Characterization of Apoptotic Cells in Equine Proximal Suspensory Desmitis

By

Christina A. Hewes, DVM

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APPROVED: _____
Nathaniel A. White II, Chairman

_____ _____
Virginia Buechner-Maxwell John L. Robertson

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Christina A. Hewes, DVM

Committee Chairman: Nathaniel A. White II, DVM, MS
Diplomate American College of Veterinary Surgeons
Theodora Ayer Randolph Professor of Surgery
Veterinary Medical Sciences

ABSTRACT

Suspensory desmitis is a common problem and affects a broad cross section of equine athletes in various disciplines. For this study, the proximal portion of the suspensory ligament was collected from 6 horses without suspensory ligament injury (16 ligaments) and 4 horses with degeneration of the suspensory ligament (11 ligaments). Specimens were collected immediately after euthanasia and placed in neutral-buffered 10% formalin. The tissue was fixed, sectioned, and stained with hematoxylin and eosin (H&E), Masson's trichrome, and for apoptosis by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique. Histological changes in the abnormal ligaments included mineralization, fibroplasias, neovascularization, collagen degeneration, and significant architecture disruption in 2 ligaments. There was a trend for increased apoptosis in the injured ligaments compared to the normal ligaments.

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INTRODUCTION

Suspensory desmitis is a common problem in equine athletes and was ranked as one of the ten most important diseases in a survey of members of the American Association of Equine Practitioners.[a] Minimal research has been performed to define the pathogenesis, histopathologic changes, and their correlation with a definitive diagnosis. One previous post-mortem study on chronic hind limb suspensory desmitis cases found hypercellularity, acellularity, hemosiderin deposits, fibrosis, collagen hyalinization, neovascularization, and intraligamentous chondroid metaplasia.[1,2] There are no other publications reporting studies on equine ligament histopathology.

Causes of ligament injury include acute overloading, laceration or overstrain.[3-5] Human injuries are often from acute overloading. Overstrain causing degeneration of the ligament is from cumulative microdamage to the region and is thought to be a common cause of injury in horses.[3,4] Acute ligament injury in humans is initially characterized by a hypertrophic vascular response and hypertrophic fibroblasts.[6] The healing tissue is disorganized with increased numbers of blood vessels, fat cells, inflammatory cells, fibroblasts, and loose connective tissue than normal ligament.[7] During healing type III and V collagen increase with decreased size of the collagen fibrils. As the healing progresses, the collagen is aligned into parallel bundles with Type I collagen increasing in amount as healing progresses.[8] In chronic injuries with ligament degeneration histological changes include collagen degeneration, fiber disorientation, increased mucoid ground substance, and an absence of inflammation.[9] Since ligaments and tendons have similar structures except for the different location of the insertions, studies on tendons provide useful information in evaluation of ligament injury.

To further understand the cellular response in ligament and tendon injury, the presence of the pro-apoptotic proteins and apoptotic cells in degenerative tendons has been investigated. Apoptosis is programmed cell death that is initiated by activation and

[a] AAEP Membership Equine Research Study, Lexington KY, 2003.

transcription of specific genes.[10-12] Apoptosis is important for embryogenesis, tissue homeostasis, and in death of virally affected and neoplastic cells to remove unwanted cells with minimal inflammation.[13,14]

Apoptosis has been found in degenerative rotator cuff tendons with an even distribution of apoptotic cells throughout the torn edges of the tendon.[15] Equine superficial digital flexor tendons had higher levels of apoptotic cells in inflamed tendons versus normal tendons.[16] The apoptotic cells were tendinocyte-like cells, as seen in the injured human rotator cuff tendons.[15,16] When medial collateral ligament cells were compared to anterior cruciate ligament cells, the anterior cruciate ligaments were more sensitive to apoptosis than the medial collateral ligament.[17] In canine cruciate ligaments, necrosis, not apoptosis, was present.[18]

To detect apoptosis the TUNEL assay is used. TUNEL is an in-situ terminal transferase-mediated, nick-end labeling assay, that detects DNA strand breaks resulting from cleavage by endonucleases in early and advanced apoptosis.[19] It can distinguish apoptotic cells from necrotic cells and has been used successfully in human ligament studies.[19,20]

The objectives of this study are to 1) define the histopathologic changes in degenerative and normal proximal suspensory ligaments and 2) identify cells undergoing apoptosis. We hypothesized that the degenerative proximal suspensory ligaments would have increased levels of neovascularization, collagen necrosis, metaplasia, apoptotic cells, and immature collagen compared to the normal ligaments.

LITERATURE REVIEW

Ligaments

Anatomy and Structure

Ligaments are dense bands of collagenous tissue that connect bones together at or near bony articulations.[5] They are composed of roughly parallel fibers of material that tighten or loosen as different forces are applied. Ligaments are covered with layers of non-ligamentous tissue which conceals the parallel collagen bundle arrangement.[5] This tissue covering though it appears appear to be insignificant, can have important function in ligaments in joint capsules.[5]

Structurally ligaments are composed of fibrocytes surrounded by a collagenous matrix. They are heterogeneous in their structure between the bony attachments with a mix of ligamentous tissue and bone. Variation exists between ligaments in different locations.[5] Ligaments are composed of tightly packed, relatively parallel collagen with few interspersed cells.[5] Three types of fibroblasts are present in tendinous and ligamentous tissue. Type I cells have thin, spindle-shaped nuclei; type 2 cells have more rounded, thick, cigar-shaped nuclei; and type 3 cells are cartilage-like cells with round nuclei and active nucleoli. Ligament consists of primarily type 2 cells arranged in columns, although there is variation between ligaments.[4,21] Cruciate ligaments have more chondroid (round) nuclei than collateral ligaments.[5] Fibroblasts/fibrocytes are responsible for matrix synthesis.[22] Since fibrocytes represent only a small percentage of total ligament volume, the fibroblasts have cytoplasmic extensions and gap junctions to communicate across long distances.[22,23]

Ligament matrix is primarily fibrillar collagen that has crimped fibers. Crimp is undulation in the matrix due to changes in orientation of the collagen which allows the ligament to have elasticity.[21] The mid-substance of the tendon is not homogenous with bundles of loose connective tissue separating fibrous material bundles.[5] This allows inter bundle shearing to occur under loading. Cross connecting fibrils in the ligament fibers

bind the longitudinal fibrils together.[5] At the bone-ligament interface, the matrix architecture changes. The cells transition from fibroblasts to fibrocartilaginous cells to permit a progressive stiffening of the ligament and minimize the transition from bone to ligament.[5]

At the bone-ligament interface, direct and indirect insertions of the ligament on bone exist with superficial and deep fibers. Direct insertions have abrupt, well defined area with sharp demarcations between the bone and soft tissue and insert at right angles.[24] Deep fibers comprise the major fiber type in direct insertions and insert in four distinct zones. Zone 1 is the ligament proper with the matrix composed of primarily type I collagen with dermatan sulfate proteoglycans and a few elastin fibers. Flat fibroblasts are present. Small vessels run parallel to the collagen fibers, but nerves are absent.[24,25] Zone 2 consists of fibrocartilage with the same matrix as zone 1. The cells change from flat fibroblasts to rounded chondrocyte-like cells lying in pairs and arranged in rows.[24] Zone 3 is mineralized fibrocartilage. A tidemark line between the mineralized and unmineralized fibrocartilage is visible in this region. The tidemark is smooth in contour, but can be irregular, and is sharply demarcated delineating the two different regions. Mineral infiltration starts at the fibrillar edge and spreads into the interior portions of the ligament.[24] Zone 4 is bone with the collagen of the ligament blended with the collagen of the bone matrix. This is mainly type 1 collagen with chondroitin sulfate proteoglycans.[24]

Indirect insertions have deep and superficial portions but the superficial portions predominating. They attach at acute angles. The superficial fibers blend with the periosteum at the insertion sites.[24] The collagen fibers are cemented into the bone with formation of Sharpey's fibers at the periosteum to help cement the periosteum to the bone.[24,26] The matrix is minimally to partially calcified. The deep fibers in this region attach with minimal transition between the bone and ligament with a non-existent or minimal fibrocartilage zone.[24]

Ligaments are two thirds water and one third solid material. Water is important for viscoelasticity and cellular functions, such as nutrient and waste distribution.[5] The matrix

is composed of collagen, proteoglycans, elastin, actin, laminins, and integrins. Collagen is 75% of the dry weight of the matrix with the majority of the collagen being type I collagen with smaller amounts of types III, V, VI, XI, and XIV.[22] When the matrix is synthesized, pro-collagen molecules are secreted through microtubules. Post translational modification occurs to form triple helical molecules that begin to form fibrils and then fibers, which are aligned in parallel. Lysyl oxidase forms the cross links within and between molecules to give the collagen strength.[22] Collagen fibers are tightly interconnected into parallel bundles to form fascicles. The fascicles are held together by loosely connected collagen.[4] The predominant proteoglycan in the matrix is dermatan sulfate, which is important for water-binding. Proteoglycans only account for 1% of the ligament by dry weight.[21] Elastin, which also only makes up 1% of the ligament by dry weight, is important for tensile resistance due to its coiled structure.[21] Fibronectin is only present is small quantities, but is important for the matrix-cell feedback mechanism.[21] Currently only 80% of the dry weight of the ligaments is characterized.[5,22]

Epiligament, a vascular overlying layer, covers the surface of ligament and merges into the periosteum at the insertion and attachment sites of the ligament. It has an increased blood and nerve supply compared to the ligament itself, with the blood vessels and nerves traveling in close proximity. More nerves than vessels are present adjacent to the bone-ligament insertion. [22] In addition to the epiligament, the blood supply to ligaments enters through the superficial layer of the ligament at specific locations and along its length.[27] Small neurovascular bundles run into the deeper tissue parallel to the collagen fibrils. The deeper ligament tissue has decreased vascularity compared to the superficial tissue, despite the presence of neurovascular elements at regular intervals. No vessels enter at bony attachments.[5] Blood and nervous supply to the ligament insertion is from surrounding bone and soft tissue.[24] For nervous supply, C-type pain fibers and proprioceptive fibers are present. Functionally ligaments can perceive pain and proprioception, but this innervation is likely disrupted with ligament injuries.[5]

Biomechanical Behavior

Ligaments are designed to function effectively in numerous positions to stabilize the structures to which they are inserted.[5,22] Three clinically significant behaviors exist in ligaments, which are structural (load-deformation) behavior, material (stress-strain) behavior, and viscoelastic (relaxation and creep) behavior.[5]

Structural behavior is the manner in which a ligament responds to load and determines the strength of the ligament. Ligaments exhibit nonlinear anisotropic mechanical behavior and are compliant under low load conditions. The compliance is from the presence of crimp in the collagen, which allows the elongation to occur without damage of the fibrils.[5] As the load increases, ligaments have increased stiffness due to recruitment of additional fibers. In this stage the fibrils start to carry the increased load instead of just elongation of the crimp. The ligaments continue to carry the load for a prolonged period of time unless the load overwhelms the strength of the fibrils, tensile failure occurs.[22] Failure can occur abruptly but frequently is initiated by failure of a few fibrils with eventual failure throughout the entire ligament if the load is not removed.[5]

Material properties of the ligament are determined by the responses to stress and strain applied to the ligament. It is similar to load deformation except the local changes in the length of the ligament and normalization of loads for the size of the ligament are reported as non-linear deformation with increased stiffness in response to increased stress and strain.[5]

Viscoelastic properties describe the non-linear manner in which ligament can absorb energy as its stretches and recovers, with viscous properties predominating at low loads and elastic at high loads.[5] When ligaments are stretched to a fixed length and held, the load decreases due to load relaxation of the viscous component of the ligament. This minimizes injury to the ligament with new equilibrium formation. If the load causes elongation to a new equilibrium or to the point of ligament failure, ligament creep occurs.[5] Creep is deformation under constant or cyclically repetitive load.[22] It allows the

ligament to further deform to absorb the load applied to it and prevent injury to the ligament.

In general increased age causes ligaments to become more compliant with losses in structural strength and viscosity. This can lead to excessive creep behavior.[5] Loss in function of ligaments also occurs with immobilization. Atrophy during immobilization with loss in strength and stiffness develops along with a catabolic state in the ligament.[28,29] Insertion sites are more likely to have avulsion fractures after immobilization due cortical bone loss.[24] Recovery from immobilization occurs faster at the bone-ligament interface than in the body of the ligament, but the reason is unknown.[5] The changes in the bone-ligament interface can reverse with increased exercise over a prolonged period of time.[24]

Exercise causes ligaments to become 10-20% stronger and stiffer than control ligaments.[30] Strength is related to the type and duration of exercise. Endurance training induced larger diameter collagen fiber bundles and higher collagen content with an increased separation force at the bone-ligament interface.[31] Since exercise increases the strength and stiffness of the ligament, the appropriate exercise for specific ligaments may be helpful to prevent injury.

Ligament Failure and Healing

Failure sites for ligaments occur in the mid portion or at the bone-ligament interface. The most common site of failure is mid portion interstitial failure with collagen disruption. The next most common site of failure is zone 4, where the collagen of the ligament is blended with the collagen of the bone matrix, with bony avulsion with subsequent injury in zone 2 (unmineralized fibrocartilage zone) and zone 3 (mineralized fibrocartilage zone) if the avulsion is large.[24] A majority of the avulsion fractures occurred in the mineralized fibrocartilage during slow extension.[32] In fast extension, ligaments failed at the mid portion of the ligament (66%), sustained an avulsion fracture of the ligament insertion site in the bone(28%), or failed in the portions of both the bone and the ligament, not at the ligament attachment site in the bone(6%.)[33] Further study revealed

increased mid portion ligament injuries in adult animals versus an increased amount of avulsion injuries in young animals, rather than injury in the midsubstance of the ligament.[34]

Causes of failure include acute overloading, laceration or overstrain. Overstrain can occur from an acute event with sudden overloading or from overload in a degenerating area, which occurs more commonly in horses. Avulsion injuries are often from acute overloading of the insertional interface.[24] Degeneration results from cumulative microdamage to the region.[3,4] Degeneration can be associated with age and exercise.[3] In equine tendons, degeneration is the first phase of tendinopathy, which weakens the tendon, instead of invoking repair. Increased loads in the degenerative tendon or ligament can lead to failure to some or all of the collagen fibrils. Physical disruption to the matrix can include fibrillar slippage, breakage of cross linking elements, fibrillar rupture, or complete separation of the tendon or ligament.[3] Partial failure in equine ligaments appears similar to a grade I ligament sprain in humans.

Ligament sprains are common in human athletes. Grade I sprains are mild with no change in ligament laxity, while Grade II sprains are moderate with a slight increase in laxity. Grade III sprains are severe with complete disruption and significant increased laxity.[21] Grade I tears, which are the most common, can be difficult to detect due to only minor changes in function. Grade I sprains can lead to further damage to the ligament if additional injury occurs.[5]

When a ligament is injured, it heals similar to a skin wound. The first phase of healing is hemorrhage with inflammation. There is pain from injury to the nerve fibers in the ligament and bleeding due to tearing of vessels in and around the ligament.[5] The ligament ends retract, a blood clot is formed, and a large number of polymorphonuclear leukocytes and lymphocytes infiltrate the injured region.[22] Increased levels of histamine cause vasodilatation and secondary increases concentrations of serotonin, bradykinins, and prostaglandins. Edema results in increased water content in the ligament. There is also increased collagen synthesis and degradation during this phase. Increases in Type III

collagen stabilize the extracellular matrix. Glycosaminoglycan, fibronectin, and DNA concentrations also increase.[8] This phase can last from hours to days.

The second phase, cellular and matrix proliferation, includes a hypertrophic vascular response with increased blood flow and vascularity.[6] Hypertrophic fibroblasts produce dense, cellular, connective tissue to heal the defects. This new tissue is disorganized with increased blood vessels, fat cells, inflammatory cells, fibroblasts, and loose connective tissue compared to normal ligament.[7] As the healing progresses, the collagen is aligned into parallel bundles.[22] The healing collagen has increased levels of type III and V both having decreased size of the collagen fibrils. Type I collagen increases in amount in this phase while glucoaminoglycan and DNA content remain elevated.[8]

In the third phase, matrix remodeling, the matrix becomes denser to fill defects but with altered matrix components. The healed matrix has elevated biglycan, vascularity, and cellularity with decreased levels of decorin protein. Altered proportions of collagen types, immature collagen cross links, small collagen fibril diameter, abnormal innervation, altered cell connections, and persistent matrix defect endure in the healed ligament.[22,35-37] There are decreased numbers of fibroblasts and macrophages compared to the healing ligament in phase 2 with flattening of fibroblasts.[8] The collagen types return to a normal ratio of type I to type III with a normal cross linking profile. At the site of the ligament scar the tissue remains slightly disorganized and hypercellular.[8]

In chronically injured ligaments, there are slight increases in total collagen mass, collagen turnover rates, and total glucosaminoglycan concentrations.[38] There are also slight decreases in collagen concentration and an altered collagen type ration with increased type III collagen.[38] Due to the abnormal matrix in healed ligaments, abnormal function occurs. Inferior creep properties, decreased stiffness, and decreased energy absorption are present.[22,39] The tensile strength can be two thirds of normal.[8] Viscoelastic properties can recover to within 10-20% of normal. Weakness in the healing ligament results from fiber misalignment, failing collagen cross-link maturation, and failure of scar collagen fibrils

to mature to normal size.[40] The loss in function is dependent on the size of the injury and the strategies used to help the ligaments heal.[22]

Since ligament does not return to normal after injury and different ligaments heal to different degrees, physical therapeutic and surgical treatments have been advocated in humans. Exercise at low cyclic loads can promote scar proliferation and matrix remodeling.[41] Initially the injured tendon or ligament is slowly loaded with a small amount of force. The magnitude of force is increased to strengthen the tendon or ligament by increasing the speed of movement or increasing the external resistance. Guidelines for rehabilitation include no pain during non-exercise and slow progression with increased exercise if no symptoms recur.[42] In active animals medial collateral ligament injuries have better quality scores and improved strength compared to quality scores in less active animals.[43] In addition to physical therapy to heal ligament injures, surgical apposition of torn ligament ends can restore function and reduce laxity in the surrounding tissues if a large gap exists between the ligament ends. If the ends are in close apposition, repair is not justified because only a small degree of structural improvement is created.[5] Research into ligament grafting is ongoing. Attaining normal ligament function after injury is difficult as it is rare to restore the strength, viscoelasticity, and proprioception found in a normal ligament.

Injured ligaments have different healing properties despite similar therapy based on their function and mechanical properties. The medial collateral ligament of the knee has improved healing compared to the anterior cruciate ligament. When the vascular response of the two ligaments was compared, both ligaments had significant increases in vascular flow after direct injury. In response to joint laxity, the anterior cruciate ligament vascular response was significantly less than the collateral ligament response.[6] The lack of a normal vascular response could be a factor in the diminished healing potential of the ligament. The midsubstance of the medial collateral ligament has improved healing and decreased laxity compared to injury at either bone-ligament attachment.[44] The bone near the insertion may limit the size of scar insertion and increase the stresses at this location.[24]

Suspensory Desmitis

Suspensory desmitis is a common problem and affects a broad cross section of equine athletes in various disciplines.[45] It is more common in thoroughbred racehorses, hunter, jumper, and dressage horses than in western performance horses or eventers.[46] The predominant site of injury in Thoroughbred and Standardbred racehorses is the suspensory body and suspensory branches.[1]

The cause of suspensory desmitis is unknown. It is likely due to failure of the ligament in sites of weakness or degeneration, similar to the flexor tendons. Increased loading of the ligament is seen in horses with long toes and low heels.[3] Increased strain in the suspensory ligament was seen with egg bar shoes and a 7 degree heel wedge.[47] At the trot, strain increased 2.42% in the suspensory ligament from the walk.[48] When a rider is placed on the back of the horse, suspensory ligament strain also increased at the walk on pavement. [48]

Clinical signs of suspensory desmitis typically include a lameness grade 2 of 5 in the front limbs and 3 of 5 in the hind limbs. Lameness in the front legs is often mild initially and not detected by many riders in the early phases of injury prior to development of an obvious lameness.[46] Acute cases have localized heat, slight edematous swelling, pain on palpation of the ligament, and rounded borders and enlargement of the suspensory ligament.[49] The clinical signs are more obvious with injuries of the suspensory body versus injuries at the origin of the ligament.[49] When evaluating the lameness associated with suspensory ligament injury, the lameness may be more evident when the horse is trotting in a circle with the affected leg on the outside and when trotting on soft ground.[2,46] Bilateral lesions are also common, which may result in loss of action rather than overt lameness.[2] Chronic injury may not cause palpable pain or swelling in affected suspensory ligaments.[49]

Diagnosis of suspensory desmitis is made with local anesthesia to help localize the lameness to a region of the suspensory ligament. Perineural analgesia of the lateral

palmar/plantar and/or the palmar/plantar metacarpal nerves can be performed, but the technique is not specific for suspensory ligament injury.[2] Injuries in the distal carpal or tarsal joints can be alleviated with the perineural anesthesia in the proximal metacarpal/metatarsal region due to diffusion of the local anesthetic, direct infusion in the joint or anesthesia of portions of the leg innervated by the same nerves.[49]

Definitive diagnosis of suspensory desmitis is made with ultrasonography to evaluate the size of the ligament, size of the lesion, and echogenicity of the fibers.[49-51] Ultrasound is performed with a 7.5-10MHz transducer with a built in fluid offset or hand held standoff pad with the hair clipped from the affected area. The suspensory ligament is scanned from its origin at the proximal metacarpus/metatarsus to its branch insertions on the proximal sesamoid bones. The transducer is oriented parallel to the ligament fibers for the sagittal (long axis) views and perpendicular to the ligament fibers for the transverse (short axis) views.[51] To image the proximal portion of the suspensory ligament on the hind limb, the probe should be placed plantaro-medially where the skin is flat and the distance between the ligament and probe is minimal.[51] The location of injury is measured in centimeters from a point of reference, such as the accessory carpal bone or point of the hock. To characterize ligament lesions, the ligament total cross-sectional area, lesion cross sectional area, echogenicity, and fiber alignment are recorded. Ultrasonographic abnormalities associated with suspensory ligament injury include enlargement of the cross-sectional area, poor demarcation of suspensory ligament borders, focal or diffuse areas or reduced echogenicity, focal anechoic core lesions, reduced regularity in fiber pattern, and focal mineralization (Figure 1a).[50,51]

Cross sectional area measurements of transverse images determine enlargement of the ligament and are important in subtle lesions. It can also be used to determine the proportion of the ligament injured at a specific location. Cross sectional area is important to evaluate the quality of the repair during the rehabilitation program. The cross sectional area should decrease as the injured ligament heals concurrent with a decrease in the lesion size.[51]

Grades for echogenicity and fiber alignment score have been reported.[51] Echogenicity is the brightness of the structure. The grades for echogenicity range from 0 to 3. Grade 0 is a normal ligament without a lesion. Grade 1 is a mild decrease in the echogenicity with a majority of the lesion isoechoic to the normal ligament. Grade 2 lesions have mixed echogenicity with 50% echoic and 50% anechoic and grade 3 lesions are anechoic. Fiber alignment is performed in the longitudinal images and evaluates the fiber arrangement in the ligament. Grade 0 has 75% or greater parallel fibers. Grade 1 lesions have 50-75% parallel fibers, while grade 2 lesions have 25-50% parallel fibers. Grade 3 lesions have 25% or less parallel fibers.[51] Echogenicity and fiber alignment scores decrease as the lesion in the ligament heals. The degree of the ultrasonographic abnormality often reflects the severity of the lameness.[51]

Radiography can be used to image injuries to the proximal portion of the third metacarpal/metatarsal bone at the site of the suspensory ligament insertion. Abnormalities include sclerosis of the trabecular pattern in the dorsopalmar/plantar view, alteration of the trabecular pattern dorsal to the palmar/plantar cortex in the lateromedial view, or entheseophyte formation in the palmar/plantar aspect of the bone (Figure 1b).[1,2,49,50] Nuclear scintigraphy can also be used to help localize the region of injury with soft tissue and bone phase, but negative results do not preclude a suspensory ligament injury.[50]

The predominate treatment for suspensory desmitis is rest with slow return to controlled exercise.[2,50] Correct foot balance is also important in the healing process.[49] Many ancillary treatments have been tried including hypothermia, hyperthermia, support bandages, local and/or systemic administration of glucosaminoglycans, local administration of corticosteroids, therapeutic ultrasound and internal blister, but no scientific research has found these treatments beneficial to healing and return to exercise.[1]

Initially horses are placed on stall rest without exercise. Support bandages are applied if swelling is present in the leg. Hydrotherapy with cold water, icing, and administration of

non-steroidal anti-inflammatory medications can also be used to decrease the swelling.[49] Exercise is started with hand walking and slowly increased if ultrasonographic healing is present. Horses generally are not allowed ad lib turn out until the lesion has healed. Ultrasonographic healing included decreased cross sectional area of the lesion and ligament and decreased echogenicity and fiber alignment scores. The prognosis for recovery from a forelimb injury to the suspensory ligament is reported as high as 90% with chronic injuries requiring prolonged rest and rehabilitation.[2] Prognosis for recovery of proximal suspensory desmitis in the hind limbs is reported from 0 to 58%.[1,45] Chronic cases in the hind limbs have a very guarded prognosis with some horses suffering from progressive lesions.[2,50] Due to the poor prognosis, many ancillary treatments have been attempted.

Ancillary treatments for non-responsive desmitis have been recommended including local and/or systemic administration of glycosaminoglycans, local administration of corticosteroids, or an internal blister.[4] Newer treatments include injection of bone marrow or ACell® into the lesion, radial or focused pressure wave therapy of the lesion, fasciotomy and neurectomy, and surgical splitting of the injured portion of the ligament.[52-56] Although there are many new treatments available, healing time is still 3-6 months.

In horses treated with an intralesional injection of ACell Vet®[i] (urinary bladder matrix powder) 81.6% of horses with proximal suspensory desmitis and 66.7% of horses with suspensory branch desmitis were sound and in work.[53]This study combined results from both forelimb and hindlimb desmitis cases, although there were 38 hind limb cases and 28 forelimb cases.[53] Most hindlimb (36/38) cases were treated with a fasciotomy in addition to the ACell Vet ® injection.[53]

In another study bone marrow injection into the ligament had a higher success rate than rest alone.[52] Horses treated with intralesional injection of bone marrow into the suspensory ligament had similar healing rates of 84% returning to full work and soundness and 8% having a mild lameness that did not prevent return to work.[52] Fifteen

of these horses had a fasciotomy if the proximal suspensory ligament was greatly enlarged. Since there were 81 forelimb cases and 19 hindlimb cases and the results were combined, it is difficult to determine the effect on hindlimb cases, since forelimb cases often have a better prognosis than hindlimb cases.[52]

Radial pressure wave therapy is another ancillary treatment used in chronic cases of suspensory desmitis. Previous reports have only 17% horses with proximal hind limb suspensory desmitis could return to work with a rest and rehabilitation program, while with 3 radial pressure wave treatment 41% horses with hind limb proximal suspensory desmitis returned to work.[55] Results in the forelimb are similar to previous reported studies with rest and rehabilitation alone. Ninety percent of horses with forelimb desmitis resolved with rest and rehabilitation, while 53% with chronic, non-healing injuries resolved in 6 months with radial pressure wave therapy.[1,55]

Surgical splitting of tendons and the suspensory ligament has been used for several decades in cases of suspensory desmitis resistant to healing using conservative treatment.[56] The goal of the surgery is to decompress the acutely affected tendon or ligament and promote new blood supply in the chronic non-healing lesions.[56,57] Experimentally created defects in superficial digital flexor tendons initially healed with a fibrovascular tissue and after 24 weeks had the appearance of normal tendon.[58] This work suggests that healing can be stimulated by surgically invading the tendon or ligament to stimulate vascular ingrowth. Chronic injuries of the suspensory ligament maintain an anechoic core lesion and a failure of normal fiber alignment.[50,56] Histopathology reports of failing suspensory ligaments and chronically injured tendons describe unorganized fibroplasia, acellular regions of necrobiosis, and lack of vascularity.[56] One previous report of suspensory branch splitting describes treating the injured ligaments with a fan-like split to open the ligament in Standardbred trotters.[58] Fasciotomy has also been performed to reduce the effect of the compartment syndrome on the healing ligament but there is no report of proximal desmoplasty and its affect on healing of suspensory desmitis.[56] Surgical splitting has improved success compared to other treatments with 85% of proximal suspensory desmitis cases returning to work.[59] Fasciotomy combined

with lateral palmar neurectomy for rear limb proximal suspensory desmitis had similar results with resolution of lameness in 95% of horses suggesting that relief of the compartment pressure is important for the success in this type of case.[54]

The site of injury is related to the prognosis. The prognosis for recovery from a forelimb injury including the branches, body, and origin of the suspensory ligament is 90% with more chronic injuries requiring prolonged rest and rehabilitation.[2] Prognosis for recovery in hindlimb branch injuries is also approximately 90% whereas lesions in the origin and body is lower at 17%.[45] Chronic proximal suspensory desmitis in the hindlimbs have a very guarded prognosis with some cases having lesions which progressively worsen.[2,50] As the suspensory weakens with progression of the injury, lack of support results in hyperextension of the fetlock (Figure 1c). In these horses the ultrasonographic changes in the suspensory ligament continue to be abnormal, ligaments continued to enlarge with a poor prognosis for return of normal conformations or regular use.

There are few studies which have examined the microscopic structure of the injured suspensory ligament. In a small number of cases of chronic hind limb suspensory ligament injury assessed on post-mortem exam, the suspensory ligament was greatly enlarged with thickening of the plantar and fascia and periligamentous tissues.[1,2] Regions of injury have hypercellularity, acellularity, hemosiderin deposits, fibrosis, collagen hyalinization, neovascularization, and intraligamentous chondroid metaplasia.[1,2] There was also evidence of compression of the adjacent peripheral nerves.[1,2]

Apoptosis

Morphology

Apoptosis is programmed cell death that is initiated by activation and transcription of specific genes.[10-12] Apoptosis is important in normal development, tissue homeostasis, defense against viral infections, and oncogenesis.[12,13] Apoptosis was first defined by Kerr when he observed characteristic histopathologic changes in dying cells without evidence of necrosis or inflammation.[60]

Apoptosis is characterized by cell shrinking, nuclear condensation, margination of the chromatin and ruffling of the plasma membrane.[61] The apoptotic cell separates from its neighbors and undergoes blebbing with condensation of the cytoplasm, causing an increase in cell density, and shrinking of the cell.[62,63] There is compaction of cytoplasmic organelles and condensation of nuclear chromatin to form dense granular caps. In the nucleus, nuclear pores disappear. The cell then divides into a cluster of membrane bounded bodies. The endoplasmic reticulum dilates and connects to the cell surface through gaping pits.[62,63] The apoptotic cells are phagocytosed by neighboring cells or nearby phagocytes. The apoptotic cells are removed rapidly without inflammation.[63,64]

Necrosis is characterized by cellular swelling with chromatin condensation, which leads to cellular and nuclear lysis with subsequent inflammation.[61] The rupture of the plasma membrane with external release of intracellular contents causes inflammation and secondary change.[65] It does not involve expression of new protein and mRNA or require ATP.[66] Some apoptotic cells undergo secondary necrosis if not phagocytosed by local cells. While undergoing secondary necrosis, the apoptotic membrane become permeable to trypan blue and propidium iodine stains, that had previously been impermeable. The cell also swells but the characteristic condensed chromatin remains in the cell. [63]

Functional Apoptosis

Apoptosis is important in developmental physiology. Apoptotic cells have been found in tadpoles during metamorphosis into adult frogs, in the formation of fetal hands, and in the formation of the adult nervous system.[12] Abnormal brain development has been seen with caspase 3, caspase 9, or Apaf-1 deficiency, while abnormal heart development is seen with caspase 8 or FADD deficiency.[67] (See Apoptosis pathways for explanation of the aforementioned molecules.) T-cell homeostasis is controlled by apoptosis to remove autoreactive lymphocytes or lymphocytes without a functional antigen receptor.[68] Virus infected cells can detect metabolic changes or foreign nucleic acid and respond with apoptosis to prevent the spread of infection or further viral replication. Apoptosis has also been found in cells with extensive DNA damage.[12] Apoptosis is also important in causing death of neoplastic cells.[14]

Apoptosis is also important in glucocorticoid induced changes in thymocytes.[62] When methylprednisolone succinate treated mouse thymocytes were studied, well organized chromatin condensation of chromatin was seen.[62]

Apoptosis Pathways

The pathway for apoptosis was discovered through the research on *Caenorhabditis elegans*. [69] The adult organism was easy to study since it was small and translucent. Once the pathway was determined, structural and functional homology was found with worm cells and human cells. For example two core genes *ced 9* and *ced 3* are homologous to the mammalian genes bcl 2 and ICE (interleukin converting enzyme), respectively. [63,69]

Apoptosis can be initiated through two different pathways, the extrinsic and intrinsic. [66,70] Induction of apoptosis can occur through ionizing radiation, hyperthermia, anti-tumor drugs, hypoxia, or growth factor deficiency.[19] The extrinsic pathway is receptor mediated through the death receptors, Fas and TNF-R1, which are located in the cell membrane. The intrinsic pathway involves the mitochondria and cytochrome C and is the target of oxidative stress.[70] Both processes are energy dependent. Hypoxia can induce apoptosis by increasing levels of p53 or p38, which are transcription factors that allow the extrinsic and intrinsic pathways to occur.[71]

The extrinsic pathway is initiated by death receptors in the plasma membrane.[72] This pathway is stimulated by extracellular hormones or agonists from the tumor necrosis factor (TNF) family. Members of the TNF family include TNFα, Fas/CD95 ligand, and Apo2 ligand/TRAIL (TNF associated apoptosis inducing factor).[69] Death receptors, Fas/CD95 and TNF-R1, bind Fas ligand and TNFα, respectively. Recruitment of intracellular Fas-associated death domain (FADD) occurs once the receptors bind their ligands.[66] The FADD binds and activates caspases. The death inducing signaling complex (DISC) is formed from the FADD, caspases, ligand, and receptor molecules. DISC activates initiator caspases, caspase 8, or caspase 10.[69] Caspases 8 and 10 activate downstream caspases 3 and 7, which cleave cellular substrate mediating the apoptotic

phenotype. TNF-RI also causes FADD to bind with TNF receptor-associated death domain protein to initiate apoptosis.[12] TRAIL also triggers apoptosis when binding ligands DR4 or DR5.[66] The extrinsic pathway can be linked to the intrinsic pathway by cleavage of Bid by caspase 8. [12]

The intrinsic pathway involves the mitochondria and starts with signals from the nucleus.[72] Triggers such as oxidative damage or ionizing radiation can cause apoptosis.[20] When stimulated by specific triggers, cytochrome C is released from the mitochondria and forms a complex with Apaf-1 (Apoptotic protease activating factor), and ATP, which is called the apoptosome.[12] The apoptosome and apoptosis-initiating factor activate caspase 9, which then causes activation of caspase 3. [12,70] Caspase 3 causes DNA fragmentation after activation of ICAD (Inhibitor of caspase-activated DNAse) and membrane blebbing after activation of ROCKI (Rho-associated kinase). PARP (poly-ADP-ribose polymerase), a DNA repair enzyme, is inhibited, allowing cytoskeleton collapse. Degradation of nuclear factor - kappa B and Bcl 2, an anti-apoptotic molecule, occurs.[12] Nuclear factor kappa B is important for immune response, inflammation, and cell cycle regulation.[73] Caspase 3 also activates caspase-activated DNAse (CAD) which cuts the genomic DNA between the nucleosome to generate the characteristic 180 base pair fragments that form the DNA ladder.[74]

The apoptotic cells are removed by neighboring cells or by macrophages. Apoptotic lymphocytes express phosphotidylserine on the outer cell surface, instead of the inner cell membrane, due to loss of membrane phospholipid symmetry and due to degradation of flippase. (Flippase is responsible for retaining phosphotidylserine in the inner leaflet of the cell membrane.).[66,68] This allows phagocytosis of the apoptotic cells prior to cell lysis.[14] The phosphotidylserine exposure also promotes procoagulant activity and production of immunosuppressive cytokines, IL-10 and TGF-β.[14,66] Macrophages have also been found to bind an abnormal or immature sugar on the outer cell surface with a macrophage lectin. Macrophage vitronectin receptors can bind a negatively charged particle on cell surface.[14,68]

Although apoptotic cells usually are cleared without inflammation, apoptotic vesicles can spread an infection if they carry microbial compounds. The bacterial molecular conserved regions, called pathogen-associated molecular patterns, can be recognized by inflammatory cells to trigger inflammation through toll-like receptor activation.[66] Following uptake by inflammatory cells, lysosomes are used to destroy the apoptotic vesicles. If the vesicle contained a *mycobacterium*, lysosomal acidification is inhibited allowing survival of the organism.[75]

Apoptosis Control

Control of apoptosis is by the anti-apoptotic Bcl-2 family, inhibitor of apoptosis proteins (IAPs), and inhibitors of the inhibitory molecules. The Bcl-2 gene is an inhibitor of apoptosis by preventing translocation of the normal physiologic signals for cell death.[12] Members of the Bcl-2 family, such as Bcl-2, Bcl-xL, and Mcl-1, inhibit apoptosis by forming heterodimers with pro-apoptotic molecules to prevent cytochrome C release by the mitochondria.[72] Bcl-2 has blocked cell death triggered by growth factor withdrawal, glucocorticoid treatment, p53 overexpression, heat shock, and overexpression of cysteine proteases.[12] IAPs, such as XIAP, IAP-1, and IAP-2, inhibit caspases at various levels to stop the apoptotic pathway.[72] XIAP inhibits the active caspase9/caspase 3 complex. Caspase O is inhibited by the caspase homologue FLIP.[74]

When death receptors are activated, anti-apoptotic molecules area also activated. TRAF2 and IKK activate nuclear factor- kappa B to activate gene expression of IAPs, Bcl-xL, and inducible nitric oxide synthase.[72] Once nuclear factor kappa B is inhibited by proteosomal inhibition, there is protein synthesis inhibition, or overexpression of the inhibitor of nuclear factor kappa B.[72] Binding of insulin, IGF, and other growth factors, activate and cause formation of the Akt/protein kinase B, which inactivates pro-apoptotic BAD and other anti-apoptotic signals.[72] C-FLIP is an important antagonist to death receptor signal transduction by inhibiting activation of caspase 8 by FADD.[76]

SMAC/Diablo molecules from the mitochondria inhibit IAPs, such as XIAP, and Bcl-2 molecules, to promote apoptosis. Other pro-apoptotic molecules from the mitochondria

which are released during cytochrome C release include AIF (apoptosis inducing factor), endonuclease G, and HtrA2/Omim which cause chromatin condensation and degradation of DNA and IAPs. Drp1, a mitochondrial protein, forms complexes with Bax and other proapoptotic Bcl-2 members to promote outer mitochondrial membrane permeabilization and further cytochrome C release.[72] Although Bid and Bax are members of the Bcl-2 family, they promote apoptosis by forming pores in the mitochondria to allow cytochrome C release. [12] Bid's activity is increased after caspase 8 cleavage. [74] The endoplasmic reticulum increases calcium stores under the influence of Bax and Bak. The calcium stores are released under oxidative stress to provide calcium for the calcium dependent mitochondrial permeability transition pores (MPT) and for activation of phospholipase A2, which also promotes MPT.[72]

Pathogens can also trigger apoptosis by stimulating the immune system. Activated lymphocytes and macrophages release TNFα, which binds to death receptors to activate apoptotic pathways.[66] Stimulated T cells can also express FASL to bind to FAS and cause apoptosis.[66] *Mycobacterium tuberculosis* activates myeloid differentiation factor 88 to cause apoptosis through the recruitment of FADD and caspase8.[66]

Different signaling pathways for apoptosis are used in different cells. Cytotoxic drugs and ultraviolet radiation cause caspase 9 and Apaf-1 mediated apoptosis in lymphocytes through mitochondrial disruption.[67] TNF activation causes caspase 8 and FADD associated apoptosis in lymphocytes through the extrinsic pathway.[67] Caspase 3 is important in activation in lymphocytes activated by either the intrinsic or extrinsic pathways.

Apoptosis Detection

Several methods exist to detect apoptosis. Characteristic morphologic changes with the characteristic chromatin and cytoplasmic condensation and typical DNA ladder are the gold standard for detection of apoptosis.[19] Flow cytometry can be used to detect light scatter properties and increased DNA sensitivity to denaturation.[19] Other methods to

detect apoptosis include antibodies to the pro-apoptotic proteins, the TUNEL assay, DNA laddering, or the Annexin V assay.

The TUNEL assay is an in-situ terminal transferase-mediated, nick-end labeling assay, that detects DNA strand breaks resulting from cleavage by endonucleases in early and advanced apoptosis.[19] The DNA ladder analyzes for DNA fragmentation with an agarose gel electrophoresis to detect fragmentation characteristic of apoptosis.[19] The ladder is more specific for apoptotic cells than the TUNEL assay.[19] The Annexin V assay has a calcium dependent molecule with a high affinity for phosphotidylserine, which is exposed on the plasma membrane surface in early apoptosis.[77]

The TUNEL assay is widely used to detect apoptotic cells. The TUNEL assay was found to label apoptotic cells that were confirmed to morphologically have apoptosis.[78] Necrotic cells are routinely not stained by the TUNEL stain.[79,80] The TUNEL method uses terminal deoxynucleotidyl transferase (TdT) to bind 3'-OH ends of DNA, which is more specific than in situ nick translation method.[81] The TdT signal is amplified by avidin-peroxidase to allow identification with light microscopy.[81] Advantages of the TUNEL assay include direct labels of the 3' hydroxyl termini of DNA strand breaks, detection of apoptotic nuclear changes prior to morphologic changes, and measurement of DNA content.[19] Pretreatment with proteinase K and use of TdT are important to have accurate labeling of apoptotic cells, which are both components of the Apop Tag Peroxidase kits.[82] Microwave pretreatment can also improve TUNEL sensitivity without changing specificity.[79] TUNEL method has been used to successfully detect apoptosis in a variety of cells from equine tendinocytes, extra ocular muscle tendons, and Schwann cells.[16,83,84] Successful staining of apoptotic rat thymic lymphocytes has been performed with the Apop Tag Peroxidase Kit.[81]

One disadvantage of the TUNEL technique include non-specific labeling of long term formalin fixed tissue. [85] The formalin can replace protons in the purine and pyramidine bases.[85] Prolonged fixation can also cause single stranded DNA to become unavailable for binding to the polymerase, which could cause detection false negative results to

occur.[85] Large, uneven tissue samples are also difficult to stain with the TUNEL kits, which can cause blanks areas in the tissue section and specifically labeled areas in other tissue regions.[79]

Apoptosis in Tendons and Ligaments

In human studies, apoptosis has been evaluated for its role in tendon degeneration.[15,77,86-89] Current research in tendinopathies has been evaluating the presence of the pro-apoptotic proteins and apoptotic cells in degenerative tendons. In tendon degeneration, histological changes include collagen degeneration, fiber disorientation, increased mucoid ground substance, and an absence of inflammation.[9] Degenerative rotator cuff tendons had twice as many apoptotic cells compared to normal subscapularis tendons.[15] There was an even distribution of apoptotic cells throughout the torn edges of the tendon.[15] All of the apoptotic cells were fibroblasts or fibroblast-like cells.[15] Increased apoptotic cells were also found in degenerative regions of peri-articular tendons than normal regions.[90]

Inflamed equine superficial digital flexor tendon had disarrangement of tendon fibers and an increase in the number of tendon fibers with a decrease in the diameter of tendon fibers.[16,91] The injured equine superficial digital flexor tendon also had increased pro-inflammatory cytokines, growth factors, and enzymes.[16,91] Increased levels of caspase 3 was also found in the inflamed tendons.[16] Inflamed equine superficial digital flexor tendons had higher numbers of apoptotic cells compared to normal tendons.[16] The apoptotic cells were tendinocyte-like cells, as seen in the injured human rotator cuff tendons.[15,16] The increased number of apoptotic cells was associated with a decrease in the number of tendinocytes.[16]

A partial laceration of the flexor digitorum profundus tendon in rabbits revealed a small amount of apoptosis in the endotenon adjacent to the laceration one week after the laceration, but not at 12 weeks.[89] The epitenon and the synovial sheath never stained positively for apoptosis.[89] Increased apoptosis, which resolved once the tendon started to

heal, appeared in the endotenon due to altered tension.[89] It appears that an acute tendon injury from a laceration is different from degeneration since there is minimal apoptosis in the acute injury.

Mechanisms for apoptosis in tendinopathy include oxidative stress that can activate cytochrome c release or activation of stress-activated-protein kinases (SAPK).[9,92] The effects of oxidative stress and mechanical strain on tendons have been studied Apoptosis can be induced by exposure to reactive oxygen species via hydrogen peroxide or superoxide.[9,87] Oxidation of important molecules may lead to the apoptosis seen in tendon degeneration.[87] Exposure to hydrogen peroxide in low concentrations led to apoptosis through cytochrome c release and caspase 3 activation, but higher concentrations caused cell necrosis.[86] Perioxiredoxins, a family of anti-oxidant enzymes, have been found to be up regulated in tendon degeneration and directly participate in oxidative stress protection by elimination hydrogen peroxide and other oxidizing molecules.[87] The level of perioxiredoxins increased in tendon cells exposed to hydrogen peroxide, but was also present in the control tendon cells.[87] Perioxiredoxins are constitutively expressed in tendon cells and can be up regulated in response to oxidative stress.[87]

In Achilles tendon tendinocytes, anoxia caused apoptosis.[71] This apoptosis was inhibited if the cells were treated with insulin like growth factor 1 (IGF-1), which activated protein kinase B to prevent phosphorylation of cytoplasmic and nuclear targets used in apoptosis.[71]

Activation of stress-activated-protein kinases occurred with increased duration and weight of the load.[9,15] This led to increased apoptosis in the tendon fibroblasts. Mechanical testing with strain applied to fibroblasts showed an increased expression of HSP 72, which mediated apoptosis.[77] HSP 72 can inhibit cell proliferation and apoptosis.[77] Cyclic longitudinal strain was applied to patellar tendon fibroblasts and caused a moderate increase in HSP 72 with a minimal increase in apoptosis after 2 days of moderate repetitive stress.[77] Another study also found that a longer stretch period

decreased the apoptotic rate in patellar tendon fibroblasts in vitro.[89] The decreased apoptotic rate is thought to be caused by stress tolerance, an inactivation of JNK1/JNK2, or a increased expression of HSP72.[10,77] These mechanisms could be important for tendon and ligament rehabilitation to decrease the number of apoptotic cells.

Other studies on injured tendons revealed increased levels of matrix metallo-proteinases 2 and 9 in inflamed tendons.[93] The apoptosis-promotor gene p53 binds the matrix metallo-proteinases 2 and 9, which may increase apoptosis and decrease collagen deposition by reducing the number of fibroblasts or up regulating collagenase activity.[16,93] This may be the reason some of the tendons and/or ligaments have poor healing.

Nitric oxide can also induce apoptosis in chondrocytes, synviocytes, meniscal cells, and anterior cruciate ligament cells.[17] When medial collateral ligament cells were compared to anterior cruciate ligament cells in rabbits with nitric oxide treatment, the anterior cruciate ligaments were more sensitive to apoptosis than the medial collateral ligament.[17] The mechanism for this difference was not found although the morphology of the cells varies.[17]

In recent studies on the histology of ruptured canine cruciate ligament, it has been found that the ruptured ligaments contained a higher number of nonviable cells, but not a higher number of apoptotic cells.[18] It was felt that the cells in the core region died due to necrosis and not apoptosis.[18] Elongation of crimping and collagen disruption in adjacent fascicles suggest progressive mechanical overload that led to fiber disruption.[18]

The increased levels of pro-apoptotic molecules and apoptotic cells in injured tissue may lead to the breakdown of tendon function.[9] Excessive apoptosis may lead to weakness of tendon tissue and increased risk of tendinopathy, which could make the tendon prone to the initial injury and prone to re-injury during the healing process.[15,16] Some injured tendons rupture during the healing process after treatment with glucocorticoids. Glucocorticoids decrease the viability of tendinocytes, suppressed cell proliferation, and

decrease the amount of collagen synthesis, but not via apoptosis.[94] This anti-inflammatory therapy appears to prevent healing, but not via apoptosis.[94]

Future of apoptosis

With the current knowledge about apoptotic pathways, apoptotic modulating therapies are being developed. Mediators to block Bcl-2 activity would be useful to allow normal apoptosis in B-cells with follicular lymphoma.[76] Anti-sense molecules are being developed to control apoptosis. An anti-sense BcL-2 binds to the BCL-2 DNA to prevent transcription of the gene, which has resulted in increased chemosensitivity of tumors to chemotherapeutic agents in mice.[76] By focusing on the death receptors, such as TRAIL, apoptosis could be targeted in cancerous cells.[76] Further understanding of apoptosis is crucial to help treat neoplasias and other injuries where apoptosis occurs. Since apoptosis appears to also be an important component in human and equine tendon injury, determining if apoptosis exists in equine suspensory ligaments is important. The applications techniques to reduce apoptosis in chronic non-healing tendonitis and desmitis may be of help with the treatment if equine suspensory ligament injuries.

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Characterization of Apoptotic Cells in Equine Proximal Suspensory Desmitis

By Christina Hewes, DVM, Nathaniel White, DVM, MS,DACVS, John Robertson, DVM, PhD, Virginia Buechner-Maxwell, DVM, PhD, DACVIM

From the Marion duPont Scott Equine Medical Center (Leesburg, Virginia), the Department of Pathobiology, and the Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and University, Blacksburg, Virginia.

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Attribution

Descriptions of the qualifications of the authors.

Christina Hewes, DVM:
Equine surgeon resident at the Marion DuPont Scott Equine Medical center, who was enrolled in graduate studies at Virginia Polytechnic and State University for a masters degree. She performed the sample collection, sectioning, staining, reviewed the slides, and prepared the manuscript.

Nathaniel White, DVM, MS,DACVS
Dr. White was head of the masters committee. He helped plan the masters project, stain the slides, review the slides, and reviewed the manuscript. He is the director and surgeon at the Marion DuPont Scott Equine Medical Center.

John Robertson, DVM, PhD
Dr. Robertson is a member of the pathology faculty in the Virginia-Maryland regional College of Veterinary Medicine. He helped plan the masters project, review the slides, and reviewed the manuscript.

Virginia Buechner-Maxwell, DVM, PhD, DACVIM
Dr. Maxwell is a member of the large animal clinical sciences faculty in the Virginia-Maryland regional College of Veterinary Medicine. She helped plan the project, provided the l;aboratory and technical assistance for the project, and reviewed the manuscript.

Abstract
Objective: To report histopathological changes and apoptosis in normal and degenerative proximal suspensory ligaments.

Animals: Proximal suspensory ligament specimens were collected at necropsy. Four horses had degenerative suspensory injury (8 ligaments) and 6 horses had normal suspensory ligaments (19 ligaments.)

Procedure: The ligaments were stained with hematoxylin/eosin (H/E) and Masson's trichrome stain to detect histopathologic changes. A TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling technique) was performed to detect apoptosis. The number of apoptotic cells per high power field was recorded in all ligaments.

Results: The injured suspensory ligaments in this study had fibroplasia, neovascularization, collagen degeneration, tissue metaplasia, and ligament fiber mal-alignment. There was a trend for increased apoptotic cells in injured suspensory ligaments.

Conclusion: The injured ligaments had histological signs of degeneration and remodeling. The apoptosis in the injured ligaments could be consistent with chronic injury and remodeling in the ligament.

Introduction

Causes of ligament injury include acute overloading, laceration or overstrain.[1-3] Human injuries are often from acute overload. Overstrain, which causes degeneration of the ligament, creates cumulative micro-damage to the region and is thought to be the common cause of suspensory ligament injury in horses.[1,2]

Ligament healing has been classified in 3 phases based on healing in the rabbit medial collateral ligament of the femoro-tibial joint.[4] Hemorrhage and edema associated with inflammation are present during the first phase. The ligament ends retract, a blood clot is formed, and a large number of cells infiltrate the injured region.[4] In humans acute injury is characterized by a hypertrophic vascular response and hypertrophic fibroblasts intially.[5] The second phase of healing is characterized by cellular and matrix proliferation.[5] This new tissue is disorganized with increased levels of blood vessels, fat cells, inflammatory cells, fibroblasts, and loose connective tissue than normal ligament.[4] The third phase of healing includes matrix remodeling where the matrix becomes denser filling defects but with altered matrix components. Altered proportions of collagen types, immature collagen cross links, small collagen fibril diameter, abnormal innervation, altered cell connections, and persistent matrix defect persist in the healed ligament.[6-9] In chronically injured ligaments, there is a slight increase in total collagen mass, collagen turnover rate, and total glucosaminoglycan concentrations.[6] There is also a slight decrease in collagen concentration and an altered collagen type ratio with increased type III collagen.[6]

The cellular response in ligament and tendon injury includes the presence of the pro-apoptotic proteins and apoptotic cells. Apoptosis, a form of program cell death, is important for embryogenesis, tissue homeostasis, and to remove unwanted cells with minimal inflammation by causing death of virally affected and neoplastic cells.[10,11] Apoptosis has been detected in degenerative rotator cuff tendons with an even distribution of apoptotic cells throughout the torn edges of the tendon.[12] The apoptotic cells were tendinocyte-like cells, as seen in the injured human rotator cuff tendons.[12,13] Equine superficial digital flexor tendons had higher levels of apoptotic cells in inflamed

tendons versus normal tendons.[13] Apoptotic cells were also found adjacent to a partial laceration in the flexor digitorum longus tendon in rabbits at one week post-injury.[14] When medial collateral ligament cells were compared to anterior cruciate ligament cells, the anterior cruciate ligaments were more sensitive to apoptosis than the medial collateral ligament. [15] In injured canine cruciate ligaments, necrosis rather than apoptosis was found.[8]

One of the techniques to detect apoptosis in tissue specimens is the TUNEL assay. TUNEL is an in-situ terminal transferase-mediated, nick-end labeling assay, that detects DNA strand breaks resulting from cleavage by endonucleases in early and advanced apoptosis.[16] It can distinguish apoptotic cells from necrotic cells and has been used successfully to detect apoptosis in human and canine tendon and ligament studies.[8,12]

Suspensory desmitis is a common problem in equine athletes. Minimal research has been performed to define the pathogenesis and histopathologic changes in relation to the clinical signs. Published descriptions of equine suspensory ligament histopathology are rare. Though front limb suspensory ligament desmitis has a good prognosis, proximal suspensory injury in the rear limbs has a poor prognosis when treated with rest or shockwave therapy.[17,18] One previous post-mortem study on chronic hind limb suspensory desmitis cases found hypercellularity, acellularity, hemosiderin deposits, fibrosis, collagen hyalinization, neovascularization, and intraligamentous chondroid metaplasia.[19,20] These lesions suggest a chronic non-healing desmitis which is unable to respond to the normal phase of ligament remodeling.

The presence or importance of apoptosis in non-healing suspensory ligaments has not been elucidated. Since apoptosis is known to play a role in cell replacement and remodeling the authors hypothesized this cell response was present in equine degenerative suspensory ligament desmitis along with histopathologic changes suggestive of chronic inflammation and failure of ligament remodeling.

The objectives of this study were to define the histopathologic changes in degenerative and normal proximal suspensory ligaments and to identify and quantify the number of cells undergoing apoptosis in normal and diseased suspensory ligaments.

Materials and Methods

Proximal suspensory ligament specimens were collected at necropsy. The ligaments from 4 horses with chronic suspensory injury (8 ligaments) (Figure 1) and from 6 horses without suspensory ligament injury (19 ligaments) were collected. One horse with a chronic suspensory desmitis had injuries in one rear limb and one front limb. In this horse enlargement was evident on ultrasound but excessive fetlock dorsiflexion was not evident. The normal ligaments were from horses with splenic rupture (1), lymphosarcoma (1), neurological disease (1), rectal tear with peritonitis (1), severe right front pastern osteoarthritis (1), and normal horse in terminal research study (1.) The specimens were collected immediately after death (within 5 minutes after lethal injection with concentrated sodium pentobarbital). The proximal one half of the third metatarsal bone was removed from the horse by sawing through the center of the diaphysis and through the distal intertarsal joint. The skin and tendons were removed from the bone. The third metatarsal bone with the suspensory ligament attachment intact was placed in neutral-buffered 10% formalin and immersed for a minimum of one week. Fixed tissues were first sectioned by band saw, and then soft tissues isolated by dissection. Longitudinal and cross sections of suspensory ligaments at or adjacent to sites of injury were prepared, and comparable sections were taken from unaffected horses for comparison.

Tissues were dehydrated and embedded in paraffin. Serial sections 3 microns thick were made and stained with hematoxylin/eosin (H/E) and Masson's trichrome stain. Sections were examined in a blinded manner; the pathologist evaluating the slides had no knowledge of clinical presentation or history. For the purposes of clarity, Masson's trichrome stain is used to differentiate mature collagen, from muscle and intercellular fibers. For this evaluation, H/E sections were first evaluated and then Masson's trichrome stained slides of the same sections were compared with the H/E sections.

Serial sections of each specimen were also stained with the TUNEL technique for apoptotic cells by use of a commercially available kit.[a, 21] All reagents were contained in the kit unless otherwise specified. Unstained ligament sections were deparaffinized and transferred to PBS solution for 5 minutes. The tissue sections were pretreated with 20 µg of proteinase K/mL[b], washed in dH20, quenched with hydrogen peroxide (3.0%), covered in 75 µg of equilibration buffer/mL, and incubated with 55 µg of terminal deoxynucleotidyl transferase (TdT) enzyme/mL for 3 hours in a humidified chamber. The reaction was terminated with stop-wash buffer, and the tissue sections were incubated with 65 µg of antidigoxigenin conjugate/mL for 30 minutes in a humidified chamber and washed with PBS solution. The tissue sections were stained with 75 µg of diaminobenzidine (DAB)/mL, washed, counterstained with methyl green, and mounted. As a detection control for the TUNEL technique, sections of thymus were taken from untreated (negative control) mice and mice treated with dexamethasone (positive control).[22,23] Thymus specimens were stained simultaneously with ligament specimens to ensure the staining technique was effective and apoptotic cells were being detected.

The TUNEL method uses terminal deoxynucleotidyl transferase (TdT) to bind 3'-OH ends of DNA, which is more specific than in situ nick translation method. [24] The TdT signal is amplified by avidin-peroxidase to allow identification with light microscopy. [24] Pretreatment with proteinase K and use of TdT are important to have accurate labeling of apoptotic cells, which are both components of the ApopTag Peroxidase kits. [25] Microwave pretreatment can also improve TUNEL sensitivity without changing specificity. [26] TUNEL method has been used to successfully detect apoptosis in a variety of cells from equine tendinocytes, extra ocular muscle tendons, and Schwann cells.[13,27,28] Successful staining of apoptotic rat thymic lymphocytes has been performed with the Apop Tag Peroxidase Kit.[24]

Stained slides for apoptosis were examined in a masked manner and scored for the presence or absence of apoptotic cells. The H&E-stained sections were used to identify abnormal cells and compare the results to the TUNEL stained slides. The ligaments were

examined for fibroplasia, tissue metaplasia, neovascularization, collagen production, hypercellularity, and ligament fiber change. Changes in the ligaments were graded as normal, mild, moderate, or severe. Mild changes were focal, while moderate to severe changes were diffuse.

In each specimen, the apoptotic cells located in each ligament were counted at 400× magnification. The mean number of cells in five 400x fields was recorded for each slide. An Altman's normogram was performed to determine study numbers. A power of 80 with a significant difference of $p< 0.05$ was used with a prevalence of apoptosis in injured ligaments of 19% with a normal prevalence of 0.5%, which was found in injured equine superficial digital flexor tendons.[13] For this criteria, 34 ligaments were needed in each group. Due to the inability to obtain more abnormal suspensory ligaments, the number of specimens for statistically significant results could not be met. A student's T-test (two tailed) was performed on the data obtained.

Results

Of the 27 specimens collected, 7 of the control ligaments and 1 of the affected ligaments were not fully evaluated due to poor slide quality. Normal ligaments had parallel alignment of light pink collagen with dark blue colored nuclei in fibroblasts (desmocytes) present in parallel rows. Normal mature collagen stained red with Masson's trichrome stain due to the thickness of the ligament sections.[29] Ground substance and fascia components had a blue stain. (Figure 4a and 4b) Abnormalities in the control ligaments included mild, focal neovascularization in 2 ligaments and multifocal cartilage inlets in 1 ligament. One ligament had mild, focal, novel fibroplasia, as seen with large area of blue staining tissue in an irregular pattern on Masson's trichrome stain. Two ligaments had moderate hypercellularity. Nine of the control ligaments had normal histology.

Histopathological changes seen on the H&E-stained slides of non-healing degenerative ligaments included fibroplasia, mineralization, neovascularization, collagen degeneration, and irregular ligament fibers in the injured ligaments. Fibroplasia was seen in 4 affected ligaments and was graded as mild (1), moderate (2), and severe (1) (Fig 3a and b).

Mineralization was multifocal in 3 ligaments and was graded as mild (2) and moderate (1) (Fig 5b). Neovascularization was mild to moderate in 1 ligament and marked in 1 ligament (Fig 7). Architecture changes were seen in 5 affected ligaments and were graded as mild (3), moderate (1) and marked/severe (1) (Fig 3 a, 3b and 7). The mild changes were focal, while the moderate to severe changes were diffuse. The marked changes also disrupted the normal architecture. Mature remodeling was seen in 1 ligament, 2 affected ligaments had small ligamentous bundles within the ligament, and 2 affected ligaments had multi-focal generalized changes to the architecture. Areas of increased cellularity were seen in the abnormal ligaments with increased numbers of round cells (Fig 3a, b). Chondrones (areas of cartilage-like round cells with cartilage material around the cells) were seen in 2 ligaments (Fig 5). One of the affected ligaments had normal histology. Evaluation of Masson's trichrome stained sections confirmed, novel fibroplasia associated with the presence of significant numbers of fibroblasts, lack of new collagen production, and generalized cell and fiber disorganization.

The control ligament stained with the TUNEL assay had few apoptotic cells.(Figure 2a) The mean number of apoptotic cells in the injured ligaments (Figure 2b) per high powered field was 12 (0.2-39.4) compared to 5 (0-44.2) in the normal ligaments (P= 0.17) on a student's T test. There was a trend for the mean number of apoptotic cells to be elevated in the injured ligaments. No relationship was seen with horses with a longer duration of clinical signs and histopathologic changes or amount of apoptotic cells.

Discussion

The histopathologic changes of the injured suspensory ligaments in this study were similar to those previously reported. The histological changes included fibroplasia, neovascularization, acellular regions of collagen degeneration, chondroid metaplasia with mineralization and, and fiber disruption and malalignment. Areas of hypercellularity with fibroplasias were adjacent to hypocellular areas with lack of fibers and diffuse collagen necrosis. These changes were consistent with repeated attempts at matrix remodeling without maturation of the tissue into normal ligament. The mineralization

present was likely due to chronic injury at the site of attachment of the ligament to the third metatarsal bone.

Chronic progressive suspensory desmitis with failure of fetlock support as seen in three of the horse in this study horses had areas of hypercellularity adjacent to acellularity, hemosiderin deposits, fibrosis, collagen hyalinization, neovascularization, and intraligamentous chondroid metaplasia.[19,20] In the injured ligaments in this study and in Dyson's study ligament scar remained slightly disorganized and hypercellular with isolation of collagen bundles distant from any blood supply and death of desmocytes or transformation into chondrocytes.[20,30] Cartilage-like tissue was produced resulting in an inelastic ligament that is at higher risk for re-injury.[31]

The lack of an increased vascular response could be a factor in the diminished healing potential of ligaments. Since neovascularization was seen in isolated portions of the injured ligaments, it could be a factor in suspensory ligament healing similar to what is found after tendon injury.[32] Location of ligament injury also affects healing. The midsubstance of the medial collateral ligament has improved healing and decreased laxity than at sites at either bone-ligament attachment. The bone near the ligament attachment may limit the size of scar and increase the stresses at this location.[33] The altered healing at bone attachments could be factors in the healing of suspensory desmitis in the horse. Because 6 of the injured ligaments were from horses with abnormal dorsiflexion of the rear fetlocks, the stress at the ligament origin may have been excessive creating a poor environment for healing or remodeling.

Multiple artifacts of preparation were seen in many sections including tissue folds, fragmentation of ligament bundles in cross section, and the presence of mineralized bone dust from sawing. While these artifacts affected section quality, they did not significantly interfere with interpretation, except small bone fragments, which could not be easily differentiated from foci of dystrophic mineralization. The artifacts in ligament cross sections frequently had artifacts due to the difficulty in obtaining a clean microtome cut perpendicular to the ligament fibers.

There was a trend for increased apoptosis in the injured ligaments versus the normal ligaments. The large range observed in ligaments considered normal may be the normal occurrence in equine suspensory ligaments or the ligaments collected from some of the horses were not normal. Since the injured ligament in the study had increased apoptotic cells and had a history of chronic injury, it is likely that the apoptosis in our cases was due to persistent degeneration and chronic injury.

The presence of apoptotic cells in the degenerative ligaments is similar to the apoptosis seen in the chronic injury in human rotator cuff tendons.[12] Degenerative rotator cuff tendons had twice as many apoptotic cells compared to normal subscapularis tendons and there was an even distribution of apoptotic cells throughout the torn edges of the tendon.[12] Apoptotic tendinocytes were also seen in partial laceration of the flexor digitorum profundus tendon in rabbits acutely, but no apoptotic cells were found 12 weeks post laceration.[14] Apoptosis may be beneficial in early healing of ligament and tendon injuries, but could represent persistent remodeling in chronically injured structures.

The TUNEL assay, which was used in this study, was used to label apoptotic cells that were confirmed to morphologically have apoptosis without labeling necrotic cells. [34,26,35] Advantages of the TUNEL assay include direct labels of the 3' hydroxyl termini of DNA strand breaks, detect of apoptotic nuclear changes prior to morphologic changes, and measurement of DNA content.[16] The disadvantage of the TUNEL technique include non-specific labeling of long-term formalin fixed tissue. [36] The formalin can replace protons in the purine and pyramidine bases.[36] Prolonged fixation can also single stranded DNA to become unavailable for binding to the polymerase, which could cause detection false negative results to occur. [36] Because some of the injured suspensory ligaments in this study were kept in formalin for extended periods, it is possible that the number of apoptotic cells detected was decreased. Similarly large, uneven tissue samples, such as the suspensory ligaments, are also difficult to stain with the TUNEL kits. Staining this

type of tissue can cause blanks areas in the tissue section and specifically labeled areas in other tissue regions.[26]

There were significant histological changes in the injured suspensory ligaments, consistent with poor healing. Necrobiosis, neovascularization, and proliferative fibrosis were consistent with a chronic inflammation in non-healing ligament, persisting in a remodeling phase. The reaction in these ligament lesions can be difficult to define as to the stage of healing because they are chronic injuries. The degenerative ligaments appear to be in phase 2 and/or 3 healing based on the rabbit collateral ligament model.[4] Although suspensory ligament injury is commonly referred to as a chronic desmitis, no inflammatory cells such as neutrophils were present in the normal or injured segments. This is consistent with the remodeling stage of ligament healing.[4] Overall, apoptotic fibroblasts tended to be increased in the injured ligaments, but the low ligament numbers may not have allowed adequate discrimination when a wide range of response injury or subclinical injury. Further evaluation of normal suspensory ligaments is needed determine if the changes in acute injury and chronic injury respond with a significant increase in apoptotic cells.

[a] Apop Tag® *In Situ* Apoptosis Detection Kit, Intergen, Co., Purchase, NY

[b] Proteinase K, Sigma, St. Louis, MO

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List of Figures

Figure 1a: Ultrasound image of proximal suspensory desmitis on the hindlimb, which is the early stage of degenerative condition of the ligament in the injured cases in the study. The image shows hypoechoic fibers and a lack of long, parallel fibers at the origin of the suspensory ligament on the proximal third metatarsal bone.

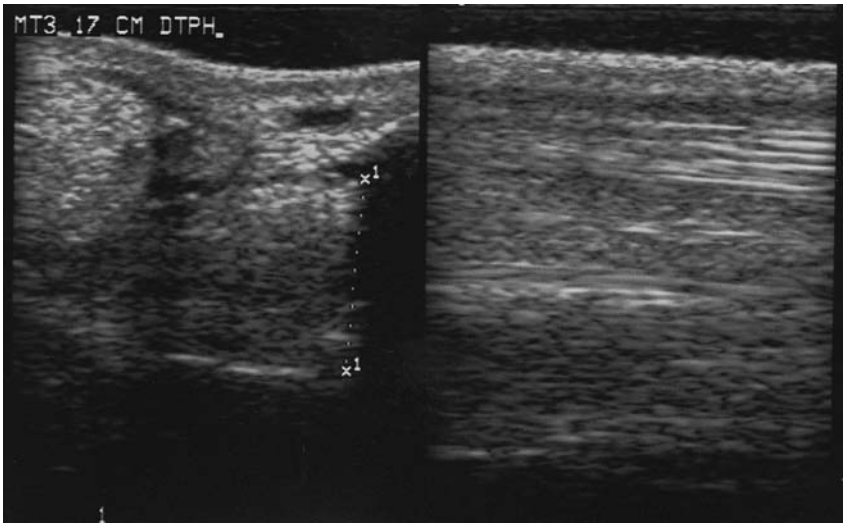


Figure 1b: A radiograph of proximal third metatarsal bone at the origin of the suspensory ligament. Note the irregular new production of bone at the ligamentous insertion site.

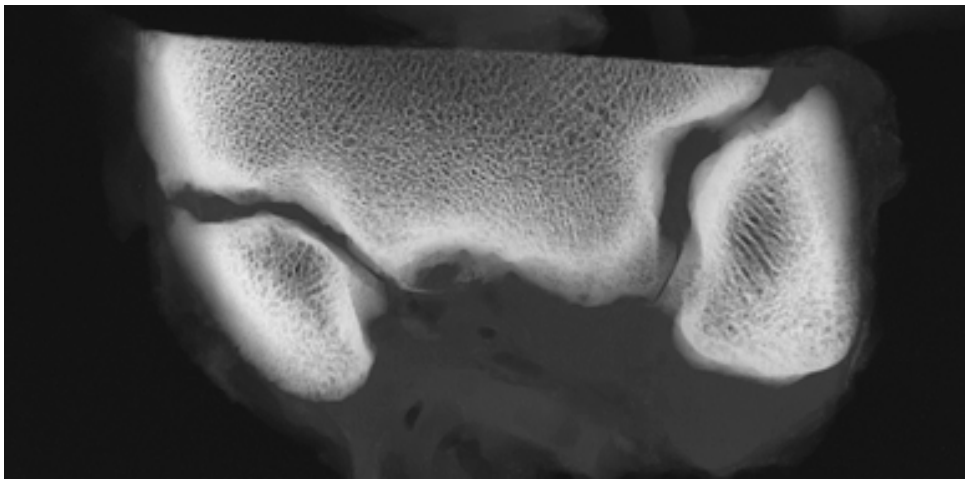


Figure 1c: Horse with degenerative suspensory desmitis of the hind limbs. The horse has the characteristic hyperextension of the fetlock joints.

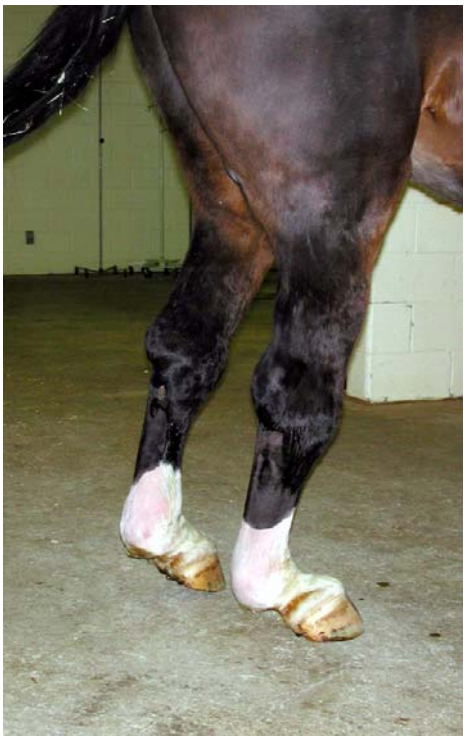


Figure 2a: Photomicrograph of a section of a normal suspensory ligament. Healthy or necrotic cells (fibroblasts) are stained light blue with the counter stain in the normal ligament.(151x)

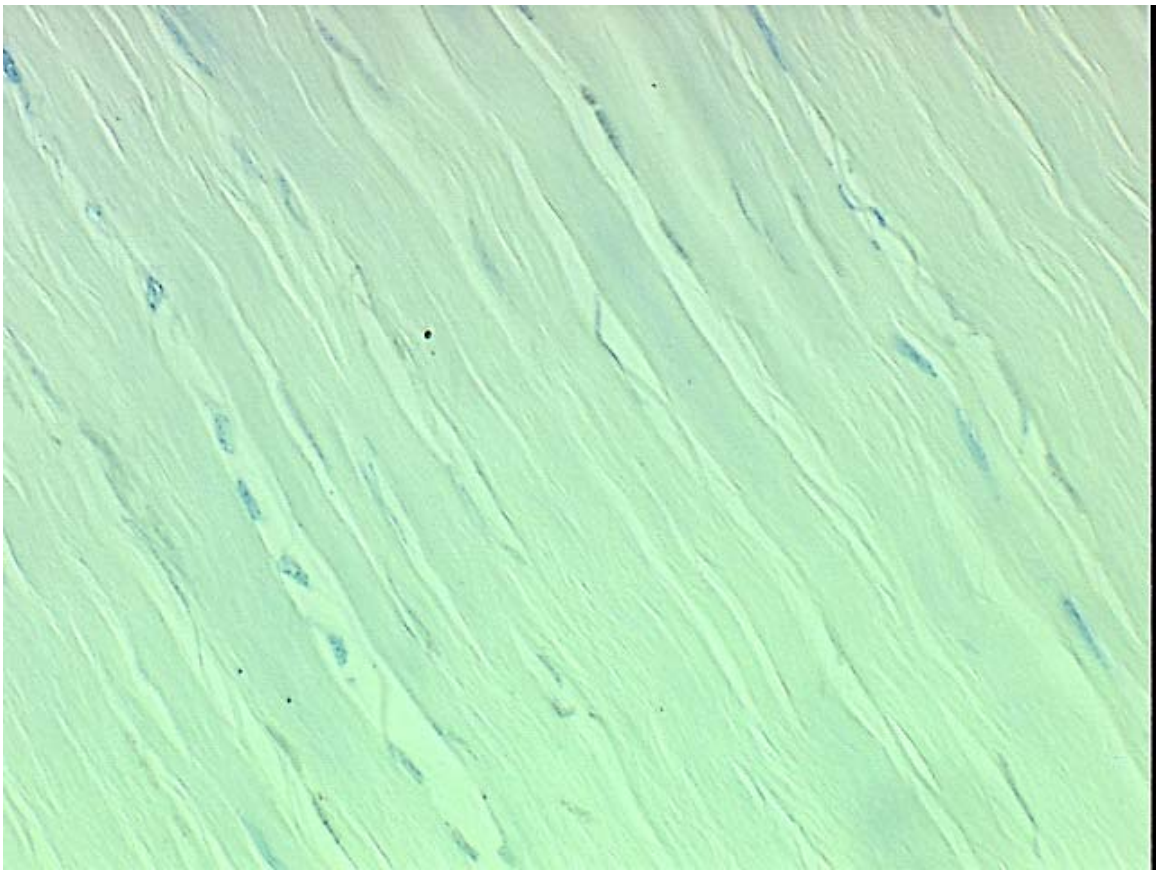


Figure 2b Photomicrograph of a section of an injured suspensory ligament. Healthy or necrotic cells (fibroblasts) are stained light blue with the counter stain in the normal ligament. In the injured ligament, darkly stained nuclei represent apoptotic cells (fibroblasts), while the light blue cells (fibroblasts) are normal or necrotic (Diaminobenzidine stain with methylene green counterstain; 151x)

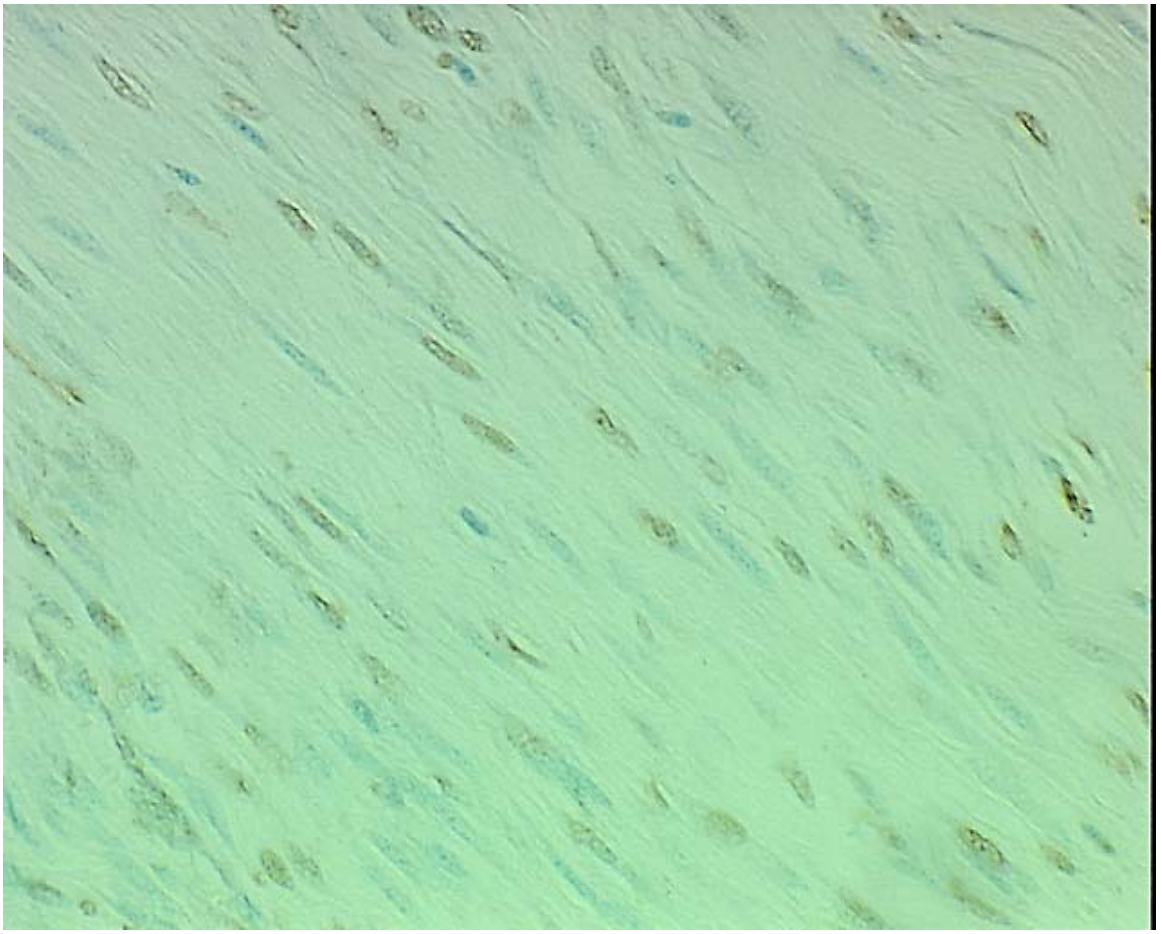


Figure 3a: Photomicrograph of an injured ligament with marked to severe disruption of the normal fiber architecture, which includes novel fibroplasia, hypercellularity and collagen degeneration are. Note the wavy appearance to the collagen and the increased number fibroblast in relation to the surrounding matrix. Figure 3a Hematoxylin and eosin stain (151x).

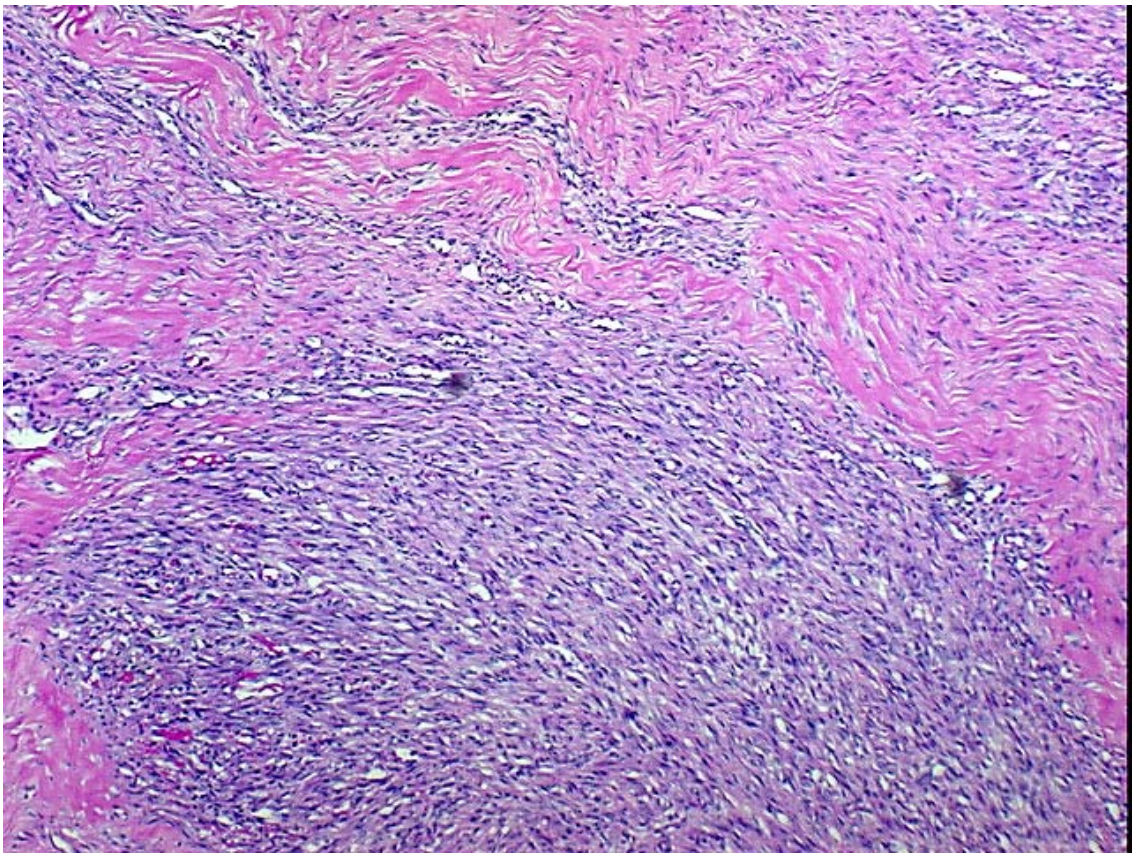


Figure 3b: Photomicrograph of an injured ligament with marked to severe disruption of the normal fiber architecture, which includes novel fibroplasia, hypercellularity and collagen degeneration are. Note the wavy appearance to the collagen and the increased number fibroblast in relation to the surrounding matrix. Figure3b: Masson's trichrome (151x).

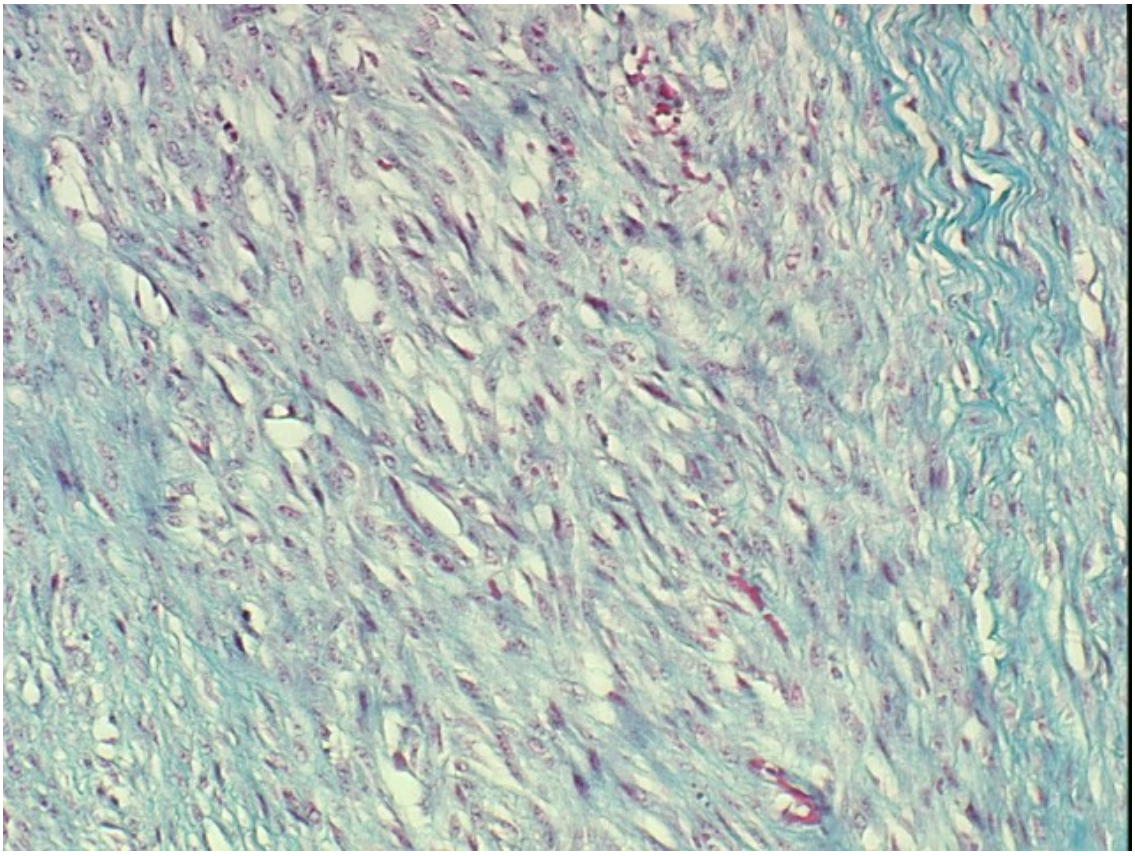


Figure 4a: Photomicrograph of a normal suspensory ligament. Figure 4a: Normal fiber structure with normal ratio of the number of fibroblasts (purple) to the pink collagen matrix, which has parallel fibers (Hematoxylin/eosin; 60x

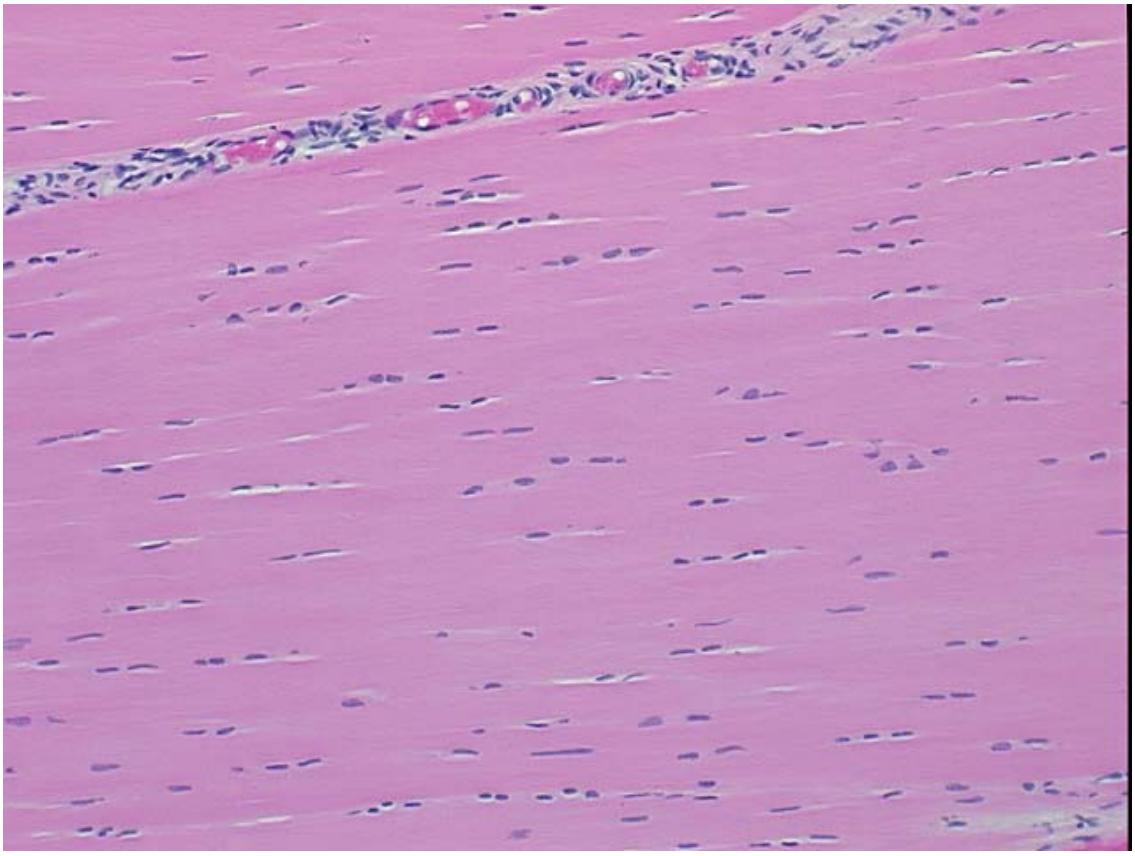


Figure 4b: Photomicrograph of a normal suspensory ligament. Figure 4b. The normal collagen is stains red due to the thickness of the sections. The ligament fibers have a parallel in orientation. There is a small amount of light blue ground substance in the vessels in the section. (Masson trichrome; 60x)

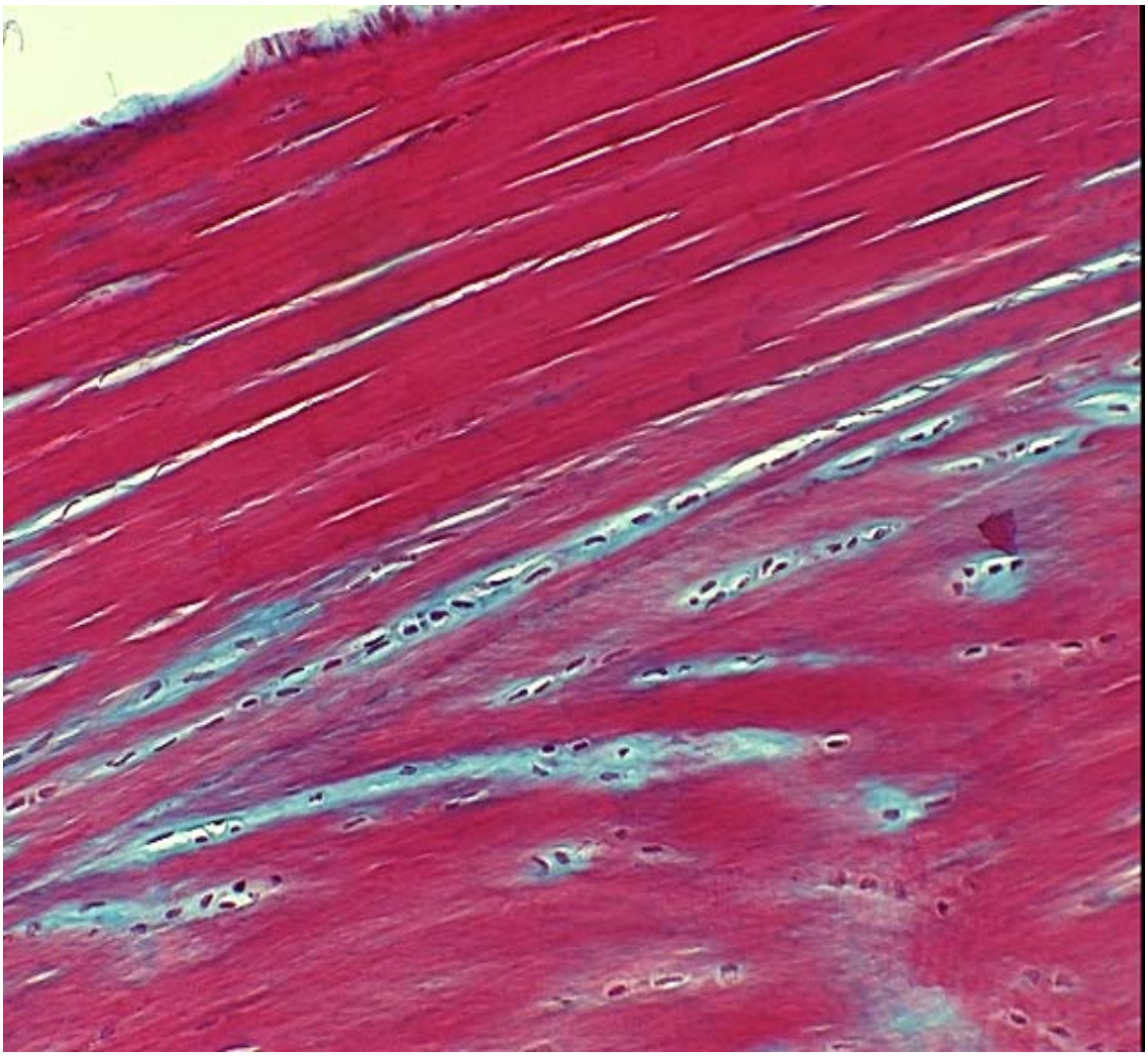


Figure 5a: Photomicrograph of a ligament with intraligamentous cartilage islets and a chondrone formation. Figure 5a: Intraligamentous chondrone formation. (Masson's trichrome stain; 60x)

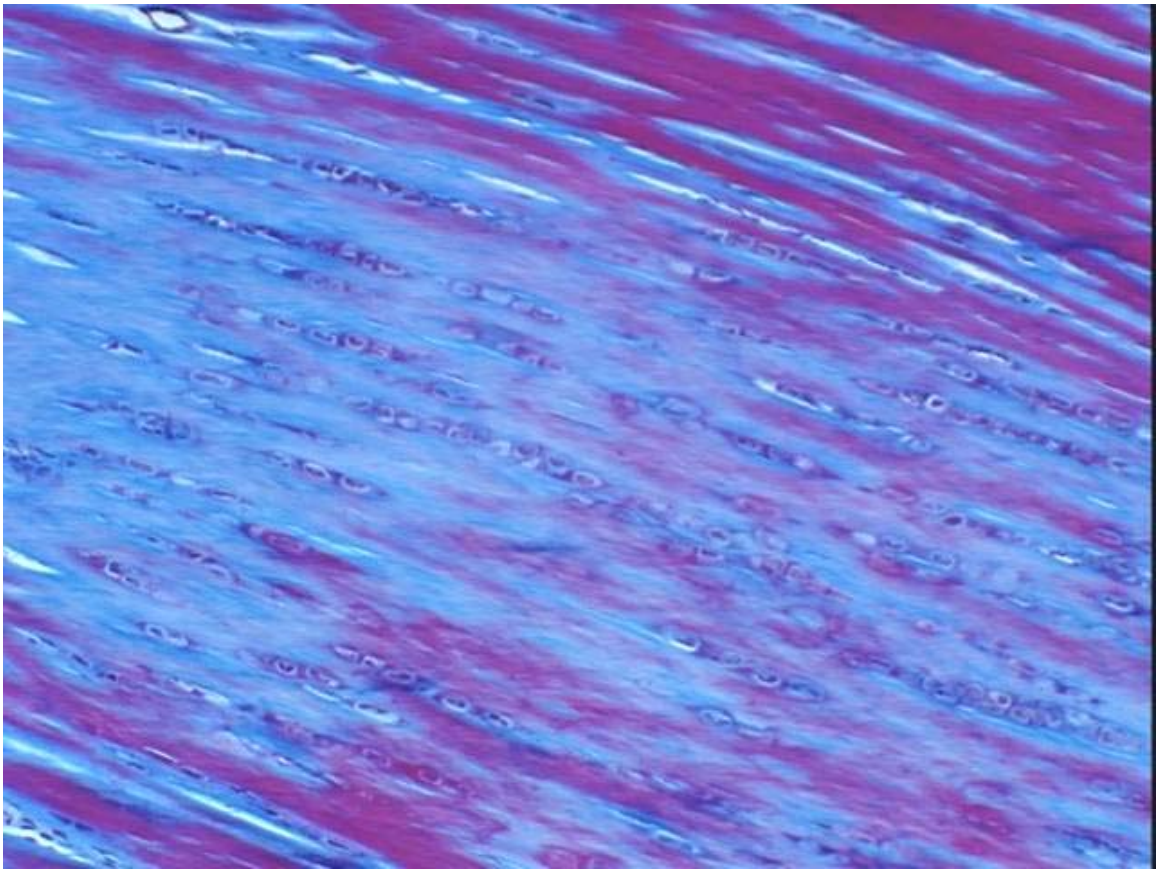


Figure 5b: Photomicrograph of a ligament with intraligamentous cartilage islets and a chondrone formation. Figure 5b: Focal chondrone formation with central mineralization (Hematoxylin and eosin; 60x).

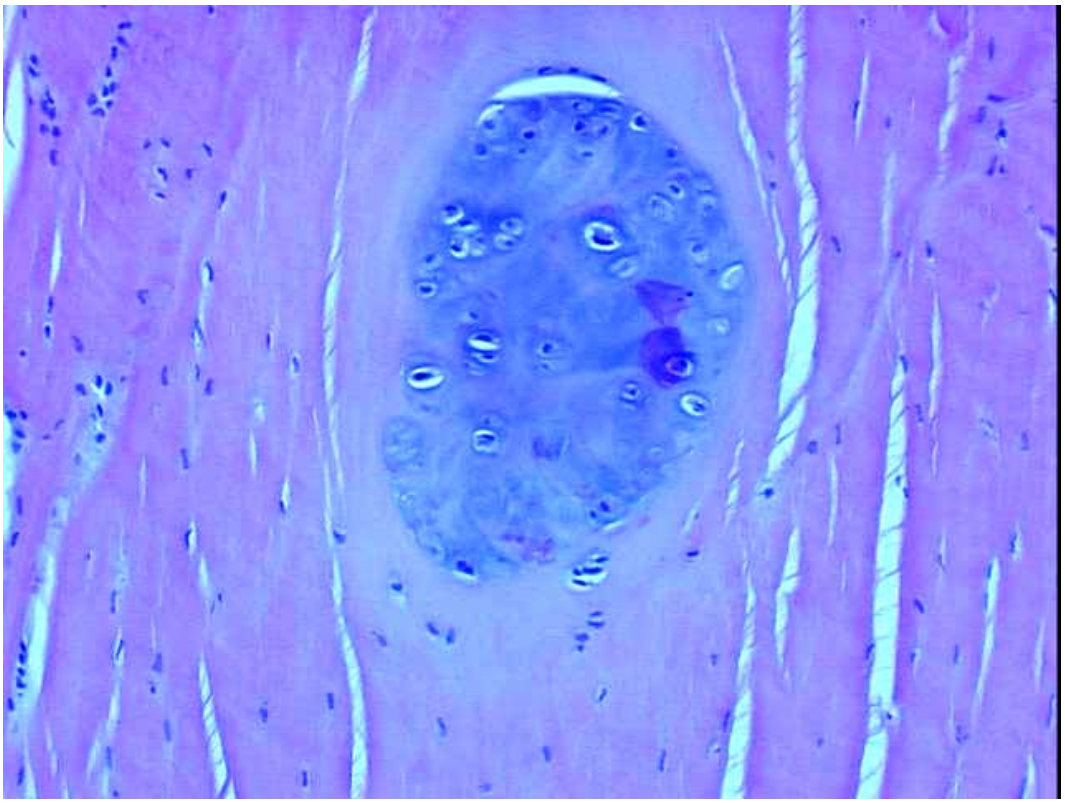


Figure 6a: Figure 6a and b: Digital image of a mouse thymus (TUNEL stain; 151x). Negative control – untreated mouse thymus. Note the light blue staining nuclei in the thymocytes and absence of dark staining nuclei.

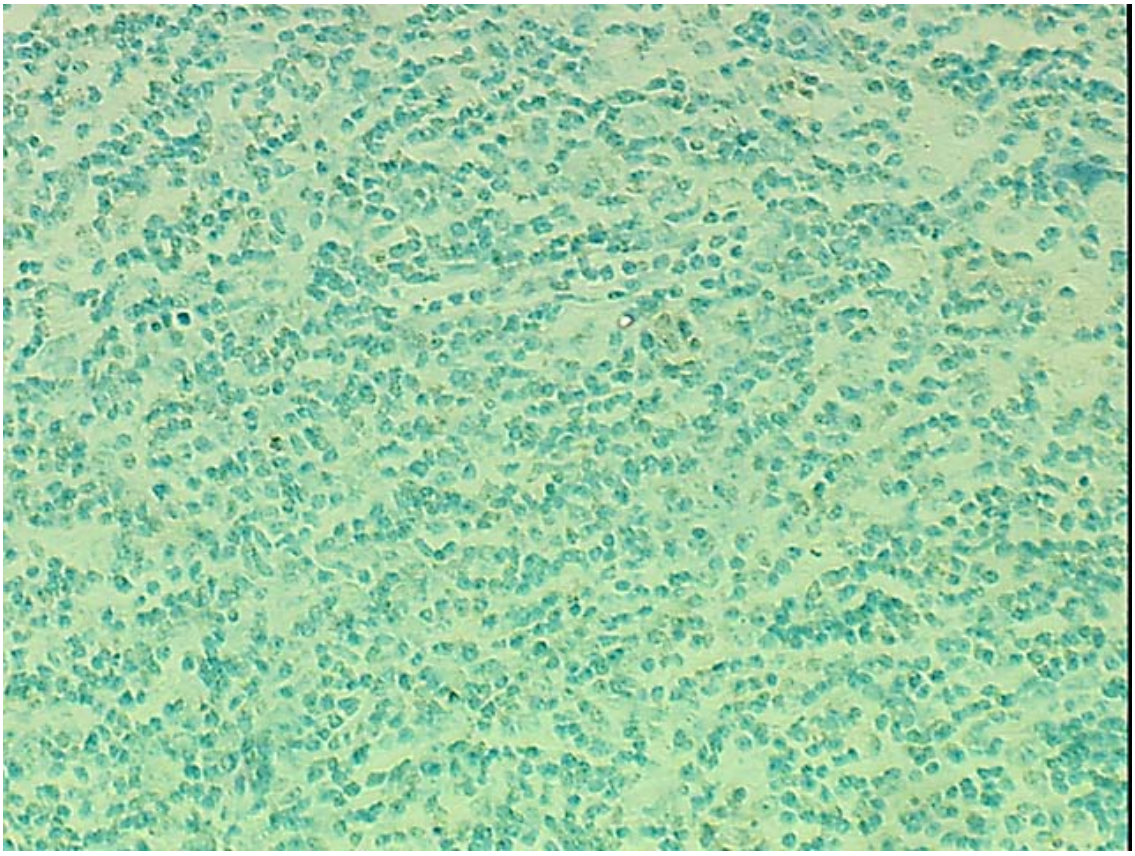


Figure 6b: Digital image of a mouse thymus (TUNEL stain; 151x). Positive control – thymus from a mouse treated with dexamethasone. Note the dark staining nuclei of the apoptotic thymocytes, which reacted with the peroxidase stain to form the dark brown color.

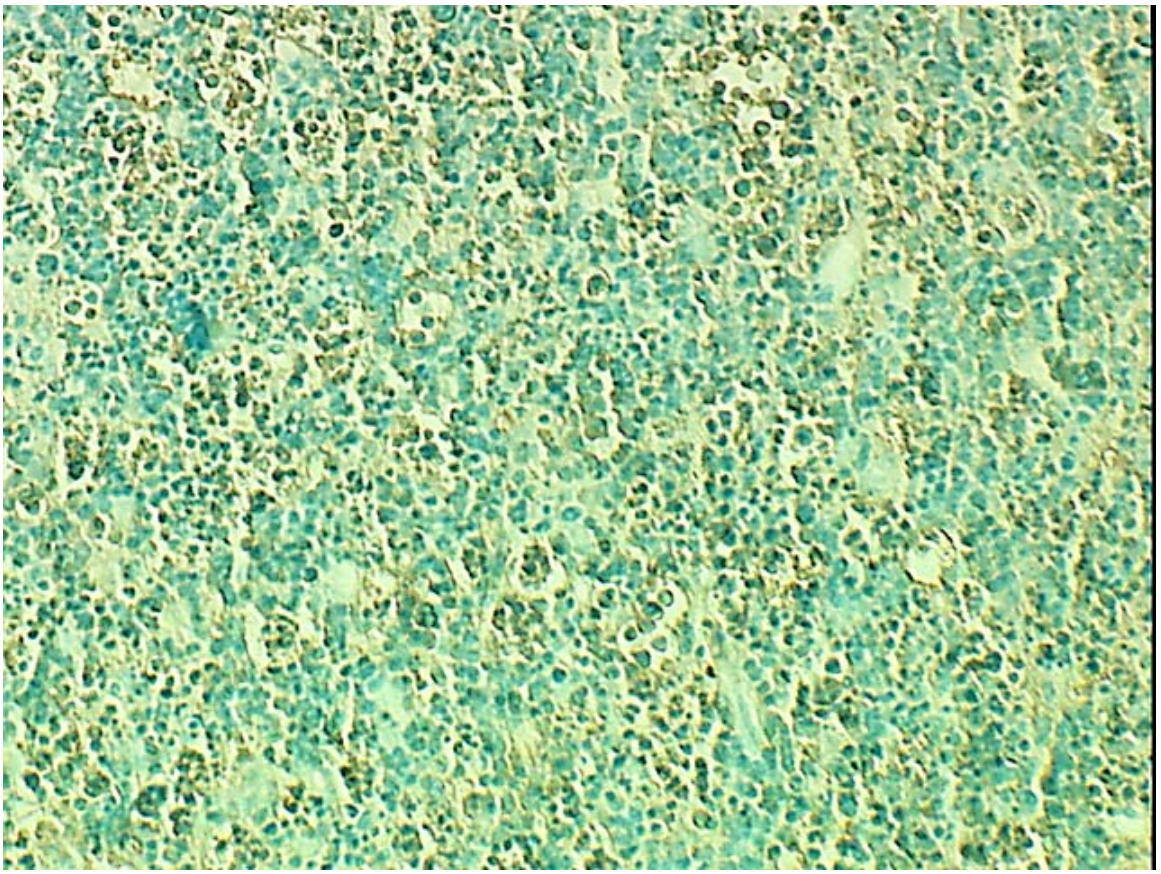


Figure 7: Focal neovascularization and fibroblast proliferation in the injured ligament. (Hematoxylin and eosin; 60x)

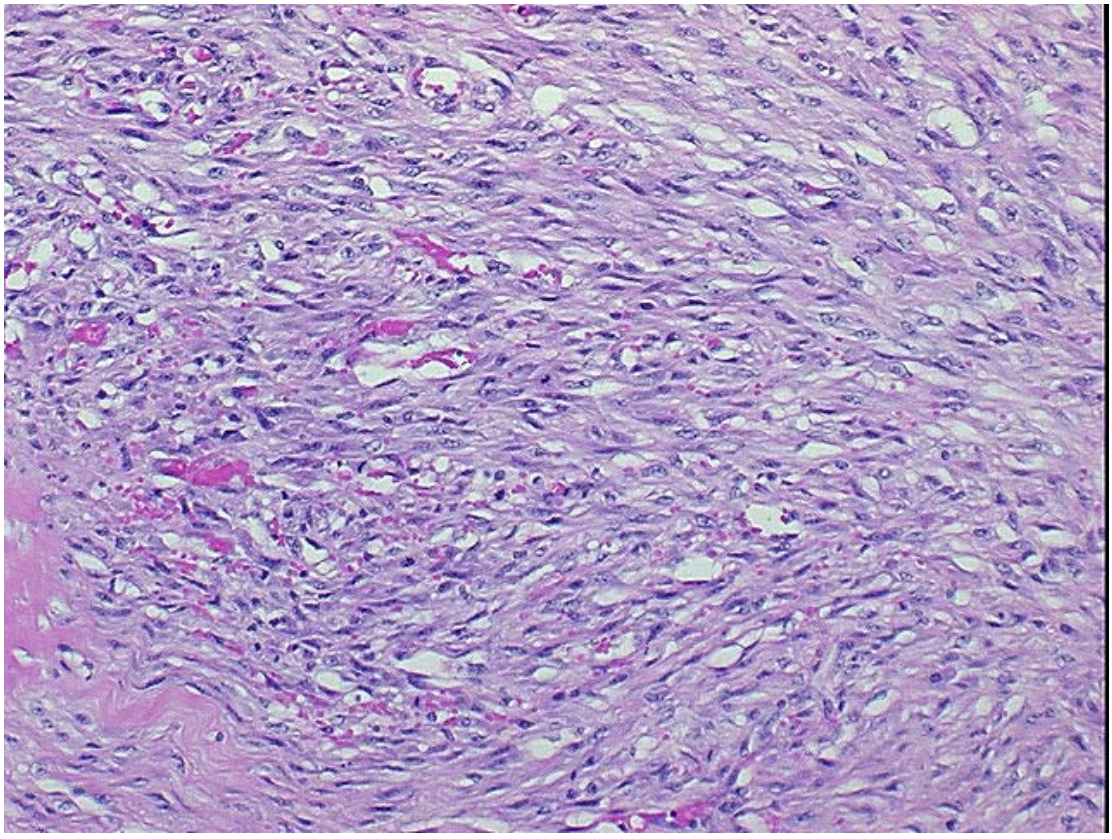
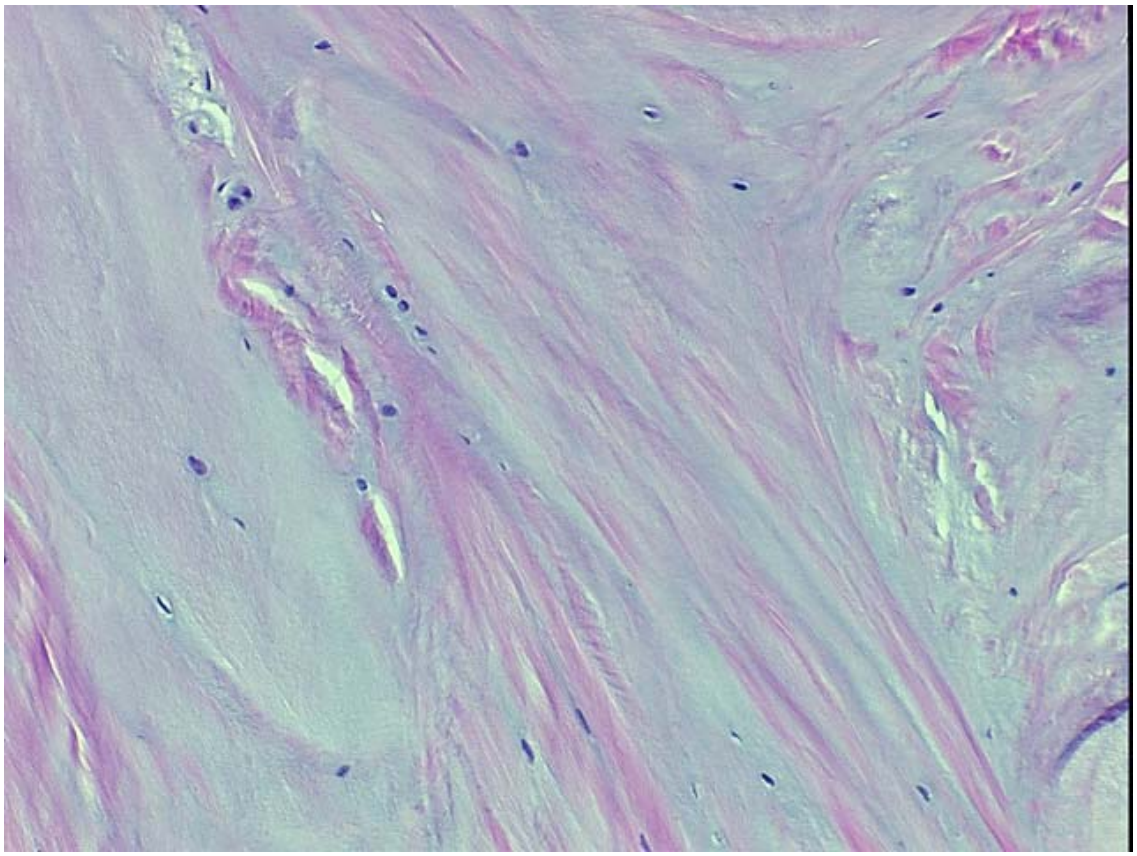


Figure 8: Photomicrograph of injured suspensory ligament. Hypocelluar region in the suspensory ligament with disoriented immature collagen. (Masson's trichrome stain; 151x)



Conclusion

There were significant pathological changes in the injured suspensory ligaments consistent with degeneration, attempted healing, and chronic scarring. The changes were represent a chronically affected ligaments which had not been able initiate remodeling in the final phases of healing. Changes included fibroplasia, neovascularization, collagen degeneration, mineralization, and lack of fiber alignment. Although suspensory ligament injury is commonly referred to as a desmitis, no inflammatory cells were present in the normal or injured segments suggesting this is a form of degeneration with a sub-acute inflammatory response. This is consistent stasis in an early remodeling stage of ligament healing rather than acute inflammation.

Apoptotic cells tended to be increased in the injured ligaments, but the small number of horses in the injured group, did not allow a fair comparison of normal versus affected ligaments. Further testing should be performed on suspensory ligaments to determine the changes in acute injuries to help develop ways to avoid persistence of the chronic lesion. Further knowledge of the cellular response during the remodeling phase of suspensory ligament healing could help develop more effective treatments for injured suspensory ligaments.