal weight. n = 4 per exposure group. Note no marked difference between this result and that of Figure 29, which was not normalized.

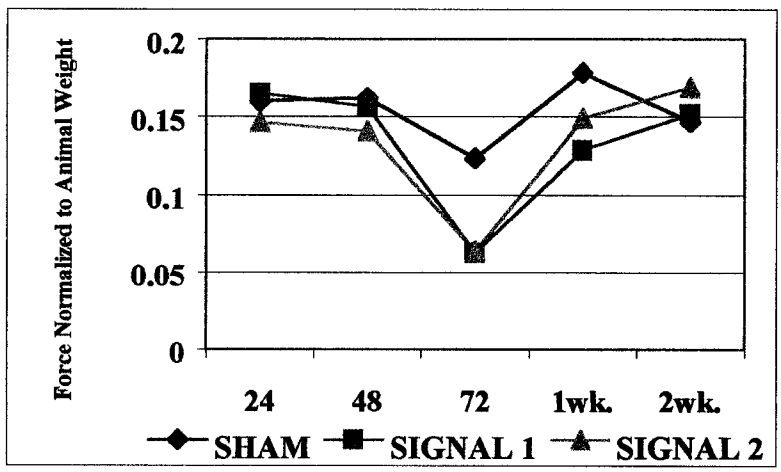
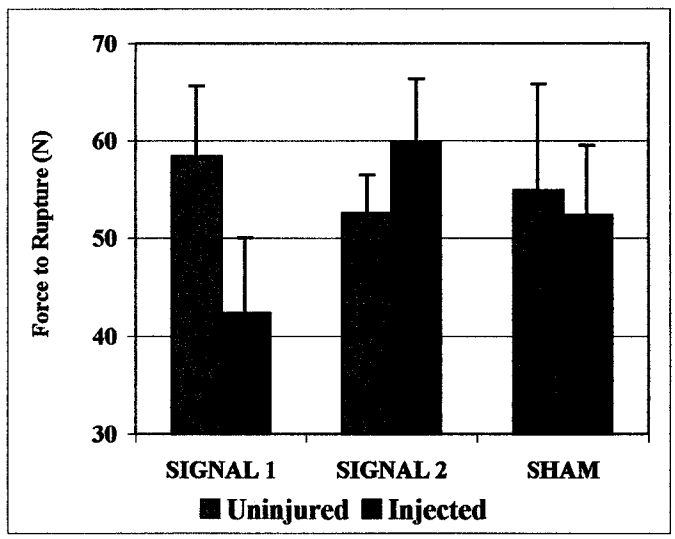


Figure 31. Force to Rupture in Uninjured vs. Injured Rat Tendon at One Week Post-Injury. Animals received collagenase injections into the right Achilles tendon and were exposed to either the TEMF or SHAM field for 30 minutes/ day for one week. Tendons were harvested one week post-injury. Results shown are the amount of force required to rupture each tendon while being pulled by a materials testing system at a rate of 1 cm/ s, as described on page 58. n = 4 per exposure group. Error bars represent SEM. There were no significant differences. Using ANOVA, F for time points (1, 18) = 0.089; p = 0.768; f for field (2, 18) = 0.269; p = 0..767 and F for interaction (2, 18) = 0.304; p = 0.741.



DISCUSSION

In vitro Studies Background

The idea that therapeutic EMFs can be used to treat inflammatory diseases has been of interest to our research group for some time. In earlier *in vitro* studies, the Jurkat cell model was used. Jurkat cells are a human T lymphocyte cell line, often used as a human T lymphocyte model. Jurkat cells are known to undergo apoptosis upon full activation, which includes activation at the T cell receptor, as well as an additional stimulatory pathway becoming activated (Nindl et al., 2003). The idea behind the initial studies was that EMFs might be capable of increasing this T cell apoptosis, thereby decreasing healing time in inflammatory diseases, as T cells will contribute to the inflammatory process.

In one study, Nindl et al. (2002) examined the effects of the TEMF field on Jurkat cell apoptosis. Both normal and activated Jurkat cells were used to determine differential EMF effects based on the cell's state of activation. Jurkat cells stimulated with anti-CD3 and PMA receive the two signals necessary to produce IL-2 and are a model for fully-activated. inflammatory T cells. The cells exposed to the TEMF (SIGNAL 1) were found to undergo apoptosis at a sixty percent greater rate than cells that were not fully activated (Nindl et al., 2002). This result suggested that TEMFs are capable of augmenting apoptosis in activated inflammatory cells. The current study was designed to test this finding *in vivo*.

Johnson et al. also examined the effects of EMFs on peripheral blood lymphocytes (PBLs), using both the EBI field and a 60 Hz sinusoidal EMF in separate experiments (Johnson et al., 2001). Human or rat PBLs respond differently than Jurkat cells to anti-CD3 stimulation. PBLs instead proliferate, due to the presence of other cell types within the culture, such as B lymphocytes, macrophages, and monocytes. Collectively, these cells provide a plethora of signals to T cells upon anti-CD3 stimulation. With the 60 Hz, 0.1 mT field exposures, it was found that the PBLs displayed increased proliferation. This same result was observed with PBL exposures to the EBI field.

The information obtained from the aforementioned studies was vital to our understanding of the possibilities for EMFs to be used as a therapeutic modality to modulate inflammatory cells. As a result of these studies, we observed that EMFs could increase apoptosis of inflammatory T cells *in vitro*. This being the case, it became clear that therapies that increase apoptosis in inflammation might lead to decreased healing times in whole animal studies. Based on this result, we focused on whole animal or *in vivo* exposure studies. In order to adequately address the effect of EMFs on whole animals, we set out to develop a model of normal healing in the rat.

Model Development

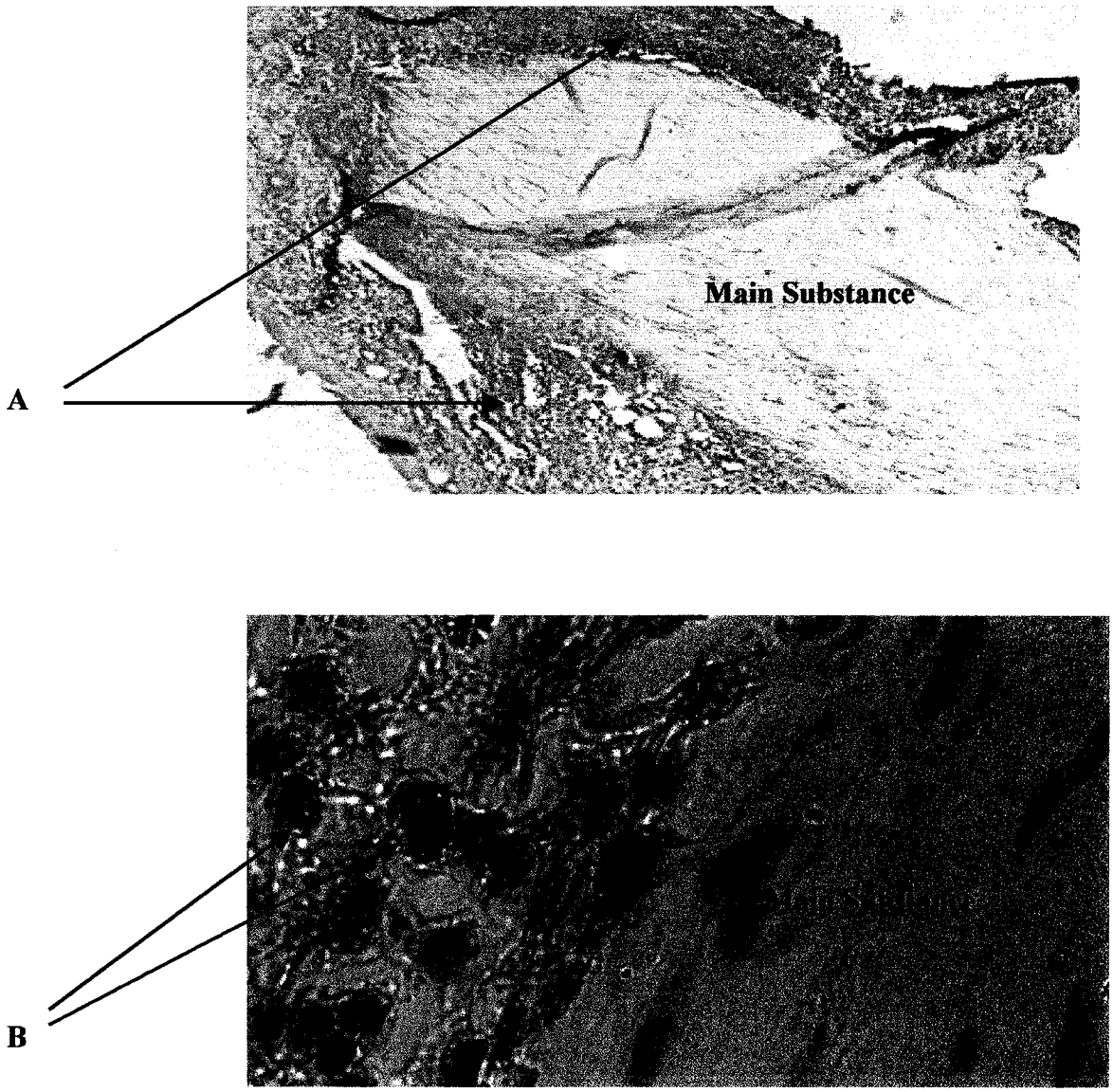
Development of this model required first determining the inflammatory process we wished to further characterize. Based on the incidence of tendon injuries within the human population, which has been shown to be as high as 56% in competitive athletes (Mazzone et al., 2002) and the lack of a treatment method which decreases healing time,

we chose tendinitis as our inflammatory disease model. We first characterized normal healing in this model, without EMF application, to determine the time course for healing without intervention. Approximate human healing times for tendinitis are known (Woo et al., 1999) but those for the rat have not been well defined. Once we had established the normal healing times for the rat, we then expanded our study to include various levels of analysis.

Our *in vivo* analysis began at the gross level, with caliper measurement of ankle swelling. To correlate the amount of hindleg swelling with actual tendon water content, we refined the caliper method and used a lyophilizer to dry the tendon. The tendon wet weight was compared to the tendon dry weight and this difference represented tendon water content. Together, tendon water content and hindlimb swelling were used to characterize normal edema formation and resolution in this rat model.

At the cellular level, we enumerated inflammatory cells within the tendon substance, as well as labeled CD3 positive cells to determine the percentage of T cells present. These analyses were done after removal of the tendon from the hind leg and dissection of the surrounding muscle tissue and bone. Our findings indicated that T cells accumulate outside the tendon substance, but that most do not penetrate the outer layers of the paratenon to enter the tendon substance at 48 and 72 hours post-injury (Figure 32). This finding was consistent with the results of Khan et al. (1996), who found the outer layers of the tendon to be more reactive and contained more cells than did the tendon substance in the early phases of healing. Results from this analysis suggest that embedment and histological analysis of the entire hind leg might provide more information about localization of T cells and their migration toward the tendon substance

Figure 32. Histological Views of $CD3^+$ Cells in Rat Tendon at Forty-Eight Hours Post-Injury. Animals were injected with collagenase into the Achilles tendon and tendons were harvested at forty-eight and seventy two hours post-injury, then embedded in paraffin, and stained for the CD3 portion of the T cell receptor, as described on page 48. (A) Rat Achilles tendon at 48 hour post-injury, 20X magnification. Note the appearance of positively staining cells surrounding the tendon area (arrows), yet not infiltrating into the main tendon substance. (B) 48 hour post-injury sample, 500X magnification. Brown colored cells are positive for CD3 (arrows).



over time. There are inherent problems with this idea. Tendon is a very difficult substance to section, due to the strength of the collagen fibrils. Decalcification is required prior to sectioning bone, which could damage the surrounding tissues if the entire hind leg was embedded. It is also possible that T cells from the surrounding area at these early time points post-injury have not yet received the necessary signals to infiltrate the tendon. Further analysis that includes additional timepoints is planned by the research group.

Once we had identified parameters at the gross anatomical and cellular level that would provide information on the course of healing, we turned our attention to the biomechanical level. Loss of tensile strength is another effect of tendon injury. The term tensile strength implies dependence on parameters such as tissue cross sectional area, and properties of the bone-tendon and muscle-tendon junctions (Mow and Hayes, 1997). In collaboration with Dr. Lee Waite at Rose Hulman Institute of Technology, we were fortunate to have access to a materials testing system. We were able to obtain measures of the force required to rupture the tendon substance without consideration of the abovementioned variables. Therefore, we report this information as the force required to rupture the tendon substance while it is gripped between two attachment points without regard to lines of force being aligned or cross sectional area of the tendon being taken into account (Fig.9). This measure will be used in each of the following sections to describe physical parameters impacted during healing and therapy.

Normal Healing Model

A quantitative model of tendinitis in the rat was developed as a means to evaluate therapies for improved healing. Based on this model, we characterized a time course for normal healing in the rat. This time course displays a return to normal levels of function, water content (via caliper and lyophilizer), and force required to rupture, by one to two weeks post-injury. Previous models have focused only on characterization of one or two parameters of healing in animal models (Khan et al., 1996; Boyer et al., 2001). In addition, earlier models were developed in larger animals (Kobayashi et al., 1999), while smaller animal models of tendinitis in the literature are lacking. The rat model has been previously described (Sandrey et al., 2002) but not quantitatively evaluated.

Examining cells that enter the tendon and their roles in healing has been a focus of some earlier studies (Khan et al., 1996; Thermann et al., 2002). Indeed, infiltration of cells and disruption of collagen fibrils are consistent with tendon injury (Sullo et al., 2001). The Thermann study in particular, involved a semi-quantitative fibroblast cell count. However, the cell numbers were not subsequently related to other parameters of inflammation. To fill in this gap in knowledge, we aimed to incorporate several parameters such as function, cellularity, tissue strength, and swelling or edema to develop a thorough, quantitative model of tendon injury and healing.

Another important gap is in our understanding of the length of time required post-injury for resolution of tendon injury. For example, in a 1997 study by Stehno-Bittel et al., the effect of casting was examined in rabbits. The only time point evaluated was fifteen days post-injury. By investigating only the two week post-injury time point, important early events in the healing process are overlooked. The method of tendon

injury, the animal model being used, and the parameters to measure healing are all variables that will contribute to a complete understanding of tendon repair.

The method chosen for induction of injury in animal models of tendinitis is of great significance. Several studies to date have used complete severing of the tendon substance (Forslund and Aspenberg, 2001; Iwuagwu and McGrouther, 1998), which in most exercise-induced or age-related injuries in humans, does not occur. More often, tendon injuries in humans are the result of several microtears due to overuse that eventually lead to enough pain for the patient to seek medical consultation (Riley et al., 1996). Age is also an important factor, as shown by a 1999 study by Almekinders et al. In this study, the effect of age on rat patellar tendon injury was examined. The youngest specimens examined showed the highest proliferation rates, via tritiated thymidine incorporation of tendon fibroblast cells. Another method used in animal models is to remove a "window' area from the mid section of the tendon substance (Iwuagwu and McGrouther, 1998). This method of injury induction is clinically similar to a form somewhere in between a complete severing model and collagenase injection models.

The enzyme-induced model of tendon injury has proven to emulate the injury that occurs naturally in humans. The healing timeline in the rat, of course, must be considered, as it will naturally be much shorter than that for a human. This is due to the increased metabolism and smaller size of the rat vs. the human. In spite of a difference in the time course, the disruption of collagen fibrils, along with cellular infiltration immediately post-injection, are similar to the clinical picture of tendinitis in a human subject (Alfredson and Lorentzon, 2000). Another option to induce a similar injury is to open the leg and tear individual collagen fibrils within the tendon at random to attempt to

mimic an overuse type of tendon injury. The ability to consistently disrupt individual fibrils in the tendons of multiple animals in one study is not realistic.

In order to ensure consistent enzyme delivery across experiments, we performed a spectrophotometric assay of enzyme activity. The FALGPA assay was used to measure collagenase activity, as complete hydrolysis of the FALGPA synthetic substrate is defined as a decrease in absorbance of 0.125 units (Van Wart and Steinbrink, 1981). This assay showed no differences between lots of collagenase used in the current study. In all samples measured for this study, this level of hydrolysis was reached within two hours, establishing the fact that we were inducing parallel injuries across experiments, and that the collagenase was enzymatically active (Figure 14).

In the model experiment we determined that an increase in cell numbers occurred by twenty-four hours post-injury (Figure 10). The peak in inflammatory cells was seen at two weeks post-injury with a trend back toward normal, uninjured levels by four weeks post-injury. Due to the increase in cell numbers observed as early as twenty-four hours post-injury, we then decided to focus in on several earlier time points in the healing cycle; forty-eight and seventy-two hours, as well as one and two weeks post-injury.

Figure 11 depicts the time course for normal healing as it relates to ankle width or swelling. It is clear that by one week post-injury, ankle widths have returned to normal without intervention. This return to normal is duplicated in functional indices as measured by the Achilles Functional Index (Fig. 12). By one week post-injury, the level of function has again returned to pre-injury, normal values.

EBI Field

Focusing on the acute phase time points, we applied pulsed electromagnetic fields to examine their efficacy as a potential therapeutic modality. The first level of analysis involved enumeration of inflammatory cells within the tendon substance. Results from this experiment showed no significant difference in inflammatory cell number between the GEO and EBI field-exposed groups for any of the acute time points measured (Fig. 13). Again, as in the model experiment, we observed an increase in cell numbers at twenty-four and seventy-two hours post-injury.

One discrepancy between Figure 10 and Figure 13 is clear. At the one week post-injury time point, the model experiment showed a cell number of 400 cells/ μm^3. In the EBI experiment in Figure 13, the one week time point displayed 50% of the cells seen in Figure 10. Further development and refining of the cell counting procedure is required to incorporate more consistency in cell enumeration.

At the gross anatomical level, as a measure of edema or hindleg swelling, we used a precision caliper to measure ankle thickness. Again, we saw no significant differences between the GEO and EBI exposed samples in terms of ankle width at any of the time points measured (Fig. 15). An increase in ankle width with collagenase-injected tendons, as compared to normal uninjected tendons can be seen at seventy-two hours post-injury. By one week post-injury, hindlimb swelling had mostly resolved. A slight increase in swelling was seen at two weeks post-injury. This is most likely a negligible increase, but may be due to an increase in proliferative fibroblast cells present to lay down new collagen (Dahlgren et al., 2002).

As hindleg swelling may not be the most precise measure of direct tendon water content (edema), we refined the method to evaluate this parameter.

We utilized a lyophilizer to dry the individual tendons and compared this dry weight to the original post-harvest tendon wet weight. As can be seen from Fig. 16, there again were no significant differences in terms of tendon water content between the GEO and EBI-exposed groups at any of the measured time points. It is interesting to note that there was an increase in tendon water content by twenty-four hours post-injury, consistent with the timing of inflammatory cell number increase seen previously. This twenty-four hour post-injury increase in water content was also observed by Lee et al. in their 1997 study examining PEMF effects on tendon healing. Also, there was a peak in tendon water content at seventy-two hours, again corresponding with this time point being of significance in regard to cell number and caliper measures from previous trials.

At this point, we had determined a seventy-two hour peak for both edema (hindlimb swelling and tendon water content), and inflammatory cell infiltration. To correlate this increase in swelling and inflammation to a functional parameter, we used the Achilles Functional Index (AFI). This method is essentially a gait analysis that determines how quickly the animals regain normal function (Murrell et al., 1992).

Using the AFI, we analyzed return to function after collagenase injection in our small animal model. Based on this information, we determined the greatest loss in function occurred at seventy-two hours post-injury. In the period between one to two weeks post-injury, all animals, regardless of treatment group, had returned to a normal level of function (Fig. 17). Analyzing each of the AFI formula's contributing parameters,

we saw a decrease in all three factors at seventy-two hours post-injury: print length, toe spread, and intermediary toe spread.

Experimental methods to determine functional recovery in animals most often are used in neurobiological studies. In a 1999 study by Hadlock et al., a comparison between two different methods of gait analysis was carried out. This study specifically examined the recovery of the peripheral nerve in the rat after injury. By comparing both walking track analysis (a method like that of the AFI) and neurobehavioral tests such as latency to withdraw from a hot surface and examination of force applied to a digital balance, they determined walking track analysis provided results that were not significantly different than those obtained using the other methods.

Video gait analysis is also a method used to establish return to function, although most studies use this method when examining nerve recovery, not tendon injuries. Comparison of video analysis in sciatic nerve-injured rats, as compared to using walking track analysis, shows a similar recovery pattern over time (Walker et al., 1994). One advantage to this method is decreased mess, as well as smaller inter-animal variability than that seen with walking track analysis. Yet, this method appears most often in nerve recovery studies, and is underutilized in tendon injury studies.

Another study by Walker et al., 1994, examined enhancement of rat sciatic nerve recovery after PEMF application. In contrast to the previous study, the walking track analysis did not yield results similar to the video analysis. In this paper, it was stated that walking track analysis may not be sensitive enough to measure differences in function after nerve injury. This statement again supports the idea that for nerve studies, video analysis might be the best method to determine differences in treatment groups in regard

to function. In contrast, walking track analysis appears to be sensitive enough to detect differences in tendon injuries and is cost and time efficient (Best et al., 1993).

The Walker study noted a more rapid return to function with PEMF application as compared to controls. It is important to note that the PEMF used in this study was a 0.3 mT, 2 Hz field. The field intensity is greater than the EBI coil used (0.12 mT) but less than the 15 mT field intensity used in the TEMF study. The TEMF coil also has a frequency of 120 Hz. The differences in field intensity and frequency may explain the fact that effects were seen in the Walker study and no significant effects were observed in the current study, as frequency windows are known to exist (Belyaev and Alipove, 2001; Binhi and Goldman, 2000).

Collagenase Dosage Trial

Once we had confirmed the utility of the cell enumeration method, the caliper and lyophilizer edema measures, and the AFI for return to function, we examined different collagenase dosages to increase the magnitude of the injury while maintaining the type of injury induced. In the severing models (Best et al., 1993; Murrell et al., 1992), the post-injury AFI reached the –100 level. Our mode of injury is much milder than the severing model, therefore even with increased concentration of collagenase, we did not observe this drastic drop in AFI values. Our model is more clinically relevant, as most tendon injuries are not complete ruptures, yet it more closely approximates overuse injuries that are the result of microtears within the tendon substance (Brukner, 1997). Results from this small trial (n = 2 animals per group) showed the 900 IU/ kg rat body weight dose to be most effective in decreasing function post-injury (Fig. 20). We observed the same

AFI trends as seen with the lower 150 IU/ kg dose used in the model and EBI exposure experiments, just at a more profound level. This result led us to use the 900 IU/ kg body weight dosage for remaining experiments.

TEMF Field

Once we had established that there was no significant effect of the EBI coil in our model, we wanted to explore a different therapeutic electromagnetic field. The TEMF coil had been used for *in vitro* studies showing an approximate forty percent reduction in T cell proliferation in field-exposed samples (Nindl et al., 2002). The TEMF coil has also been shown to decrease back pain and is currently in Phase II clinical trials (Harden et al., 2002).

Experiments utilizing the TEMF coil involved rats with tendon injury being exposed to either SIGNAL 1 or SIGNAL 2 or the control (SHAM-exposed treatment group) for thirty minutes per day. At the cellular level, we evaluated the specific cell types present using a monoclonal antibody to the T cell receptor, CD3. We focused on the forty-eight and seventy-two hour time points, as these are the time points that have proven to be important in terms of cellular infiltration and edema in prior experiments. Results from the enumeration of CD3 positive T cells show no significant difference in terms of relative percentage of total inflammatory cells between SHAM exposed and SIGNAL 2 exposed samples (Fig. 22). Relative percentage of $CD3^+$ T cells in the pool of total inflammatory cells is a more effective way to analyze $CD3^+$ cell numbers. This is due to the fact that normalizing the number of T cells to the total cell number will give a

more defined result. Expressing the number of $CD3^+$ T cells as a percent of total inflammatory cells removes individual variability by utilizing an internal standard.

We observed that T cells did not penetrate the tendon substance, but instead, were present in high numbers outside the tendon substance (Fig. 32). It is possible that T cells exert their effects via releasing cytokines outside the tendon which then travel inside to affect inflammation. This result supports findings in a 1996 study by Khan et al. in which fewer cells were present in the tendon substance than in the surrounding sheath at early time points post-injury.

Results from the TEMF experiment (Figures 23, 24, and 25) led us to believe the SHAM may not be the best control for our purposes. Normally, the ideal control for a given EMF is exposure to the putative effective field that has been cancelled out by the coil configuration, so that no magnetic field is delivered to the experimental subjects. In some experiments, the SHAM-exposed animals exhibited similar patterns of healing as those of the SIGNAL 1 and SIGNAL 2-exposed group (Fig. 23, 24, and 25). To further investigate the possibility that the SHAM exposure was exerting some type of effect, we compared the GEO-exposed (Earth's magnetic field as a control) data with the SHAM-exposed data using both the caliper edema and the AFI as the chosen levels of analysis.

In regard to caliper edema and AFI, the GEO exposure plotted along with the SHAM showed the SHAM was different from the other type of control (geofield of the Earth) (Figs. 33 and 34). Hindlimb swelling as measured by caliper edema in the TEMF-exposed vs. SHAM-exposed animals showed a peak in ankle width at seventy-two hours post-injury with a trend back to normal by one week (Fig. 23). However, there was no significant difference between the signal-exposed groups and the SHAM at any of the

Figure 33. Caliper Measurements of Rat Ankles in SHAM-Exposed vs. GEO-Exposed Animals. Animals received collagenase injections into the Achilles tendon and were exposed to the SHAM or the GEO field. The change in ankle width from pre-injection to pre-harvest was determined through use of calipers, as described on page 52. Results are expressed as the difference between pre-injection and pre-harvest ankle widths for each of the time points indicated. $n = 4$ per exposure group. Error bars are representative of SEM. Using ANOVA, F for time points $(5, 36) = 18.2$; $p < 0.00001$; F for field $(1, 36) = 26.4$; $p < 0.00001$; and F for interaction $(5, 36) = 1.63$; $p = 0.178$. * 2 week significantly different $p = 0.014$.

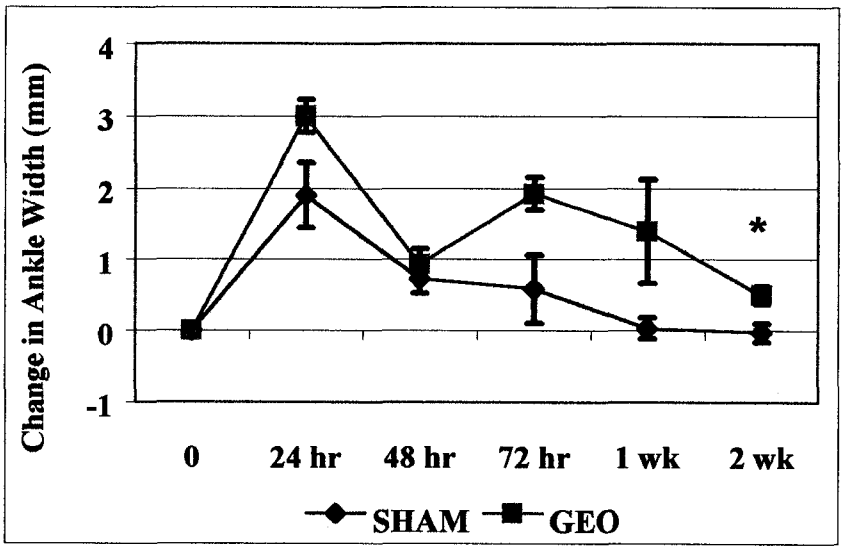
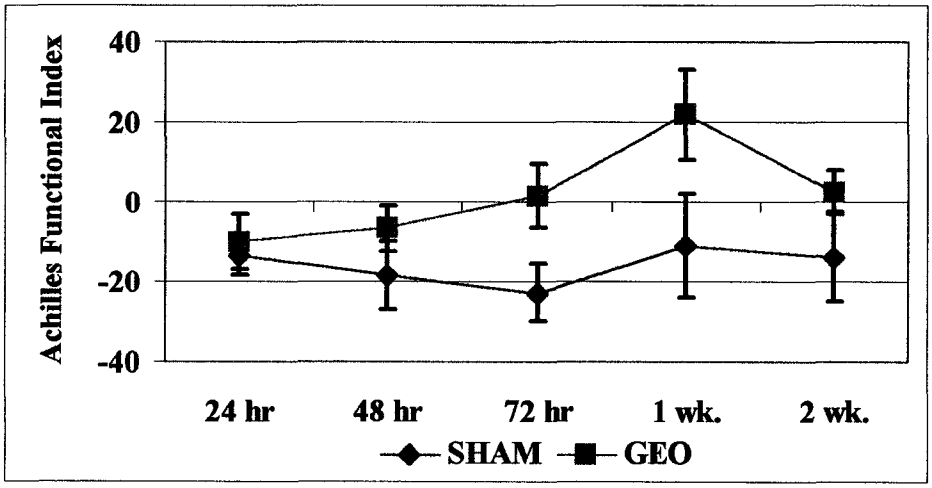


Figure 34. Achilles Functional Index in SHAM-Exposed vs. GEO-Exposed Animals. Animals received collagenase injections into the right Achilles tendon and were exposed to either the TEMF or SHAM field. Gait analysis runs were completed pre-injection and at the subsequent time points pre-harvest as described on page 52. Results are expressed as Achilles Functional Index (AFI) units. n =4 per exposure group. Error bars are representative of SEM. There were no significant differences between SHAM-exposed and GEO-exposed animals at any of the measured time points. For 24 hr, $p = 0.959$; 48 hr, $p = 0.411$; 72 hr, $p = 0.125$; 1 wk, $p = 0.192$; and 2 wk, $p = 0.759$.



measured time points. In contrast, GEO-exposed samples when compared to SHAM displayed differences (Fig. 33). Using the Bonferroni correction factor to correct for the possibility of a Type I error due to performing a total of three tests on this data set, the p value for Figures 33 and 34 was set to a level of $p = 0.05/3$ or $p = 0.017$. The only statistically significant difference was found at the 2 week time point, with GEO being different from SHAM at a level of $p = 0.014$. It is possible that the SHAM exposure is having an effect on inflammation early on, due to increasing vasculogenesis that aids future healing. The 2001 study by Williams et al. observed a decrease in mouse tumor volume after TEMF exposure. This effect of the TEMF coil on vasculogenesis may be variable, based on the fact that the animals in the Williams study did not begin TEMF exposures until one week post-tumor implantation. Timing of EMF application post-injury may prove to be of utmost importance in determining the effect of the field on processes such as changes in vascular permeability and growth of new blood vessels that contribute to healing.

Using the lyophilizer method to confirm the caliper edema results, we found SIGNAL 2 to be more effective in reducing water content at the forty-eight hour time point, although the difference was not statistically significant. There were no differences between the SHAM and SIGNAL 1-exposed samples at any of the measured time points, again lending validity to the idea that the SHAM is not a true control for this system (Fig. 24). A peak in water content occurred twenty four hours post-injury, and this peak correlates with the decreased function and increased cellularity observed at this time point across experiments. Based on the timing of inflammatory processes, this increase in water content at twenty four hours post injury is most likely due to changes in the

vasculature that have altered blood vessel permeability and allowed interstitial fluid to move into the damaged area.

As a measure of function regained over time, we again employed the AFI. Based on the AFI analysis and original investigation by Murrell et al., (1992) when an animal is injured, the print length should increase and the distance between toes should decrease, thereby resulting in a negative AFI. Correspondingly, a zero or positive AFI value correlates with a healthy or uninjured animal, although we observed a range of approximately –20 to +20 for uninjured subjects in our study. Figure 25 shows no significant difference in functional ability in any of the three exposure groups. SIGNAL 2 exposed animals display a higher level of function across all time points. It is possible that SIGNAL 2 is increasing healing in these animals via promotion of organization of the collagen fibers, leading to increased tendon strength, as seen with the SIGNAL 2 exposed animals in Figure 29, which display a more rapid return to normal function after the 72 hour time point than the SHAM and SIGNAL 1 exposed groups.

A range of print lengths was observed within one animal during one functional analysis run. Due to this variability in print length from a single subject within one measurement, we also investigated the use of actual foot length in place of the print length measure in the AFI formula (Fig. 27). Utilizing this change to the original AFI formula does not significantly alter the outcome of the overall functionality. Other studies have determined an effect of gait velocity, animal weight, and genetic strain on print length (Parker et al., 1990; Dellon et al., 1991). Future studies to correlate the functional index to parameters such as force to rupture and cellular infiltration will decide if this is true for the model presented in this study.

With the TEMF coil, we observed a drop in function at seventy-two hours post-injury with a corresponding return to normal levels by one week post-injury. We also compared the GEO-exposed AFI results and determined the SHAM once to be an ineffective control (Fig. 34). The GEO-exposed animals displayed a more negative AFI early on, yet AFI values were not significantly different from the other exposure groups by forty-eight hours post-injury. It is important to note that the SIGNAL 2-exposed animals displayed the best function in terms of the AFI at each of the time points observed. The AFI graph of the SHAM vs. GEO data (Figure 34) again shows the two fields exhibit differences in regards to function, although these differences were not significant for any of the time points using the Bonferroni adjusted p value of $p = 0.017$.

As a final means of assessing healing, we used a materials testing system to determine the force required to induce tendon breaking (force to rupture). Results from this parameter show a drop in force to rupture at seventy-two hours post-injury and a return to normal levels by two weeks post-injury (Fig. 29). The SHAM-exposed samples exhibited stronger breaking strength than either the SIGNAL 1 or SIGNAL 2 groups, at all the measured time points except one and two weeks post-injury. A decreased tensile strength early in the healing process may not necessarily be detrimental. This is due to the fact that increased vascularity and infiltration of cells is necessary to achieve healing within the tendon (Chan et al., 2000). The disruption of collagen fibers and neovascularization that occur upon injury would naturally mean that the tendon has lost the strength it once had when the collagen triple helix structure was in place prior to injury. It is possible that TEMF signals 1 and 2 increase vascularity early in the healing process. This would in turn be measured as decreased tendon strength, due to the

increased blood vessels causing misalignment of the collagen fibrils within the tendon substance.

Previous studies have shown PEMFs to be effective at increasing angiogenesis. One study in particular, by Roland et al., used an arterial loop model in rats to determine effects of exposure to a PEMF of either 0.01 mT or 0.2 mT at 27.12MHz frequency. Quantification of neovascularization of the arterial loop after either PEMF or control exposures confirmed the hypothesis that PEMFs can increase vascularization in an *in vivo* rat model. Although the frequency (27.12MHz) used in the Roland study is much greater than that of the TEMF coil used in the current study (120 Hz), it is possible that similar effects could be observed.

SUMMARY

The successful development of a quantitative, thoroughly characterized model of a mild form of rat tendinitis was achieved by the current study. Based on this model, it is clear that in the rat, healing occurs as early as one to two weeks post-injury. The cellular infiltration study designed to characterize the timing of the chronic and acute phases displayed a peak in cell numbers at two weeks post-injury (Fig. 10). By four weeks post-injury, cell numbers had begun a trend back toward those of normal, uninjured tendon. This result can be explained by the infiltration of fibroblasts later in the healing phase of inflammation, which might lead to this peak in cell numbers at two weeks post-injury.

Further focusing on the acute phase is of great importance when examining potential therapies. Inhibiting development of the chronic phase can prevent the loss of function that occurs concomitantly with this phase progression from acute to chronic. We therefore focused our attention on the period up through two weeks post-injury.

It is apparent that when using a collagenase-induced form of tendon injury in the rat, natural healing begins to take place as early as one to two weeks post-injury. This conclusion was made because the comprehensive methods used to assess healing all reveal resolution by this time point. The natural healing results without EMF exposure, all display this trend back toward normal by 1 to 2 weeks post-injury in following levels of analysis: caliper measurements (Fig. 11, 15, 23) and AFI data (Fig. 12, 17, 25).

As early as twenty four hours post-injury, animals display a decrease in function (Fig. 12, 17, 25). Cell numbers also increase by twenty four hours post-injury (Fig. 10,

13), along with an increase in hindleg swelling via caliper (Figs. 15 and 23) and tendon water content via lyophilizer (Figs. 16 and 24). Rapid infiltration of PMNs is known to occur after tissue injury and their infiltration into the tissue leads to an accumulation of fluid from the surrounding interstitium that leads to the edema observed in both the caliper and lyophilizer analyses. This increased amount of fluid and cellular infiltration disrupts the collagen fibers within the tendon. This fiber disruption leads to decreased function, as the tendon substance is no longer intact with its organized meshwork of collagen.

Between forty eight and seventy two hours post-injury, the tendons have lost some of their initial strength (Fig. 29). This may be attributed to changes occurring in cells infiltrating or exiting the area, previous to incoming fibroblasts that arrive later to replace damaged collagen. The functional ability of the animals continues to stay low through the seventy two hour post-injury time point, when it begins a trend back toward normal by one to two weeks post-injury. The seventy two hour time point is characterized by an increase in cell numbers (Fig. 13), a decrease in the amount of force required to rupture the tendons (Fig. 29), a decrease in function (Figs. 17 and 25), and an increase in hindleg swelling via caliper (Figs. 15 and 23), which makes this time point a critical one in the healing time course in our model.

By one to two weeks post injury, all measured variables have returned to normal ranges, except for the inflammatory cell number. This can be explained by the infiltration of fibroblasts, which occurs later in the healing process and might have caused this peak at this later time point.

The EBI field was ineffective at decreasing healing time in our model of rat tendon injury. Only one statistically significant difference was observed between EBI and GEO-exposed animals. This occurred at the one week time point in the Achilles Functional Index. Decreased function in the EBI-exposed group, as compared to the GEO-exposed group was observed (Fig. 17). This result might be indicative of the EBI causing increased cellular infiltration and collagen fiber disruption that leads to a decreased level of function in the observed animals. This field has a very long off time (around 200 ms) between pulses, which may contribute to its ineffectiveness. The majority of results at all levels of analysis, showed no significant differences between the EBI and the GEO-exposed samples at any of the observed time points.

A trend of more rapid healing appeared upon observation of all the data from the TEMF exposure experiments. SIGNAL 2 displayed trends of decreased water content and increased function as compared to SIGNAL 1 and SHAM-exposed animals (Fig. 24, 25, and 27). In the AFI, SIGNAL 2 maintained a higher functional level than SIGNAL 1 or SHAM groups across the entire time post-injury (Fig. 25). Additionally, the force to rupture and water content data (Figs. 29 and 24) began a trend back toward normal more rapidly than the SIGNAL 1 or SHAM exposed groups. The difference in off times between pulses (SIGNAL 1 vs. SIGNAL 2) could contribute to the trend seen for SIGNAL 2 exposed animals. SIGNAL 2 has a shorter off time than does SIGNAL 1. It is possible that the decreased off time in SIGNAL 2 provides for significantly more of the signal to be delivered to the tissue than does SIGNAL 1. This may lead to the trend toward more rapid healing observed with SIGNAL 2.

The best control for the TEMF exposures is unclear. The statistically significant difference seen between the SHAM and GEO exposures in the caliper edema (Fig. 33) indicate the SHAM control is different than the GEO control. The SHAM setting is done via the standby position on the TEMF generator, while the GEO control is a true control, involving no electrical systems. It is possible this difference in the controls contributes some residual effect in the SHAM samples that is not observed in the GEO animals. Further experiments to fully characterize the SHAM field will be conducted by the research group. Including a GO control group for every experiment in the future would also be advised, as this group would provide data on what occurs under normal conditions of healing.

Overall, normal tendon healing in the rat appears to resolve by one to two weeks post-injury. The acute phase time points display an increase in cell number as early as twenty four hours after injury, accompanied by fluid entering the area of damage. This edema is caused by changes in the permeability of the vasculature upon injury and increased permeability of blood vessels. The seventy two hours post-injury time point appears to be when the animals experience the greatest loss of function. This is due to an increased amount of fluid and cells present in the injured area, which inhibit the normal weight bearing and gliding of the tendon. The force required to rupture the tendons also decreases at this seventy two hour time point, indicative of this loss of tendon fiber integrity due to cellular infiltration and edema. In our model, animals have begun a return toward normal pre-injury levels of function, water content, swelling, and tendon strength by one to two weeks post-injury.

Some discrepancy occurred in the edema data, in that the caliper and lyophilizer time courses did not always match. This is due to the fact that the caliper method is measuring the entire hindlimb. It is taking into consideration the amount of swelling in the surrounding tissue as well as the tendon. The lyophilizer method is examining directly the amount of tendon water content.

Based on the results of the current study, future experiments abound. The first of which includes further refining the cell enumeration protocol to deliver more consistent results. Another important addition to the model would be an increased number of time points. Due to the decrease in function and healing observed at many levels of analysis at seventy two hours post-injury, accompanied by a return to normal by one to two weeks, adding time points 96, 120, and 144 hours would add important information to the timeline for normal healing.

Another possible modulation of the current study would be to induce a repetitive injury. Many animal studies of rheumatoid arthritis have used a repeated injection type injury (Luross and Williams, 2001). Due to the similar matrix destruction observed in rheumatoid arthritis and tendinitis, using a repeated injection model of collagenase-induced tendon injury could more directly emulate the actual overuse injury that occurs in humans. The one injection collagenase model, as used in this study, leads to development of the hallmarks of tendinitis, including collagen fiber disruption and cellular infiltration. Microtraumas that occur in athletes and lead to tendinitis might be better induced via repeated small volume collagenase injections over a time course to slowly develop the injury.

Growth factor administration might be another alternative route to decrease healing time in tendinitis. In earlier studies in our laboratory, systemic cytokine levels were measured twenty-four hours post-injury in control and EBI-exposed animals. Increased levels of TGF-β mRNA were observed in samples from both the controls and the EBI-exposed samples. TGF- β is an anti-inflammatory cytokine and has also been shown to increase collagen production (Isomaki and Punnonen, 1997). Administration of TGF- β post-injury may act to enhance therapeutic EMF effects.

A further possible future direction would be to investigate the potential therapeutic effect of cryotherapy over the acute phase of healing. This conservative method is often the first line of defense prescribed by doctors when a patient presents with tendon pain. The timing of cryotherapy is of vital importance and is usually effective only in the acute phase of tendon healing (Curl et al., 1993). Cryotherapy in humans is recommended only intermittently within the first few days post-injury and acts to decrease pain and edema (Knight, 1990). Its effects are to decrease blood flow and inflammation, which if extended too long into the post-injury period, may be counterproductive to healing. Based on this timeline for cryotherapy application in humans and the much quicker resolution time in the rat, cold therapy applied to a rat tendinitis model might be limited only to the first few hours post-injury.

Heat application is also used to treat tendon injuries, yet in contrast to cryotherapy, is used in later stages of the inflammatory process (Houglum, 1992). It is thought that applying heat during the later phase of tendon injuries might lead to accelerated healing via heat-induced vasodilation. Heat also increases tissue metabolism, thereby increasing waste removal at the cellular level. This waste removal occurs due to

increased blood flow bringing antibodies, leukocytes, nutrients, and enzymes to the area (Rivenburgh, 1992). Cryotherapy followed by heat therapy in the later stages of healing, accompanied by exposure to the TEMF field, may lead to further enhancement of the SIGNAL 2 trend of a more rapid return to function seen in some levels of analysis within this paper.

Evaluation of potential therapeutic modalities demands a broad approach that incorporates many levels of analysis to derive a complete picture of healing and its acceleration by application of specific interventions. Application of EMFs to decrease inflammatory processes requires modulation of frequency and intensity in order to achieve the most robust effect for the model system under investigation, as well as strict control of all variables. Timing of EMF exposure post-injury may also be of importance. Further studies to more accurately characterize specific cellular aspects of EMF effects on inflammatory processes will yield vital information that can be applied in the clinical setting to accelerate healing. Studies that delineate possible EMF effects on chemoattractant or adhesion molecules will lead to a better understanding of the potential role for EMFs in the inflammatory process and the recruitment of cells from the surrounding area to begin the healing process. Although trends of decreased healing time with the TEMF SIGNAL 2 exposures were observed, the EMFs used in the current study were not capable of markedly altering the time course for normal healing in a mild, collagenase induced form of tendon injury, as used and characterized in this paper.

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