# Characterization of equine tendon fibroblasts grown in tissue culture: A model for use in the study of the equine superficial digital flexor tendon and its response to injury and medication



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# CHAPTER 1. GENERAL INTRODUCTION

## Thesis Organization

This thesis is presented in alternate format and contains two journal papers. The first paper is titled "Development of an *in vitro* model for the study of the response of equine tendon fibroblasts to injury and medication." The second paper is titled "The effect of polysulfated glycosaminoglycan on production of proteoglycan by equine tendon fibroblasts in monolayer culture." Each paper contains its own summary, introduction, materials and methods, results, discussion and references. The Literature Review is designed as a review of the literature that created the ideas to pursue these projects. Additional information on specific materials and methods and related results not described in either paper have been included in Appendix A. Additional electron micrographs not in the papers have been included in Appendix B.

## Introduction

Tendinitis is a common condition affecting all disciplines of equine athletes (17,25,33). Tendinitis of the superficial digital flexor (SDF) tendon is commonly referred to as "bowed tendon" and is characterized by fiber disruption and inflammation within the tendon. Recent advances in the diagnosis and treatment of tendinitis have improved the prognosis for this type of injury; however, the pathophysiology of degenerative tendon injuries is incompletely understood. Investigations into the etiology, prevention, management, early detection and treatment of tendinitis are ongoing and will continue to advance the state of the art in this area.

The SDF tendon is of special interest because of its high frequency of injury. Thoroughbred racehorses have a particularly high incidence of SDF tendinitis, especially of the forelimbs. Injuries to the SDF tendon account for an estimated 8% to 30% of all racing injuries (20,25,46,47). Mechanical overstretching or repeated microstrains from racing at high speeds are the primary causes of flexor tendon injuries. The degree of disruption of the tendon fibers varies from mild stretching to complete avulsion and tearing. With the increased availability of diagnostic ultrasound, the evaluation, documentation and reassessment of

tendon injuries has become routine in veterinary medicine (18,20). Ultrasonographic examination of tendon injuries provides valuable information about the type and degree of tendon damage in an individual case (20). Ultrasound evaluation of injured tendons has provided new insights into the pathophysiology of tendinitis (20). Despite these improvements in the early detection and serial evaluation of damaged tendons, a consistently successful treatment regimen has yet to be developed.

## Anatomy

Tendons are the collagenous continuation of an associated muscle and form the mechanism of attachment of the muscle to the bone at both the origin and insertion of the muscle. There are three main structures that support the metacarpo/metatarsophalangeal joints of the horse: the deep and superficial digital flexor tendons and their associated check ligaments and the suspensory ligament. These support structures are able to sustain high loads and strains during the course of normal daily exercise. *In vitro* studies have demonstrated that strains of approximately 3% result in reversible, elastic lengthening of the tendon (25,36). As strains increase, the tendon behavior becomes viscoelastic until failure occurs at strains between 8% and 20% (13,25,55). The strains that have been measured in the SDF tendon of galloping Thoroughbreds, around 16%, is very close to the yield and failure strains measured *in vitro* (25,49).

Tendon is a dense regular connective tissue composed of an extracelluar matrix of four main macromolecules: collagen, elastin, proteoglycans and connective tissue glycoproteins. The extracelluar matrix is produced and maintained by tendon cells that are present in low numbers within the matrix. The tenocytes are arranged in rows between the collagen bundles. Three types of tenocytes have been described, but not thoroughly characterized: (1) cells with flattened cigar shaped dense nuclei, (2) cells located in linear groupings with more rounded dense nuclei and (3) immature cells with less dense open nuclei (25).

The collagen fibers and ground substance are arranged in a complex manner that is visible on both a macroscopic and a microscopic level. The collagen fibers are densely packed, arranged in parallel and embedded in ground substance or proteoglycans. In a mature animal, tendon fibers demonstrate a larger diameter and an increase in the number of

non-reducible collagen cross-links (25). This arrangement of collagen fibers is what creates the high tensile strength of tendon. Collagen comprises approximately 80% to 90% of the dry weight of tendon (25,38). In normal adult equine tendon, type I collagen fibers predominate (10). A small portion of type III and V fibers are also present associated with basement membranes and the intra tendinous vasculature (10).

The endotenon is a thin sheet of loose connective tissue which serves to arrange the collagen fibers into bundles or fascicles in a hierarchical manner. The endotenon carries blood vessels, lymphatics and nerves into the body of the tendon. It arises from the epitenon, a thicker connective tissue layer which surrounds the entire tendon (38,41). In the straight areas of the limbs where the tendon is not surrounded by a tendon sheath, the tendon is enclosed in a layer of vascular connective tissue. This outer layer is known as the paratenon and allows the tendon to move beneath the skin (41). Specialized types of fibroblasts are located within the extracellular matrix of the paratenon, epitenon and endotenon and lend each its unique function (10). Tendon healing occurs via both intrinsic and extrinsic mechanisms (2,34,48,55). The populations of fibroblasts within the connective tissue framework as well as those within the tissue parenchyma are capable of responding to tendon injury and initiating tissue repair (19,32,34).

Proteoglycans form a small but vital percentage of the dry weight of tendon (up to 5%) (3). Proteoglycans are complex macromolecules formed by the addition of one or more glycosaminoglycan (GAG) side chains to a protein core. These side chains are polymers of repeating disaccharides. Proteoglycans may assemble around a molecule of hyaluronic acid to form giant aggregates responsible for the viscoelastic nature of tendon. They provide resilience and flexibility to the connective tissue matrix. Studies in other species of animals have shown that the distribution of individual GAGs appears to be regulated based on the tensional forces placed on the tendon (54). In areas of high tension such as the mid-metacarpus, the predominant GAG is dermatin sulfate. Where compressional forces predominate, chondroitin sulfate is the major GAG. Although these studies have not been repeated in the horse, the consistency across species suggests that one could expect to find a similar distribution in the horse.

**Literature Review**

Traditional modes of treatment for tendinitis have included medical and surgical therapy (4,12,15,18,21,24,26,27,42,56). Treatment is aimed at decreasing inflammation within the tendon, preventing further trauma to the damaged tissues, stimulating the healing process and preventing recurrence of the injury after the horse goes back into training. Whether medical or surgical treatment (or a combination of the two) is selected, enforced rest with gradually increasing controlled exercise is the cornerstone of successful treatment (4,14,29). With traditional modes of therapy, retrospective studies show that approximately 40% to 60% of injured horses return to athletic soundness following injury to the SDF tendon (16,20).

Collagen turnover in normal tendon is slow (9). It is estimated that tendon renews all its collagen approximately every six months (48). The repair and maturation phases of tendon healing start at about four days and last for as long as six months (22,48). During this time new collagen fibers are produced and realigned along the lines of stress. Type III collagen is initially present in increased proportions to Type I collagen compared to normal tendon and results in decreased tensile strength of the healing tendon (21,57). As the maturation phase of wound healing progresses, Type III collagen is gradually replaced with Type I collagen (22,48). Up to eight months or more than a year may be required before an athlete is ready to return to competition (16,22,29,39). In studies following surgical treatment of SDF tendinitis, six to 19 months were required to get horses back to racing (16,29).

Because of the inherently slow healing of tendinous tissues, extended periods of rest and rehabilitation are required regardless of the treatment method(s) chosen. Given the prolonged rest period and guarded prognosis for return to racing, SDF tendinitis represents a significant economic loss to the equine industry. Losses occur in the form of veterinary costs, prolonged layup time and training costs incurred during rehabilitation. Recurrent tendinitis or severe injury may result in early retirement from athletic function (28,47).

The focus of research in the field of tendon injuries is aimed at the early detection of tendinitis prior to catastrophic failure, improving the speed and quality of tendon healing and preventing reinjury. Few controlled studies have been performed to objectively evaluate the various treatments for tendinitis that have been reported. Recent studies have focused on the

prospective evaluation of the most current therapies in order to provide objective data for comparison. The long term objective of research in the area of tendon healing is to develop methods of modulating tendon healing, minimizing the recovery period and maximizing the quality of the healed tissues.

Modulation of tendon healing through the use of local or systemic medications such as polysulfated glycosaminoglycan (PSGAG), hyaluronic acid, ß-aminoproprionitrile fumarate and growth factors is an area of significant research interest (15,18,21,37,42,43,50). Studies have traditionally been aimed at evaluation of response on the macroscopic level. The specifics of tendon healing on a cellular/biochemical level are incompletely understood. Further investigation into the biochemistry and cellular response of normal equine tendon fibroblasts is essential in order to gain a further understanding of the pathways stimulated in the promotion of tendon healing.

Tendon cells have been cultured *in vitro* from chickens, rabbits, cattle and humans (1,8,11,44,51,53). Equine tendon explants have been used on a limited basis to investigate the response to insulin-like growth factor 1 (37) and in a pilot study to optimize tissue culture conditions (45). Oxidative energy metabolism and the effects of hypoxia, free radicals and hyperthermia have also been investigated using equine tendon fibroblasts cultured *in vitro* (3). These data have yet to be published in a widely distributed form. To the authors' knowledge there is not any established model by which the characteristics of equine tendon cells may be studied *in vitro* for application to the clinical problem of SDF tendinitis.

Polysulfated glycosaminoglycan has been studied extensively for its effects on cartilage (5-7,23,30,31,52,58). Polysulfated glycosaminoglycan is a polymeric chain of repeating units of hexosamine and hexuronic acid, highly sulfated glycosaminoglycans. It is structurally similar to the glycosaminoglycans produced by mesenchymal cells such as tendon fibroblasts. It is thought to promote the synthesis of cartilage matrix components and collagen (23). It is also a potent inhibitor of the lysosomal hydrolases and neutral metalloproteases that are responsible for cartilage matrix and collagen destruction (5,31,35,40).

The effects of PSGAG on tendon healing have been investigated on a limited basis in the horse and the rabbit (25,38,42). Two of these studies used a collagenase induced model of SDF tendinitis (38,42) and a third followed the progress of clinical cases of acute tendinitis

in horses (25). Systemic administration of PSGAG significantly improved the rate and quality of tendon healing in horses (42). Local (intra- and peri-lesional) treatment of collagenase injury in rabbits decreased swelling, restored tissue organization and alignment and protected collagen fibers from degeneration (38). There was a reduced but measurable effect following systemic administration of PSGAG in the rabbit (38). Clinical trials following local injection of PSGAG in the horse have resulted in a rapid reduction of pain and swelling along with encouraging overall results (25). The mechanism of action involved in the improved tendon healing is uncertain. Controlled studies on a molecular level are limited. The development of a reliable cell culture model may help investigate the effects of PSGAG on the biochemical products of cultured equine tenocytes. The information gathered via the *in vitro* studies may subsequently be applied to the larger clinical problem of tendinitis.

The origin for this thesis was the need for a better understanding of tendon response to injury and to pharmacological manipulations. The development of an *in vitro* model was necessary to study the tendon response on a cellular level. The major objective of this thesis was the development of a reliable model for the evaluation of equine tendon fibroblasts in cell culture. In addition, the model was used to study the effects of one particular medication, polysulfated glycosaminoglycan (PSGAG), on the production of proteoglycan by the equine tendon fibroblasts.

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# CHAPTER 2. DEVELOPMENT OF AN IN VITRO MODEL FOR THE STUDY OF THE RESPONSE OF EQUINE TENDON FIBROBLASTS TO INJURY AND MEDICATION

A paper accepted by Veterinary and Comparative Orthopaedics and Traumatology

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**Summary:**

Equine tendon fibroblasts were isolated from explants of superficial digital flexor tendon, subcultured and maintained in monolayers. The cells were characterized by light microscopy, electron microscopy and radiolabel studies for proteoglycan production. Two predominant cell morphologies were identified. The cells dedifferentiated toward a more spindle shape with repeated subcultures. Equine tendon fibroblasts were successfully cryopreserved and subsequently subcultured. The ability to produce proteoglycan was preserved.

The isolated cells were identified as fibroblasts, based on their characteristic shape by light microscopy and ultrastructure and the active production of extracellular matrix proteins. Abundant rough endoplasmic reticulum and the production of extracellular matrix products demonstrated active protein production and export. Proteoglycans were measurable via liquid scintillation counting in both the cell-associated fraction and free in the supernatant. This model is currently being utilized to study the effects of polysulfated glycosaminoglycan on tendon healing. Future uses include studying the effects of other pharmaceuticals, such as hyaluronic acid, on tendon healing.

## Introduction:

Injuries involving the digital flexor tendons are a common cause of lameness in performance horses. It is estimated that 7-30% of injuries incurred by racehorses are related to the digital flexor tendons (20,25,40,41). The superficial digital flexor (SDF) tendons in the forelimbs are most commonly affected (32).

Equine tendon heals slowly and the structure and function of healed tendon is often

suboptimal (20,25,35). Extended periods of rest and rehabilitation are needed, regardless of the treatment method chosen (4,13,30). Recurrent tendinitis, or severe injury, may result in early retirement from athletic function. Given the prolonged healing time and a guarded prognosis for return to previous athletic performance, SDF tendinitis represents a significant economic loss to the equine industry.

A number of treatments for SDF tendinitis have been developed and used (4,11,14,17,18,21,24,27,29,30,37). Few, if any, have shown consistently superior results to rest alone. New methods of modulating tendon healing, aimed at improving the quality and speed of the healing process, continue to be investigated (14,18,20,21,34,36,37). Local or systemic medications such as polysulfated glycosaminoglycan (PSGAG) and hyaluronic acid (HA) have been studied *in vivo* (18,21,36,37,42). The beneficial effect of PSGAG on tendon healing reported by Redding is encouraging (37); however, the mechanism of action is unknown. Improved understanding of the cellular responses to medications, such as PSGAG, will help advance the treatment of tendinitis.

The responses to injury and treatment have been measured using ultrasound and histopathology (11,20,21,37); however, tendon healing is incompletely understood at a molecular level. Further investigation into the biochemistry and cellular responses of normal equine tendon fibroblasts to injury and medication is essential to further understand the biology of equine tendon healing.

Tendon cells have been cultured *in vitro* from chickens, rabbits, cattle and humans (1,7,9,38,43,44). Equine tendon explants have been used on a limited basis to investigate the response to insulin-like growth factor 1 (34) and in a pilot study to optimize tissue culture conditions (39). Oxidative energy metabolism and the effects of hypoxia, free radicals and hyperthermia have also been investigated using equine tendon fibroblasts cultured *in vitro* (3). These data have yet to be published in a widely distributed form. To the authors' knowledge there is not any established model by which the characteristics of equine tendon cells may be studied *in vitro* for application to the clinical problem of SDF tendinitis.

Our goal was to develop an *in vitro* model for investigating the effects of available pharmaceuticals on tendon healing at a cellular level. The specific objectives of this study were to describe certain morphological and biochemical characteristics of equine tendon

fibroblasts grown *in vitro*.

**Materials and Methods:**

The middle one third of the SDF tendons were harvested aseptically from the forelimbs of nine horses (two to 14 years old) immediately following euthanasia. Horses were euthanatized for reasons other than tendinitis. Evaluation of the health of the tendons was made based on lack of abnormalities on physical examination and gross appearance of the tendon on cross section. There were five geldings, two mares and two stallions. Breeds represented included one pony, one Thoroughbred, five Quarter Horses and two American Paint Horses. The tendon segments were placed in Hank's balanced salt solution[a] for transport to the laboratory for further processing.

Tendon fibroblasts were isolated via primary explant culture (15). The paratenon and epitenon were removed by trimming the outer 1-2 mm of tissue from all four sides of the tendon specimen. The tendon was minced into 1-2 $mm^3$ fragments of tissue which were placed 5-6 mm apart in 25 $cm^2$ flasks. Growth medium [MCDB 105[a] or Dulbecco's Modified Eagle's Medium, high glucose[b] (DMEM), 10% foetal bovine serum[c] (FBS), 1% gentamicin[a] and 20 ng/ml recombinant human insulin-like growth factor[d]] was added to each flask to cover the tissue fragments (explants). The flasks were incubated in 5% $CO_2$ and humidified air at 37°C for 14 days. Growth medium was changed every four to five days.

On day 14 the growth medium and explants were removed. Fibroblasts were trypsinized with 0.05% trypsin with 0.2% ethylenediaminetetraacetic acid in saline A (ATV). The flasks were rinsed with ATV to remove serum. The ATV was discarded and fresh ATV was added to each flask. The flasks were placed on the 37°C heating block for 30-60 seconds and the ATV was immediately removed and discarded. Then the flasks were replaced on the

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[a]Sigma Chemical Co., St. Louis, MO

[b]GIBCO Laboratories, Grand Island, NY

[c]Summit Biotechnology, Fort Collins, CO

[d]Fisher Scientific, Itasca, IL

37°C heating block for an additional one minute, until the cells were released. The sides of the flask were rapped intermittently to aid in cell detachment. Medium was added to the flask to arrest the trypsinization and the newly trypsinized cells were seeded to new 25 $cm^2$ flasks or 12 well plates. Subcultures were performed using the same trypsinization protocol. Following trypsinization, each flask was divided into two new flasks (split 1:2). Medium was changed every four to five days. The monolayers were studied as described below.

In preparation for light and transmission electron microscopy (TEM), the monolayers were then rinsed with phosphate buffered saline (PBS). For light microscopy, the cells were fixed with 95% ethanol for five minutes and stained with 1.2% aqueous crystal violet solution. For TEM the cells were fixed in 3% glutaraldehyde for 15 minutes then scraped into a pellet while in the 3% glutaraldehyde. The cell pellet was processed in a standard protocol for TEM (28) and the samples were examined by TEM[e].

Cryopreservation was performed using complete monolayers of equine tendon fibroblasts. These cells were also trypsinized, as previously described. Cells were suspended in growth medium supplemented with 10% dimethyl sulfoxide at approximately 6.0 X $10^6$ cells/ml in cryovials. The vials were placed in a cell-freezing container (Nalgene Cryo 1°C Freezing Container[a]) to allow the cells to freeze at 1°C/min to -70°C. After 24 hours at -70°C the cryovials were transferred to liquid nitrogen storage.

The cells were thawed rapidly in a 34°C water bath and the cell suspension (1 ml) from one cryovial was added to 5 ml of culture medium containing 10% FBS, 1% gentamicin and 30 $\mu$l/ml of 10% lactalbuminhydrolyzate[a] in a 25 $cm^2$ flask. The flasks were pre-coated with either 5 $\mu$g/ml of recombinant human fibronectin[a] or collagen[f]. All of the spent medium was exchanged with standard growth medium 12-24 hours after thawing and standard subculture techniques were employed thereafter.

Proteoglycan production was measured in two distinct fractions: cell-free (supernatant) and cell-associated (from the monolayer). First passage cells from five of the nine horses were seeded into 12 well plates and incubated in 5% $CO_2$ and humidified air at

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[e]Hitachi, San Jose, CA

[f]Vitrogen 100™, Flow Laboratories, McLean, VA

37°C for 48 hours. Next the cells were incubated with growth medium containing 40 $\mu$Ci/ml of $Na_2{}^{35}SO_4$[g] for 24 hours at 37°C in 5% $CO_2$ and humidified air. Proteoglycan production was determined by guanidine hydrochloride (GnHCl) extraction, cetylpyridinium chloride (CPC) precipitation, and liquid scintillation counting (5,6). The supernatant fraction was removed from each well and stored at 4°C in individual microcentrifuge tubes. Proteoglycans were isolated from the cell-associated fraction by extraction in 4*M* GnHCl (0.05*M* sodium acetate and protease inhibitors, pH 5.8)[a] (5,6). Extractions were performed for 48 hours at 4°C. Precipitation of proteoglycans was completed by spotting 100 $\mu$l of GnHCl extraction solution or supernatant on filter paper and dipping the filter papers in 0.1% CPC[a] in 0.3*M* sodium chloride for 30 minutes. Unincorporated isotope was removed by three sequential washes in the same solution.

Each filter paper was placed in an individual glass scintillation vial containing scintillation fluid[d]. Proteoglycan content was determined using a liquid scintillation counter[h]. Results were reported per well of a 12 well plate (4 $cm^2$). Counts were converted from counts per minute to disintegrations per minute (DPM) based on the efficiency of counting of the machine as calculated via a control sample. Cell-associated and supernatant fraction counts were multiplied by factors of two and 10 respectively to adjust for the portion of the total volume that was spotted onto the filter paper.

**Results:**

The cells migrated out of the explants from all of the horses and formed a visible halo of cells by ten days following the initiation of cultures. They continued to migrate and divide, expanding the diameter of the halo through 14 days. By 14-17 days in culture, adequate numbers of cells were in monolayers to permit proceeding with trypsinization. The cells were able to establish secondary monolayers within three days following trypsinization. The population of cells migrating out of the explants was morphologically consistent with fibroblasts. None of the cells appeared to possess phagocytic characteristics (i.e. no cells

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[g]ICN Pharmaceuticals, Irvine, CA

[h]Packard Instrument Company, Meriden, CT

were found to contain nuclei or nuclear detritus in cytoplasmic vacuoles).

By light microscopy, two primary shapes of fibroblasts were identified. The cells along the more sparsely populated margin of the halo were stellate in shape (Figure 2.1). Each cell had several cytoplasmic processes extending from the center of the cell. Those in the more densely populated areas demonstrated an elongated spindle shape (Figure 2.2). Both types of cells had large centrally located oval nuclei with several large nucleoli. Other types of cells were not identified. As maintenance of the cultures continued, the cells dedifferentiated toward the more spindle-shaped type of cell. The time required to form a secondary monolayer was prolonged to four to five days.

Electron microscopy confirmed that the cells were fibroblasts. The cell morphology, and ability to produce extracellular matrix products, are characteristic of fibroblasts. Rough endoplasmic reticulum was abundant and it was characterized by distended cisternae containing finely granular material. Numerous membrane-associated pinocytotic vesicles were common. Proteoglycan and collagen were present in the extracellular space adjacent to the cell membrane. Proteoglycan appeared as an amorphous mass of material. Collagen fibers were identified as linear filaments aligned with each other and parallel to the cell membrane (Figure 2.3).

The equine tendon fibroblasts were very sensitive to trypsinization by routine techniques. Standard protocols for the trypsinization of epithelial cells resulted in complete detachment of the tendon cells and subsequent poor viability. By reducing the contact time with the ATV from three to five minutes to 30-60 seconds, the cells remained viable for multiple subcultures and demonstrated the capacity for continued cell division up to ten subcultures. After the fourth subculture, an increasing number of cells failed to reattach following each subsequent trypsinization. Attachment was improved significantly by coating the culture dishes with collagen[f] prior to seeding.

Initial efforts at cryopreservation of the cells resulted in low cell viability. Cell counting was not performed. Losses resulted primarily from failure to attach to the flask when human recombinant fibronectin[a] was used to coat the flasks. Pretreatment of the plasticware with collagen[f] rather than fibronectin improved attachment dramatically. Viability rates were nearly 100% and cells were successfully subcultured for up to nine passages after

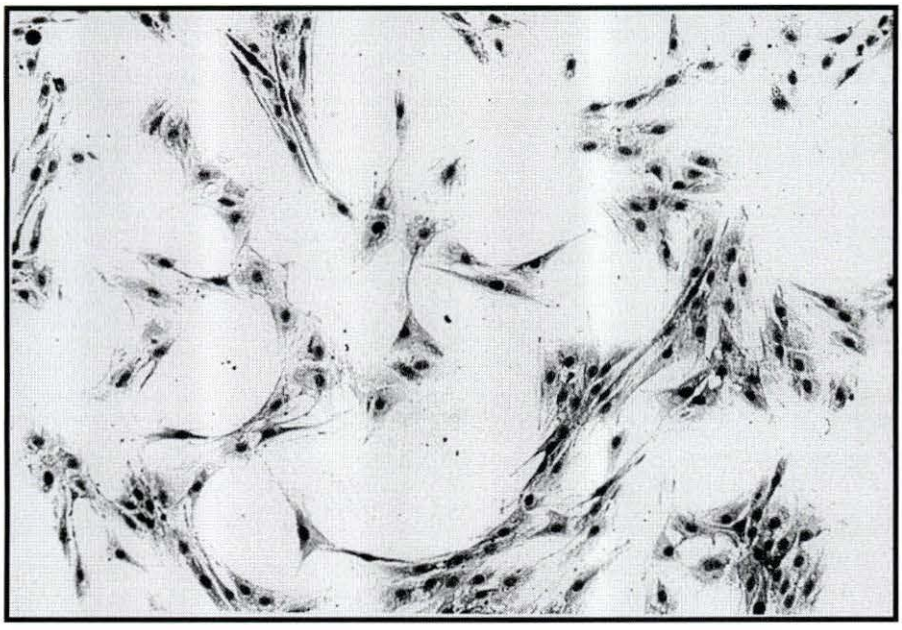

Figure 2.1. Photomicrograph of equine tendon fibroblasts demonstrating the stellate cells characteristic of the more sparsely populated areas (crystal violet stain, approximately 70x).

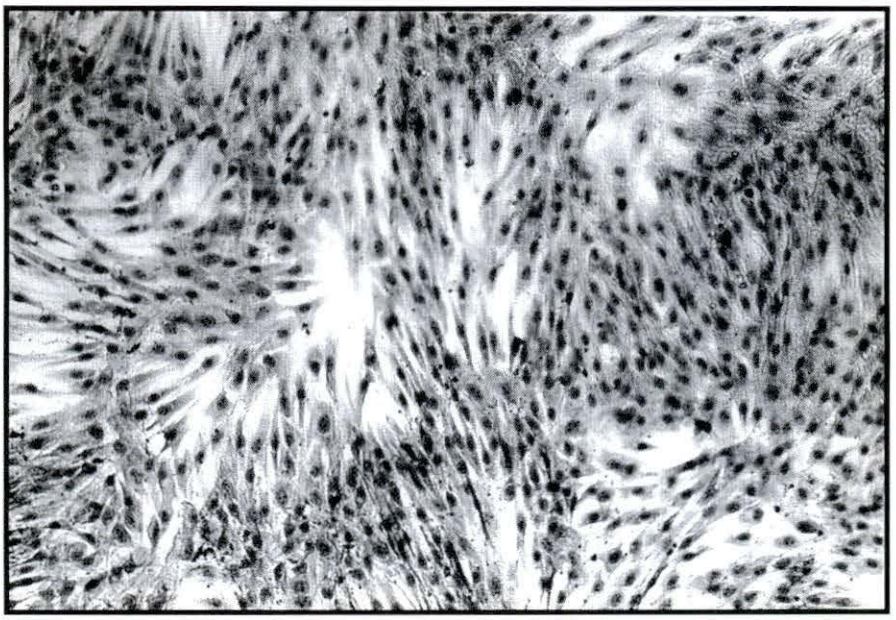

Figure 2.2. Photomicrograph of equine tendon fibroblasts demonstrating the spindle shape cells characteristic of densely populated areas (crystal violet stain, approximately 70x).

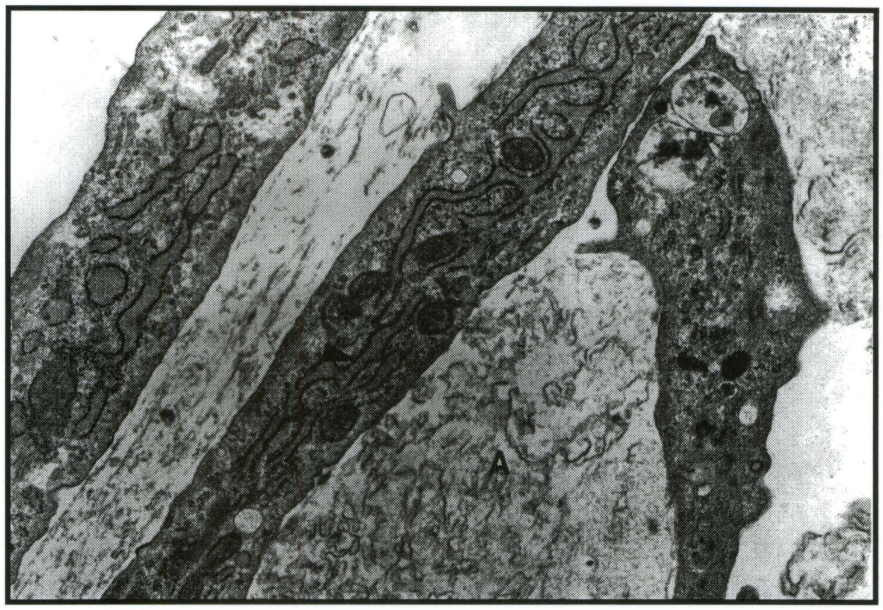


Figure 2.3. Transmission electron micrograph of equine tendon fibroblasts. Note the abundant rough endoplasmic reticulum with dilated cisternae containing granular material (arrow head) and extracellular matrix between the cells (A) (approximately 13 X $10^3$x).

thawing. Thawed cells maintained their characteristic fibroblastic appearance and their ability to produce proteoglycan.

Radiolabel studies confirmed that the equine tendon fibroblasts were capable of producing proteoglycan in measurable amounts. Proteoglycan was present in both the cell-associated fraction and in the supernatant (Table 2.1).

Table 2.1. Sulfated proteoglycan content in the cell-associated and supernatant fractions from cultures of tendon fibroblasts after labeling with $Na_2{}^{35}SO_4$

| Block # | Fraction | DPM x $10^3$ |
|---|---|---|
| Block #1 | Cell-Associated<br>Supernatant | 11.6<br>28.6 |
| Block #2 | Cell-Associated<br>Supernatant | 14.8<br>41.3 |
| Block #3 | Cell-Associated<br>Supernatant | 31.1<br>98.3 |
| Block #4 | Cell-Associated<br>Supernatant | 25.1<br>82.0 |
| Block #5 | Cell-Associated<br>Supernatant | 23.5<br>89.0 |

**Discussion:**

Our results are consistent with those reported in cattle (43,44), chicken (38), rabbit (1,9) and humans (7) with regard to the ability to isolate viable fibroblasts from tendon tissue and maintain them in cell culture. Cells were isolated from the tissue via out-growth of migrating cells. This technique was selected over tissue digestion methods in order to maximize the numbers of viable cells isolated. Enzymatic degradation of tendon is laborious, requiring many hours of processing (9,44). Poor cell viability following enzymatic degradation has been reported (9). The migration technique worked well. The system described is simple and yields a more than adequate number of healthy, metabolically active cells.

Regardless of the method employed to isolate cells, there is a degree of inherent selection for a certain cell type. When enzymatic degradation is used, only those cells that survive the disaggregation technique and adhere to the substrate or survive in suspension are propagated. The migration technique inevitably selects for the subset of cells contained within the explant that are capable of migration. Although there are advantages and disadvantages of any culture system, we feel that the selection by migration is beneficial in this model, because fibroblast migration is an accepted portion of the tendon healing process (2,7,19,26). Radiolabel studies have documented fibroblast migration from a site distal to an injury (31). We speculate that the cells capable of migration from the tissue explants are the same cells that are responsive to injury *in vivo* (7).

The equine tendon fibroblasts isolated in this study were very sensitive to trypsinization. Prolonged contact with ATV resulted in decreased attachment and cell viability. Through repeated trials the protocol presented here in consistently removed the cells from the plastic, without irreversible cell damage or loss when the trypsin was removed and discarded. Scraping the cells off the flasks, followed by centrifugation, resulted in poor cell viability and excessive cell clumping due to the trauma of the scraping and the centrifugation.

Fibroblasts are known to be anchorage dependent (15). Standard polystyrene culture dishes are a suboptimal environment for attachment. Collagen and fibronectin are available commercially for coating of the plastic culture dishes prior to cell seeding. In particular in our laboratory, we observed improved attachment using a commercially available collagen product[f]. Coating the plates for the initial explant phase was unsuccessful because the tissue fragments did not remain adhered to the plastic; the detached cells lacked a surface for migration.

Fibroblasts can vary in shape based on location, the nature of the substrate to which the cell adheres, or in response to different culture conditions (12,15,23,31). When viewed by light microscopy, cells in our culture system demonstrated two distinct shapes. It is uncertain whether these shapes represent two distinct cell types or simply two morphological alterations of the same population of cells. The cell morphology appears to be heavily influenced by the cell density. They start out in the stellate form in early cell cultures, immediately following attachment and during migration out of the explants. As the cell density increases and the

cultures become more mature a gradual transformation to the more spindle shape is noted.

Three types of tenocytes have been described in normal equine tendon. They have not yet been characterized (25). Identification of unique cell surface markers would be useful to further characterize the cell types described *in vitro* and in normal equine tendon (16). The *in vitro* populations of cells could then be compared to the *in vivo* population of tenocytes. The identification of individual cell types may aid in the further subculture of cells to study their individual responsibilities with regard to tendon healing. This is important because distinct cell types may produce different cell products and have different roles in the healing process.

Equine tendon fibroblasts survived multiple cell generations; however, repeated subculturing has several disadvantages. First, the tendon fibroblasts become increasingly dedifferentiated with progressive subculturing. Second, each successive passage of cells creates a larger difference between the *in vivo* and *in vitro* characteristics of the fibroblasts, with regard to their metabolic activity. Third, this system does not allow for the influence of the extracellular tendon matrix environment or the effect of mechanical forces to help maintain the differentiated cell type (15,22).

Multiple factors act on individual cells to produce a specific phenotype. In tissue culture systems, these factors may be altered and cells may change their phenotype. Dedifferentiation is defined as the loss of specific phenotypic properties associated with the mature cell *in vivo* (15). When these phenotypic changes occur it can either be an adaptive process (the differentiated phenotype may be regained) or a selective process (a precursor cell has been selected over the differentiated cell type).

Maintaining phenotype and preventing dedifferentiation is an important issue in tissue culture systems that are a model for a clinical problem. Our model, as described, did not allow for clonal selection of phenotypically distinct populations of tenocytes because the cells rapidly dedifferentiated toward one phenotype. Cell surface markers may help identify and sort the different cells.

There are two possible sources of the cells that have been isolated in this model. They may be actual tenocytes from the tissue parenchyma, they may originate from the endotenon or be a combination of these two sources may be represented. Care was taken to remove the paratenon and epitenon in order to avoid contamination of the cultures by cells from these

sources. The endotenon is an intricate network of connective tissue fascicles that organize the tendon fibers into bundles. Because of the nature of this intrinsic tendon structure, it is impossible to remove all of the endotenon and any remaining endotenon could act as a cell source.

The cultured cells were successfully cryopreserved. This means that tendon fibroblasts can be stored for future investigations. The value in cell storage is the ability to accumulate experimental material from clinical cases in sufficient numbers over an extended period of time to obtain statistically significant results. Cryopreservation allows for simultaneous studies to be done on cell lines isolated from different horses, ensuring that experimental conditions are the same for each cell line. For example, cell lines could be compared between young and old horses or lines from horses with SDF lesions which had healed well compared to those from horses with lesions which healed poorly.

Proteoglycan concentrations were approximately 2.5-3.5 times higher in the supernatant than in the cell-associated fraction. Proteoglycan measured in the cell-associated fraction can be intracellular or incorporated into the extracellular matrix immediately adjacent to the cells (45). Additional studies would be necessary to determine the relative amounts of intracellular and matrix-associated proteoglycans in the cell-associated fraction (45). Based on our results, it is presumed that the majority of extracellular proteoglycans produced by cultured tenocytes are distributed as soluble molecules in the supernatant.

There are several advantages of *in vitro* cell cultures in the investigation of clinical tendinitis: 1) Cell culture provides a homogeneous population of cells replicating uniformly for assignment to treatment groups, 2) *In vitro* models simplify biological effects and allow precise environmental control, 3) Monolayer culture isolates fibroblasts from the influences of the extracellular matrix, other cell types and cell products present in explant systems, and 4) Cellular responses can be monitored on a biochemical and ultrastructural level. In addition, *in vitro* research can be less expensive because of the reduced costs of housing and maintaining animals. Finally, the need for animal sacrifice is reduced, which is ethically appealing.

In conclusion, equine tendon fibroblasts can be grown as monolayers and these tendon fibroblasts are capable of extracellular matrix production. This model will be valuable in future studies of certain aspects of tendon healing. Further work is required in the area of the

quantitation and characterization of collagen production. There is also the need to further characterize the tendon cell types encountered. This model will be used to investigate the effects of pharmaceuticals including PSGAG and HA on tendon fibroblast metabolism and matrix production.

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# CHAPTER 3. THE EFFECT OF POLYSULFATED GLYCOSAMINOGLYCAN ON PRODUCTION OF PROTEOGLYCAN BY EQUINE TENDON FIBROBLASTS IN MONOLAYER CULTURE

A paper submitted to Veterinary and Comparative Orthopaedics and Traumatology

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**Summary:**

Equine tendon fibroblasts were isolated from tissue fragments of superficial digital flexor tendon, subcultured and maintained in monolayers. The cells were treated with two dosage regimes of polysulfated glycosaminoglycan (PSGAG). The high dose group received 0, 25, 50 or 200 $\mu$g/ml of PSGAG and the low dose group received 0, 0.3, 1 or 2 $\mu$g/ml of PSGAG. Proteoglycan production was measured via liquid scintillation counting following radiolabel with $^{35}SO_4$ (40 $\mu$Ci/ml). Counts were measured in both the cell associated fraction and the supernatant fraction.

Addition of PSGAG to the culture system did not adversely affect cell viability at any treatment level. Cells in all treatment groups produced measurable amounts of proteoglycan in both the cell associated and supernatant fractions. There were no differences between treatment means in any of the four groups (high dose cell-associated, high dose supernatant, low dose cell-associated, low dose supernatant). No linear trend in proteoglycan production was documented between treatment groups in either the cell-associated or supernatant fractions for either of the two dosage regimes. The significance of the lack of effect is uncertain. PSGAG may influence tendon healing in a manner other than by increasing proteoglycan production.

## Introduction:

Tendinitis of the digital flexor tendons is a common problem in athletic horses. The high frequency of injury and the extended healing time required for rehabilitation of equine tendon injuries have stimulated an interest in developing treatments that will improve the rate and quality of tendon healing. One of the treatments currently being investigated is the use of

polysulfated glycosaminoglycan (PSGAG). Systemic administration of a commercially available PSGAG[a] improved the quality and rate of tendon healing in a collagenase-induced tendinitis model (22). In another report, it was suggested that intramuscular or peri-lesional injection of PSGAG[b] in horses benefited tendon healing (12) and in a single case report it was suggested that intralesional PSGAG[b] in the horse was beneficial to healing (24). In humans, PSGAG[c] has been used successfully to treat Achilles peritendinitis by local injection (26). The mechanism of action of PSGAG with regard to tendon healing is uncertain.

Polysulfated glycosaminoglycan is a polymeric chain of highly sulfated glycosaminoglycans. The major component is chondroitin sulphate (5). It has a molecular weight of 2,000 to 16,000 daltons and is extracted from bovine lung and trachea (5). Polysulfated glycosaminoglycan has been studied extensively in several species for its effects on cartilage (3,5,6,7,9,11,15,25,27-29). It is marketed for the treatment of degenerative joint disease and is reported to promote the synthesis of hyaluronic acid (3,20,23,30), cartilage matrix components and collagen (11). Polysulfated glycosaminoglycan also inhibits the action of several catabolic enzymes such as neutral metalloproteases (10,15,19).

The similarities in matrix constituents between cartilage and tendon suggest that some of the beneficial effects of PSGAG documented in cartilage studies may also be expected to occur in tendon. The ground substance of both tendon and cartilage are constructed of proteoglycan complexes of glycosaminoglycan side chains attached to a protein core and linked by another protein to hyaluronate. The primary differences between cartilage and tendon lie in the distribution of specific glycosaminoglycans and the orientation of the collagen fibers.

The purpose of this study was to investigate the effect of PSGAG on the production of proteoglycans by equine tendon fibroblasts and the mechanism of action by which PSGAG may improve tendon healing. A previously described *in vitro* model was utilized to

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[a]Adequan, Luitpold Pharmaceuticals, Shirley, NY

[b]Adequan, Panpharma, Hayes, UK

[c]Arteparon, Luitpold-Werk, Munich, Germany

accomplish this goal (8).

**Materials and Methods:**

The middle one third of the superficial digital flexor tendons were harvested aseptically from the forelimbs of five horses (two to 14 years old) immediately following euthanasia. Horses were euthanatized for reasons other than tendinitis. Evaluation of the health of the tendons was made based on lack of abnormalities on physical examination and gross appearance of the tendon on cross section. There were two geldings, one mare and two stallions. Breeds represented included three Quarter Horses and two American Paint horses. The tendon segments were placed in Hank's balanced salt solution[d] for transport to the laboratory for further processing.

Tendon fibroblasts were isolated via primary explant culture as described in a previous study (8). Following removal of the paratenon and epitenon, the tendon was minced into 1-2 $mm^3$ fragments of tissue which were placed into 25 $cm^2$ flasks. Growth medium (Dulbecco's Modified Eagle's Medium, high glucose (DMEM)[e], 10% fetal bovine serum[f], 1% gentamicin[d] and 20 ng/ml recombinant human insulin-like growth factor[g]) was added to each flask to cover the tissue fragments. The flasks were incubated in 5% $CO_2$ and humidified air at $37^0$ C for 14 days. Growth medium was changed every four to five days.

On day 14 the growth medium and explants were removed. Fibroblasts were trypsinized as previously described (8) and seeded into 12 well plates at an approximate density of $1.5 \times 10^4$ cells/$cm^2$. The cells were reincubated in 5% $CO_2$ and humidified air at $37^0$C for 48 hours until a rapidly dividing monolayer covered approximately 80% of each well. The standard growth medium was exchanged with fresh medium containing PSGAG[a] in concentrations ranging from 0 to 200 $\mu$g/ml. The concentrations of PSGAG were grouped

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[d]Sigma Chemical Co., St. Louis, MO

[e]GIBCO Laboratories, Grand Island, NY

[f]Summit Biotechnology, Fort Collins, CO

[g]Fisher Scientific, Itasca, IL

into high and low dosage regimes of four treatment levels each: (1) high dose (0, 25, 50 and 200 $\mu$g/ml) and (2) low dose (0, 0.3, 1 and 2 $\mu$g/ml). Each treatment was performed in quadruplicate. Five horses were used for the high dose groups and three horses were used for the low dose groups. The cells were incubated in the presence of PSGAG[a] for 48 hours and then labeled with 40 $\mu$Ci/ml of $Na_2{}^{35}SO_4$[h] for an additional 24 hours at 37$^0$C in 5% $CO_2$ and humidified air (8).

Proteoglycan production was measured in two distinct fractions: cell-free (supernatant) and cell-associated (from the monolayer) by guanidine hydrochloride extraction and cetylpyridinium chloride precipitation (6-8). Proteoglycan content was determined using a liquid scintillation counter[i]. Results were reported per well of a 12 well plate (4 cm$^2$) in disintegrations per minute (DPM) based on a factored adjustment for the proportion of the total volume spotted onto the filter paper (8).

Statistics were performed on the means for each treatment level. Differences in proteoglycan production at the four levels of PSGAG treatment (within the high or low treatment group) were tested using the analysis of variance procedure, each horse comprising an experimental block. The presence of a linear trend with dose was also tested for each of the four groups of results (high dose cell-associated, high dose supernatant, low dose cell-associated, low dose supernatant). Significance was established at $p<0.05$.

**Results:**

Cells were successfully harvested from all five horses utilized in this study. The cells migrated out from the tissue fragments in adequate numbers by 14 days in culture to seed the necessary number of monolayers for control and treatment groups. Secondary monolayers were established within two days. Addition of PSGAG to the culture medium did not adversely affect cell viability at any treatment level. The cells maintained the two primary shapes described in a previous study following treatment with PSGAG (8). Subjectively, cells treated with PSGAG appeared to proliferate more rapidly than the cells in the control wells.

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[h]ICN Pharmaceuticals, Irvine, CA

[i]Packard Instrument Company, Meriden, CT

Cells in all eight treatment groups produced measurable amounts of proteoglycan in both the cell-associated fraction and in the supernatant (Tables 3.1, 3.2, 3.3 and 3.4). There were no differences between treatment means in any of the four treatment groups (high dose cell-associated, high dose supernatant, low dose cell-associated, low dose supernatant). No linear trend in proteoglycan production was documented between treatment groups in either the cell-associated or supernatant fractions for either of the two different dosing regimes. The variations between horses was very large. Counts were generally 3.5 times higher in the supernatant fraction than in the cell-associated fraction for each treatment.

**Discussion:**

The use of PSGAG in the treatment of degenerative joint disease has been studied extensively in the dog, horse, pig, human, chicken and rabbit (1-3,6,7,11,16,31). Polysulfated glycosaminoglycan is reported to stimulate the biosynthesis of extracellular matrix proteoglycans and has a chondroprotective effect (2,10,13,15,18). Studies evaluating the effects of PSGAG on equine cartilage have shown inconsistent results. One *in vitro* study demonstrated both increased cell replication and increased production of extracellular matrix when normal and arthritic equine cartilage was treated with PSGAG (11). Subsequent studies were unable to reproduce Glade's results (6,7). Polysulfated glycosaminoglycan caused a small but significant decrease in proteoglycan synthesis and failed to prevent cartilage degradation in normal and osteoarthritic cartilage tissue explants (6,7).

Despite these mixed results *in vitro*, PSGAG has shown promise clinically in the treatment of osteoarthritis (3,13,27) and more recently in the treatment of tendinitis in humans and horses (12,21,22,26). Our results failed to support increased proteoglycan production as the mechanism by which PSGAG modulates tendon healing. We were unable to document any effect on proteoglycan production by equine tendon fibroblasts incubated with various levels of PSGAG.

Polysulfated glycosaminoglycan may influence tenocyte metabolism by a mechanism other than the stimulation of total proteoglycan production. *In vitro* studies have shown PSGAG to stimulate the synthesis of collagen, hyaluronic acid and proteoglycan (3,5,11,20,30). No effect on proteoglycan production was measured in this study. Perhaps

Table 3.1. Sulfated proteoglycan content in the cell-association fraction after labeling with $Na_2{}^{35}SO_4$. All counts are reported as DPM X $10^3$. SEM = 8.6.

| Level of PSGAG ($\mu$g/ml) | Block #1 | Block #2 | Block #3 | Block #4 | Block #5 | Treatment Means |
|---|---|---|---|---|---|---|
| **0** | 14.8 | 30.9 | 25.1 | 23.4 | 42.0 | **27.2** |
| **25** | 13.7 | 23.6 | 26.9 | 20.3 | 54.6 | **27.8** |
| **50** | 13.3 | 25.4 | 29.2 | 22.3 | 38.2 | **25.7** |
| **200** | 12.0 | 25.5 | 23.0 | 23.1 | 32.8 | **23.3** |

Table 3.2. Sulfated proteoglycan content in supernatant fraction after labeling with $Na_2{}^{35}SO_4$. All counts are reported as DPM X $10^3$. SEM = 2.0.

| Level of PSGAG ($\mu$g/ml) | Block #1 | Block #2 | Block #3 | Block #4 | Block #5 | Treatment Means |
|---|---|---|---|---|---|---|
| **0** | 41.3 | 100.5 | 82.0 | 88.6 | 136.9 | **89.9** |
| **25** | 54.5 | 93.1 | 95.0 | 128.5 | 83.5 | **90.9** |
| **50** | 51.1 | 105.4 | 86.8 | 114.6 | 82.1 | **88.0** |
| **200** | 48.9 | 90.0 | 69.2 | 142.2 | 75.8 | **85.2** |

Table 3.3. Sulfated proteoglycan content in cell-associated fraction after labeling with $Na_2{}^{35}SO_4$. All counts are reported as DPM X $10^3$. SEM = 12.8

| Level of PSGAG ($\mu$g/ml) | Block #1 | Block #2 | Block #3 | Treatment Means |
|---|---|---|---|---|
| **0** | 31.3 | 23.4 | 42.0 | **32.2** |
| **0.3** | 36.2 | 21.5 | 33.4 | **30.4** |
| **1** | 31.1 | 21.4 | 34.2 | **28.9** |
| **2** | 27.2 | 19.8 | 29.4 | **25.5** |

Table 3.4. Sulfated proteoglycan content in supernatant fraction after labeling with $Na_2{}^{35}SO_4$. All counts are reported as DPM X $10^3$. SEM = 1.5.

| Level of PSGAG ($\mu$g/ml) | Block #1 | Block #2 | Block #3 | Treatment Means |
|---|---|---|---|---|
| **0** | 96.1 | 88.6 | 136.9 | **107.2** |
| **0.3** | 104.8 | 128.5 | 80.2 | **104.5** |
| **1** | 102.5 | 114.6 | 77.9 | **98.3** |
| **2** | 106.4 | 142.2 | 78.5 | **109.0** |

PSGAG exerts its beneficial effects on tendon healing through the modulation of collagen or hyaluronic acid synthesis. Polysulfated glycosaminoglycan may influence tendon healing by inhibiting the activity of the collagen degrading enzymes such as lysosomal enzymes, neutral proteases, stromelysin and elastase (2,13,15,18,19). Data collection in this study was limited to the measurement of total proteoglycan production. Further studies investigating the production of collagen and hyaluronic acid and the inhibition of proteolytic enzymes will be valuable.

Normal tissues were selected for use in this model; however, it is possible that damaged tendon tissue would be more responsive to treatment with PSGAG. Arthritic cartilage was more sensitive to the effects of PSGAG than normal cartilage (11). An *in vitro* study investigating the effects of PSGAG on hyaluronic acid (HA) production by human synovial fibroblasts demonstrated a much greater stimulation of HA synthesis in rheumatoid cells than in normal cells (23).

The effects of PSGAG as investigated within the constraints of this model are limited by the nature of the model. Factors that are not reproduced in the laboratory may have a positive influence on the cells *in vivo*. By measuring proteoglycan production in clinical cases of tendinitis treated with intramuscular or perilesional PSGAG, a positive effect may be demonstrated.

The optimal dose of PSGAG required for tendon healing is not known. Drug concentrations in the microgram per milliliter range were selected over those in the milligram per milliliter range for this study. These dosages were based on previous *in vitro* studies involving equine cartilage (6,7). We were successful in growing the cells at these concentrations (25, 50 and 200 $\mu$g/ml).

Doses for the second, lower dose regimen, were extrapolated from the tissue levels measured in serum and synovial fluid following intramuscular injection of tritium-labeled PSGAG (4,14). Levels of PSGAG that are achieved in tendon following intramuscular injection have not been established; however, for the purposes of this study they were assumed to be similar to serum and synovial fluid. Levels of PSGAG in serum and synovial fluid peaked at two hours post-injection at 1.96 and 0.33 $\mu$g/ml respectively. Levels of 0.3 - 1 $\mu$g/ml are reported to be adequate to stimulate proteoglycan synthesis in diseased articular

cartilage (17). Based on this information, levels of 0.3, 1 and 2 $\mu$g/ml PSGAG were selected for the lower dosing regimen. No difference in effect was seen with either the high or low dosage regimes. The lack of effect reported in this study may be related to the selection of drug concentrations that are not optimal for influencing an increase in proteoglycan production.

The apparent lack of effect of PSGAG on proteoglycan production may be an indication that PSGAG is influencing tendon healing in a manner other than by an increase in total proteoglycan production. Further studies are necessary to measure collagen production, matrix degradation and cell division in an effort to identify the mechanism by which PSGAG improves tendon healing.

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# CHAPTER 4. GENERAL CONCLUSIONS

The research presented in this thesis establishes the groundwork for further investigations into the modulation of tendon healing. We have established that equine tendon fibroblasts can be consistently isolated, maintained, cryopreserved and subcultured using the explant culture system described. The cultured tendon fibroblasts were capable of producing proteoglycan in measurable amounts both before and after cryopreservation. Evidence of collagen production was demonstrated via electron microscopy.

Two primary cell morphologies were documented. The cell shapes appeared to be influenced by the surrounding cell density. In areas of decreased cell density, stellate cells were characteristic. As the monolayers became more complete, the cells adopted a spindle shape. The precise implications of this observation are uncertain; however, future work characterizing the cells using surface markers and monoclonal selection will provide more specific information with regard to the specific cell types present within tendon. An understanding of the cell types and their individual functions in the maintenance of normal tendon matrix will improve the ability to modulate tendon healing.

Much of the initial time and effort expended in completing the work for this thesis was in working out the details of the culture system. The cells were unusually sensitive to trypsinization, necessitating the investigation of alternate techniques to minimize cell loss and to maximize cell adherence following trypsinization. The techniques that have evolved produce consistent results with good cell viability. The basic culture model work provides the basis from which other studies may be designed.

The study investigating the effect of PSGAG on proteoglycan production by the cultured tenocytes is the first of what will, hopefully, be many such studies. We were able to demonstrate that the cells maintained their viability and ability to produce proteoglycan following addition of PSGAG to the culture medium. At the doses selected, we were unable to show any effect on proteoglycan production. We did find a very large variation between individual horses in the amount of proteoglycan produced and in the response of the individual populations of cells to the PSGAG. Perhaps with a larger number of replications we may have been able to demonstrate a statistically significant trend.

The model developed and described will serve as a research tool in better understanding the physiology of tendon healing. Future studies involving the effect of PSGAG on collagen production, tenocyte replication and hyaluronic acid production will be necessary to elaborate the mechanism of action of this drug on tendon healing. The effects of PSGAG on diseased tendon tissue is also of interest. Other potential medications may be evaluated in a similar manner utilizing this model. A more thorough understanding of tendon healing is essential before we can expect to successfully improve the quality and rate of tendon healing via pharmacological or physical therapy means.

# APPENDIX A. SUPPLEMENTAL MATERIALS AND METHODS

## Media and stock solution preparation

Hank's Balanced Salt Solution (HBSS), MCDB-105 medium (MCDB), Dulbecco's Modified Eagle's Medium (DMEM) and insulin-like growth factor 1 (IGF-1) were mixed and prepared as per enclosed manufacturers' instructions. They were filter sterilized where indicated, stored in the refrigerator and opened only in the laminar flow hood to maintain sterility. The IGF-1 was stored in 0.5 ml aliquots at -20$^0$ C and thawed as needed.

Growth medium was used for all culture conditions and was prepared fresh each day prior to planned laboratory procedures. The total amount of growth medium required for the day was calculated and appropriate volumes of fetal bovine serum (FBS), gentamicin and IGF-1 were added to the calculated volume of stock medium (MCDB or DMEM) to achieve concentrations of 10%, 1% and 20 ng/ml respectively. One 0.5 ml aliquot of diluted (1$\mu$g/ml) IGF-1was added per 24 ml of medium to achieve 20 ng/ml. The prepared growth medium was warmed to 36$^0$ C in a water bath prior to use.

## Guanidine hydrochloride extraction/cetylpyridinium chloride precipitation

Stock solutions of protease inhibitors were made ahead of time and used to make a fresh solution of 4*M* guanidine hydrochloride with protease inhibitors prior to each extraction. A solution of 4*M* guanidine hydrochloride with protease inhibitors was made by mixing 3.82 g guanidine hydrochloride in 4 ml of distilled water and adding the following stock solutions: 250 $\mu$l sodium acetate (2*M*), 100 $\mu$l phenylmethyl-sulfonylfluoride (1m*M*), 1.0 ml disodium EDTA (10m*M*), 100 $\mu$l benzamidine (1m*M*) and 100 $\mu$l N-ethylmaleide (1m*M*). The solution was completed by adding 0.131 g $\sum$-aminocaproic acid in the powder form to make a 100 m*M* solution of $\sum$-aminocaproic acid. The total volume was increased to 10 ml and the pH was adjusted to 5.8 using 1N sodium hydroxide. Stock solution preparation for the protease inhibitors and the cetylpyridinium chloride precipitation are described in table A.1.

Table A.1. Mixing instructions for stock solutions. RT = room temperature, FS = filter sterilize, $dH_20$ = distilled water, QS = bring volume up to, NaOH = sodium hydroxide.

| Stock Solution | Weight | Diluent/Volume | Storage | Comments |
|---|---|---|---|---|
| 0.3*M* sodium chloride (NaCl) | 19.5 g | 1 liter $dH_20$ | RT | FS |
| 0.1% cetylpyridinium chloride (CPC) | 500 mg | 500 ml 0.3*M* NaCl | RT | warm prior to use to resuspend |
| 2*M* sodium acetate | 16.4 g | 100 ml $dH_20$ | FS<br>RT | pH to 5.8 using glacial acetic acid |
| 100 m*M* phenylmethyl-sulfonylfluoride | 1.74 g | 10 ml methanol | -20$^0$ C | |
| 10m*M* disodium EDTA | 18.6 g | 500 ml $dH_20$ | FS (0.2$\mu$)<br>4$^0$ C | pH to 5.8 using 10N NaOH |
| 1m*M* benzamidine hydrochloride | 156 mg | Add 9.4 ml $dH_20$<br>Mix/QS to 10 ml | 2 ml aliquots<br>-20$^0$ C | |
| 1m*M* N-ethylmaleide | 125 mg | Add 8-9 ml $dH_20$<br>Mix/QS to 10 ml | RT | |

Initially, a protocol was devised to precipitate all proteins in the supernatant using trichloroacetic acid (TCA) prior to the guanidine hydrochloride extraction of the proteoglycan. Two hundred microliters of cold 60% TCA was added to 1 ml of medium to achieve a 10% solution. This solution was incubated for one hour or overnight at 4$^0$ C. Samples were kept on an ice bath at all times. The samples were centrifuged at 4$^0$ C for five minutes in the microcentrifuge. The supernatant was drawn off and discarded. One hundred microliters of the 4*M* guanidine hydrochloride protease inhibitor solution was added to each tube and mixed. Tubes were placed on a spinner in the refrigerator at 4$^0$ C for 48 hours. The samples were then spotted on filter paper for the CPC precipitation as described for the cell-associated fraction in Chapter 2. Further investigation obviated the need for TCA precipitation. The proteoglycans were extracted and precipitated directly to from the supernatant as described in Chapter 2.

**Cell attachment factors /collagen coating of plasticware**

Standard culture flasks and dishes provide a suboptimal environment for fibroblast attachment. Following trypsinization, a large number of cells were lost because they were unable to anchor themselves to the plastic of the flasks. Several commercial products were evaluated in an effort to improve cell attachment. Vitrogen 100, a commercial collagen product, and five other attachment factors were evaluated.

In the laminar flow hood, enough Vitrogen 100 was added to each flask or well to completely coat the bottom using slow gradual swirling motions so as not to fragment the collagen fibers. The excess liquid was removed by pipetting and the flask or plate was allowed to sit uncovered overnight in the laminar flow hood until completely dry. The container was then rinsed with sterile distilled water and allowed to dry in the laminar flow hood overnight. Coated containers were sealed and stored in the refrigerator until used. The additional preparation of rinsing the flasks was necessary prior to usage to insure a dry surface for explant adherence. Use of phosphate buffered saline (PBS) to rinse the flasks should be avoided because the dried salt forms a potentially cytotoxic layer on the surface of the plastic when the PBS dries.

Each of the commercial plates that were evaluated were coated with one of five different attachment factors: (1) human fibronectin, (2) poly-D-lysine, (3) rat tail collagen type I, (4) mouse collagen IV and (5) mouse laminin. Cells trypsinized following explant removal were seeded into these six well plates and incubated in growth medium for three days. After three days the cells were fixed and stained with 1.2% aqueous crystal violet solution as described in Chapter 2 and this Appendix. Density of cell populations were evaluated by light microscopy for cell adherence and general morphology.

The Vitrogen 100 worked consistently well. The fibroblasts adhered well and readily formed a monolayer. The results with the other commercial products varied. Attachment with the mouse laminin was poor. Fair attachment was noted with the rat tail collagen and the mouse collagen. Attachment was good with the poly-D-lysine. The human fibronectin resulted in the best cell viability and anchorage of the five commercially coated plates. In all plates but the human fibronectin and poly-D-lysine, the cell morphology was unusual. This may have been a sign of an adverse reaction to the attachment factor. Based on these

observations, it was concluded that continued use of the Vitrogen 100 was the best choice based on the flexibility of coating plates and flasks individually as needed rather than purchasing pre-coated plates.

**Tendon harvesting and cell culture**

Equine limbs were prepared aseptically. The hair was clipped circumferentially from the mid-carpal region to the pastern of both forelimbs and the excess loose hair was removed with the vacuum. A latex exam glove was placed over the hooves and the legs were suspended from an IV pole to allow circumferential aseptic preparation of the clipped area. Each metacarpal region was scrubbed for five minutes in a standard manner alternating between chlorhexidine and alcohol.

The skin over the palmar metacarpal region was incised aseptically and dissected back using a number #10 scalpel blade. Once the paratenon was exposed, the process was repeated on the opposite forelimb. The mid-metacarpal region of the superficial digital flexor (SDF) tendon was dissected free from the deep digital flexor tendon and was transected proximally and distally using a #20 scalpel blade. Following transection, the tendon was immediately placed in sterile HBSS in a sterile specimen container, sealed and transported to the laboratory for further processing.

The tissue explants were prepared in the laminar flow hood. The tendon segments were removed one at a time from the HBSS and rinsed in PBS to remove adherent red blood cells. They were then immersed in 0.2% sodium hypochlorite for five seconds to disinfect the surface and rinsed a second time in PBS. The epitenon was removed from all four sides of each tendon segment using a sharp flame sterilized razor blade. The central core region was then cut sequentially into longitudinal strips of tendon 2-3 mm in width. Cut strips were stored in culture medium in a separate petri dish to avoid desiccation. The strips were minced into 1-2 $mm^3$ tissue fragments and stored in culture medium.

The tissue fragments were removed five to six at a time and briefly set on a piece of sterile filter paper to absorb excess moisture and increase adherence to the flask. Tissue fragments were placed one at a time 5-6 mm apart into 25 $cm^2$ flasks using a flame sterilized metal spatula. A total of 30-40 tissue fragments were placed in each flask. Approximately 20

flasks were set per horse based on the number of cells required for each experiment. Set up of 24 well plates was performed similarly with one tissue fragment centered in each well.

When the medium was changed, approximately 60% of the total volume of medium was exchanged. New medium was added gently to the side of the flask or the well so as not to disrupt the tissue fragments or cells. Special care was taken not to disturb the tenuous attachment of the tissue fragments. On day 14 the tissue fragments were removed without disrupting the cells using a rubber cell scraper, removed using a pipette and discarded. The cells were trypsinized.

**Trypsinization of equine tendon fibroblasts**

The standard laboratory trypsinization protocol for fibroblasts was modified in an effort to maximize cell detachment and minimize cell trauma. A number of alternative protocols were tested and the modification described produced the best results. The original protocol is described for completeness.

The flask surfaces, including the top, were rinsed gently using 2 ml of porcine pancreatic trypsin with ethylenediaminetetraacetate (EDTA) in saline A (ATV). The ATV was removed immediately and a fresh 2 ml of ATV was added to cover the entire bottom surface of the flask. The flask was capped and placed on a $37^0$ C heating block and gently swirled periodically for three to five minutes until pin-point holes could be seen in the halos of cells. The flask was rapped against the palm of the hand to loosen the cells. Ideally, the cells would have been loose but not detached at this stage. The ATV was to be removed and 5 ml of growth media added to the 25 $cm^2$ flask. The media would be pipetted up and down several times gently to break up the clumps of cells and transferred to clean labeled 25 $cm^2$ flasks and reincubated at $37^0$ C and 5% $CO_2$.

The standard trypsinization protocol as described above was apparently too strong for the equine fibroblasts. The cells were readily released from the flask and were floating free in the trypsin. Removal of the trypsin would have also removed the cells. As a result, the protocol was modified to retrieve the cells. The cells and trypsin were pipetted into a 6 ml snap cap tube and centrifuged for three minutes at 1000 RPM to form a cell pellet. The excess trypsin was removed leaving the cell pellet. Growth medium was added to the tube

and gently pipetted three to four times to break up the cell clumps. The media and cells were added to clean 25 $cm^2$ flasks and were reincubated.

The final modification that was utilized successfully throughout the later studies and worked well. The growth medium and tissue fragments were removed. The flask was rinsed with 2 ml ATV and this ATV was discarded. A fresh 2 ml of ATV was added and the flask was placed on the $37^0$ C heating block for 30-60 seconds. The ATV was removed and the "dry" flasks were placed on the heating block for another two to four minutes. The flasks were rapped occasionally in the palm of the hand and the progress of the trypsinization was reassessed at 30-60 second intervals. When the cells were loose, 2 ml of growth medium was added per flask and pipette up and down two to three times. The cells were then combined in one of the two flasks trypsinized at the same time and stored in the incubator temporarily. When all of the flasks had been trypsinized, the cells were swirled, combined and distributed to the necessary plasticware for monolayer seeding.

**Electron and light microscopy**

Cells that were to be fixed in a monolayer were prepared in a similar fashion to that described in Chapter 2. Rather than scraping the cells, the glutaraldehyde was removed and the bottom of the well was punched out using a #10 cork borer from the outside. The disks were placed in a scintillation vial in fresh glutaraldehyde and stored in the refrigerator overnight. Those monolayers that were seeded on well inserts were fixed in a manner similar to that just described for the wells. The insert membranes were removed using scissors in place of the cork borer. These techniques were less than ideal for viewing the cells under EM due to the paucity of cells spread out over a large area and difficulties in processing the plastic discs for EM. The pellet method is preferable.

Crystal violet staining for light microscopy was performed as described in Chapter 2. The staining process itself with the filtered crystal violet was performed for 15 minutes. Gamori's trichrome stain was used to specifically stain for collagen. The monolayer of cells was fixed by covering with $56^0$ C preheated Bouin's solution for 30 minutes. The excess Bouin's was discarded and the monolayers were cooled and washed under running tap water until the yellow color disappeared. One rinse was performed with distilled water. The

monolayers were covered with Weigert's iron hematoxylin solution for ten minutes after which the excess Weigert's solution was discarded. A two minute rinse under running tap water was followed by a rinse with distilled water. Finally, the monolayer was covered with Gamori's trichrome stain for 15 minutes. The excess trichrome was discarded and the monolayer was covered with 0.5% glacial acetic acid for one minute. The monolayers were allowed to dry. The colors in the stain and the structures they represent are displayed in Table A.2.

Table A.2. Key for Gamori's trichrome stain.

| Structure stained | Color |
|---|---|
| Muscle fibers | Red |
| Collagen | Blue |
| Nuclei | Blue to ink |

The results of the trichrome stain were disappointing. In the areas of dense cell populations, there were large accumulations of blue stain. It was difficult to determine whether these were areas of abundant collagen deposition or whether the stain accumulation was artifact from adherence to the cells themselves. The coloring may well have been true collagen deposition, but the final product was not useful for photography or measurement.

**Proteoglycan staining**

The presence of proteoglycan in the guanidine extraction solution and subsequently on the filter paper following precipitation was tested with several different stains. Thymol/napthol (T/N) stain was made using 0.5 g thymol, 0.5 g napthol, 95 ml 95% ethanol and 5 ml concentrated sulfuric acid. Coumassie brilliant blue (1%) (CBB) was premade as a stock solution. Toluidine blue (TB) was made using 100 ml 0.1N acetic acid (94.3 ml $dH_20$ plus 5.7 ml glacial acetic acid (17.4N) with 0.2% toluidine blue.

The filter paper was spotted with two separate spots. Both Whatman 3MM and CF/A filter paper was used. One spot each of 10 $\mu$l and 1$\mu$l of guanidine extraction solution was spotted on the paper. The spots were allowed to dry and the proteoglycan was precipitated using CPC and allowed to dry again. The filter paper was dipped in the stain five times for a

total of about five seconds. The CBB and TB were rinsed with $dH_20$ from the squirt bottle and destained in acetic acid for 10-15 minutes. The T/N strips were placed on foil and placed in the $120^0$ C oven for ten minutes. All strips confirmed the presence of proteoglycan on the filter paper, validating the guanidine extraction and CPC precipitation protocol.

**Cell labeling with $Na_2{}^{35}SO_4$**

Ideally the cells were labeled during the log phase of growth when they had created approximately 80% of a solid monolayer. The $Na_2{}^{35}SO_4$ was ordered in 2.0 mCi doses in a 2 $\mu$l volume. Following delivery from Environmental Health and Safety, the manufacturer's reference date was recorded on a card in Room A and the $Na_2{}^{35}SO_4$ was stored in the glass cabinet in the same room for future use. Prior to the first use, 198 $\mu$l of sterile distilled water was added to the isotope vial to achieve a concentration of 10 $\mu$Ci/$\mu$l. At this dilution, 4 $\mu$l was used per milliliter of medium to achieve a final concentration of 40$\mu$Ci/ml. Whenever working with the isotope standard radiation safety protocols were observed.

**Scintillation counting**

Proteoglycan content was determined using a liquid scintillation counter. Background counts were recorded using a glass scintillation vial containing scintillation fluid and a plain piece of filter paper. Counter efficiency was estimated using 100 $\mu$l of C14-leucine spotted onto a piece of filter paper and placed in a glass counting vial with scintillation fluid. The background counts were performed on the same channel as the control and treatment samples.

**Dilution of PSGAG**

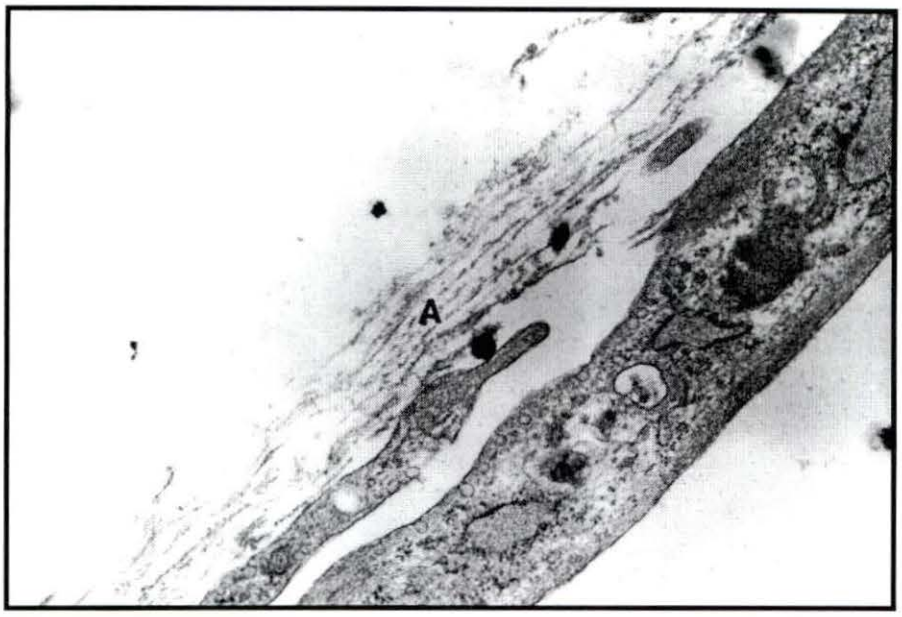
The intra-articular form of Adequan (IA) was utilized as PSGAG for all treatments. The stock solution of 250 mg/ml was diluted 1:10 with sterile distilled water (100 $\mu$l PSGAG added to 900 $\mu$l $dH_20$) in a microcentrifuge tube. This created a concentration of 25 $\mu$g/$\mu$l. A second 1:10 dilution was performed to achieve a concentration of 2.5 $\mu$g/$\mu$l. Appropriate volumes of these stock solutions were added to the medium to achieve the desired concentrations.

**Materials**

All chemicals and biochemicals were obtained from Sigma Chemical Company (St. Louis) with the following exceptions: DMEM, 1L packets of powder (GIBCO Laboratories, Grand Island, NY), FBS, premium grade (GIBCO Laboratories, Grand Island, NY and Summit Biotechnology, Fort Collins, CO), rhIGF-1 (Sigma Chemical Company, St. Louis, MO and Fisher Scientific, Itasca, NY), Vitrogen 100 (Flow Laboratories, McLean, VA), $Na_2SO_4$ (ICN Pharmaceuticals, Irvine, CA), $^{14}C$-leucine (ICN Pharmaceuticals, Irvine, CA), PSGAG (Adequan, Luitpold Pharmaceuticals, Shirley, NY), commercially coated six well plates (Fisher Scientific, Itasca, NY).

# APPENDIX B. ELECTRON MICROGRAPHS

(a)




(b)

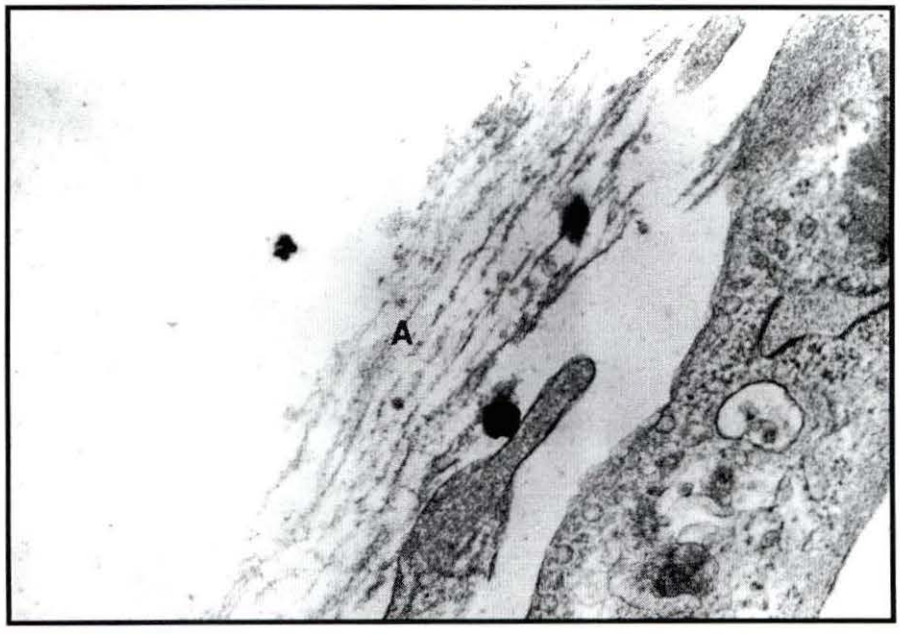
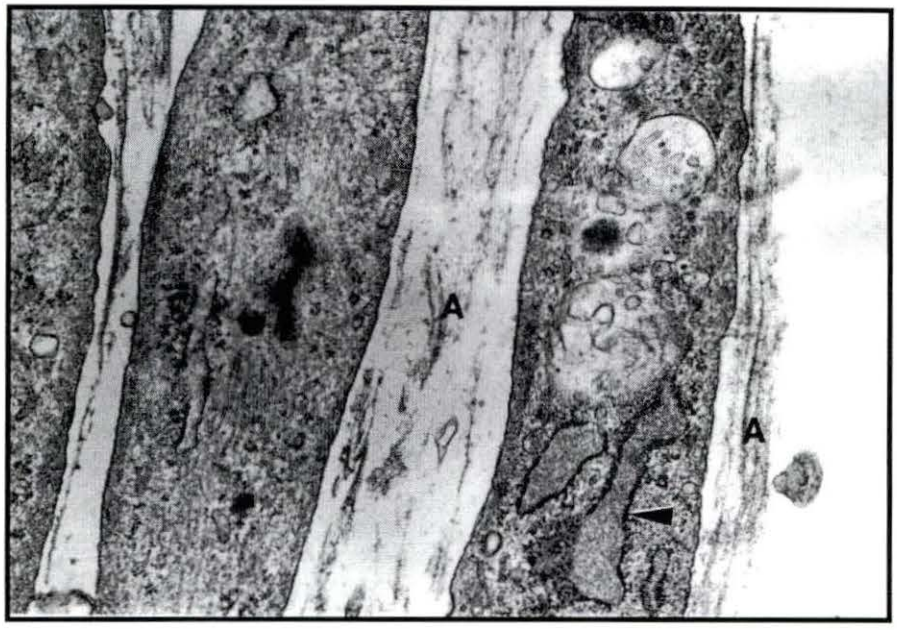



Figure B.1. Transmission electron micrographs of equine tendon fibroblasts. (a) approximately 23 X $10^3$x and (b) approximately 33 X $10^3$x. Note the extracellular matrix products (collagen and proteoglycan) accumulated outside the cells (A).

(a)




(b)

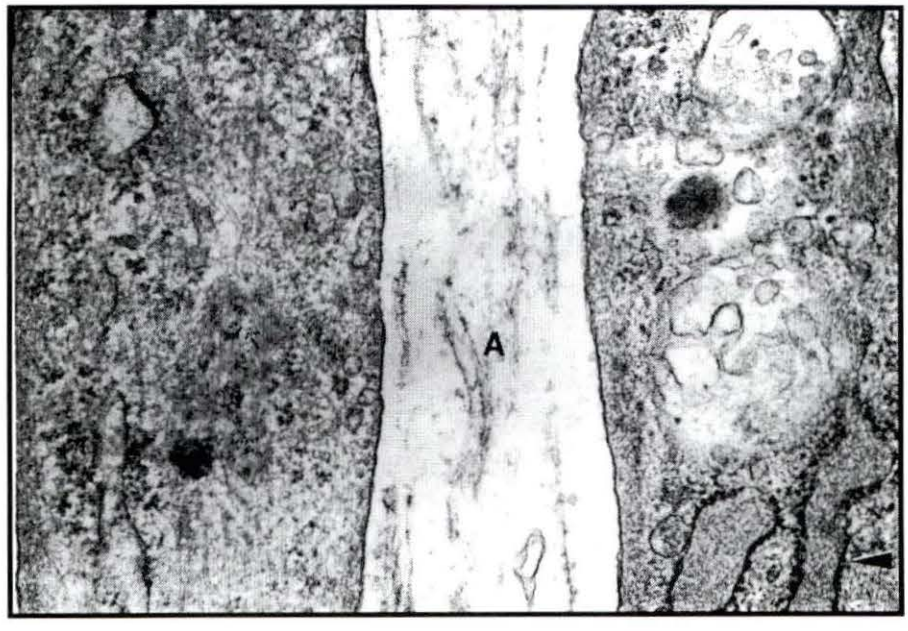
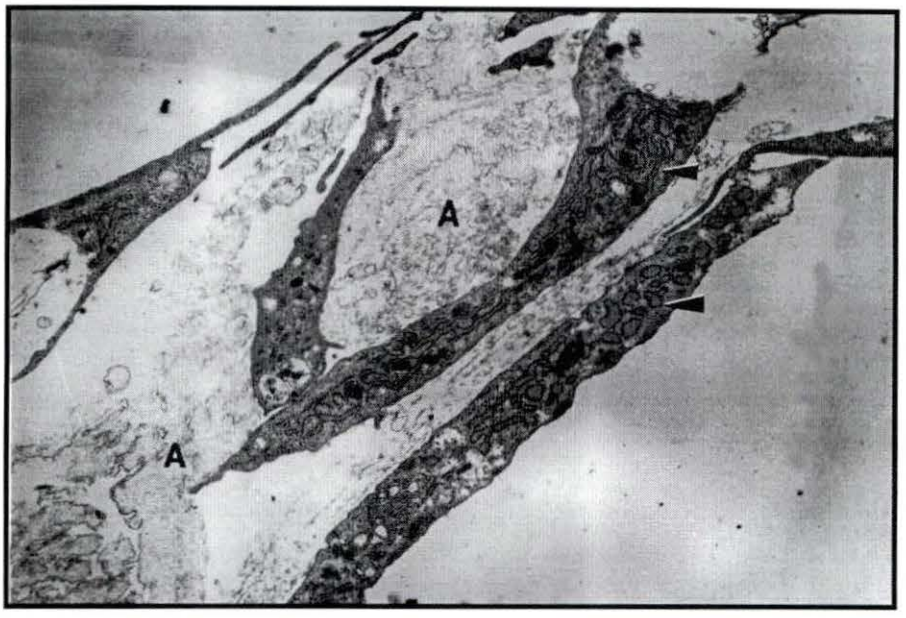



Figure B.2. Transmission electron micrographs of equine tendon fibroblasts. (a) approximately 23 X $10^3$x and (b) approximately 33 X $10^3$x. Note the abundant rough endoplasmic reticulum with dilated cisternae containing granular material (arrow head) and extracellular matrix between the cells (A).

(a)




(b)

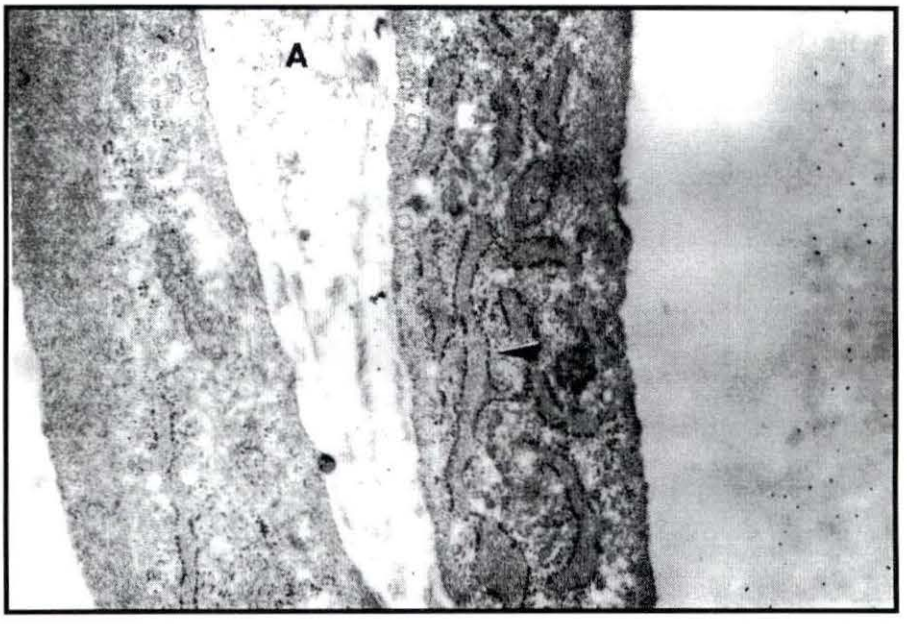
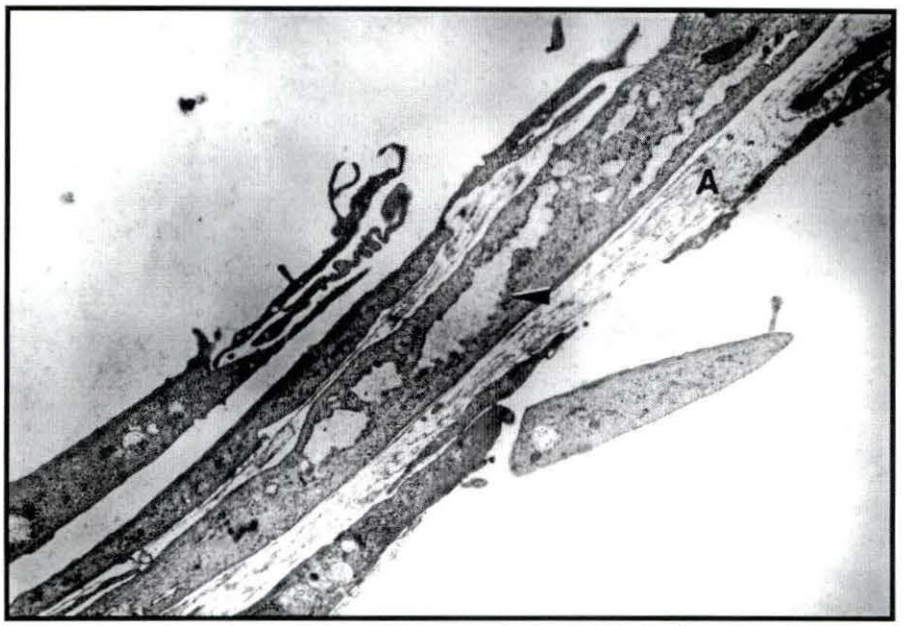



Figure B.3. Transmission electron micrographs of equine tendon fibroblasts. (a) approximately 8 X $10^3$x and (b) approximately 13 X $10^3$x. Note the abundant rough endoplasmic reticulum with dilated cisternae containing granular material (arrow head) and extracellular matrix between the cells (A).

(a)




(b)

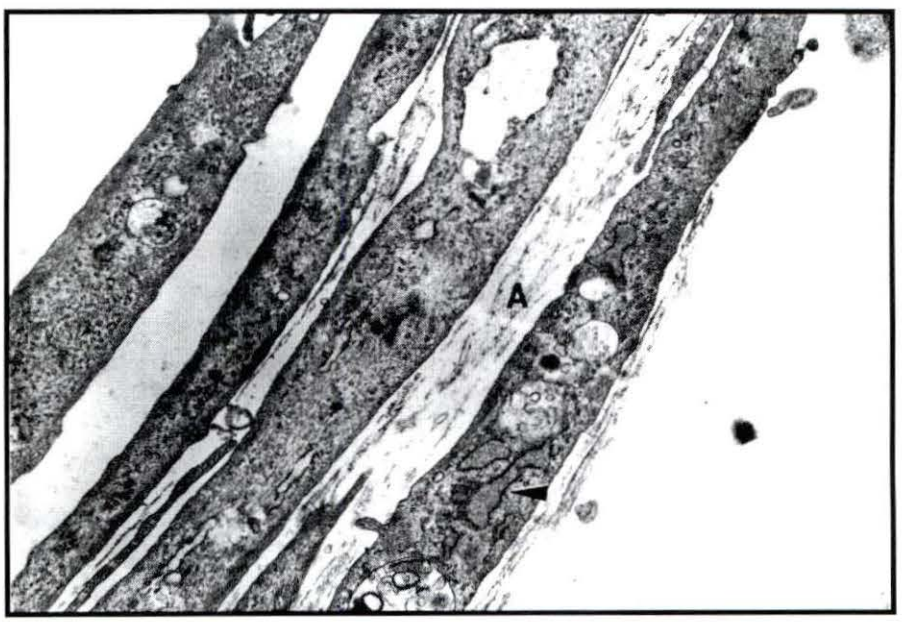



Figure B.4. Transmission electron micrographs of equine tendon fibroblasts. (a) approximately 8 X $10^3$x and (b) approximately 23 X $10^3$x. Note the abundant rough endoplasmic reticulum with dilated cisternae containing granular material (arrow head) and extracellular matrix between the cells (A).

## ACKNOWLEDGMENTS

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