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AN *IN VITRO* MODEL TO STUDY THE EFFECTS OF STRESS-DEPRIVATION ON THE MATERIAL PROPERTIES OF CONNECTIVE TISSUE: A BIOMECHANICAL AND HISTOLOGICAL ANALYSIS.

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ABSTRACT

AN *IN VITRO* MODEL TO STUDY THE EFFECTS OF STRESS-DEPRIVATION ON THE MATERIAL PROPERTIES OF CONNECTIVE TISSUE: A BIOMECHANICAL AND HISTOLOGICAL ANALYSIS

By

Amardeep Kaur Hoonjan

Immobilization is a widely used clinical method of rehabilitation of soft tissue injury. However, it has many disadvantages including alterations in the material properties and tissue atrophy. While experimental studies have examined some of the deleterious effects of immobilization, these *in vivo* investigations are limited by the inability of models to be standardized. The goal of this study was to evaluate the effects of immobilization on the material properties of connective tissue using an *in vitro* model. The ability of cyclic loading to reverse the immobilization-induced alterations in material properties of the tissue was also investigated.

Biomechanical and histological properties degenerated with increasing periods of immobilization. Cyclic loading of the tissue minimized these changes. Cyclic loading of previously immobilized tissue was able to arrest the detrimental immobilization-related alterations. The decrease in material properties seen with immobilization is thought to be a cell-mediated event, secondary to a lack of mechanical stimulus.

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I. INTRODUCTION

Immobilization or some form of physical support to minimize load or stress has, on various connective tissues, been a mainstay of therapy for many musculoskeletal diseases and injuries. By eliminating stress, the injured tissue is protected from any disruptive forces, enabling the weakened area to heal, mature and strengthen. Immobilization of the injury site helps to prevent the dislocation of wound edges, protects the developing tissues, and prevents disruption of new blood vessels.

Despite the need for protection of the injury site, some stress is required to allow for effective wound maturation and subsequent increase in strength. The relationship between stress and connective tissue is best described by Wolff's law (78) which essentially states, "...that the form of tissue being given, the tissue elements will increase or decrease themselves to reflect changes in the direction or magnitude of the applied stress." Thus, if a tissue is not subjected to normal mechanical stresses, because of partial or total immobilization, the strength of the tissue diminishes[20, 40, 41, 56, 64 82, 84]. Therefore, a dilemma exists in determining how much stress is required for maintaining tissue homeostasis, without jeopardizing wound healing.

The detrimental effects of immobilization (stress-deprivation) on connective tissue have been documented extensively by many experimental and clinical studies[2, 3, 6, 8, 11,15, 17, 18, 20, 24, 30, 32, 34-36, 40, 41, 43, 44, 51, 53-56, 62, 63, 65, 66, 72-76, 80, 82, 84]. These include atrophy [20, 24, 40, 41, 56, 82, 82], change in microstructure [2, 20, 32, 62, 75, 76], decrease in extracellular matrix [20, 34, 75, 83], loss of water

content[20, 34, 75, 83], and alterations in the material properties of the tissue [3, 6, 11, 168, 32-35, 44, 51, 56, 63, 66, 73, 83]. The changes in the material properties of these tissues, brought on by stress-deprivation, is manifested by a loss in tensile strength[2, 6, 11, 15, 16, 18, 34, 35, 40, 43, 44, 52, 55, 56, 62, 73-75, 83, 84], tensile stiffness [2, 11, 32, 34, 35, 43, 44, 53, 55, 56, 62, 75, 83, 84], and distensibility of the tissue [15, 34, 40, 44, 56, 73-75, 83, 84].

While stress-deprivation has been shown to be detrimental to connective tissue the role of exercise in preventing these changes or reversing them is poorly understood. Some investigators have found exercise to result in the recovery of lost tissue strength following immobility [11, 15, 16, 23, 34, 35, 40, 41, 43, 44, 51, 52, 55, 56, 65, 66, 71, 73, 74, 80, 82, 84]. Other studies have documented little beneficial effect of exercise or controlled motion as a rehabilitative method for injured and immobilized tissue [8, 16, 55, 66, 72, 74]. Despite this work, the effects of exercise or mobilization are poorly understood. Following varying periods of immobilization, the exercise (remobilization) period documented has ranged for 3 weeks to 12 months [41, 43, 44, 51-56, 80, 82, 84]. These studies fail to distinguish how much exercise is beneficial and to what extent the exercise may become detrimental.

While clinical research has shown that exercise and controlled motion can reverse some of the detrimental effects associated with immobilization, these studies do not always have precise scientific control and standardization. Much of the experimental research regarding the effects of immobilization and mobilization on connective tissue is done utilizing animal models. Factors such as body weight, caloric intake, and, to a certain extent, activity of the animal are difficult to standardize, this may lead to erroneous interpretation of the results of various exercise programs.

Thus, an experimental model which allows for explicit control of as many parameters as possible is needed.

The primary purpose of this thesis is to examine the effects of stress-deprivation on the material properties of connective tissue, by biomechanical and histological evaluation of an *in vitro* model. In addition, the effects of cyclic loading in maintaining the normal material properties of tendons as well as arresting or reversing the effects of previously immobilized tendons will be examined. This will be achieved using an *in vitro* tissue culture model[32] which allows precise control of the loading (stress) history.

The main topics explored in this thesis can be designated into three hypotheses:

> H_1: Stress-deprived tendons *in vitro* will experience a change in their material properties with time. This alteration will be reflected by a decrease in the tensile modulus of the tissue.

This will be tested by examining the tensile modulus of stress-deprived tendons *in vitro* over weekly intervals spanning a total of 8 weeks. The second part of the thesis is two-fold. It involves the evaluation of mechanical stimuli via cyclic loading on maintaining the normal material properties of the tendon. The respective hypotheses are as follows:

> H_2: Cyclic loading (mobilization) of tendons *in vitro* will reduce loss of material properties (tensile modulus).

H_3: The harmful effects (i.e. the decrease in material properties) of stress-deprivation in tendons can be reversed to a certain extent by a specified cyclic loading regime.

The second hypothesis will be tested by examination of the tensile modulus of the tendons involved in a mobilization procedure *in vitro*. These values will be compared with controls and stress-deprived tendons to observe any significant trends. The final hypothesis will be examined by determining the tensile modulus of tendons that have been involved in a cyclic loading procedure following stress-deprivation for an identical period of time.

II. LITERATURE SURVEY

This section is presented in a manner so as to provide sufficient background and rationale to support the goal behind the topic of this thesis. It will discuss the effects of stress-deprivation, the effects of mechanical stimuli (controlled motion and/or exercise), and various experimental models used to date.

Of all the tissues in the body, the connective tissues are thought to have the greatest mechanical strength. It is known that connective tissue requires a certain degree of mechanical stress to remain healthy and retain this strength, however the precise amount of stress required to maintain homeostasis is not known. An overview of some of the studies that have been done over the past 20 - 30 years reveals an extensive search for the exact mechanism by which connective tissue maintains itself.

Stress-Deprivation (Immobilization)

Immobilization of connective tissues (bones, ligaments, tendons) following injury has been a mainstay of therapy for many musculoskeletal diseases. Immobilization protects the injured tissue from disruptive forces, especially during the early period of the healing response, in order to ensure effective healing. Immobilization allows for stabilization of wound edges, which in turn permits the in-growth of new blood vessels and the normal progression of wound repair (17, 18).

Despite the benefits of immobilization in the initial healing process of injured connective tissue, it is well documented that immobilization results in degradative changes within the tissue[2, 3, 6, 8, 11,15, 17, 18, 20, 24, 30, 32, 34-36, 40, 41, 43, 44, 51, 53-56, 62, 63, 65, 66, 72-76, 80, 82, 84]. These changes are attributed to the fact that some form of mechanical stimuli is necessary for the normal maintenance of healthy tissue (78). Immobilization has been seen to have detrimental effects on various aspects of tissue morphology, biochemistry, and biomechanical properties [3, 6, 11, 20, 30, 34, 35, 44, 56, 62, 66, 72, 76, 82, 84].

The effects of immobilization and stress-deprivation on the mechanical integrity and tensile properties of soft connective tissue have been documented by many groups over the past two decades. A majority of the investigators have reported decreased mechanical properties with increased periods of immobilization, in both ligament and tendon.

Noyes (56) examined the effects of immobilization on the mechanical and histological properties of knee ligaments. The immobilized group showed a 31 % decrease in linear slope, and a 40 % decrease in maximum load to failure with tensile loading to failure, when compared to the controls. The energy stored to failure was significantly less than control, and the elongation to failure increased with the immobilized groups. Amiel (6) and associates showed similar decreases in stiffness and strength of the lateral collateral ligaments in rabbits immobilized for 9 weeks.

Larsen and co-workers (41) studied the effects of 4 weeks of immobilization on the tensile stiffness of the anterior and posterior cruciate ligaments in rats. They saw a 25 % and 38 % decrease in stiffness, respectively, when compared to controls.

Loitz and coworkers (44) observed the effects of short-term immobilization on the biochemical and biomechanical properties of rabbit Achilles tendon. While a significant decrease in the linear tensile stress was seen, the tensile stiffness did not differ notably for the 3 week immobilization period. However, overall they found the short immobilization period (3 weeks) to result in a mechanically inferior tendon.

Many studies have correlated the biochemical changes occurring with the changes in tensile properties secondary to immobilization [2, 6, 11, 16, 34, 43, 44, 75, 84]. The significant changes have been associated with alterations in collagen content, synthesis, and cross-linking [6, 34, 35, 43, 44, 75, 84]. Collagen turnover serves as an important indicator of fibroblastic activity in terms of collagen dynamics. Increases in soluble collagen cross-links is often seen in immobilized connective tissue [6, 34, 35, 75, 84]. An increase in cross-links is usually associated with decreased strength of the tissue (75); the increase in cross-links are thought to cause interference of movement in and between the collagen molecules by inhibiting the free gliding of the collagen fibers (84). However, contrary to this some studies have reported a decrease in collagen cross-linking with long term immobilization (6).

A significant increase in collagen synthesis and degradation with immobilization was reported by Woo et al. (82) in a study which examined the effect of immobilization on Medial Collateral Ligament (MCL)-bone complexes. The groups that were immobilized for 9 weeks and 12 weeks time showed significant decreases in the stiffness, and ultimate load values. In another related study, Woo et al. (83) observed the biomechanical changes in rabbit MCL-bone complexes following 9 weeks stress-deprivation. Stress-deprivation was seen to result in linear slope, ultimate load, and energy absorption capacity values approximately one third that of the contralateral controls.

In an immobilization study done to observe biomechanical and ultrastructural properties of the rat medial collateral ligament, Binkley and Peat (11) saw a 56.7% decrease in the tensile stiffness and a 60 % decrease in linear stress after 5 weeks. A decrease in collagen synthesis, as indicated by a decrease in small diameter collagen fibers in the immobilized tissue was also observed.

Harwood and Amiel (35) examined the different metabolic responses for the periarticular tissue in rabbit knees, with 9 and 12 weeks of immobilization. They demonstrated that stress-deprivation secondary to internal immobilization had profound affects on collagen metabolism of the patellar tendon, medial collateral ligament, and anterior cruciate ligament (ACL). They found that the ACL was the most susceptible to immobilization-related changes. These results are similar to those reported by Larsen et al. (41).

Woo et al. (84) examined the biomechanical and biochemical effects of immobilization in order to gain insight on why joint stiffness occurs with immobilization. They found significant decreases in water and glycosaminoglycan (GAG) content after 9 weeks of immobilization, with no changes in total collagen. GAG content is examined as an indicator as to what is occurring in the ground substance, or extra-cellular matrix, of the tissue. A correlation was made between hexosamine content (GAG indicator) and the torque required to move the joint. It was found that with the loss of hexosamine, the torque required to move the joint increased.

Tencer and associates (72) studied the effect of immobilizing rabbit knees in full extension versus traditional flexed position. The knees immobilized in full extension

showed degeneration of the articulating surfaces; those immobilized in flexion showed no deterioration. However there was no difference seen in joint stiffness between the two groups.

In some cases, usually after tendon surgery in which suturing has been done, immobilization or partial immobilization as a rehabilitative measure has been seen to result in better healing than its counterpart of controlled passive motion [14, 37]. Similar results have been seen with the healing capacity of articular cartilage [8, 17, 18, 54]. Cast immobilization was examined as a rehabilitative measure for the healing of articular cartilage by Athanasiou and associates (8). They reported cast immobilization to result in stiffer cartilage and better healing of articular cartilage than what was seen with a controlled passive motion regimen.

Namba et al. (52) evaluated the effect of immobilization on joint stiffness. After having induced intra-articular defects in the talar joints of rabbits, one joint was immobilized while the other was treated with continuous passive motion (CPM). The immobilization resulted in significantly higher joint stiffness than pre-injury, whereas with CPM the joint stiffness was maintained at levels similar to pre-injury levels. These results only reinforced those seen by Salter and associates (65) in which CPM was seen to prevent joint stiffness.

Pyne and associates (62) found that immobilization of ligament under tension helped in preserving the strength and stiffness of the tissue. Medial collateral ligaments of rabbits were immobilized with a valgus tension, and compared to immobilization of other groups maintained in positions of flexion and extension, for an 8 week period. The valgus tension resulted in a ligament which has higher stiffness, cross-sectional area, and breaking strength than the groups immobilized in flexion or extension.

In a similar type of study, Schaberg et al. (66) compared the effects of non-stressed immobilization, stressed immobilization and mobilization on the alignment of collagen fibers in healing ligament. Stressed immobilized ligaments demonstrated better collagen fiber alignment that both mobilized and non-stressed immobilization groups

Walsh an associates (76) evaluated the effects of immobilization on cell function of ligament in growing rabbits. They demonstrated a 25% decrease in cell number in all immobilized medial collateral ligaments, with a 50-75% decrease in collagen synthesis following 2 weeks of immobilization. Plasminogen activator, which can potentially degrade extracellular matrix components was seen to increase with 4 weeks immobilization.

Gamble, Edwards and Max (20) evaluated the cellular events responsible for ligament atrophy following immobilization by investigating enzymatic activity during this time. The enzymes evaluated were located in the cytoplasmic, mitochondrial, and lysosomal compartments of the cell. They saw enzymatic changes due to immobilization which caused cellular metabolism to switch from an anabolic, synthetic state to a catabolic, degradative state.

Hannifin and Arnoczky (32) observed stress-deprivation to result in a progressive decrease in the mechanical properties of tendon. An alteration from the normal spindle-shaped fibrocyte was observed upon stress-deprivation. This change in cell shape, number, and tissue morphology was theorized to be a result of the stress-deprivation.

Thus, it has been established that stress-deprivation is accompanied by detrimental changes in tissue morphology. Because it is well known that connective

tissues require some form of mechanical stress to maintain tissue homeostasis, the use of various exercise regimes to attenuate the effects of immobilization has been increasing. The effects of mobilization and exercise on tissue health are discussed below.

Mobilization (Exercise) / Remobilization

The phenomenon of increased stresses resulting in increased collagen synthesis in connective tissue has been shown in many studies (66, 75). Woo (84) provided a general relationship between the level of stress and the maintenance of tissue homeostasis. He documented that decreased stresses result in tissue atrophy and inferior mechanical strength; while increased stresses result in hypertrophy of tissue and mechanical properties somewhat superior to normal controls.

Exercise is accepted as beneficial in the prevention of muscle atrophy, maintenance of joint motion, and promotion of circulation [18, 27]. Numerous studies have evaluated the effects of exercise on connective tissue [16, 41, 73, 74, 84]. The biological notion of continuous passive motion (CPM) was developed as an alternative rehabilitative method by Salter (65) in the late 1970's. This type of therapy, which involves passive application of motion to the afflicted area, has been proven to stimulate healing and regeneration of articular tissues. In a series of basic research studies conducted by Salter and co-workers, it was determined that CPM is a well-tolerated, seemingly painless method which stimulates healing and regeneration of articular cartilage, prevents joint stiffness, and permits normal healing of arthrotomy wounds. CPM has been seen to decrease and in some cases completely avoid the deleterious effects of immobilization [8, 23, 32, 42, 43, 51, 65, 68, 71, 80]. It becomes obvious why the use of CPM has so rapidly increased and become almost standard procedure in

certain rehabilitation programs in the clinical community. Both CPM and other forms of exercise will be discussed in this section.

It has been suggested that mobilization for connective tissue structures be initiated in the very early stages of the repair phase of healing (18). Tipton and his associates (40, 73, 73) were among the first to document the importance of mechanical stimuli in the maintenance of the mechanical integrity of ligaments. They saw an increase in tissue strength with increased training, while immobilized ligament showed significantly lower strengths. They were able to see differences between different periods of exercise; more exercise correlating with greater ligament strength.

When Tipton et al. (73) induced injury and subsequent repair to the medial collateral ligaments of dogs, those which has undergone the regimen of 6 weeks of immobilization followed by 6 weeks of exercise showed strength values similar to the control, non-injured ligaments.

Woo, Gomez, Woo, and Akeson (84) examined the effects of 3 months of moderate exercise versus 12 months of the same with swine extensor tendons. Short term exercise showed minimal effects on mechanical and structural properties of the tendon. However 12 months of exercise increased both the strength and stiffness of the tendons. Dahners and Burroughs (16) found an exhaustive exercise regime to be no more beneficial than a normal enforced exercise regime on the healing of ligament.

Noyes (55, 56) examined of the effects of immobilization with subsequent remobilization on the mechanical and histological properties of knee ligaments. The decreased mechanical properties of the bone-ligament-bone complex due to 8 weeks of

immobilization could only be reversed by a 12 month remobilization period. Thus, a year of remobilization was required before the normal, healthy material properties of the tissue returned.

Woo et al. (82) examined the effect of a similar protocol of immobilization followed by remobilization with MCL-bone complexes. Following both 9 week and 12 week immobilization periods, the groups which were remobilized for 9 weeks showed a rapid return to mechanical properties similar to controls. Larsen and coworkers (41) saw similar results when they studied the effects of 4 weeks of immobilization on the elastic stiffness of rat anterior and posterior cruciate ligaments, followed by subsequent remobilization for 6 weeks. The tensile stiffness of the ligaments was no different than that of the controls. It is apparent that exercise does can reverse some of the effects of immobilization.

Salter et al. (65) have conducted a series of studies in which there was histological evidence that faster healing and regeneration of articular cartilage occurs with CPM. It was also demonstrated that CPM is significantly superior to both immobilization and intermittent active motion in stimulation of healing and regeneration of articular tissues. However, Athanasiou et al. (8) found cast immobilization to result in more favorable healing of articular cartilage than with CPM.

Moran, Kelley, and Salter (51) found CPM to result in early repair of full-thickness laceration of the patellar tendon; a better result than what was seen with immobilization or intermittent activity. Loitz et al (44) found 3 weeks of CPM to result in a tendon with mechanical properties intermediate to those of 3 week immobilized tendons and control tendons. However, the elastic stiffness of the 3 week immobilized

group and the 3 week CPM group were comparable, at 79% and 76% of controls respectively. Thus, CPM was seen to counterbalance the immobilization-related biomechanical alterations in the tendon.

Woo et al (80) also showed positive effects of CPM on tendon repair. The tendons which underwent varying periods of CPM following immobilization showed a higher rate of repair than those tendons immobilized continuously for the same period of time ranging from 3 weeks to 12 weeks.

Namba et al. (52) evaluated the effect of immobilization and CPM on joint stiffness. After having induced intra-articular defects in rabbit ankles, CPM was able to maintain joint stiffness similar to pre-injury levels.

It is apparent that the frequency of CPM may affect the rate of repair as well as the tensile properties of the tissue being rehabilitated. In 1990, Woo et al. (71), using canine flexor tendons, demonstrated a higher frequency CPM regimen to exhibit higher values for linear slope, ultimate load, and energy absorption than that of lower frequency CPM protocol. Thus, there is evidence that CPM, applied after certain periods of stress-deprivation, results in the return of normal mechanical properties despite the differences in time of immobilization and CPM times for the studies.

Common Experimental Models and Procedures

The research done in this thesis makes it is imperative to have an understanding of the various models that have been utilized to study the effects of mechanical stimuli on connective tissue. The choice of an *in vitro* tissue culture model should become clear

upon evaluation of the other immobilization and mobilization methods used in the literature.

There are not many methods by which joints are immobilized for experimental evaluation. The most common method of immobilization has been with extra-articular pin-fixation of the joint, while it is in a flexed position [11, 20, 35, 43, 44, 73, 83, 84]. The general procedure entails the insertion of a stainless steel pin through both of the articulating bones of the joint, thus forcing the knee joint to be immobile in a hyper-flexed position. However, such flexion can place extreme tension on portions of the cruciate and collateral ligaments. While this method results in immobility of the knee joint, portions of the soft tissues may be stressed position when the joint is flexed. Thus, depending on which tissue is being evaluated, there is a distinct possibility that some tissues are under tension.

Another manner by which tissue is immobilized is by the application of a cast [41, 56, 74, 80]. Cast immobilization is not as prevalent as pin-immobilization. The cast can be applied to the entire limb, the entire body, or just limited to the area of injury. If the animal is allowed activity while being casted, the casts have a tendency of loosening. Thus, midway through the treatment the cast needs to be changed. For both pin immobilization and cast immobilization, there is little control of the amount of stress or the lack of stress that the tissue experiences due to the gross nature of both methods.

Mobilization methods vary more with the type of study and the animal model being used. Many of the current studies are utilizing CPM as a remobilization technique [23, 43, 44, 51, 52, 65]. However, the cycles and duration of CPM are not standardized nor well documented. Some studies have relied strictly on cage activity for mobilization of the animals [55, 56, 82]. This method of mobilization does not allow for evaluation of

the loads being experienced by the tissues in any manner. The same predicament occurs with running animals on a treadmill [73, 74, 84], manual flexion of joints [55, 80], and swimming protocols [41].

Thus, upon evaluation of the possible methods by which to study the effects of mechanical stimulus on connective tissue, none provided the type of experimental control as the use of an *in vitro* tissue culture model. This model provides for the complete lack of stress for the evaluation of stress-deprivation effects; it also allows for the precise control of the amount of load and the method of loading for evaluation of the effects of loading on the tissue.

This experimental model in also based on the concept that diffusion is the effective process in providing nutrients to certain animal tissues allowing them to be maintained in a tissue culture environment. Connective tissues have been successfully maintained and evaluated in a tissue culture environment [9, 22, 26, 28, 31, 34, 42, 47, 49, 57, 59, 64, 68]. Previous work has shown that whole tissue segments can be favorably maintained in a chemically defined, cell-free environment in the absence of a circulating blood supply or a bathing synovial fluid. The principle behind tissue culture lies in the ability to provide an *in vitro* nutritional environment for the explanted tissue which approximates, as closely as possible, that which occurs in the intact animal *in vivo*. The canine FDP tendon was selected as the tissue of choice for this study. Because a portion of the FDP tendon derives its nourishment from diffusion of synovial fluid in the tendon sheath, it was a perfect choice of tissues for the tissue culture model used in this study.

The FDP tendon has avascular and vascular regions (24, 58). The avascular D-region (58) was the portion of the tendon used in this study. There has been considerable controversy regarding the relative roles of vascular perfusion and synovial fluid diffusion as nutritional sources for flexor tendons; many have investigated this route by observing how the FDP tendon heals (22, 28, 46, 47, 49). The flexor retinaculum synovial bands, or vinicula, provide blood supply to flexor tendons (67). Several studies have demonstrated that synovial fluid diffusion provides sufficient nutrition for the FDP tendon (22, 28, 31, 38, 47-50) both with tissue culture models and with intact, *in vivo* studies.

Manske and associates first evaluated the nutrient pathways to flexor tendons approximately fifteen years ago (50) and determined that hydrogen molecules were transported to and across the synovium, and subsequently into the flexor tendon by diffusion; this occurred in the absence of vascular attachments. In studies following this one, Manske and Lesker and others (38, 47, 49) were able to demonstrate similar results of synovial diffusion *in vitro*, via the assistance of flexor tendon healing in various animal species. Cell proliferation and increased collagen content was the indicating factor in progressive healing in culture.

Graham et al. (28) found flexor tendons to be fully capable of healing in a tissue culture environment. Similarly, Gelberman and associates (47) were able to successfully exclude any vascular contributions to the healing process of injured flexor tendons of four animal species by demonstrating that tendons could heal on their own in tissue culture. Hooper and his associates studied the nutrient pathways of flexor tendons by evaluating clearance of a radionucleide in canine flexor tendons with an *in vivo* study (38). They saw similar results to Manske, Graham, and Gelberman. This study

demonstrated that flexors tendon receive a majority of its nutrition from the surrounding tissues via diffusion. The high content of radionucleide in the tissue suggested that this was more than could possibly be transported by the surrounding system of vessels.

From the literature it is apparent that the alterations occurring due to immobilization following injury have deteriorating consequences for the uninjured soft tissue present. As tissue culture models have been observed to be suitable for the maintenance of whole connective tissue segments, it is feasible to use such a model to study the alterations occurring due to stress-deprivation *in vitro*.

III. MATERIALS AND METHODS

This chapter provides a general description of the experimental methods and techniques that were utilized to perform the specified tests, collect and analyze the data, and evaluate the histological results. All of the biomechanical testing and histology was done in the Laboratory for Comparative Orthopaedic Research (LCOR) in the College of Veterinary Medicine at Michigan State University. Methodology for all three phases of the study is provided, with a emphasis on tissue culture technique and biomechanical testing.

Tissue Evaluated - Flexor Digitorum Profundus Tendon

The canine flexor digitorum profundus tendon (FDP) was the connective tissue chosen for this study. The FDP has been utilized in many previous studies and is well-suited for this *in vitro* model because of it's nutrient pathways. The FDP tendon consists of distinct avascular and vascular regions (Figure 1). The avascular region has been shown to receive a majority of its nutrition via diffusion of small solutes from the surrounding synovial fluid [21, 22, 28, 31, 46, 47, 49, 50].

The Okuda D-region (58) of the FDP tendon was isolated for this study. Okuda documented the biomechanical, histological, and biochemical properties of the canine FDP tendon (58). The tendon was divided into five regions dependent upon these

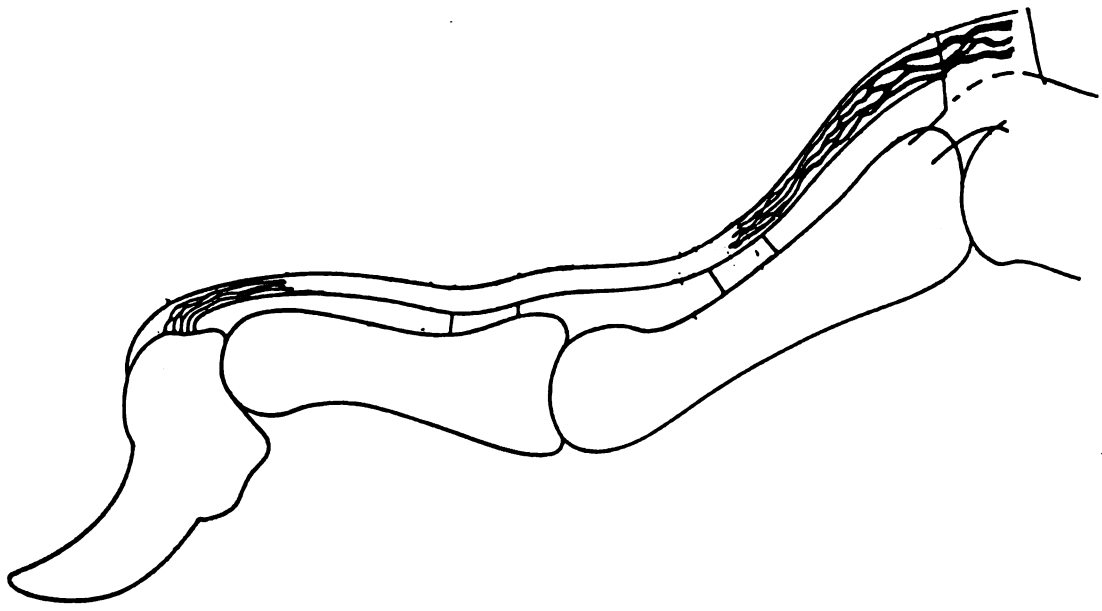


Figure 1: A schematic depiction of the avascular and vascular regions of the canine flexor digitorum profundus tendon (21).

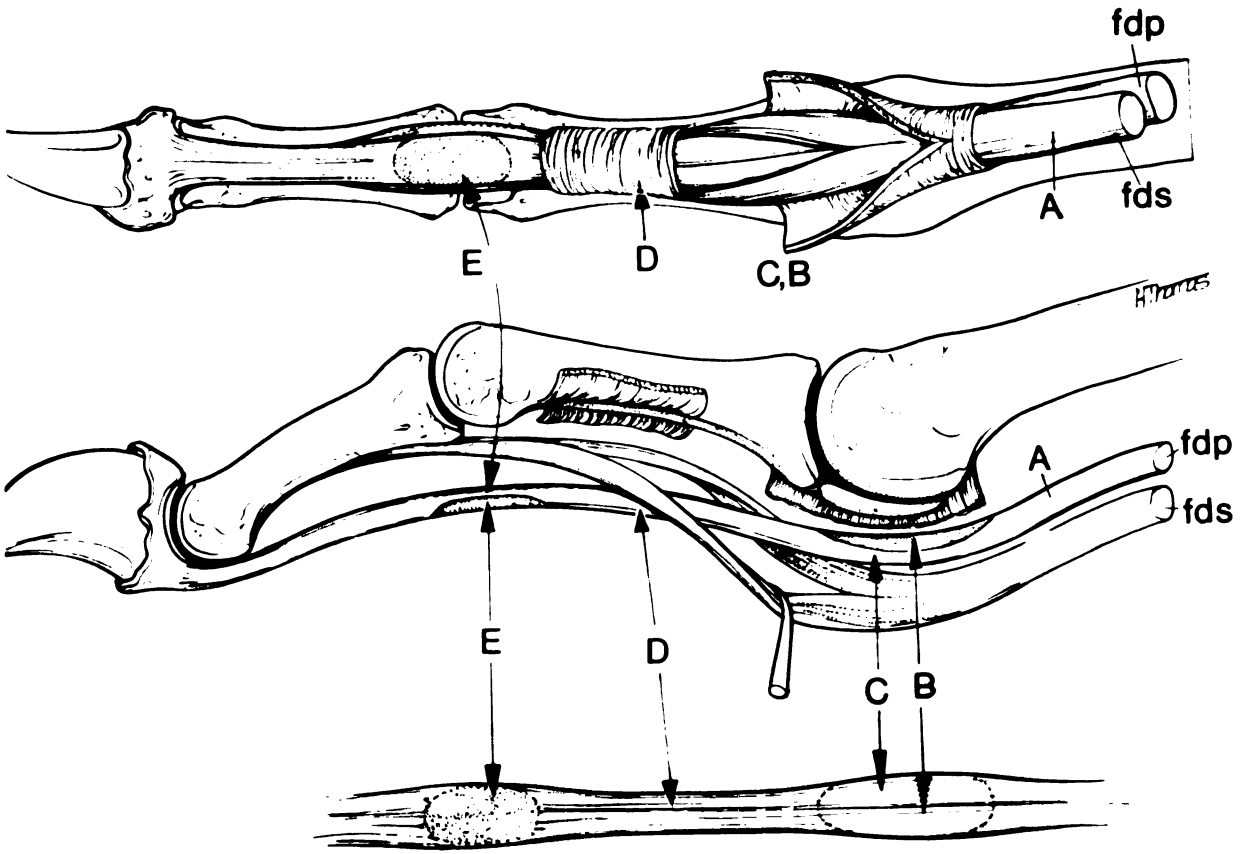


Figure 2: Anatomical drawing of the ventral (top) and lateral views (bottom) of the canine flexor digitorum profundus (FDP) and the flexor digitorum superficialis (FDS) tendons, with reference to the distinct regions as determined by Okuda and co-workers (58).

characteristics (Figure 2). The Okuda D-region consists of dense, hypocellular collagen bundles, located between two strictly fibrocartilaginous areas (Okuda B/C- and E-regions). Being a predominantly tendinous area within a tendon sheath, the Okuda D-region was well suited to maintain in culture and to apply tensile load in culture.

The tendons were obtained from dogs being utilized in other unrelated studies, and from dogs being used in the College of Veterinary Medicine teaching laboratories. The dogs were adult mongrels of mixed age and sex. The animals were euthanized with an intravenous injection of sodium pentobarbital (Fatal Plus). Immediately following euthanasia, the tissue was harvested and four FDP tendons were taken from each of the four limbs in every animal.

Dissection Technique:

A lateral incision was made along the foot and the foot pad in order to exposed the flexor digitorum superficialis (FDS) tendons. The FDS tendons were then transected proximally and reflected distally in order to expose the underlying FDP tendons, sheath, and pulley systems. The synovium and sheath was cleared from the tendon before transection. The FDP tendons were then transected approximately 3 mm proximal to their distal attachment on the third phalanx and pulled gently in the proximal direction, through the sheath. Subsequently, the proximal end of the FDP tendons was transected and placed in Dulbecco's modified Eagle medium solution containing a 10 % concentration of an antibiotic/antimycotic solution (penicillin/streptomycin); this solution is the antibacterial wash solution. The tendons were then taken to the tissue culture facility of the LCOR where the remainder of the tissue preparation was carried out in a tissue culture hood. The common proximal attachment of the FDP tendons

was trimmed off in order to separate the four individual tendons, prior to the antibacterial wash sequence. The Okuda A-region and the distal-most portion of the Okuda E-region were also trimmed (Figure 3).

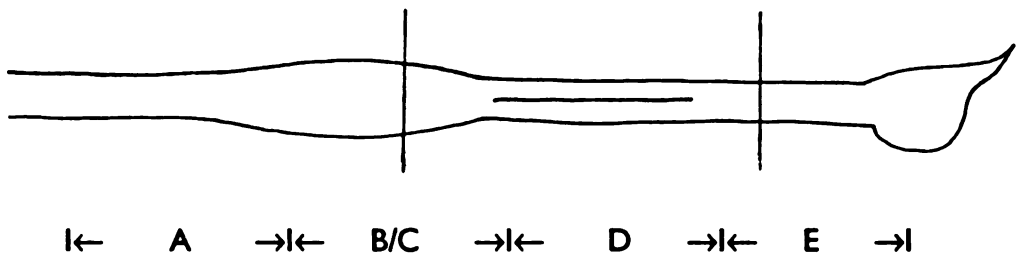


Figure 3: Schematic depiction of the canine flexor digitorum profundus tendon, the vertical lines indicating the regions at which trimming was done, with reference to the Okuda regions (26).

Antibacterial Wash Sequence:

After harvest, the individual tendons were rinsed in a wash solution of Dulbecco's modified Eagle medium (DMEM) containing 10 % antibiotic/antimycotic solution (Gibco Laboratories, Grand Island, New York). All tendons that were to be utilized for zero time controls were rinsed once in the wash solution and then immediately frozen at - 80 ° C until time of tensile testing. The experimental tendons were taken through the washes prior to tissue culture protocol. The tendons were

taken through a series of three ½ hour washes in 100 mm x 20 mm petri dishes (Corning Glassworks, Corning, NY), with approximately 40 ml of wash media. This procedure was done to ensure the removal of external contamination prior to tissue culture. The entire wash procedure was conducted in an aseptic environment under the tissue culture hood. Tendons were placed into tissue culture immediately following this wash sequence.

Tissue Culture Techniques

The *in vitro* model for stress-deprivation and subsequent loading entailed the maintenance of the FDP tendons in a tissue culture environment. The process is technically referred to as "organ culture" as the culture is of a three-dimensional, undisaggregated tissue which has all of the histological features of the tissue *in vivo*. In this study a complex, defined media, M-199 (Gibco Laboratories, Grand Island, New York), was utilized. Previous *in vitro* studies done with flexor tendons have utilized medium M-199 with supplements [22, 32, 47]. Thus, M-199 medium has been shown to successfully maintain tendons in a tissue culture environment. The media was stored at 4 ° C until the tissue culture media was to be made. The culture media was prepared by adding vitamin and other nutrient supplements to the M-199 media. Fetal bovine serum (FBS) and ascorbate were the nutritional supplements utilized. FBS (Gibco Laboratories, Grand Island, NY) provides growth factors and cytokines to the media in proportions similar to what the tissue would normally get *in vivo*. Ascorbate (Sigma, St. Louis, MO) is a vitamin C supplement which is necessary in providing a substrate for collagen synthesis in tissue culture. Antibiotic/antimycotic (ab/am) solution (Gibco Laboratories, Grand Island, NY) was added to the media solution as an antibacterial and anti fungal supplement. The culture media was prepared under the tissue culture hood in sterile

conditions, using warm (37°C) solutions. At the time of culture media preparation, the solutions and M-199 media were filtered through a 0.20 μ tissue culture filter (Nalgene, Rochester, New York). The protocol for making 500 ml of M-199 tissue culture media is given below:

435 ml of M-199 media (87 %)

5 ml of antibiotic/antimycotic solution (1 %)

10 ml of ascorbate (2 %)

50 ml of fetal bovine serum (10%)

Once the media was made, it was stored in the 37 ° C water bath until the time of its use. Multi-well (six 10 ml wells per dish) plastic culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) were used to maintain the tendon while in tissue culture. Approximately 9 ml of tissue culture media, at 37 ° C, was pipetted into each well in the dish. The tendons were placed into the wells with sterile forceps under the tissue culture hood (Figure 4). Following this step, the dishes were placed in a tri-gas, humidified incubator (Forma Scientific, Inc., Marietta, Ohio) which was maintained at 37.0 ° C with a 10 % CO_2. The culture media was changed every other day under sterile conditions.

Tissue Preparation

The examination of the first hypothesis involves the evaluation of material properties of tendons that have been deprived of stress or immobilized for different time periods. *In vitro* stress-deprivation was achieved by maintaining the tendons in tissue culture for specified periods of time. The experimental animals were divided into

groups based on the duration of tissue culture immobilization. The tendons were separated into the following experimental groups:

(i) 1 week immobilized in tissue culture

(ii) 2 weeks immobilized in tissue culture

(iii) 3 weeks immobilized in tissue culture

(iv) 4 weeks immobilized in tissue culture

(v) 5 weeks immobilized in tissue culture

(vi) 6 weeks immobilized in tissue culture

(vii) 8 weeks immobilized in tissue culture

The time zero controls for each animal in this phase of this study were frozen immediately following harvest, as noted previously. A summary of the tendons utilized for each experimental group in this stress-deprivation portion of the study is provided in Table 1. At the end of each experimental time period, the respective tendons were removed from tissue culture and frozen at - 80 ° C in individual freezer tubes until the time of tensile testing. In order to maintain tissue integrity the tissue must be deep frozen at temperatures below - 39 ° C (13).

For each animal used in each experimental group, a time zero control group was established. A control group was required in order to attain a normalized data set. Thus, each experimental tendon has it's own set of control tendons. This procedure serves to minimize the effects of variations in tendon size, age, sex, and breed between the animals used. In this manner, the parameters evaluated in the "experimental" tendon group are normalized when compared to the same parameters in the respective control group; both groups having similar tendon size and being from one animal, thus eliminating any changes occurring due to age, sex, and breed.

Figure 4: Photograph showing the freshly-trimmed canine FDP tendons in the tissue culture dish with culture media, prior to incubation.

TABLE 1: Tendon Summary for T_0 Controls and Stress-Deprived Groups

STRESS DEPRIVATION	CONTROL (T_0) PERIOD	EXPERIMENTAL[a]
1 WEEK	n = 18	n = 25
2 WEEKS	n = 27	n = 31
3 WEEKS	n =13	n = 10
4 WEEKS	n = 15	n = 15
5 WEEKS	n = 12	n = 8
6 WEEKS	n = 18	n = 8
8 WEEKS	n = 19	n = 9

[a] The disparity in the number of tendons used per experimental group is a result of the pooling of data from all three phases of the study.

Freeze-Thaw Procedure:

It has been hypothesized that the deterioration of material properties in tendon due to varying periods of stress-deprivation is probably a cell-mediated response. In order to explore this theory, a subset of tendons in which the cells had been killed were examined.

The cells were killed using a freeze/thaw technique. The tendons were taken through a series of freeze and thaw cycles immediately after harvest. The tendons were frozen in liquid nitrogen (- 196 ° C) for approximately 10 seconds and subsequently "thawed" in warm (37 ° C) wash media for approximately 30 seconds. This cycle was repeated five times in order to ensure complete cell death. This freeze/thaw procedure was shown to result in complete cell death due to destruction of the cell membrane due to ice crystal formation (23, 24). Following the freeze/thaw cycles, the tendons were taken through the same wash procedure as the tendons from previous experimental groups. After the washes the tendons were placed in tissue culture for the specified stress-deprivation periods. The time zero control tendons were frozen in the - 80 ° C freezer immediately after harvest.

Four animal groups were utilized for a freeze/thaw protocol to kill the cells before immobilization in tissue culture. The four animals were each divided into the following experimental groups:

(i) zero time controls,

(ii) 2 weeks stress-deprivation (freeze/thaw),

(iii) 4 weeks stress-deprivation (freeze/thaw)

Once the tendons in the experimental groups had been in tissue culture for their specified time periods, they were removed and frozen in the - 80 ° C freezer until the time of tensile testing.

The second and third phases of this study involve mobilization and re-mobilization of the respective tendons. Mobilization was the cyclic loading of the tendon immediately after harvest. Re-mobilization entailed the cyclic loading of a tendon previously immobilized for a specific time period. The loading of the tendons in tissue culture was accomplished by suspending a specified weight from the tendon in a test tube of culture media. Loading was done by securing the suspended tendon to a specially designed cyclic passive motion machine. The tendon hanging and loading procedure is described in the following section.

Tendon Hanging Procedure:

The tendons are clipped on both the fibrocartilaginous Okuda B/C and E-regions, both adjacent to the Okuda D-region test area. Small (9 mm) metal clips (Autoclips, Becton Dickinson, Parsippany, NJ) were used for this purpose. The distal metal clip was attached to a 50 gram (0.49 N) stainless steel calibration weight via nylon suture thread (Surgilon, American Cyanimide Company, Pearl River, NY). The proximal metal clip was used to secure the loaded tendon to the motion device, with nylon thread. This entire procedure was done in the hood using sterilized equipment. Sterile 50 ml conical test tubes (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were filled with 47.5 ml of M-199 culture media. 5 mm holes were made in the center of the test tube caps (sterilized prior to loading), allowing for full excursion of the tendon during the cyclic loading regime. The loaded tendons remain in the media-filled test tube both during loading and during rest.

A load of 50 grams was chosen after evaluating the effects of both lower and higher loads on the tissue (32). This load has been seen to result in tissue strains

approximately 0.5 % (as determined by stress-strain curves of tensile loading of the canine FDP tendon). This strain falls well within slow, passive physiological strain levels.

Cyclic Loading Procedure:

Tendons were loaded using a specially designed cyclic loading devices (Figure 5). The device consists of an arm attached to a rotating cam which was motorized by a small, slow speed electric motor (Edmund Scientific Co., Barrington, NJ) which had a set frequency of 1 rotation per minute. Tendons were loaded for 2 hours a day at 5 days a week. This frequency and duration of loading was gleaned from previous CPM studies.

The loaded tendon was suspended in the 50 ml test tube of culture media from the arm of the device. As gleaned mentioned above, the device allowed for a loading/unloading cycle of 1 rotation per minute via the rotating cam which raised and lowered the suspended tendon at this frequency. This frequency was equivalent to 30 seconds of rest (when the arm traveled through the lower hemisphere of the rotation area) and 30 seconds of loading (when the arm traveled through the upper hemisphere of the rotation area). A schematic depicting this set-up is shown in Figure 5. This loading/unloading frequency was achieved by adjusting the length of the suture thread before loading. All cyclic loading periods began with the tendon having a small pre-load (i.e. the suture thread was not slack, as when it was at rest, yet it was not fully tensioned) with the device arm at a "3 o'clock" or "9 o'clock" position. This set-up ensured proper 30 second loading and 30 second unloading of the tendons. At the end of each loading period, the tendons were brought to a rest position with the arms at a "6 o'clock" stance (Figure 6). Similar to the previous phases of this study, the zero time controls from these groups were rinsed with 10 % ab/am solution of DMEM and

immediately frozen at - 80 ° C. Four experimental groups were established to evaluate the second and third hypotheses of this study, the groups were as follows:

(i) 2 weeks stress-deprived (immobilized),

(ii) 2 weeks loaded (mobilized)

(iii) 2 weeks stress-deprived (immobilized) / 2 weeks loaded (re-mobilized),

(iv) 4 weeks stress-deprived (immobilized)

Each experimental group consisted of tendons from more than one animal. A summary of the tendons in each animal group is provided in Table 2. The tendons involved in the second phase of the study were mobilized or loaded for a period for 2 weeks in a tissue culture environment . The tendons were taken through the wash procedure and then immediately started on the loading regimen previously described. After the loading period, the tendons were removed from tissue culture and frozen in the - 80 ° C freezer.

The third phase of the study entailed stress-deprivation of the tendons for a 2 week period immediately followed by a loading (re-mobilization) period for the same time. Following this 4 week program, the tendons were removed from tissue culture and frozen in the - 80 ° C freezer.

TABLE 2 : Animal Summary for Loaded (Mobilized & Re-mobilized) Tendon Groups

EXPERIMENTAL GROUP	CONTROL (T_0)	EXPERIMENTAL
2 WEEK LOADED	n = 22	n = 19
2 WEEK STRESS-DEPRIVED	n = 27	n = 31
2 WEEK STRESS-DEPRIV./ 2 WEEK LOADED	n = 18	n = 19
4 WEEK STRESS-DEPRIVED	n = 15	n = 15

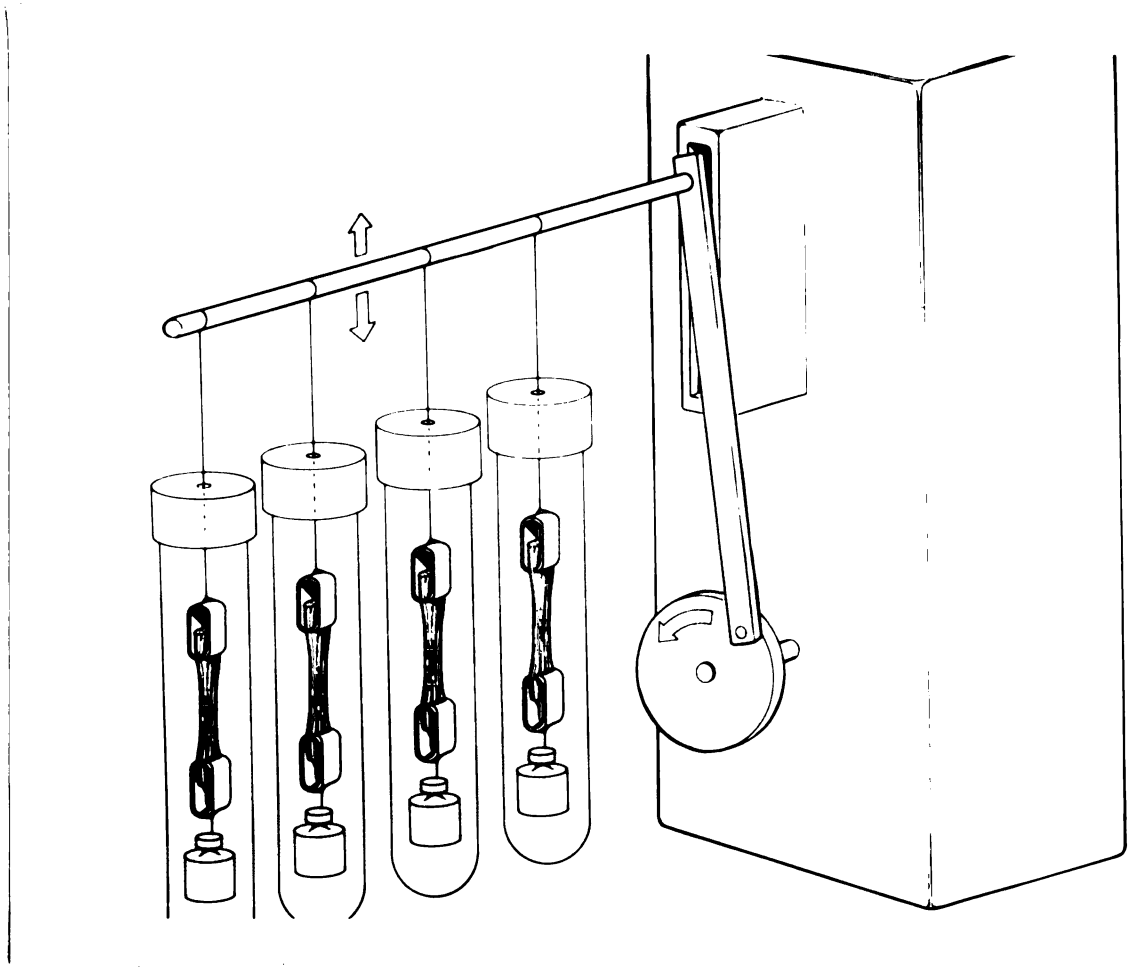


Figure 5: A schematic depicting the cyclic loading apparatus used for mobilization and remobilization of the FDP tendons in tissue culture.

Figure 6: Photograph of the FDP tendons fastened to the cyclic loading device while in tissue culture environment.

Biomechanical Testing of Tendons

The structural properties of every tendon were determined from tensile testing. Acquisition of tendon mechanical data was accomplished with a servo hydraulic mechanical testing machine (Instron 8501 M) aided by a micro-computer (Commodore Amiga 2500).

Tissue Preparation:

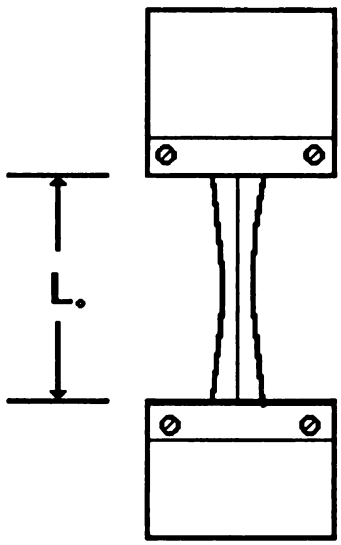
The individual tendons were removed from the - 80 °C freezer and allowed to thaw at room temperature (for ~ 10 minutes). The tendons were kept in M-199 media to maintain hydration before testing. Once the tendon had thawed, it was trimmed at the Okuda B/C and E-regions to provide a sufficient gripping area for testing purposes. All measurements were taken after the tissue was secured in the grips.

Once the grips were fastened to the Instron attachments, the initial gauge length (l_0), width, and thickness measurements were taken. These measurements were taken with hand-held digital calipers (Mitutoyo Corporation, Tokyo, Japan). All measurements were taken with an approximate load of 0.098 N (10 g) on the tendon. This load was required to eliminate any slack in the tendon. The tendon was kept moist throughout the measurement procedure. This was done by dousing the tendon with the M-199 media. A schematic diagram depicting all points at which measurements were taken is shown in Figure 7. The tendon was kept in a saline (0.9%) bath, at room temperature, while testing (Figure 8). Because no changes have been seen in the mechanical properties of collagenous tissue over the temperature range of 0 to 37 ° C (13, 15), this temperature was thought to be sufficient during the testing period.

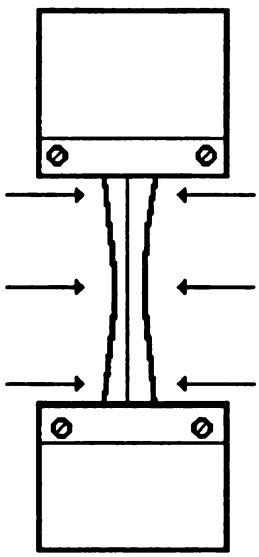
Tensile Test Parameters and Procedure:

As noted above, all tensile tests were performed using an Instron 8501-M mechanical testing machine. A 25 lb. load cell (Data Instruments, Acton, MA) was utilized for testing. All tendons were tested to a 9.81 N (1000 g) load at strain rate equivalent to 0.5 % of the tendon gauge length per second. This rate was derived from previous studies (60) and from testing done prior to this experiment (unpublished data). In preliminary tests, this rate resulted in the best response of the tendon upon observing the load-deformation graph - i.e. it allowed for sufficient linear region prior to reaching the 9.81 N final load limit. All tendons were tested with no pre-conditioning. A data acquisition program was provided with the required parameters prior to testing in order to sample data at a rate of 80 hertz. Upon completion of the test, the tendon was removed from the grips, the gripped portion removed, and the test-region of the tendon fixed in 10 % buffered formalin for histology.

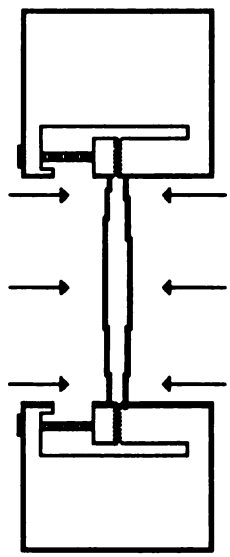
The tensile testing data was saved directly to floppy disk in order for subsequent manipulation on a computer spread-sheet program. Three columns of data were obtained: time (in seconds), an amplified position of the actuator (in volts), and load readout through the load cell (in volts). This data was later manipulated, with the aid of scaling factors, to obtain values that would provide the required load-deformation plots for data analysis.



Initial Length Measurement



Width Measurements



Thickness Measurements

Figure 7: Schematic depiction of all points at which measurements were taken on the FDP tendon prior to mechanical testing. All measurements were taken with an approximate load of 0.098 N (10 g) on the tendon.

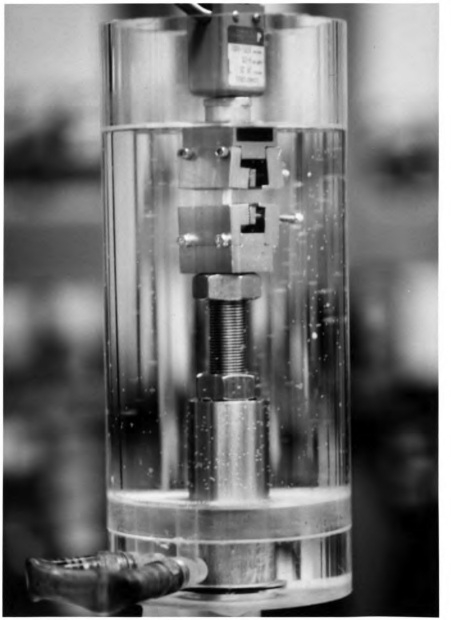


Figure 8: Photograph of the FDP tendon fixed in the grips within the bath apparatus prior to tensile testing.

Histology

Following the mechanical testing each tendon was trimmed (leaving only test region - the Okuda D-region) and put in 10% buffered formalin for fixing of the tendon. The tendons were fixed for at least 24 hours before any tissue processing could be done. The standard tissue processing procedure of dehydration, and infiltration was done prior to embedding the tendon samples in paraffin. The paraffin blocks were oriented such that longitudinal sections of the FDP tendon could be cut. 5.0 μ thick sections were cut on a rotary microtome and mounted on glass slides. These sections were then stained with a standard Hematoxylin and Eosin (H & E) staining procedure. This protocol resulted in the cell nuclei retaining blue stain, while the cytoplasm and collagen fibers retaining a pink stain.

The stained tendon sections were observed with a light microscope (Olympus BH-2, New Jersey Scientific, Middlebush, NJ). Cell shape, relative cell number (cellularity), and the status of cell nuclei were observed with standard light microscopy. Collagen fiber alignment was evaluated using polarized light microscopy. The birefringent nature of collagen allowed for viewing of the sinusoidal crimp pattern in the individual collagen fibers, as well as the relative alignment of the fibers.

Data Analysis

The raw data that was obtained from each tensile test was evaluated with the assistance of a computer spread-sheet program. This allowed for manipulation of the raw data into the functional, scaled data required for a load-deformation plot. The scaling factors were utilized, specifically, to convert the load and deformation values (in volts) to the metric units required for proper interpretation and analysis of the data. The load was recorded in grams, while the deformation was recorded in millimeters.

Load-deformation curves were developed directly from the computer spread-sheet program. The load-deformation plots allowed for the calculation of the stiffness values (K) for each tendon tested. The stiffness values for all tendons were computed manually directly from the load-deformation output graph. A sample load-deformation graph is shown in Figure 9. This graph is not an actual plot of a canine FDP tendon tensile test, it is a schematic depiction.

Calculation of Stiffness (K):

The stiffness of the tendon was calculated directly from the load-deformation curve. The slope of the load-deformation curve designates the stiffness of the tendon, i.e.:

stiffness = change in load / change in deformation

This is shown in Figure 9. A tangent to the load-deformation curve was drawn with in the linear region. The tangent was drawn in a manner such that the largest portion of linearity in the curve's linear region was utilized. The stiffness values were recorded in units of N/mm.

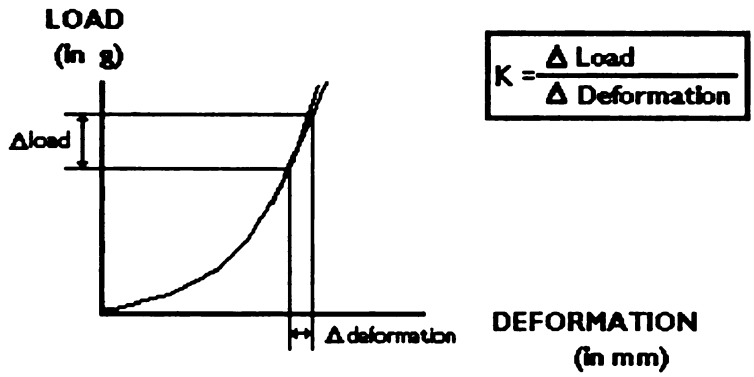


Figure 9: Sample load-deformation curve indicating regions at which stiffness (K) measurements would be taken for further calculations and analysis.

The stiffness represents a structural property. The modulus of elasticity or tensile modulus is based on a linear relationship between stress (force per unit area) and strain, which translates into the stress being directly proportional to the strain for elastic materials. For biological materials, which are viscoelastic in nature, the tensile modulus is used to represent the "elasticity" of the material. Structural properties of a tissue can be converted to material properties by utilizing individual tissue characteristics. In this manner the stiffness (K) of a tendon can be converted to a material property, the tensile modulus (E), by the use of the initial gauge length (l_0) and the cross-sectional area (A) of the respective tendon. A brief derivation of the equations used for the calculation of the tensile modulus in this study is provided below. Detailed calculations are provided in the Appendix.

CALCULATIONS:

A summary of the symbols:

F = force or load	... in N
δ = deformation	... in mm
l_0 = initial gauge length of tendon	... in mm
A = cross-sectional area of tendon	... in mm^2
K = stiffness coefficient of tendon	... in N / mm

E = tensile modulus of tendon ... in mega Pascal
σ = stress (= force/unit area) ... in mega Pascal
ε = % strain

The load-deformation curve the coefficient of stiffness, K, is obtained:

$K = F / \delta$ ... in N / mm^2 (1)

$\Rightarrow$ To obtain the tensile modulus, E, of the tendon

$E = \sigma / \delta$

where $\sigma = F / A$
$\varepsilon = \delta / l_0$

$E = (F / A)(\delta / l_0)$ ... in N / mm^2

$\Rightarrow$ The tensile modulus (E) value is obtained in N / mm^2, it is converted to Pascals.

$1 N / m^2 = 1$ Pascal

$\Rightarrow$ Following conversion of N / mm^2 to $1 N / m^2$ Pascals can be used as the functional unit.

Once the value for the elastic modulus (E) for each experimental tendon was calculated, it was expressed as a percentage of it's respective control tensile modulus value. The time zero control tensile modulus was expressed as a mean value of all zero time control tensile modulus in the respective experimental group.

$\Rightarrow$ if Econtrol = tensile modulus of the control group (in MPa)

if Eexperimental = tensile modulus of the experimental group (in MPa)

$\Rightarrow$ Then the reported values for each experimental group were expressed as:

Eexperimental (%) = Eexperimental (in MPa) / Econtrol (in MPa)

Eexperimental = E as a % of control

In this manner, each experimental tendon was normalized by a control from the same animal. Thus, all results are expressed with the experimental tensile modulus as a percentage of its individual control.

Statistical Analysis

Following the collection and processing of the mechanical data, statistical analysis was done for all of the data. To evaluate the changes in tensile modulus with the increasing periods of stress-deprivation, a one-way analysis of variance (ANOVA) was done. Multiple confidence intervals were utilized to determine statistical significance in this method. In this manner, the effect of time (corresponding to increased periods of stress-deprivation) on the material properties of the respective tendon groups can be observed. This test was used only to evaluate the data from the stress-deprivation group.

The Student's t-Test was utilized to determine if any change was occurring between the control groups and the respective experimental groups, as well as between the different experimental groups. For this evaluation, the mean elastic modulus for a particular experimental group was expressed as a percentage of the respective control tensile modulus. In this manner, a mean value for the tensile modulus a percentage of it's control as well as the standard deviation was obtained. Statistical analysis was done both with each raw data (percentage of control tensile modulus values) value, as well as with each mean and standard deviation within the group value. The t-Test was done using 99th percentile confidence interval, with "p" values below 0.05 indicating validation of the hypothesis - or statistical significance.

IV. RESULTS

The results listed in this chapter are written in direct relation to the stated hypotheses of this study.

H_1: Stress-Deprivation Study

Tensile testing of all tendons in this portion of the study resulted in a classic load-deformation curve (Figure 10). The tensile modulus (E) was calculated from the linear region of the load-deformation curve using the method described in the materials and methods chapter. The tensile moduli for each control and stress-deprived tendon group is shown in Table 3. Because of the variation in size, age, sex, and breed of the animals utilized, the values for the tensile modulus were normalized by their respective control tensile moduli. This allows for valid comparisons between each of the experimental groups, despite being from different animals. All values for the experimental tensile modulus were expressed as percentages of individual zero time control tensile modulus:

$$E \text{ as } \% \text{ of control} = E_{experimental} / E_{control}$$

The mean tensile moduli of each group, expressed as a percent of control results are presented in Figure 11. The effect of stress-deprivation over time on the tensile modulus was determined by performing a one way analysis of variance (ANOVA)

statistical test. The effect of time on stress-deprivation was shown to be statistically significant with a p value of 0.000. However, statistical significance between certain groups was not significantly different as determined by the Student's t-Test. The modulus of elasticity for the 2 weeks and 3 weeks stress-deprived groups were statistically the same, as were the tensile modulus of the 5 weeks, 6 week, and 8 weeks stress-deprived groups. However, the tensile modulus for the 1 week stress-deprived group was statistically different from the 2 week stress-deprived group; similarly the 3 week stress-deprived group showed statistical significance with the 4 week stress-deprived group. Finally, the 4 week stress-deprived group was statistically different from the 5 week stress-deprived group.

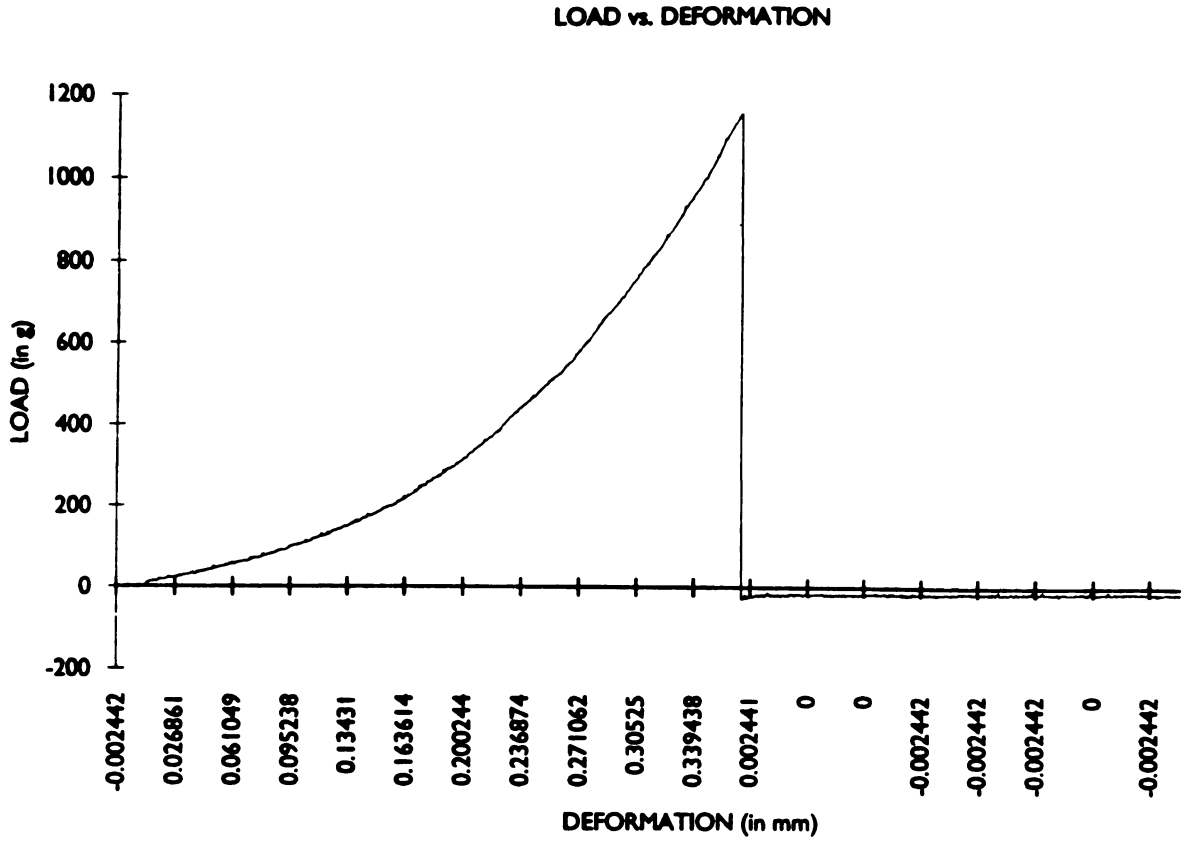


Figure 10: Load-deformation curve of time zero control canine FDP tendon. Depicts the classic load-deformation response of tendon.

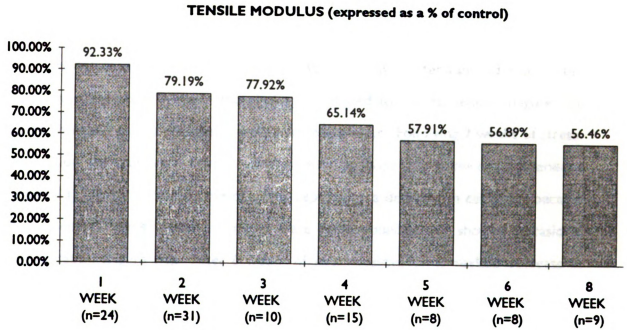


Figure 11: Histogram depicting the time-dependent change in the tensile modulus (E) with increased periods of stress-deprivation.

TABLE 3: SUMMARY OF THE ELASTIC MODULUS VALUES FOR TENDONS IN STRESS-DEPRIVATION STUDY

STRESS DEPRIVATION PERIOD	ELASTIC MODULUS OF STRESS-DEPRIVED GROUP (in MPa)	n
T_0 control	87.37 ± 7.04	88
1 week	80.71 ± 1.03	24
2 weeks	67.05 ± 5.70	30
3 weeks	69.88 ± 1.25	10
4 weeks	55.49 ± 0.97	16
5 weeks	50.44 ± 1.66	8
6 weeks	50.40 ± 1.86	8
8 weeks	50.18 ± 5.77	9

Histological assessment of the stress-deprived tendons demonstrated progressive alteration in tissue morphology compared to normal tendon (Figure 12). These changes included changes in cell shape and number. Following 2 weeks of stress-deprivation, the cells were morphologically similar to those seen in the control tendons (Figure 13). With increased time of stress-deprivation a decrease in cellularity became more evident. By 4 weeks cell nuclei were more rounded and showed occasional fragmentation (Figure 14). After 6 weeks of stress-deprivation a marked decrease in cellularity was observed (Figure 15). In addition, a majority of the collagen fibers demonstrated a random alignment differing markedly from the typical parallel orientation observed in the control tendons. Following 8 weeks of stress-deprivation, very few cells were observed. The few cells which remained showed evidence of degradation (Figure 16), and collagen alignment varied with some areas depicting normal crimp, other areas showed erratic crimp patterns.

Freeze-Thaw Tendon Group:

The tendons which were taken through the freeze-thaw procedure prior to the immobilization *in vitro* were evaluated in a similar manner. Tensile testing again resulted in classic load-deformation curves, with no deviation seen in either groups. The tendons that were freeze-thawed and subsequently maintained in tissue culture for 2 weeks had an tensile modulus of 99.3% of the respective time zero controls. Similarly those tendons freeze-thawed and stress-deprived for 4 weeks had an tensile modulus of 100.2 % of their respective controls (Figure 17). Both the 2 week and 4 week freeze-thawed/stress-deprived groups were shown to be

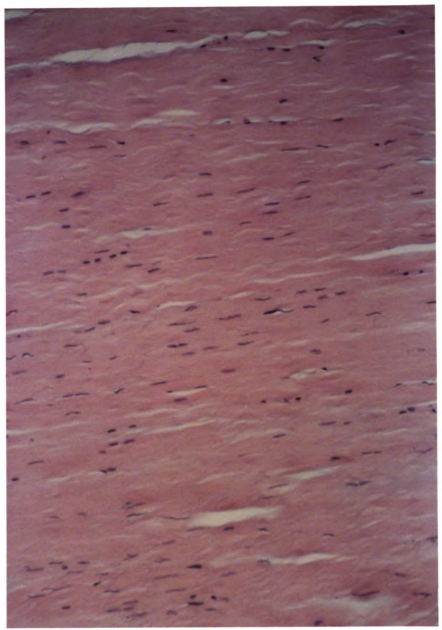


Figure 12: Histological appearance of H&E stained time zero control canine FDP tendon, as seen with light microscope (at 40X magnification).

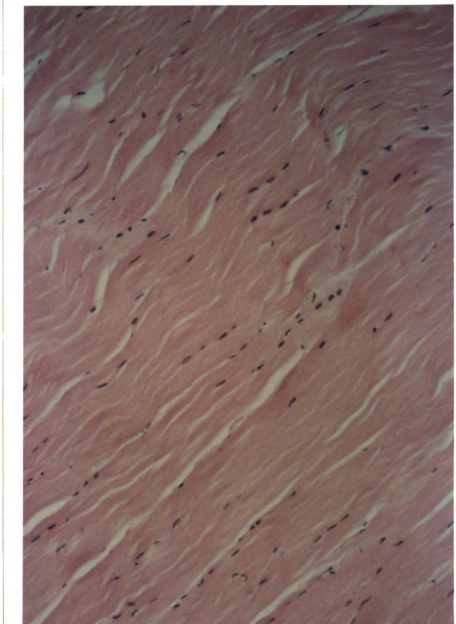


Figure 13: Histological appearance of H&E stained canine FDP tendon after 2 weeks of stress-deprivation. Observed with light microscope at 40X magnification.

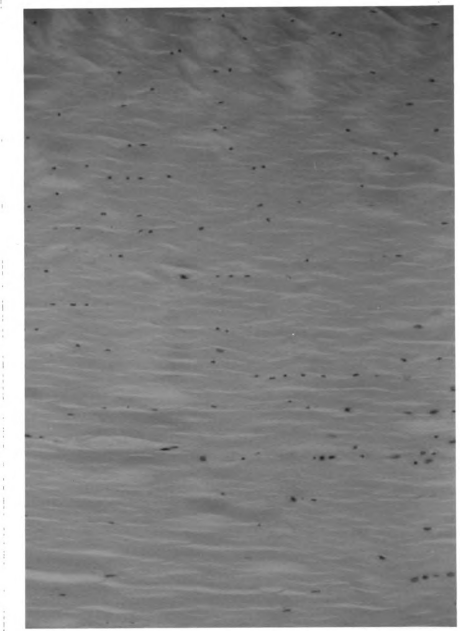


Figure 14: Histological appearance of H&E stained canine FDP tendon after 4 weeks of stress-deprivation. Observed with light microscope at 40X magnification.

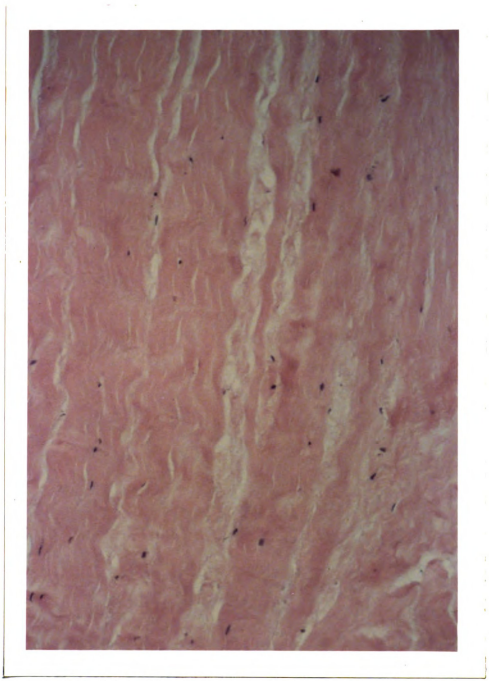


Figure 15: Histological appearance of H&E stained canine FDP tendon after 6 weeks of stress-deprivation. Observed with light microscope at 40X magnification.

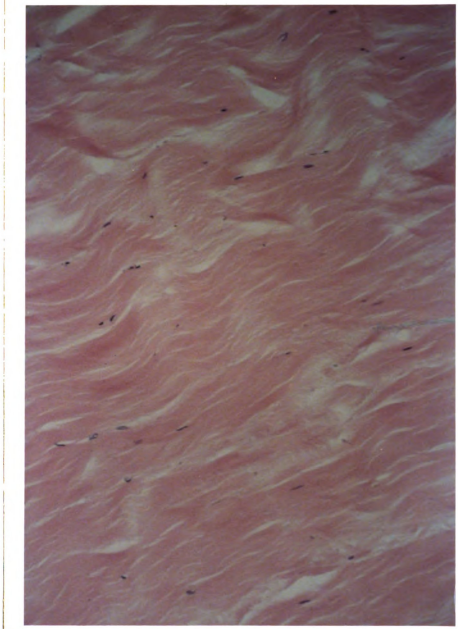


Figure 16: Histological appearance of H&E stained canine FDP tendon after 8 weeks of stress-deprivation. Observed with light microscope at 40X magnification.

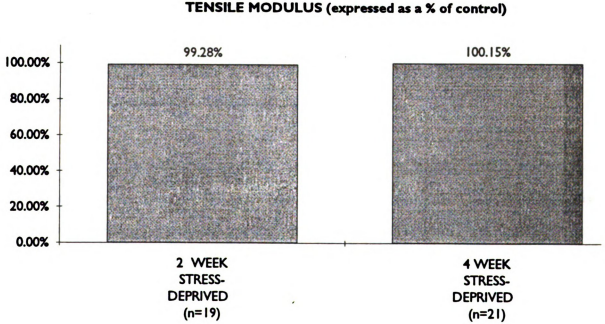


Figure 17: Histogram depicting the effect of freeze-thaw technique with subsequent stress-deprivation on the tensile modulus of the canine FDP tendon.

statistically the same ($p>0.05$) when compared to their respective time zero controls, by the Student t-Test. Similarly, the 2 week and 4 week values were statistically the same ($p>0.05$)

Histological evaluation of the 2 week and 4 week freeze-thawed, stress-deprived tendons demonstrated a complete absence of cells (Figure 18). However collagen orientation and alignment did not appear to be adversely affected.

H_2: Mobilization Study

This portion of the investigation examined the effects of a controlled program of cyclic loading, on the material properties of the tendons maintained *in vitro*.

Using the previously described cyclic loading regimen, the tensile modulus for the tendons mobilized for 2 weeks was 84 % of their individual controls. While this elastic modulus value was higher than that shown by the 2 week stress-deprived tendon group it was not statistically different (Figure 19).

Histological appearance of the tendons cyclically loaded for 2 weeks was comparable to that seen in the zero time control tendons (Figure 20). However, the cellular morphology and collagen crimp pattern in the 2 week mobilized specimens was better than that observed with the 2 weeks immobilized group. The tendon showed high cellularity, with elongated spindle-shaped within normal, parallel oriented collagen fibers. Thus, maintenance of cellular morphology was seen despite the slight diminishing of mechanical properties.

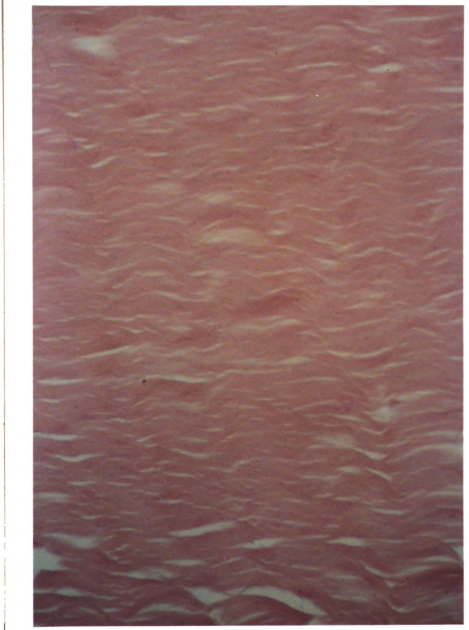


Figure 18: Histology (H&E stained) of canine FDP tendon following freeze-thaw technique and 2 weeks stress-deprivation. Complete absence of cellularity is seen. (Observed with light microscopy, 40X magnification).

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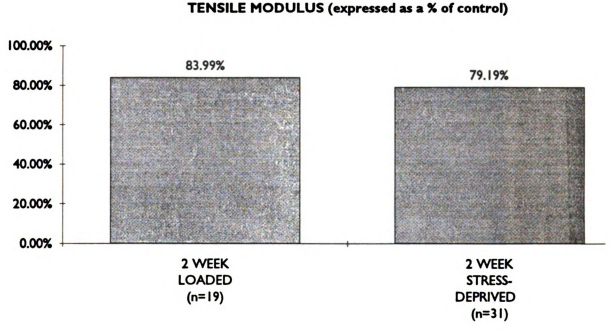

Figure 19: Histogram depicting the variations seen between tendon groups stress-deprived for 2 weeks and those cyclically loaded for 2 weeks.

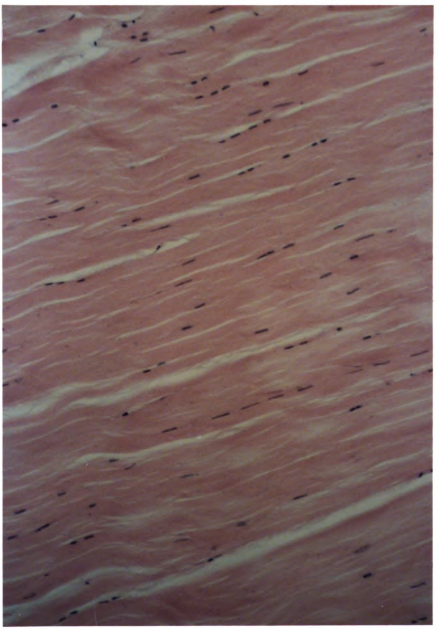

Figure 20: Histological section of canine FDP tendon cyclically loaded (with 50 gram load) for 2 weeks in tissue culture. Viewed with light microscopy at 40 X magnification.

H_3: Stress-Deprivation / Remobilization Study

The third hypothesis explored in this thesis was done to determine the ability of cyclic loading to reverse or arrest the degenerative changes (decreased tensile modulus, change in cell morphology) associated with stress-deprivation.

The tensile modulus of tendons stress-deprived for 2 weeks then cyclically loaded for 2 weeks was found to be 80.8% of their individual controls. While this value is higher than tendons stress-deprived for 2 weeks it was not statistically different. However the tensile modulus for the remobilized tendons was significantly higher than for the group stress-deprived for 4 weeks (Figure 21)

Histological evaluation of the remobilized tendons showed a slightly different cellularity as compared to the control tendons. However, the majority of the cells in the tendon were spindle-shaped and appeared healthy (Figure 22). This is in contrast to the tendons deprived of stress for 4 weeks which demonstrated a distinct decrease in cellularity and alterations in cellular morphology.

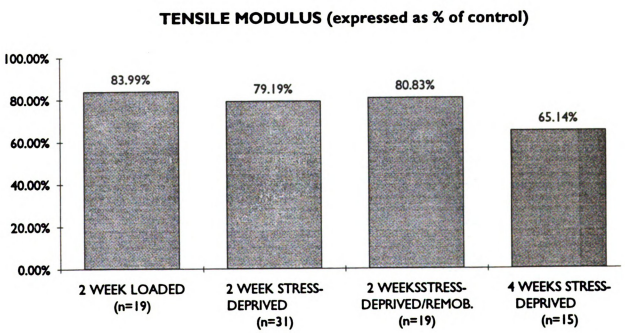

Figure 21: Histogram comparing the effects of 2 weeks cyclic loading, 2 weeks stress-deprivation, 2 weeks stress-deprivation/cyclic loading, and 4 weeks stress-deprivation on the tensile modulus of canine FDP tendon.

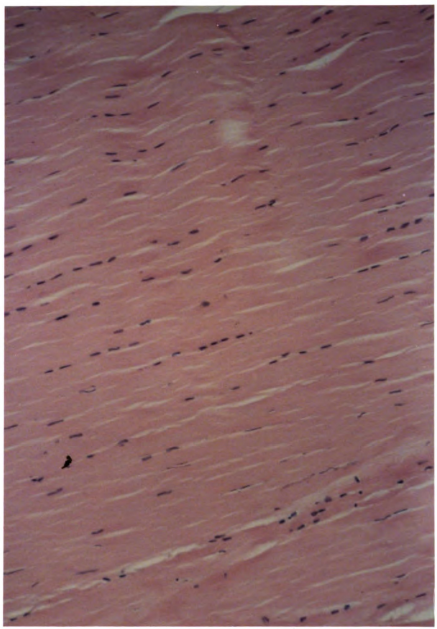

Figure 22: Histological section (H&E staining) of canine FDP tendon deprived of stress for 2 weeks and then cyclically loaded (with 50 gram load) for 2 weeks in tissue culture. Viewed with light microscopy at 40 X magnification.

IV. DISCUSSION

The results of this stress-deprivation study are indicative of the same trend in decreased material properties as seen in previous investigations[2, 11, 32, 41, 43, 44, 54-56, 62, 75, 82]. The time-dependent decrease in the tensile modulus of the tendons deprived of stress for 0 - 8 weeks is a clear indication of the injurious effect of immobilization. A sharp decrease in tensile modulus (~ 13%) was observed as early as after 2 weeks of stress-deprivation. This indicates that alterations secondary to lack of stress occur soon after the procedure is commenced. From the onset of stress-deprivation, the material properties of the tendon were seen to decrease significantly, finally leveling off at approximately 6 weeks. The interesting circumstance of this level of stress-deprivation was seen upon histological evaluation.

Histological assessment indicated that the fibrocytes began to deteriorate after 4 weeks of stress-deprivation. The change in cell shape observed can be interpreted in two ways: either the previously spindle-shaped cells have now changed their shape due to a lack of mechanical stimuli to provide them with a direction for alignment (parallel to the direction of the mechanical stimuli), or new cells have arisen which are not morphologically the same as the previous fibrocytes. It is probably more likely for the cells to be changing their shape in response to their mechanical environment. It was also observed that cellularity decreases with increased times of stress-deprivation becoming significant after 4 weeks. This reduction in cellularity correlated well with the significant decreases in tensile modulus at 4 weeks and 5 - 8 weeks.

To determine if the above noted immobilization-related changes were cell mediated, the tendon cells were destroyed by freezing and then maintained in tissue culture. The results revealed no significant change in the material properties had occurred. This suggests that the cells do indeed play an active role in tissue homeostasis. This is especially apparent when comparing the tensile moduli of the tendons stress-deprived for 2 weeks and those freeze-thawed with subsequent stress-deprivation for 2 weeks; similar results were seen upon comparison of the 4 week groups (Figure 23). The 2 week stress-deprived group was significantly different than the 2 week stress-deprived (freeze/thaw) group; similarly the 4 week stress-deprived group was significantly different from the 4 week stress-deprived (freeze/thaw) group. This disparity in the tensile modulus appears to be due to the elimination of any cellular effect. This result would correlate well with the decreased elastic modulus observed at 4 - 8 weeks immobilization, when cellularity appears to diminish at a quick rate.

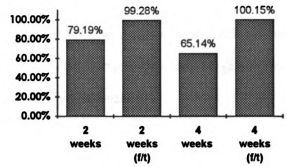

Figure 23: The tensile modulus of the 2 week stress-deprived and 4 week stress-deprived groups as a % of their respective controls. Column graph depicts the effects of absence of cells (freeze/thaw group) on the tensile modulus of stress-deprived tendon.

The lack of change in material properties with the absence of cellularity leads to the conclusion that stress-deprivation causes specific changes to occur at the cellular level. It is evident that the application of mechanical stress to connective tissue has a beneficial effect. However, the distinct manner by which stress works is not well understood. Few have postulated that stretch-activated ion channels on the surface of cells are responsible for this benefit derived from mechanical stimulus. The channels allow for a flux of ions to proceed through the cell membrane. The ion flux may be responsible for stimulating cell metabolism, which is required for tissue health. The stretch-activated ion channels are closely associated with the cellular cytoskeleton; thus upon application of mechanical stress it would appear that cells would deform. This translates to the direct deformation of the cytoskeleton. It is perhaps this deformation that causes the activation of these ion channels. Thus, the basis of the time-dependent, immobilization-related degradation may be explained by studying what occurs at the cellular level of the tissue. Previous studies done with cyclic straining of connective tissue *in vitro*[26, 42, 68, 69] have shown that mechanical strain activates cellular responses which result in increased cell proliferation and changes in protein synthesis. By studying strains at the cellular level, correlation between what is occurring at the global, tissue level and at the cell surface may help in gaining insight into the biological basis of connective tissue injury and healing.

Following 6 weeks of immobilization - the typical immobilization period for connective tissue deformities and/or injuries - a 45 % decrease in the tensile modulus was seen in this study. As previously mentioned, the cellularity of the tendon was also low. This may imply that similar alterations in morphology may occur *in vivo* after 6 weeks of immobilization. The fact that clinical physiotherapy, following injury and

immobilization, can sometimes take up to 12 months before resulting in any increase in strength or ease in mobility shows the damaging impact of a long immobilization period.

Gamble et al. (20) found chemical alterations to occur after 2 weeks of immobility when they evaluated the enzymatic changes in the ligament of rabbit. Morphological changes in the tendon after 4 weeks of immobilization were a result of a decrease in GAG content, due to enzymatic degradation. This result is consistent with the first great decrease in material properties and cellularity observed in the first portion of this thesis investigation. This study has shown the deleterious effects of stress-deprivation on the material properties of tendon; Loitz (44) found similar results with short-term immobilization periods, noting a marked decrease in linear tensile stress. Similar results were seen in other studies by Akeson[3], Amiel[6], and Woo[83].

The evaluation of the effect of mechanical stimuli (cyclic loading) on the material properties was studied in the second and third hypotheses of this study. The effect of immediate mobilization (H_2) on the material properties of tendon was evaluated in order to gain insight as to what occurs in the healthy tissue when the injured joint is mobilized immediately. Tissue morphology resembled that of untreated, healthy tendon, whereas the material properties were less than the controls. Other studies have seen a protocol of CPM to result in a tissue that is mechanically superior to an immobilized tissue (44). While the cyclic loading regime utilized in this study resulted in an increase in material properties, it was not a significant increase. The reason for this is unclear but may be due to the limited number of specimens examine.

The effect of mobilization of tendons previously deprived of stress (remobilization) was the focus of the final hypothesis of this study (H_3). The aim was to arrest the immobilization-related decreases in tensile modulus and cellularity by application of controlled motion. The cyclic loading regime utilized in this study resulted in a mechanically superior tendon following remobilization for 2 weeks. The significance of this result is that remobilization was able to stop the immobilization-related alterations, while retaining cellularity similar to that of the 2 weeks stress-deprived group, after 4 weeks in a tissue culture environment. From this study it was concluded that the controlled cyclic loading regime had a counter-balancing effect on the harmful consequences of immobilization for 2 weeks. The results of this part of the study indicate that the deteriorating effects of immobilization can be stopped and, perhaps reversed with increased remobilized periods.

Thus, in regards to mobilization studies, this research was able to repeat, to a certain extent, with an *in vitro* tissue culture technique, the results of previous studies in which immobilized tissue was found to recover with the application of cyclic loading [23, 41, 51, 52, 56, 65, 73, 74, 80, 82, 84].

Exercise, in many forms, has been shown to result in tissue than that tissues which are mechanically superior to immobilized tissue. Tipton and his associates were among the first to document the benefits of exercise [73,74]. A majority of the studies done examining the benefits of exercise or controlled motion on connective tissue have either been *in vivo* animal model studies. Thus there is not any substantial prior data to base the results of this *in vitro* study, except via inferences to the above mentioned studies. CPM has been seen to cause an increase in tissue stiffness with respect to an

immobilized control tissue (44). The CPM tissues in Loitz's study (44) showed tensile modulus values between those of the immobilized and the control groups. Similar results were seen in this study.

It may be possible that controlled mobilization is more effective in rehabilitating immobilized tissue, when applied for longer periods of time. Larsen (41) found it required 6 weeks of remobilization to successfully remove the harmful effects of a 4 week immobilization period. Others have found longer immobilization periods followed by remobilization for the same time to result in superior tissue strength. Woo (82) found a 9 week immobilization period, followed by a 9 week remobilization period to result in increased tendon strength, as compared to controls. Further investigation is required to examine the effects of a longer remobilization period, utilizing an *in vitro* tissue culture model.

Because the tendons examined in this study had never been damaged or surgically repaired, the results are pertinent for the assessment of immobilization and remobilization effects on healthy tissue. Also, from a clinical viewpoint, the duration of a rehabilitative program required to overcome the adverse effects of immobilization alone would be much shorter than that required to overcome the combined effects of surgery and subsequent immobilization. Therefore, realistically, the remobilization portion of the rehabilitation may need to be longer than the immobilization period.

It is probable that varying levels of mechanical stimuli are needed for the maintenance of tissue homeostasis, for the repair of injured tissue, as well as for tissue that is remodeling. The *in vitro* model utilized in this study allowed for the precise control of the loading parameters, such as frequency and duration, in exploring the

effect of mobilization on the material properties of connective tissue. This may allow for the designing of more proficient rehabilitation procedures upon further investigation.

Future Research Directions

Now that it has been established that the decrease in tensile modulus in a time-dependent phenomenon, the next logical step is to evaluate how this process can be stopped or reversed utilizing controlled passive motion. It is evident that 2 weeks of remobilization is able to stop, if not fully reverse the detrimental changes occurring after 2 weeks of immobilization. The increasing of the remobilization periods by increments of weeks may be the next manner in which to sequentially document the effects of an immobilization-remobilization protocol.

Another parameter to be examined is the effect of different frequencies of the cyclic loading regimens. The frequency used for the mobilization studies in this research was arbitrarily chosen; in order to commence the study a frequency was required and 1 rpm was randomly chosen. Upon comparing slow and fast frequencies of CPM, faster frequency has recently been documented to result in more favorable tissue tensile properties (71). This result is worth exploring, as the 1 rpm frequency utilized in this study may well be a factor in the decrease/regaining of material properties of the cyclically loaded tendons. Similarly it would be worthwhile to explore different durations of cyclic loading of the tissue.

Conclusions

1. Stress-deprivation results in a progressive degradation of the material properties of tendon. This is based on evaluation of the tensile modulus of the tissue.

2. For short time periods, cyclic loading of healthy tissue results in tissue morphology similar to that of the non-treated, control tissue. However, normal material properties were not maintained with short-term cyclic loading.

3. Cyclic loading of previously immobilized tissue appears to arrest the harmful effects associated with short-term immobilization. However, *significant* reversal of these alterations was not seen in this study.

V. APPENDIX

APPENDIX

CALCULATION OF TENSILE MODULUS:

A summary of the symbols:

F = force or load	... in N
δ = deformation	... in mm
l_0 = initial gauge length of tendon	... in mm
A = cross-sectional area of tendon	... in mm^2
K = stiffness coefficient of tendon	... in N / mm
E = elastic modulus of tendon	... in mega Pascal
σ = stress (= force/unit area)	... in mega Pascal
ε = % strain	

From the load-deformation curve:

$K = \Delta F / \Delta\delta$ ... in g/mm^2 (1)

$E = \Delta\sigma / \Delta\varepsilon$ ... in g / mm^2 (2)

$\sigma = F / A \quad \Rightarrow \quad \Delta\sigma = (\Delta F)(1 / A)$ ...in g / mm^2 (3)

↖ constant

and $\Delta\varepsilon$ = change in length of tendon / original length of tendon
= deformation / original length

$\Rightarrow \quad \Delta\varepsilon = \delta / l_0$ ... in mm / mm (4)

↖ constant

Upon substitution of equations (3) and (4) into equation (2):

$\Rightarrow \quad E = \Delta\sigma / \Delta\varepsilon = (\Delta F / A)(\delta / l_0)$
$= (\Delta F)(l_0) / (\delta)(A)$
$= [(\Delta F)/(\delta)][(l_0)/(A)]$

... $K = (\Delta F)/(\delta)$

$\Rightarrow \quad E = (K)*[(l_0)/(A)] = (K)(l_0) / (A)$ ... in g / mm^2

Once the value for "E" was obtained in the units g / mm^2 , the conversion was done into the units of Pascals, as follows:

$$E = (K)(l_0) / (A) = (g/mm)(mm/mm^2) = g/mm^2$$

$$E = 1 \times 10^6 \text{ kg} / m^2$$

...conversion to Newtons:

$$g = 9.81 \text{ m/s}^2$$

$$\Rightarrow \quad E = (1 \times 10^6 \text{ kg} / m^2)*(9.81 \text{ m/s}^2)$$

$$= 9.81 \times 10^6 \text{ (kg-m/s}^2) / (m^2)$$

... 1 kg-m/s^2 = 1 Newton

$$\Rightarrow \quad E = 9.81 \times 10^6 \text{ N/m}^2$$

... 1 N/m^2 = 1 Pascal

$$\Rightarrow \quad E = 9.81 \times 10^6 \text{ Pascals} = 9.81 \text{ mega Pascals (m-Pa)}$$

VI. LIST OF REFERENCES

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