AN *IN VIVO* EVALUATION OF A NATURALLY DERIVED CYTOCOMPATIBILE AND ARCHITECTURALLY OPTIMIZED TENDON ALLOGRAFT IN THE HORSE

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ALLOGRAFT IN THE HORSE

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 4

The anatomy of the equine *flexor digitorum superficialis* tendon
The histology of the equine *flexor digitorum superficialis* tendon
The biochemistry of the equine *flexor digitorum superficialis* tendon
Allograft processing techniques associated with tendon tissue
The immunology of musculoskeletal allograft transplantation

III. METHODOLOGY 25

Experimental subjects
Allograft preparation
Surgical technique of allograft implantation
Ultrasonographic measurement of tendon cross sectional area
Histological processing of autograft and allograft material

IV. FINDINGS 32

Ultrasonographic examination of treated tendon
Gross pathological examination of treated tendon
Histological scoring of treated tendon
Descriptive histological analysis of treated tendon

V. CONCLUSIONS 47

Study limitations
Conclusions based on ultrasonographic data
Conclusions based on histological data
Final remarks

REFERENCES 53

LIST OF TABLES

Table Page

1. 40

LIST OF FIGURES

Figure | Page

1 28
2 33
3 34
4 36
5 38
6 43
7 44
8 45
9 46

CHAPTER I

INTRODUCTION

The athleticism of the horse has been both admired and exploited for millennia. To our eyes there can be no doubt that the equine musculoskeletal system represents an elegant phylogenic solution to the problem of rapid and efficient locomotion. Through the process of selective breeding, humankind has modified the prehistoric equine form to fulfill our militaristic, agrarian and recreational endeavors. However, this relationship cannot be considered completely altruistic. Along with the benefits of careful husbandry have come disease processes that have never been observed in wild equids.

Of considerable importance amongst these afflictions has been musculoskeletal injury (MSI). Having recognized the detrimental effect of equine MSI on the endeavors of man, considerable resources have been invested in the treatment, prevention and understanding of these conditions. In fact, the historical development of the veterinary profession closely parallels society's need for educated professionals familiar with the diagnosis and treatment of equine MSI.[1]

The use of musculoskeletal allografts to facilitate healing has a long history in human orthopedic surgery. [2] Recent advances in the physico-chemical techniques used to process connective tissue allografts have dramatically enhanced their clinical utility. Dedicated human tissue banks now provide appropriately sourced and processed cadaveric tissue for allotransplantation.[3] In the United States, connective tissue allograft distribution from tissue banks increased from 7,525 tendons in 1993 to approximately 750,000 tendons in 1999.[4] Allograft tendons which are commonly sourced and supplied include: bone-patellar tendon-bone

(BPTB), Achilles tendon, semi-tendinosis, gracillis and posterior tibialis tendons.[5] These materials have found a wide variety of clinical applications, including: anterior cruciate ligament (ACL) reconstruction, collateral ligament repair, proximal hamstring reconstruction, Achilles tendon repair and reconstruction of the rotator cuff of the shoulder.[6-9]

Despite numerous extensive experimental studies utilizing allograft techniques in companion animal species, the use of musculo-skeletal allografts in clinical veterinary orthopedic surgery has been limited. Commercial organizations exist to supply canine tendon, fascia and BPTP as well as equine osteoinductive demineralized bone matrix (DBM) and osteoconductive cancellous bone chips.[10] Soft tissue for equine allotransplantation is not yet clinically available. To date, no study has evaluated processing techniques that might enable successful allotransplantation of equine tendon. If achievable, this type of biomaterial may have clinical utility.

In horses, lacerations of the flexor tendons are common, potentially career ending injuries.[11,12] Primary suturing of transected flexor tendons has been advocated by some authors.[13,14] *In vitro* studies have indicated improved healing characteristics after primary suture repair.[15] Clinically, necrosis or surgical debridement may result in significant tendon defects that are not amenable to primary suture repair. In the equine FDS tendon, endogenous repair tissue lacks the biomechanical characteristics of normal tissue.[16] This functional disparity is believed to make the affected region susceptible to re-injury. A variety of synthetic materials have been proposed to augment tendon healing.[17-19] None of these have gained widespread clinical acceptance. Using an equine model, Reiners *et al* (2002) demonstrated the potential for autograft *extensor digitorum lateralis* (EDL) tendon to augment healing of transected *flexor digitorum profundus* (FDP) tendon.[20] Clinically, this approach would necessitate a second surgical site and may not provide an adequate amount of tissue of inappropriate dimensions. These problems would be solved by the availability of an appropriate allogeneous biomaterial.

Recently, Whitlock *et al* (2007) described a physico-chemical process for the decellularization and architectural optimization of avian FDP tendon.[126] This particular protocol included: immersion in hypotonic solution, trypsin digestion, peracetic acid and non-ionic surfactant treatment followed by freezing and lyophilization. Tendon tissue treated using this method exhibited excellent *in vivo* biocompatibility and retained 75% of its ultimate tensile strength. We hypothesized that, with modification, a similar protocol could be used to process cadaveric equine FDS tendon and produce an equine-specific biomaterial for clinical use. The objective of this study was to employ two variations of this physico-chemical protocol to process equine FDS tendon in order optimize its biocompatibility when implanted into normal equine subjects.

CHAPTER II

LITERATURE REVIEW

2.1 The gross anatomy of the equine forelimb *flexor digitorum superficialis* tendon

The equine FDS tendon is an elegant refinement of form and function, the anatomy of which has been well described.[21] In the forelimb, the FDS tendon originates from the medial epicondyle of the humerus and inserts on the *scutum medium*; a fibro-cartilaginous plate palmar to the second phalanx. In a cadaveric study of forelimb muscles in seven thoroughbred horses, Brown *et al* (2003), reported an average FDS tendon length of 549.4mm, a muscle volume of 227.4 cm^3 and a resting muscle length of 356mm.[22] This compared with the neighboring humeral head of the FDP which had a length of 481.6mm, a muscle volume of 524.5cm^3 and a resting muscle length of 354.3mm. The FDS tendon shares a number of muscle fascicles with the humeral head of the FDP as well as a common short tendon of origin.[22]

In the region of the antebrachium, the muscular portion of the FDS adopts a more caudal and medial position to the more substantial musculature of the FDP. In contrast to the humeral head of the FDP, the single headed FDS muscle is exceedingly fibrous and highly pennate, having eight to twelve strong longitudinal aponeuroses running through the length of its substance.[21] Seven to eleven centimeters proximal to the antebrachiocarpal joint, a tendinous band known as the superior or proximal check ligament arises from the caudo-medial aspect of the radius. This structure courses caudo-distally to insert on the medial aspect of the FDS just proximal to the antebrachiocarpal joint.[23] In the proximal carpus, a tendinous band unites the FDS and FDP tendons near the musculo-tendinous junctions in an unknown proportion of horses.[24]

Extending from near the origin of the superior check ligament, both the rounded FDS and FDP tendons and the median nerve pass within the synovial structure known as the carpal canal. At this point muscular tissue may still be evident within the tendinous FDS structure. In the mid-metacarpal region the FDS tendon emerges distally from beneath the thick fascia associated with the palmar carpus and is enveloped within a thin paratenon. In the mid-metacarpal region, the tendon is somewhat crescent shaped with its medial border being more blunt and rounded.[25] In the distal third of the metacarpal region, the FDS and FDP tendons become enveloped in a second synovial structure, the digital sheath. Just proximal to the metacarpophalangeal joint the FDS tendon elaborates a ring-like extension which encircles the FDP tendon. Known as the *manica flexoria*, this structure ensures anatomic alignment of the tendons as they pass over the proximal sesamoid bones.[23] At this level the FDS tendon is thinner, broader and concave on its dorsal aspect. From the proximal sesamoid bones distally, the FDS tendon becomes thinner axially before bifurcating and becoming two discrete tendons inserting abaxially on the fibro-cartilaginous structure on the palmar aspect of the second phalanx.

The blood supply to the FDS tendon has been well described.[27] Like other musculo-tendinous structures, important nutrient vessels arise proximally within the musculature and distally at the insertion. In a cadaveric study Kraus-Hansen *et al* (1992) demonstrated an anastomosing network of vessels within the mid-metacarpal region of the FDS tendon supported by two major abaxial longitudinal arteries giving a ladder-like arrangement of vasculature.[27] A key finding was the relatively avascular nature of the core of the FDS tendon in the mid-metacarpal region. In the same paper, the authors demonstrated *in vivo* that the contribution of vasculature associated with the mid-metacarpal paratenon was negligible. The authors also identified a third nutrient arterial branch of the median artery running in the distal third of the accessory ligament. Distally, near the distal border of the palmar annular ligament, a branch of each abaxially located digital artery

also supplies the FDS tendon.[24] Within the digital tendon sheath a sagittal adhesion to the proximal digital annular ligament also carried branches of the digital artery.

In contrast to the arterial supply, the innervation of the FDS tendon has not been well described. Like other forelimb flexors the FDS musculature receives innervation from the median nerve (C8-T2) and ulnar nerve (T1-2).[21] The relative importance of each remains undetermined. In the metacarpal region large medial and lateral palmar nerves (originating primarily from the median nerve) course distally, dorsally to the deep digital flexor tendon. A communicating branch crosses obliquely over the palmar aspect of the FDST in the mid-metacarpal region.[24]

In the distal limb, palmar branches of the medial and lateral digital nerves supply innervation to the FDS tendon. Although likely to follow the path of accompanying vessels, the exact nature of this innervation has not been described. The relative importance of intra-tendinous innvervation versus paratendinous innervation remains to be determined. However, the use of perineural analgesia to locally desensitize FDS tendon would suggest that unlike vasculature, extra tendinous nerves play a more important role.[27]

2.2 The microscopic anatomy of the equine forelimb *flexor digitorum superficialis* tendon

Tendon exhibits a hierarchical micro-anatomic structure.[29] Currently the nomenclature assigned to each subdivision lacks standardization and varies between authors.[30,31] Generally, sub-microscopic aggregations of collagen fibrils become visible microscopically as fibril bundles are separated from each other by an investment of endotenon (40-500nm). Fibril bundles are aligned with the long axis of the tendon. When viewed using polarized light microscopy, fibril bundles exhibit an in-phase waveform known as crimp.[30] This waveform is believed to straighten with mechanical loading, thereby contributing to tendon elasticity and potentially acting as a mechanical safeguard to excessive loading.[31] The fiber bundle crimp pattern is most prominent in

neonatal equine FDS tendon and has been observed to decline with age and exercise, especially in the central core region.[32-34]

The endotenon elaborates a thin reticular network within the tendon delineating individual fiber bundles and macroscopically visible tendon fascicles (50-300µm).[29] The connective tissue framework of the endotenon distributes limited blood vessels, lymphatics and nerves to central regions from the peripheral tendon where they are comparatively well developed. The endotenon is believed to allow fiber bundles and fascicles to slip relative to one another, thereby contributing to the overall elasticity of the tendon structure.[29] Superficially, the endotenon is contiguous with the enveloping epitenon on the tendon surface. The relatively disorientated, crisscrossing fiber bundles of the epitenon form a dense connective tissue sleeve. In the carpal sheath proximally, and in the digital sheath distally, the epitenon is enclosed within true synovial structures. In the mid-metacarpal region, a fine connective tissue sleeve called the paratenon facilitates gliding function.[30]

Tenocytes are the fibroblast-like cells responsible for production of extra-cellular tendon matrix.[35] They are arranged longitudinally between collagen fibril bundles within fascicles.[29] Most tenocytes are situated remote from blood vessels located within the endotenon. Elaborate cytoplasmic processes afford connections with matrix components and other tenocytes.[36] Gap junctions between tenocytes may facilitate the transfer of nutrients and coordinate synthetic responses to mechanical stimuli.[37] Webbon (1978) classified tenocytes on the basis of their nuclear morphology.[26] Type-1 tenocytes have elongate spindle shaped nuclei while type-2 tenocytes have more rounded nuclei. Ultra-structural examinations of type-2 tenocytes have found a greater amount of rough endoplasmic reticulum and Golgi apparatus indicative of a greater synthetic capacity[37]. Immuno-histochemical studies have also indicated higher levels of expression of certain proteins including pro-collagen.[38]

With age and exercise there is an increase in the proportion of type-1 tenocytes. This coincides with an overall reduction in cellularity.[39] It is assumed that type-2 tenocytes convert to quiescent type-1 phenotype. However the evidence for this conversion is only indirect. Type 3 tenocytes are cartilage like cells with round nuclei and prominent nucleoli. This cell type is a normal finding close to the fibro-cartilaginous FDS tendon insertion.[24] However with age, chondroid metaplasia may develop with tendinous compression in the metacarpal region and where the FDS tendon traverses the palmar aspect of the metacarpo-phalangeal joint.[26] A separate population of fibroblastic cells is associated with the paratenon, epitenon and endotenon. These cells are believed to play important roles in maintaining tendon extra cellular matrix (ECM). Using *in situ* immunohistochemical techniques, Cauvin (2001) revealed transforming growth factor beta-3 (TGF-β3) associated with endotenon fibroblasts and not intra-fascicular tenocytes themselves.[41]

2.3 Ultra-structure and biochemistry of the equine *flexor digitorum superficialis* tendon

The primary biochemical components of tendon are water, collagen fibers, and proteoglycans.[29] In tendon, collagen is the most abundant protein of the ECM comprising approximately 75% of its dry weight.[30] Collagens are a diverse super-family of proteins which possess a characteristic quaternary structure consisting of three polypeptide α-chains arranged in a right-handed triple helix.[41] Each α-chain is itself arranged in a left handed helix with a pitch of 18 amino acid residues per turn. Assembly of the triple helical quaternary structure requires an amino acid sequence in which every third residue is glycine $(Gly\text{-}X\text{-}Y)_n$.[42] The internal positioning of small glycine residues allows for their close packing in the center of the triple helix. The bulky side chains of the X-Y residues, principally prolene and lysine, are arranged eccentrically on the outside of the helix.

Collagen is divided into various types on the basis of its structure, splice-variants, non-helical domains, assembly and function.[43] The fibril forming collagens (types I, II, III, V and XI) form the structural building blocks of tissues.[41,42] In tendon fascicles, type I collagen predominates.[29,44] Collagen types III, IV and V function primarily as components of the reticular endotenon and basement membranes.[44] The triple helix of type I collagen consists of two identical α1(I) chains and an α2(I) chain. The rod-like triple helical core, 300nm in length and 1.5nm in diameter, is flanked by non-helical, extended n-terminal and c-terminal telopeptides.[43] Telopeptides lack the triple helical conformation but are important in fibril formation.

Collagen is synthesized intra-cellularly as pro-collagen which contains N-terminal and C-terminal globular pro-peptides.[42] These peptides contain asparagine linked oligosaccharide units composed of N-acetylglucosamine and mannose that may function in secretion or in prevention of premature (intracellular) fibril formation.[46] The coordinated removal of propeptides by termini specific metallo-proteinases (procollagen aminoprotease and procollagen carboxypeptidase) is a requirement for fibril formation to occur.[47]

Positive staining of fibrillar collagen molecules with phosphotungstic acid followed by uranyl acetate gives a characteristic banding pattern visible with electron microscopy.[48] This technique and modeling studies have identified rigid and flexible domains in collagen types I, II and III.[49] Regions of high flexibility appear to coincide with sequences devoid of proline and hydroxyproline.[50,51] This would suggest that the collagen triple helix can be considered a composite with regions of varying stiffness. This variation may be important for fibril formation, final fibril diameter and crimp formation.

The assembly and maintenance of tendon collagen as network of cross-liked fibrils is believed to require cellular regulation and is responsive to mechanical stimuli.[52] The degree of adaptive response however, may be dependant upon tendon function and species.[53] *In vivo* fibrillogenesis in developing tendon has been extensively studied in the chick embryo. The early stages of

molecular assembly occur within intra-cellular vesicles which are secreted into deep cytoplasmic recesses.[56] N-propeptides remain attached until fibril segments with diameters of 20-30nm are formed.[56] N-propetide cleavage coincides with an increase in fibril diameter. The C-propeptide has been observed in fibrils of between 30-100nm and appears to play a role in the initiation and growth of fibrils. Later removal of the C-propeptide is also accompanied by further lateral fibril growth.[54,55] In 14 day old chick embryos, Birk *et al* (1997) observed fibril segments in extracytoplasmic channels that were 10-30µm in length.[57] Mature fibrils then grow in length at a constant diameter by end to end fusion of the intermediate species.

The assembly of collagen molecules into fibrils under physiologic conditions is entropically favorable.[58] The interaction between the non-polar surfaces of collagen molecules extrudes water molecules from the interior of the super-molecular assembly. Within the fibrils, collagen molecules are aligned parallel to each other and exhibit a characteristic 67nm periodicity (D period) when collagen fibrils are negatively stained with phosphotungstic acid and examined using electron microscopy.[59] This observation is a function of a gap region of 40nm between axial collagen molecules and a quarter stagger arrangement between each molecule and those adjacent to it.

Collagen cross-linking provides tensile strength and resistance to proteolysis.[60] Cross-linking of nascent fibrils requires deamination of lysine or hydroxylysine residues by lysyl oxidase forming allysine (2-amino-6-oxo-hexanoic acid) and hydroxyallysine (2S,5R)-2,6-diamino-5-hydroxyhexanoic acid).[61] Divalent cross links are formed between the telopeptide region of one collagen molecule and the helical region of an adjacent molecule.[57] The quarter stagger model of fibril arrangement indicates that aldehydes within N and C terminal telopeptides cross-link with (hydroxy)lysine residues at positions 930 and 87 respectively.[48] A variety of divalent and trivalent cross links can be formed dependant on the reacting species. In fetal equine FDS tendon two forms of reducible cross-link hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine

(DHLNL) predominate. HLNL is undetectable a few months after birth but DHLNL remains detectible, albeit at low levels, up to 3 years of age.[62] These reducible bonds are intermediates that mature into non reducible stable forms.[63] Limited studies suggest that the primary mature cross-link of the equine FDS tendon appears to be the trivalent hydroxypyridinum compound, hydroxylysyl pyridinoline (HP).[64] This is similar finding to other load bearing tendons such as human patella tendon and rabbit Achilles tendon.[60]

In the equine FDS tendon, collagen fibril diameter varies spatially. Watanabe *et al* (2007) identified small fibrils (<100nm) predominating in the musculo-tendinous junction and large fibrils (>200nm) at the tendino-osseous insertion.[66] In the mid-metacarpal region collagen fibrils exhibited an intermediate distribution between these two extremes. The exact mechanism by which collagen fibril diameter is regulated is not well understood. One proposed mechanism involves the timing of extra-cellular enzymatic cleavage of amino-propeptides from pro-collagen.[43] Proteoglycans (see below) may act to shield amino-propeptides on the fibril surface from amino-propeptidases.[67] Other hypotheses implicate other fibrillar collagen types, such as III and V, as potential regulators of fibril diameter. Type V collagen filaments may act as nucleation sites for type I collagen fibrillogenesis.[68] Alternatively, due its longer helix or because of uncleaved terminal propeptides, critical concentrations of type V collagen in a fibril may inhibit further lateral additions of type-1 collagen. Similarly, the delayed cleavage of the amino propeptide of type III collagen present in embryonic tendon may slow lateral fibril growth. An interesting observation in the Equine FDS tendon is that type V collagen distribution appears heterogeneous.[66] The highest concentrations of type V collagen are found at the myotendinous junction where the smallest collagen fibrils are located. The fibril associated collagens (Types XII and XIV) may also play a role in regulation of type I collagen fibril diameter in tendon.

Despite comprising less than 1% of tendon dry weight proteoglycans are believed to be very important in the structure and function of tendon.[69] Proteoglycans (PGs) are complex molecules consisting of a protein core to which a glycosaminoclycan (GAG) moiety is covalently attached. Two

families of PG, based on molecular weight, have been identified in tendon.[70] The small PGs (~40kDa) of tendon include decorin, biglycan, fibromodulin and lumican. These molecules are characterized by leucine rich repetitive (LRR) sequences through which small PGs interact with cells, collagen fibrils or other ECM molecules such as epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β).[71] Small PGs are divided into four classes based on, amongst other characteristics, the number of LRR sequences present. The N-terminal domain of small PGs is usually substituted with either one (decorin) or two (biglycan) chondroitin/dermatan sulfate side chains, giving the molecule polyanionic properties. The primary functions of the small PGs specific to tendon can be defined as (1) regulators of collagen fibril diameter and (2) contributors to tendon mechanical properties by providing interfibrillar linkages.[72]

The role of small PGs as determinants of collagen fibril size has been examined in gene-knockout studies in mice. Decorin, the primary small PG of tendon, has been hypothesised to inhibit the lateral fusion of fibrils and organize fibril orientation within the ECM.[73] Danielson *et al* (1997) reported large coarse irregular collagen fibrils with axial variations in diameter in tail tendons from decorin deficient mice.[74] When Zhang *et al* (2005) examined the weight bearing *flexor digitorum longus* (FDL) tendon in the same decorin deficient murine model, a population of abnormally large fibrils was observed.[75] The small PGs display a high degree of homolgy in their respective primary structures. The results of small PG gene knockout studies suggest that a degree of functional overlap exists between these molecules, especially within classes. Tendons from decorin deficient mice, for example, displayed an enhanced expression of biglycan as measured by semi-quantitve immunoblot analysis.[76] Similar compensations have been observed between the class 2 small PGs, fibromodulin and lumican.[77] However, the phenotypic effect of specific small PG deficieny is not consistent. For example, in a population of fibromodulin deficient mice, Ezura *et al* (2002) identified an increased frequency of small diameter fibrils (<65nm) and enhanced lumican expression.[78] The relative importance of these four small PGs in the equine FDS tendon is not well understood. In a small sample of young adult thoroughbreds,

Watanabe *et al* (2005) identified the highest concentrations of decorin in the proximal part of the mid metacarpal region of the FDS tendon.[79] This finding corresponded with an increase in the mass average diameter of collagen fibrils and a decrease in the collagen fibril index in the decorin poor distal metacarpal region.

Scott (1992) described a model of structural interaction between the GAG side-chains of small PGs and collagen.[80] Subsequent *in vitro* ultra-structural studies have demonstrated a specific periodic interaction of decorin and its GAG side-chain with type I collagen fibrils.[81,82] The GAG side-chains of decorin may extend away from the fibril surface and connect with GAGs of adjacent small PGs on other collagen fibrils or even the surface of adjacent fibrils themselves. These linkages are non covalent in character and involve hydrogen bonding, hydrophobic and electrostatic interactions. Small PGs have been identified as providing the visco-elastic properties characteristic of the initial deformation of tendon with load.[73,80] However, examination of this hypothesis is problematic as removal of specific proteoglycans also alters collagen fibril morphology. In an *in vitro* model of self-assembling type-1 collagen, the addition of decorin increased the tensile strength of un-cross-linked fibrils.[81] Zhang *et al* (2006) reported a reduction in strength and stiffness of decorin deficient *flexor digitorum longus* (FDL) tendons harvested from gene-knockout mice.[73] The role of small PGs in the mechanical properties of the equine FDS tendon is not clear.

Of the large proteoglycans (~200kDa), aggrecan and versican are the most abundant in tendon.[72] These molecules consist of complex core proteins with multiple central globular domains extensively liked to chondroitin or dermatan sulphate. The N-terminal G1 globular domain interacts with the free GAG hyaluronan.[83] Abundant GAGs provide large anionic surfaces resulting in a stiff extended molecular form that retains large amounts of water. These molecules primarily provide resistance to compressive forces and as such are concentrated in areas of tendon compression. In a biochemical analysis of bovine flexor tendons, Vogel *et al* (1985) identified only 10% of PGs isolated from the

tensile region as being large PGs.[84] Furthermore, the aggrecan of the tensile region of bovine flexor tendon appears to lack the G1 globular domain and lacks keratan sulphate side-chains, indicating a different function from the agreccan of compressed tendon or articular cartilage.

Cartilage oligomeric matrix protein (COMP) is a non-collagenous glycoprotein that is found in abundance in the tensile region of the equine FDS tendon.[85] Like other members of the thrombospondin group of proteins, COMP exhibits a pentameric structure with 5 identical arm-like sub-units united by a coiled coil domain at their N-termini.[86] The C-terminal globular domains on each subunit binds type I collagen in tendon as well as type II and type IX collagen in cartilage.[87] The role of COMP in tendon remains uncertain although, a role in fibril formation has been proposed.[88] The distribution of COMP in the equine FDS tendon has been well described.[85] The highest concentrations are found in the tensile (mid-metacarpal) region. COMP levels peak at 2 years (~10mg/kg wet weight) and then decline after 5 years of age (~2mg/kg wet weight). The relationship between COMP levels and mechanical properties of the equine FDS tendon however is not completely clear. In horses of the same age, a positive correlation was found between COMP levels and mechanical properties.[89] In foals, COMP appears to be up-regulated in response to mechanical load and exercise.[90] However, this effect does not seem to occur in juvenile or adult horses. These findings would suggest a role for COMP as an organizer of fibril structure in the ECM of the developing equine FDS tendon.

2.3 Allograft processing techniques associated with tendon tissue

The post-harvest processing of tendon allograft tissue has several functions. Of primary importance are those processing techniques designed to decrease the antigenic stimulus of the allograft to the host.[91] Also important are processing techniques that ensure allograft sterility and facilitate storage.[92] Additional steps may be taken to improve the architectural and bio-integrative qualities of the allograft tissue.[93] A key consideration is that the methods employed do

not significantly alter those key properties of the native allograft that make it a desirable biomaterial in the first place.

Deep freezing is the most common form of connective tissue processing and storage used in tissue banks.[4] Grafts treated by deep-freezing can reportedly be stored for up to 5 years.[94] Freezing of connective tissue to -80°C destroys significant numbers of donor cells and denatures MHC antigens.[95] In a canine model of ACL repair, Arnoczky *et al* (1986) demonstrated that fresh patella tendon allografts from subjects with dissimilar MHC antigenic profiles incite a significant intra-articular inflammatory response.[96] When the patella tendon allograft was frozen to -196°C for a minimum of four weeks prior to implantation, the allograft treated joints healed in a similar manner to those joints receiving an autograft control. Despite its apparent effectiveness and widespread use, protocols for the freeze treatment of connective tissue allografts vary widely. [97-99] Several experimental studies report histological evidence of residual cellular material in freeze-treated tendon tissue. This may explain instances where, despite freezing, allografts appeared to elicit deleterious immunological reactions.[100]

Deep freezing alters the architecture of native tendon tissue. As well as denaturing tendon cells, ice crystals that form during the freezing process expand the interfibrillar space.[99] In an *in vitro* evaluation of human posterior tibial tendons, Giannini *et al* (2008) demonstrated that tendon tissue frozen at -80C for 30 days exhibited increases in mean fibril diameter and cross sectional area and a decrease in collagen fibril density as measured by calorimetric analysis.[101] These structural changes corresponded with changes in the biomechanical properties. Freeze treated tendons exhibited a decrease in ultimate load, ultimate tensile stress and ultimate tensile strain. These findings are in agreement with other studies of human biceps brachii tendon and rabbit patella tendon.[102,103] Conversely, other studies comparing the mechanical properties of fresh and freeze treated anterior cruciate ligament tissue in dogs and monkeys respectively report no

significant differences.[104] Discrepancies in the rate of freezing, rate of thawing, duration of freezing and the type of tissue make direct comparisons difficult.

In vivo studies examining only the effect of freezing on the incorporation of allograft tendon tissue are uncommon. Most studies choose to compare a fresh-frozen allograft to a fresh autograft control. This type of experimental investigation attempts to answer the clinical situation that arises in human orthopedic surgery. In a rat patella hemi-tendon transplantation model utilized by Maeda *et al* (1997), the tensile strength of patella tendons implanted with fresh autografts was significantly greater than those receiving freeze treated autografts at 8 weeks.[105] Using the same model with radio-isotope labeled tendon (^{3}H-proline) Nagano *et al* (1996) determined that, at 12 weeks, the amount of old collagen in the frozen autograft treated tendons was significantly lower than tendon receiving the fresh autograft.[106] These findings suggest that freezing facilitates collagen turnover resulting in a functionally weaker construct initially. This may have implications for the healing and rehabilitation of clinical cases where frozen connective allografts are used.

Freeze drying (lyophilization) in a physical process in which a material is frozen, the atmospheric pressure is then lowered and sufficient heat is added to allow water molecules to directly sublime from the solid to the gas phase. Freeze drying protocols are reportedly more complicated than deep-freezing and require prior ethanol treatment for blood/lipid removal.[92] Subsequent lyophilization reduces tissue moisture to around 5%. This allows the graft to be vacuum packed and stored at room temperature for a period of between 3-5 years.[94] In a cadveric study of human patella tendon tissue, Bechtold *et al* (1994) reported greater ultimate tensile stress and strain values for frozen than for freeze-dried tendon.[107] Whether this difference is clinically significant is not known. The lengths of time freeze dried allografts are left to re-hydrate likely influences the subsequent biomechanical properties of the graft. Few studies have examined the

immunological effects of lyophilization. Arthroscopic biopsy samples from a group of 21 human patients receiving a freeze dried fascia lata allograft ACL replacement revealed a progression of biologic incorporation very similar to that seen with fresh-frozen allograft ACL replacement.[108] Studies of cortical bone allografts would suggest that the process of lyophilization further decreases antigenicity beyond that which is achieved with freeze treatment alone.[92]

One of the most potentially significant disadvantages to allograft usage is the risk of disease transmission. Freezing techniques are not effective in eradicating viral pathogens such as human immunodeficiency virus (HIV) and hepatitis.[109] The disease transmission implications of the use of equine allograft technologies as yet remain unexamined. Gaseous ethylene oxide (EO) is an alkylating agent that kills micro-organisms through the inactivation of DNA, RNA and protein.[110] The clinical use of EO for allograft sterilization has been anecdotally associated with accumulation of toxic by-products, ethylene chlorohydrin and ethylene glycol. In a case series of 109 patients receiving an EO sterilized BPTB allograft, Jackson *et al* (1990) reported a cellular intrasynovial inflammatory reaction in 7 patients (6.4%)[111] Removal of the allografts resulted in resolution of the reaction. In the one graft that was subsequently analyzed, high levels of ethylene chlorohydrin were detected using gas chromatography. Roberts *et al* (1991) reported complete graft dissolution in 8 of 36 patients (25%) receiving a freeze dried EO sterilized BPTB allograft.[112] The authors' clinical assessment was that EO treatment of the allograft tissue was the cause. Unfortunately, no analysis of EO residues was carried out. A subsequent *in vitro* study demonstrated synoviocyte production of interleukin-1 in response to exposure to EO sterilized BPTB wear particles.[113] These findings are in disagreement with earlier clinical reports of safe, effective bone and fascia lata sterilization with EO.[114] These discrepancies are probably due to differences in EO processing techniques, the allograft material involved and the pre-operative aeration and rehydration or rinsing techniques employed.

Gamma irradiation alters nucleic acid resulting in genomic dysfunction and generates free radicals from water molecules.[115] The bactericidal properties of gamma irradiation are currently utilized in the United Kingdom in allograft processing.[109] The latter effect is diminished if lyophilization has been carried out prior to irradiation.[95] A practical result of this is that much higher doses of radiation are required to achieve sterility if the allograft is in a frozen or freeze-dried state. Fideler *et al* (1995) reported statistically significant reductions in ultimate tensile stress, strain energy, and Young's modulus in fresh-frozen allografts after 2.0 Mrad of radiation.[116] Doses of 4.0 Mrad have been reported as necessary to inactivate HIV from BPTB allografts.[117] For this reason gamma irradiation is not recommended where allograft mechanical strength is a critical issue.

Per acetic acid (PAA) has several ideal qualities for allograft sterilization. The bactericidal qualities of PAA are due to generation of the hydroxyl radical (HO•) in solution which oxidizes cellular carbohydrates, lipids and catalyses hydroxylation of phenylalanine to 3-hydroxyphenylalanine (m-tyrosine).[118] The break-down products of PAA are relatively innoccuous (hydrogen peroxide and acetic acid). A PAA treated small intestinal submucosa allograft retained its native GAG as well as the resident growth factors; tumor necrosis factor-beta (TNF-β), basic fibroblast growth factor (BFGF) and vascular endothelical growth factor (VEGF).[119] While this has not been determined for tendon, this may help retain desirable biomechanical and bio-intetegrative properties of the allograft. Starke (1984) initially established the suitability of PAA for the sterilization of Achilles tendon allografts.[120] Subsequent studies have demonstrated no effect on *in vitro* biocompatibility and only limited detrimental effects on tendon biomechanical properties.[121] In an ovine model of ACL replacement, Scheffler *et al* (2008) reported delayed remodeling of a PAA treated FDS allograft compared to non PAA sterilized allografts and autografts.[122] An inferred consequence of this was the inferior biomechanical properties observed at 3 months. For the reasons outlined previously, these

findings are unlikely to be the result of toxic by-products. The authors suggested that unspecified ultrastructural (possibly oxidative) graft changes may have delayed the bio-integrative process. Further investigation is required to determine the exact ultrastructural changes that result from PAA treatment.

Non-ionic detergents are desirable decellularization agents because of their effect on ECM is purportedly limited to lipid-lipid and lipid-protein interactions. The most studied non-ionic detergent is polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether, known commonly by its trade name, Triton X-100 (Triton X-100, Sigma-Aldrich Corporation, St. Louis, MO). The effectiveness of Triton X-100 as a decellularization agent appears to be dependant upon the type of tissue and the duration of its exposure. Cartmell and Dunn (2000) examined the effectiveness of Triton X-100 treatment using rat-tail tendon.[123] Treatment of tendons with 1% Triton X-100 for 24 hours did not effectively remove cells. In rat tail tendon, Hu *et al* (1997) demonstrated disruption of the normal collagen D-period and a 50% reduction in tensile strength after Triton X-100 treatment.[124] In contrast, treatment of porcine ACL with Triton X-100 suffered no significant loss of biomechanical properties.[114] Triton X-100 may function most effectively as part a multi-step decellularization process. Using avian FDP tendon tissue, Whitlock *et al* (2007) reported excellent decellularization utilizing a protocol that included immersion in hypotonic solution, trypsin digestion and a PAA-Triton X-100 solution. Tendon tissue treated using this method exhibited excellent biocompatibility and retained 75% of its ultimate tensile stress.[126] In contrast to non-ionic detergents, ionic detergents such as sodium dodecyl sulphate (SDS) and Triton X-200 are highly effective at removal of cellular components. However, such compounds are associated with loss of collagen integrity and removal of GAG.[127]

Enzymatic protocols for allograft decellularization commonly include a combination of proteases, nucleases and chelating agents.[127] Trypsin is a proteolytic enzyme that cleaves the

peptide bond on the C terminal side of arginine and lysine, provided the next residue is not proline.[128] Trypsin cannot cleave intact helical collagen and as such has been used as an indicator of collagen degradation. The addition of chelating agents such as ethylenediaminetetra-acetic acid (EDTA), will bind the divalent cations (Ca^{2+}, Mg^{2+}) necessary for cellular attachment to the ECM.[129] The addition of nucleases to a decellularization protocol may facilitate the removal of DNA and RNA. Trypsin has been used as part of a multi-step decellularization tendon allograft processing technique. Chong *et al* (2009) reported the complete decellularization of rabbit flexor tendons using a freeze-thaw cycle followed by trypsin and Triton X-100 treatment.[130] Tensile testing showed no effect on biomechanical properties. While not specifically examined in tendons, prolonged exposure of porcine heart valve tissue to Trypsin/EDTA resulted in substantial loss of GAG and fibronectin resulting in a decrease in tissue ultimate tensile strength.[131] In a tendon derived allograft where biomechanical strength may be important, it would be desirable to determine ideal exposure times to ensure decellularization without adverse biomechanical effects.

2.4 The immunology of musculoskeletal allograft transplantation

In transplantation medicine, the transfer of cellular allogenic tissue between genetically dissimilar individuals will usually elicit an immunologic rejection response.[132] Despite the advent of highly effective surgical transplantation techniques, this remains a significant clinical impediment. In solid organ transplantation the most vigorous rejection reactions are associated with donor-host disparity within the major histocompatibility complex (MHC) antigens.[133] Currently, the evidence for the presence of MHC antigens on tenocytes is indirect. Using two strains of inbred rats, Minami *et al* (1982) demonstrated that the cellular component of tendon was the primary determinant of immunological reactivity.[134] A further finding was that, after freezing, tendon cells lost their ability to elicit an immune response as measured by a complement

dependant cytotoxicity test. These observations further illuminated histological studies in which allotransplantation of fresh tendon resulted in immunologic rejection and implant failure while deep-freezing prior to implantation was associated with remodeling and incorporation by the host.[135]

Upon implantation, decellularized tendon allografts can be infiltrated by cells with a fibroblastic morphology and subsequently remodeled.[136] For this reason tendon allograft materials are commonly decellularized and implanted as ECM. Collagen itself is considered to be a weak antigen.[137,138] However, the interpretation of immunological reactions to collagenous implants is often complicated by the presence of non-collagenous proteins, cells and cell-remnants. Immunologic reactions to decellularized tendon allografts have been reported. In an experimental study of ACL replacement, Vasseur *et al* (1987) reported the presence of anti-donor dog leukocyte antigen in the synovial fluid but not in the serum of all subjects receiving a BPTB ACL allograft.[139] Thompson *et al* (1992) identified a humoral immune response characterized by production of antidonor IgG in 38% of human subjects receiving a BPTB allograft for ACL reconstruction.[140] However, when this group was compared with autograft recipients, no significant difference in clinical outcome was identified. This type of occult immune response may be why some studies report delayed remodeling of allograft tissue when used for ACL replacement.[142,143]

Lynn *et al* (2003) identified three classes of antigenic determinants in collagen as (1) epitopes associated with the intact collagen triple helix; (2) epitopes associated with the amino-acid sequence rather than the 3 dimensional conformation; and (3) epitopes associated with the terminal telopeptide sequences.[138] The helical component of the tropocollagen is believed to be highly conserved, with amino acid sequences varying only by a small amount, even between species.[43] Centrally located epitopes in the helical region may be hidden from the immune system

and only become exposed to interacting antibodies when the triple helix is unwound. This may have an immunological implication if allograft collagen is degraded during processing. In contrast, the terminal telopeptide regions may exhibit considerable heterogeneity and as such have been implicated in some studies as major antigenic determinants.[144] However, in order to maximize any immune response to transplanted ECM, most reported studies involve xenotransplantation and therefore the true situation with regards to allotransplantation remains unclear.

Chemical cross-linking of allograft collagen, using isocyanate, glutaraldehyde or cabodiimide, has been proposed as a method for enhancing allograft biomechanical properties and slowing its rate of degradation.[145,146] Subsequent studies have identified an unspecified yet detrimental immunological response to chemically cross-linked collagen. Valentin *et al* (2006) examined porcine small intestinal submucosa (PSIS) ECM based xenografts in a rodent abdominal wall defect model.[147] Xenografts derived from acelluar porcine small intestinal submucosa (PSIS) cross-linked with either isocyanate or carbodiimide exhibited low grade inflammation, minimal scaffold degradation and fibrous encapsulation. Conversely non cross-linked PSIS xenografts exhibited rapid remodeling with minimal inflammation resulting in improved biomechanical characteristics in the repair tissue.

The host immune response to allotransplantation is regulated by specific phenotypes of T-lymphocytes. These sub-populations are believed to determine graft acceptance or rejection based upon the prevailing cytokine profile.[148] Currently three helper T-lymphocyte phenotypes have been identified and have been designated Th1, Th2 and Th17.[149] Classically, allograft rejection involved a predominance of Th1 derived cytokines (IL-2, IFN-γ, TNF-α).[150] Aggarwal *et al* (2003) reported the expression of IL-17 in response to IL-23 by a separate T–lymphocyte lineage designated Th-17.[151] IL-17 has been implicated in rejection of solid organ transplants. Th2

derived cytokines (IL-4,IL-5, IL-6 and IL-10) are believed to inhibit Th-1 mediated cytotoxic T-lymphocyte and delayed type hypersensitivity responses.[152] However, this paradigm is likely overly simplistic. For example, the prototypic Th-2 cytokine, IL-4, can be both immunosuppressive and immunostimulatory.[153]

Few studies have examined the effect of allograft ECM scaffolds in terms of the host Th1/Th2/Th17 lymphocyte response. Allman *et al* (2001) examined the cytokine response to subcutaneously implanted acellular xenogenic PSIS.[154] Graft site cytokine analysis showed an increase in IL-4 and an absence of IFN-γ as measured by reverse transcriptase PCR. The predominant antibody response was of the non complement fixing IgG_1 isotype. Furthermore, sequential implantation of PSIS xenografts 28 days apart did not elicit a second set rejection reaction. No evidence of Th1 cytokines at the secondary graft site was found and the antibody response remained exclusively the IgG_1 isotype. Unfortunately, similar studies examining cytokine profiles associated with allotransplantation (decellularized ECM) are not readily available.

Macrophages play a key role in ECM degradation and by extension allograft remodeling.[152] As with lymphocytes, macrophages phenotypes have been identified on the basis of their functional properties, cell surface markers and the prevailing cytokine products.[155] The M1 macrophage phenotype produces the pro-inflammatory cytokines IL-12 and TNF-α. Conversely the M2 macrophage phenotype produces the Th-2 limited cytokine IL-10 as well as TGF-β. Using a rodent abdominal wall defect model, Valentin *et al* (2009) identified diverging macrophage phenotypes in response to chemical cross linkage of collagen in a PSIS derived xenograft.[156] At both graft sites, a $CD68^+$ population of mononuclear cells was present during the first 4 weeks. However, at 16 weeks, the non-cross linked xenograft exhibited a predominantly $CD163^+$ response (M2 profile). In contrast, the cross linked xenograft exhibited a predominant $CD80^+$ and

$CCR7^{+}$ response (M1 profile). Knowledge of the specific cell surface markers for different phenotypes of equine macrophages and lymphocytes would be useful in evaluation of host response to allotransplantation.

CHAPTER III

METHODOLOGY

3.1 Experimental subjects

Ten horses (7 mares and 3 geldings) were obtained for the purposes of this study. Based on dental examination the experimental subjects were aged between 5 and 15 years. All experimental subjects weighed between 445 and 520 kilograms. A physical examination carried out on each experimental subject prior to the commencement of the study revealed no abnormalities.

3.2 Allograft preparation

Two unrelated quarter-horse mares of approximately 10 years of age acted as donors for allogenous tendon tissue. Euthanasia was carried out by barbiturate overdose. Immediately after death, the distal forelimbs were clipped and aseptically prepared. The forelimb FDS tendons were surgically excised from the level of the accessory carpal bone to the level of pastern. Once removed, the FDS tendons were stripped of all paratenon and extraneous tissue and shipped overnight on dry ice to the Wake Forest Institute for Regenerative Medicine (Wake Forest University, Winston-Salem, NC).

All harvested tendon tissue was stored at −80°C until processing. Two variations of a previously published protocol were employed to carry out tendon decellularization and oxidation.[126] Tendon tissue from the first donor horse (denoted type-1 allografts) was processed according to the following protocol: Each FDS tendon was sectioned transversely in half prior to processing. One liter of DNase-free/RNase-free, distilled

water was added to each sample. After 24 hours, the water was discarded and the cycle was repeated. At the conclusion of the second cycle, the water was discarded and 500ml of 0.05% trypsin (Sigma-Aldrich Corporation, St. Louis, MO), 4.0mmol sodium bicarbonate, and 0.5mmol ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Corporation, St. Louis, MO), prepared in Hank's balanced salt solution was added. After 1 hour, the trypsin solution was discarded and 500 ml of Dulbecco's modified Eagle's medium high-glucose containing 10% fetal bovine serum (DMEM-FBS) (Valley Labs, Winchester, VA) and an antibiotic cocktail of 100IU/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B was added in order to halt trypsin digestion of the sample and eliminate any microbial contamination.

After 24 hours, the DMEM–FBS solution was discarded and 1L of the DNase-free/RNase-free distilled water was added. After 24 hours, the washing solution was discarded and 500 ml of 1.5% peracetic acid (PAA) solution with 2.0% non-ionic detergent (Triton X-100) (Sigma-Aldrich Corporation, St. Louis, MO) in distilled, de-ionized water (diH_2O) was added for 2 hours. The solution was discarded and three 12 hour immersions with 1L of diH_2O were performed. At the end of the third immersion, the sample was removed and placed into a clean, sterile polyethylene container and frozen for 24 hours at −80°C. The sample then was removed from the −80°C freezer and freeze-dried (Labconco, Freeze Dry System, Kansas City, MO) for 24 hours before being returned to the freezer and stored at −80°C prior to shipment to the Oklahoma State University College of Veterinary Medicine at room temperature.

Tendon tissues from the second donor horse donor horse (denoted type-2 allografts) were processed with the following modifications to the previously described protocol:

1. The period of immersion in PAA-Triton X-100 solution was increased from 2 to 4 hours.

2. The total number of 12 hour washes in diH_2O after PAA-Triton X-100 treatment was increased from 3 to 6.

3.3 Surgical technique of allograft implantation

The surgical technique employed was based on a technique previously described by Fackelman (1973).[167] All experimental subjects received pre-operative broad spectrum antimicrobials (potassium penicillin; 22,000IU/kg IV and gentamicin; 6.6mg/kg IV) as well and NSAID analgesia (flunixin; 1.1mg/kg IV). General anesthesia was induced with ketamine (2.2mg/kg IV) and diazepam (0.1mg/kg IV) after pre-medication with xyalzine (1.1mg/kg IV) and was maintained using isoflurane delivered in 100% oxygen. The experimental subjects were randomly assigned to either right lateral or left lateral recumbency prior to surgery. Both forelimbs distal to the accessory carpal bone were clipped, aseptically prepped and draped prior to surgery. A 15cm skin incision, orientated proximo-distally was made on the palmaro-medial aspect of the proximal metacarpus (Figure 1). A 10cm longitudinal incision was then made in the paratenon exposing the epitenon of the FDS tendon. Using a number 11 scalpel blade the palmar midline of the FDS tendon was scored to a depth of 2mm for approximately 6cm in length. With a #11 scalpel blade orientated at 45 degrees to the palmar aspect of the tendon, a longitudinal incision was made into the tendon from the palmar midline, 6cm in length. A second incision, orthogonally orientated, was then made in a similar fashion. The resulting effect was to create an envelope within the FDS tendon.

Figure 1

Figure 1: Intra-operative photographs demonstrating the surgical approach to implantation. Top: A 15cm incision orientated proximo-distally on the proximal palmar metacarpus through skin and paratenon. Middle: A longitudinal incision is made with a #10 blade orientated 45 degrees to the sagittal plane. Bottom: Positioning graft in the pocket created within the FDS tendon. Closing sutures have been pre-placed at the margins of the tendon.

Prior to implantation, tendon allografts were soaked for 12 hours in a solution of 250ml of isotonic saline with 1000mg of amikacin at room temperature. Allografts were trimmed intra-operatively to dimensions of 60mm by 2mm by 6mm. The sized allograft was then placed in the pocket within the FDS tendon. The epitenon on the palmar aspect was then closed with 6 simple interrupted sutures of 3-0 (USP) polyglactin-910. The paratenon was then closed with 3-0 polyglactin-910 using a simple continuous suture pattern. The skin margins were opposed using 2-0 nylon in a simple interrupted suture pattern. Half-limb bandages were placed on both operated limbs prior to recovery.

In the first 4 experimental subjects operated upon, one limb received a type-1 allograft while the contra-lateral limb functioned as a sham-operated control. In the second group of 4 experimental subjects, one FDS tendon received a type-2 allograft and the contra-lateral limb received an autograft harvested from the EDL tendon of the uppermost hindlimb at the level of the tarsus according to a previously published surgical technique.[110] Prior to implantation the paratenon was removed and the autograft was trimmed to the same dimensions as the allograft.

3.4 Post-operative management of experimental subjects

All experimental subjects recovered from anesthesia without complication. Intravenous potassium penicillin at the previously mentioned dose-rate was given every 6 hours for 24 hours post-operatively. For analgesia and anti-inflammatory effects, flunixin was administered (1.1mg/kg IV q12hrs for 3 days followed by 1.1mg/kg IV q24hrs for 3 days) for 6 days post-operatively. Half limb bandages were maintained on operated limbs for two weeks and were changed every second day. Skin sutures were removed from all surgery sites at 10 days post-operatively. No complications relating to healing of surgical sites were observed. All experimental subjects were confined to a hospital stall until bandages were removed. After this

time, the experimental subjects were housed in 3 meter by 3 meter stalls until the conclusion of the study.

3.5 Ultrasonographic examination of treated tendons

All ultrasonographic examinations were carried out using a MyLab® 30 CV ultrasound system (Biosound Easote, Inc. Indianapolis, IN) with a 15MHz linear tendon probe. Ultrasonographic cross-sectional images of the forelimb FDS tendons were obtained prior to study commencement and then at 2 weekly intervals for the duration of the study. Digital images were obtained from the transverse level of the accessory carpal bone at 2cm intervals to the level of the metacarpophalangeal joint. Cross sectional area (CSA) measurements of FDS tendons were made using an integrated software package (Biosound Easote, Inc. Indianapolis, IN). In all experimental subjects, images obtained at 12cm and 14cm distal to the base of the accessory carpal bone (DACB) corresponded to the proximal and distal aspects of the implantation site. These two locations were used to obtain CSA measurements for comparison between treatment groups.

3.6 Gross and histological examination of treated tendons

Twelve weeks postoperatively, all experimental subjects were euthanized by barbiturate overdose and the forelimbs were sectioned at the level of the radiocarpal joint. After removal of the skin, any adhesions between the paratenon and FDS tendon were documented photographically. The FDS tendons were then excised from the level of the accessory carpal bone to the metacarpo-phalangeal joint and the amount of tendinous reaction was documented photographically. Full thickness longitudinal sections were made corresponding to transverse levels 12 and 14cm DACB respectively. These sections were embedded in paraffin and cut to a thickness of 5μm using an ultramicrotome and mounted on glass slides. The mounted sections were then stained with hematoxylin and eosin (H&E, Sigma-Aldrich Corp. St. Louis, MO).

A subjective scoring system was utilized to examine the effects of sham surgery, allotransplantation and autotransplantation on the FDS tendon. Each slide was scored by two pathologists blinded to the treatment groups.

3.7 Statistical analysis

All statistical analyses were carried out using PC SAS Version 9.2 (SAS Institute, Cary, NC). For ultrasonographic (CSA) data, analysis of variance assuming a repeated measures model with an autoregressive covariance structure was carried out. A comparison of means was done using protected pair-wise t-tests. For histological scores from the subcutaneously implanted allograft-autograft pair data, two factor analysis of variance was undertaken, examining the effect of treatment (allograft versus autograft) at given time period (1, 5 or 10 weeks). For histological scores from tendon implanted allografts and autografts, data were also assessed using a two factor analysis of variance. All data were considered significant at $P \leq 0.05$.

CHAPTER IV

FINDINGS

4.1 Ultrasonographic examination of treated tendons

The CSA of all operated tendons was observed to increase post-operatively. (Figure 2) At 2 weeks, there was no significant difference in CSA between treatment groups (sham surgery, autograft, type-1 allograft and type-2 allograft). By 6 weeks, a significant difference in CSA was observed between all treatment groups. This difference between groups was present at every observation from this time-point forwards. From the 4 week time-point, the sham-operated tendons decreased in size until by the conclusion of the study, they were approximately 10% larger than their pre-operative measurements. At this point no tendon lesion was identifiable ultrasonographically.

The greatest increase in size was observed in the tendons implanted with the type-1 allograft. At the conclusion of the study tendons in this group were over 100% larger than their pre-operative measurements. In this group, the CSA did not appear to have stabilized by the conclusion of the study. Ultrasonographically, the type-1 allograft remained clearly visible within the host tendon, surrounded by a well demarcated zone of hypoechogenicity (Figure 3). From the initial observation at 2 weeks, the allograft itself was not observed to change in size. The allograft remained more echogenic than peripheral areas of host tendon throughout the study. Marked thickening of the paratenon and subcutaneous tissues was also evident.

Figure 2

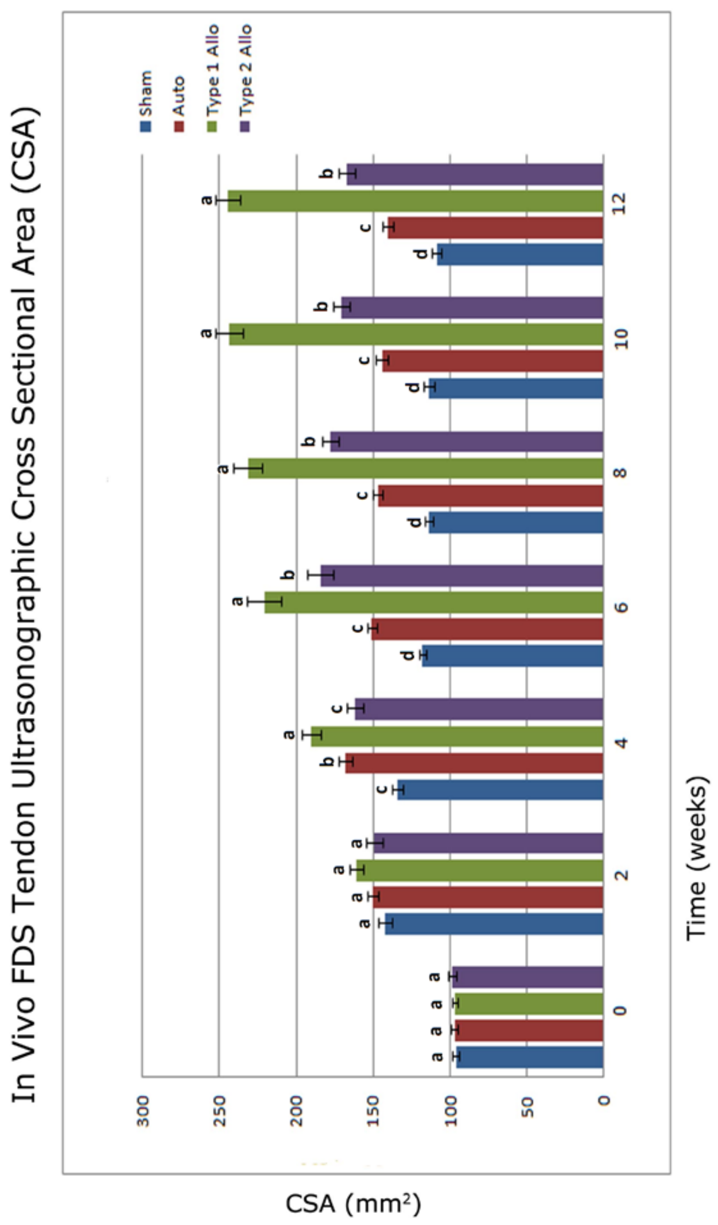

Figure 2: Bar graph showing mean ultrasonographic cross-sectional area values obtained for treatment groups, measured at two week intervals. Lines on bars indicate 1 standard deviation above and below mean values.

Figure 3

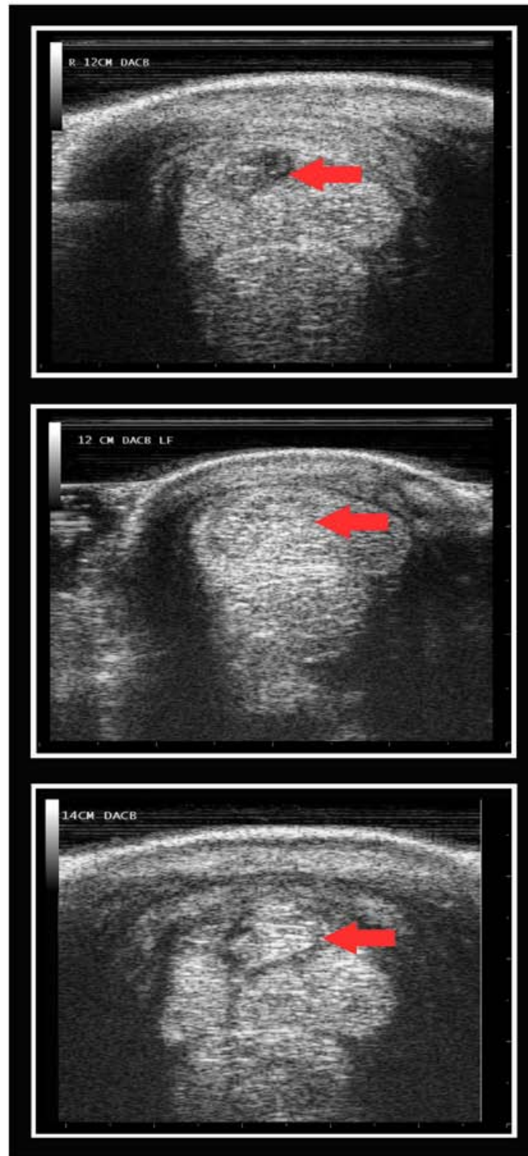

Figure 3: Transverse sonograms taken from the proximal palmar metacarpus (12-14cm DACB) of implanted limbs at 12 weeks. Red arrows indicate the location and appearance of each type of implant within the FDS tendon. Top: Autograft. Middle: Type-2 allograft. Bottom: Type-1 allograft.

Tendons which received an EDL tendon autograft also decreased in CSA from the 4 week time point onwards. By the conclusion of the study, the autograft group was the closest in CSA to the sham-operated group although still significantly larger in CSA. Ultrasonographically, the implanted autografts appeared uniformly hypoechoic relative to the surrounding host tendon. A similar degree of paratendinous and subcutaneous reaction was observed to that observed in the type-1 allograft group. However, they were more similar in size to the autograft group than the type-1 allograft group although still significantly larger. Ultrasonographically, the type-2 allografts appeared incorporated into the host tendon to a degree similar to that seen in the autograft group. Similar to the autograft and type-1 allograft, the type-2 allograft was not observed to change in size significantly for the length of the study. Of the implanted tendons, the type-2 allograft group appeared to have the smallest amount of paratendinous reaction and that respect was most similar ultrasonographically to the sham-operated group.

4.2 Gross pathological examination of treated tendons

All operative sites healed uneventfully and no evidence of post-operative complications was found at necropsy. Sham operated limbs exhibited subtle tendinous and paratendinous thickening in the proximal metacarpal region. Dissection of the skin and subcutaneous tissues revealed mild thickening of the paratenon associated with the surgical site. In all sham-operated limbs a small number of focal adhesions were present between the paratenon and the epitenon. When the tendon was sectioned, the operative site was difficult to locate grossly.

Implantation of type-1 allografts was associated with marked paratendinous and tendinous thickening (Figure 4). Extensive paratendinous fibrosis resulted in large substantial adhesions between the palmar aspect of the FDS tendon and the paratenon. The tendon itself exhibited marked fibrosis around the allograft which remained discernable as a whitish structure within the

Figure 4

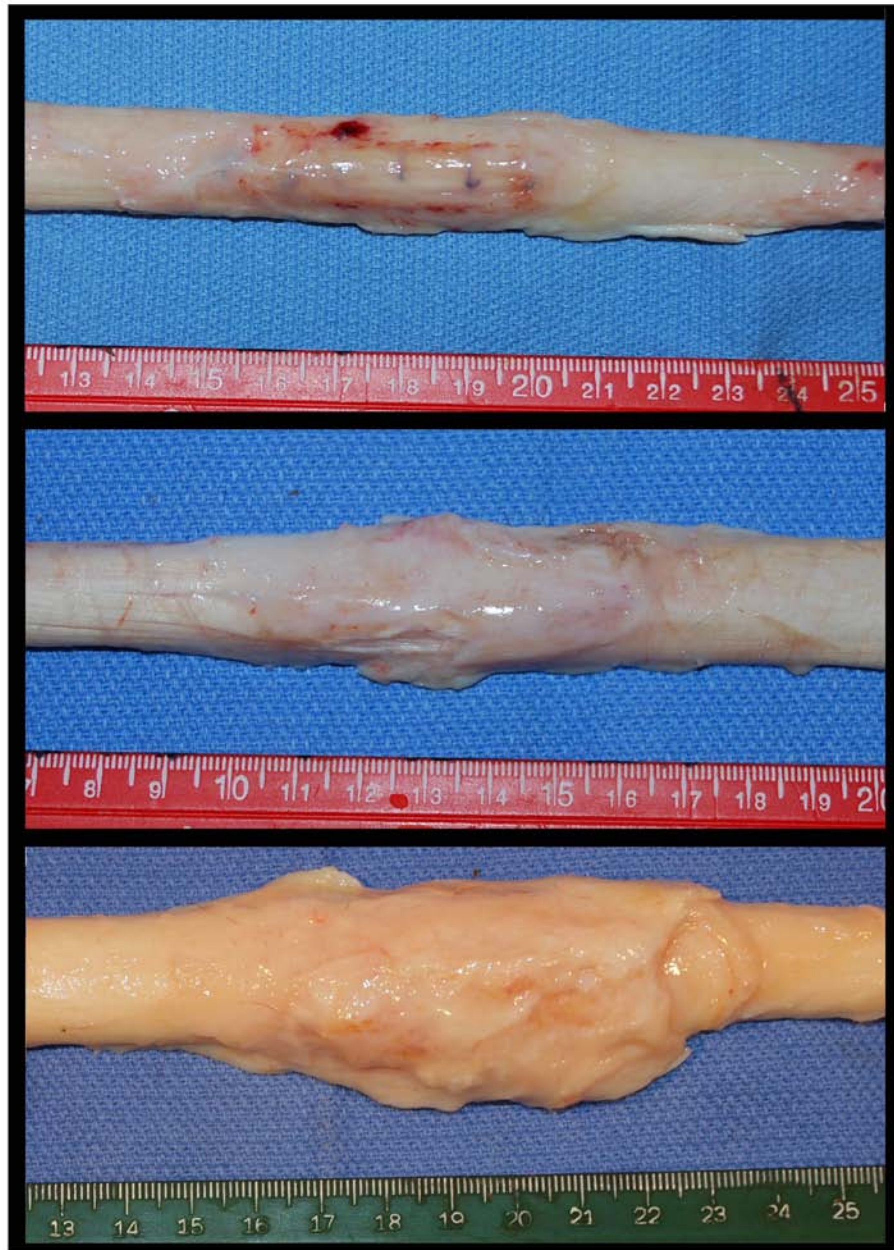

Figure 4: Post mortem photographs of the implanted regions of the FDS tendons harvested 12 weeks post-operatively. Top: Autograft. Middle: Type-2 allograft. Bottom: Type-1 allograft. A significant amount of tendinous and paratendinous reaction is associated with the type-1 allograft.

host tendon when sectioned (Figure 5). The allograft was adherent to the surrounding fibrous tissue and tendon but could be separated from the host tendon with minimal force.

Grossly, limbs implanted with type-2 allografts exhibited a similar amount of paratendinous thickening and fibrosis to the sham-operated tendons. Adhesion formation between the paratenon and epitenon was mild and considerably less extensive than the adhesions observed in limbs receiving the type-1 allograft. On transverse section, the allograft could be visualized as a white region within the cream colored surrounding host tendon. In contrast to the type-1 allograft, the type-2 allograft appeared well integrated and required dissection to remove it from the surrounding host tendon.

Limbs implanted with autografts exhibited a similar amount of paratendinous thickening and fibrosis to the limbs implanted with the type-2 allograft. Adhesion formation between the epitenon and paratenon at the surgical site appeared intermediate between that seen in limbs receiving the type-1 allograft and sham-operated limbs. On transverse section, the autograft could be consistently visualized as an area of reddish discoloration within the host tendon that corresponded with the ultrasonographic images of the area obtained ante-mortem, post-operatively. In contrast to the type-1 and 2 allograft treated limbs, the autograft tissue could not be readily dissected from the host tendon.

Figure 5

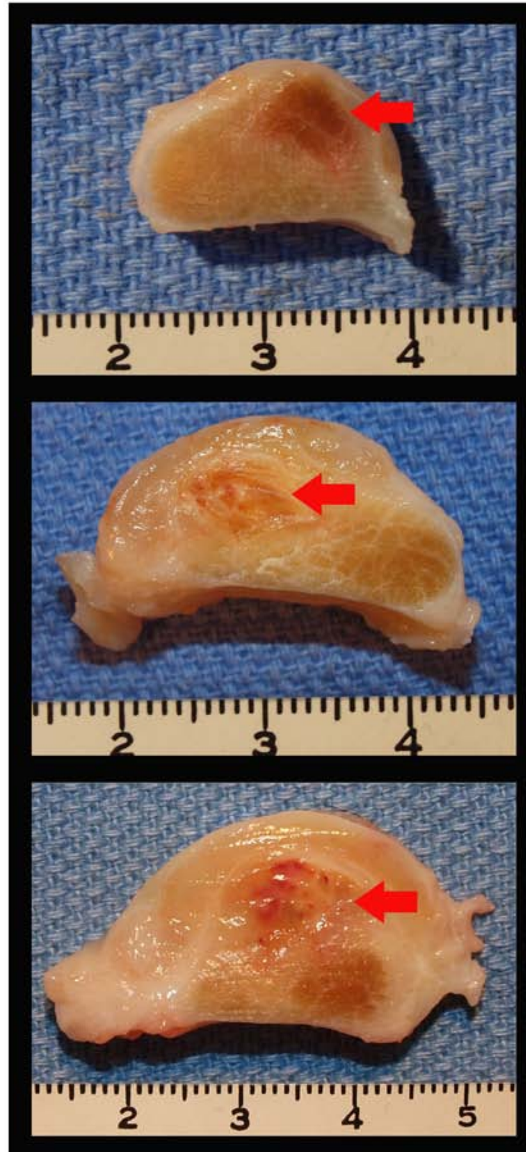

Figure 5: Transverse sections of implanted FDS tendons corresponding to regions 12-14cm DACB. Arrows indicate the location and appearance of each implant in situ. Top: Autograft. Middle: Type-2 allograft. Bottom: Type-1 Allograft.

4.3 Histological scoring of treated tendons

The highest scores for lymphocyte density were given to implanted tendons receiving the type-1 allograft (Table 1). Lymphocyte density scores for type-2 allografts and autografts were not significantly different. Conversely, the highest scores for neutrophil density and macrophage density were given to the autograft implanted tendons. No difference in score for macrophage density was recorded between type-1 and type-2 allograft implanted tendons. Type-1 allograft implanted tendons scored higher for neutrophil density than type-2 allograft implanted tendons. Autograft treated tendons received the highest scores for degree of neo-vascularization while tendons implanted with the type-2 allograft scored higher in this characteristic than those receiving the type-1 allografts, although this finding was only significant in the distal location of the implantation site. The greatest scores for reactivity (width of reaction) were consistently assigned to the type-1 allograft treated tendons. No significant difference was found in scores for width of reaction between type-2 allograft treated tendons and autograft treated tendons.

Table 1

Means and standard errors for histological scores given to treated tendons at 12 weeks.

An explanation of scoring methodology is given in Appendix A.

Location in tendon	Treatment	Lymphocyte density (0-3 scale)	Macrophage density (0-3 scale)	Fibroblast density (0-3 scale)	Neutrophil density (0-3 scale)	Neovascularization (0-3 scale)	Organization of collagen at interface (Haphazard=1; Linear=2)	Width of reaction at interface (μm)
Proximal	Auto	1.4 (±0.2)b	2.9 (±0.2)a	1.8 (±0.2)a	3.0 (±0.0)a	2.9 (±0.1)a	1.0 (0.0)b	308.75 (±30.73)b
	Sham Surgery	0.3 (±0.2)c	0.1 (±0.1)c	0.8 (±0.2)b	1.9 (±0.1)b	1.1 (±0.1)b	1.8 (±0.2)a	40.63 (±9.75)b
	Type 1 Allograft	2.8 (±0.2)a	1.4 (±0.2)b	0.5 (±0.0)ab	0.1 (±0.1)c	0.6 (±0.2)b	1.0 (±0.0)b	2725.00 (±232.80)a
	Type 2 Allograft	1.4 (±0.2)b	1.0 (±0.0)b	1.0 (±0.2)b	1.5 (±0.2)b	1.0 (±0.0)b	1.4 (±0.2)ab	477.50 (±29.99)b
Distal	Auto	1.5 (±0.2)b	2.4 (±0.2)a	1.3 (±0.1)b	2.7 (±0.2)a	2.8 (±0.2)a	1.0 (±0.00)b	354.38 (±35.53)b
	Sham Surgery	0.4 (±0.2)c	0.3 (±0.2)c	1.0 (±0.1)b	1.4 (±0.2)b	1.3 (±0.2)b	1.8 (±0.2)a	45.63 (±4.86)b
	Type 1 Allograft	3.0 (±0.0)a	2.0 (±0.0)a	0.5 (0.0)a	0.1 (±0.1)c	0.5 (±0.2)c	1.1 (±0.1)b	2975.00 (±243.30)a
	Type 2 Allograft	1.4 (±0.2)b	1.1 (±0.1)b	1.1 (±0.2)b	1.3 (±0.2)b	1.4 (±0.2)b	1.8 (±0.2)a	345.00 (±36.99)b

Means and standard errors for histological scores and measurement values given to FDS tendons implanted with either autograft, type 1 allograft, type 2 allograft or sham operated.

Scores labelled a,b or c indicate significant difference groups at the same location ($P<0.05$)

Scores labelled ab indicate no significant difference with groups labeled a or b ($P>0.05$)

4.4 Descriptive histological analysis of treated tendons

By 12 weeks post-operatively, the sham-operated tendons appeared to have returned to a near normal histological appearance. Subtle loss of normal fascicular architecture (fascicular thickening and inter-fascicular fibrosis) was apparent at the surgical site. A mild increase in the

cellularity and vascularity within the operated region was also apparent. The predominant cell type exhibited a fibroblastic morphology. Small numbers of lymphocytes were also present.

In the autograft implanted tendons, the normal linear fascicular arrangement of tendon ECM was largely replaced by randomly orientated highly cellular fibrous connective tissue (Figure 7). However, small regions retaining the linear fiber orientation of the original allograft could be observed, scattered randomly throughout the autograft. Significant numbers of macrophages and lymphocytes were observed throughout the autograft and at the graft-host interface. Abundant diffuse neovascularization was observed.

Histologically, the type-1 allograft implanted tendons exhibited a marked fibrous reaction that surrounded the allograft within the host tendon and proliferated through to the surface of the adjacent palmar epitenon. This tissue appeared to be an encapsulating reaction around the implanted allograft. At the graft-host interface abundant cellular infiltration, composed primarily of lymphocytes, and smaller numbers of macrophages, with the occasional multi-nucleate giant cells was observed. Within the graft itself, few strands of linearly arranged infiltrating fibrous tissue could be observed accompanied by very limited neovascularization. Where the neovascularization was present it was invariably associated with lymphocytic infiltration. However, the center of the graft appeared largely acellular and uninfiltrated.

In contrast to the type-1 allograft, the type-2 allograft elicited a fibrous reaction of similar width to that seen in the autograft treated tendons. However, the reaction around the type-1 allograft appeared more organized and non-encapsulating, with linearly arranged bundles running parallel to the fiber-bundles within the graft itself (Figure 9). In contrast to the autograft, the implanted type-1 allograft retained its fascicular architecture of linearly arranged fiber bindles, similar to normal tendon. At the graft-host interface a cellular infiltration consisting of moderate numbers of lymphocytes and macrophages was observed. Within the substance of the graft, small

numbers of cells with well defined fibroblastic morphology, intimately associated with the fiber-bundles of the allograft, were present (Figure 9, insert). As with the type-1 allograft, limited neovascularization of the graft had occurred.

Figure 6

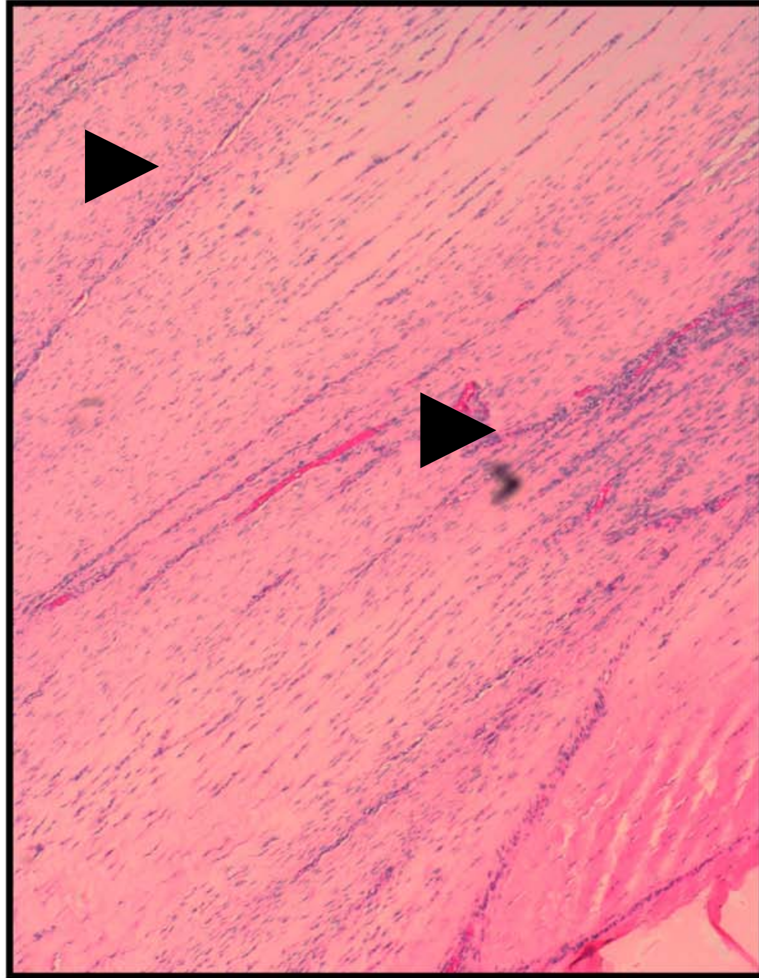

Figure 6: Photomicrograph of longitudinally sectioned FDS tendon taken from site of sham surgery. Arrow indicates a region of increased cellularity and vascularity (20x, H&E stain).

Figure 7

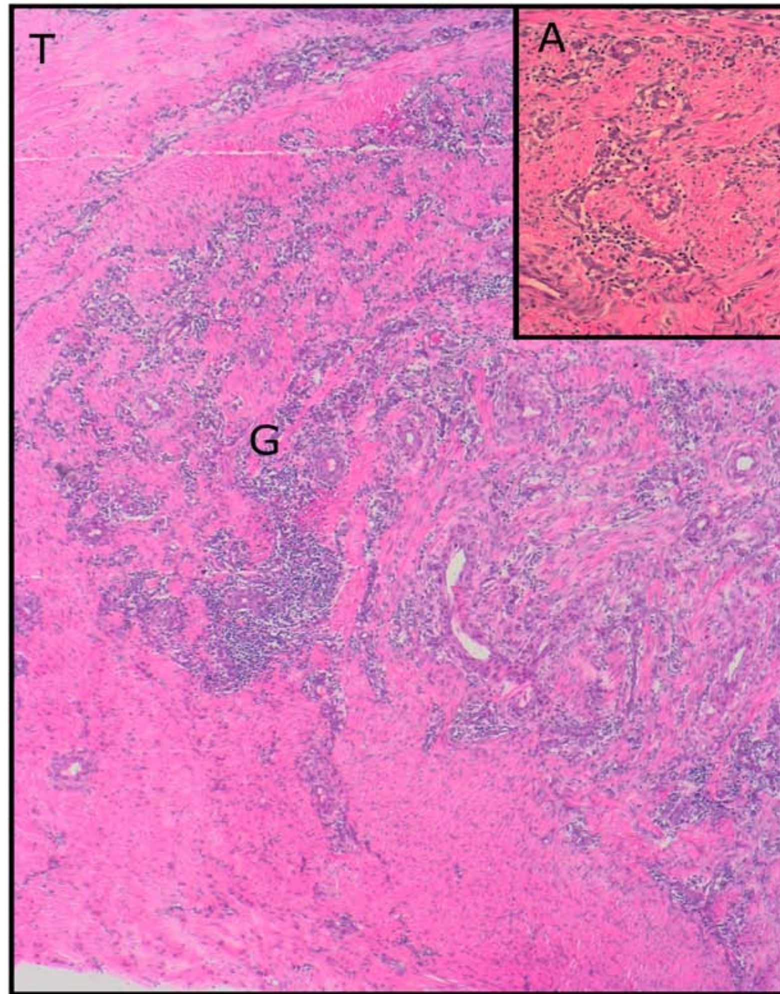

Figure 7: Photomicrograph of longitudinally sectioned FDS tendon taken from a site of autograft implantation. T: A region of host tendon. G: A region of autograft demonstrating breakdown of the graft and its replacement by disorganized collagen fibrils. (20x, H&E stain) A: High power magnification of region corresponding to G (200x, H&E stain).

Figure 8

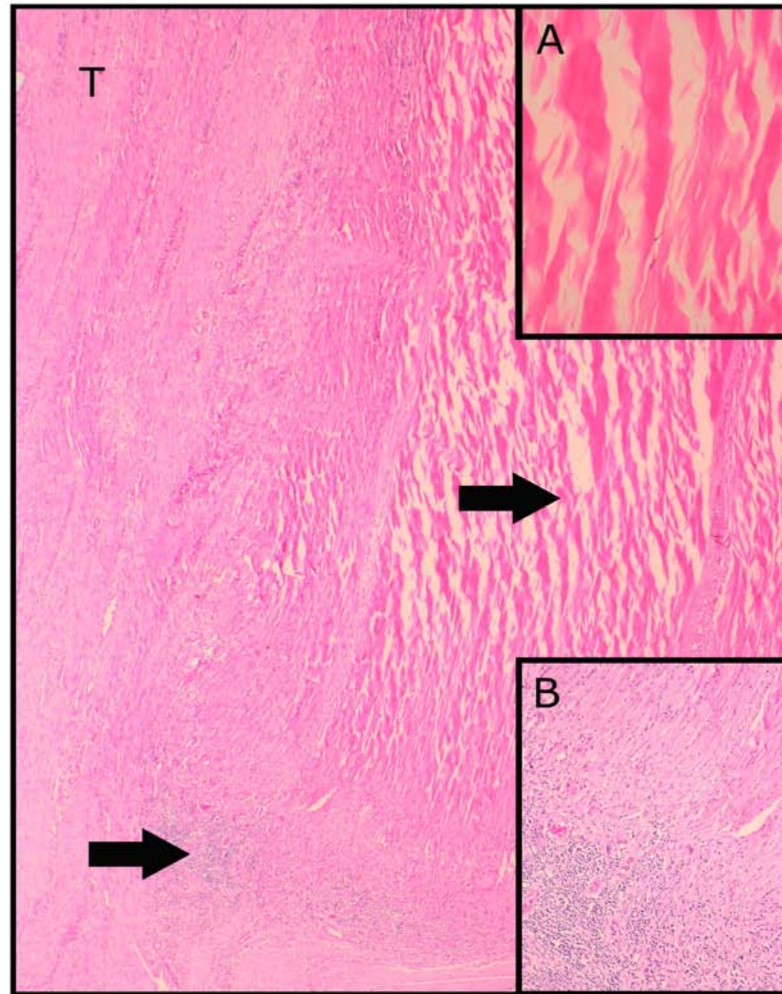

Figure 8: Photomicrograph of longitudinally sectioned FDS tendon taken from a site of Type-1 allograft implantation. T: Region of host tendon. Top arrow: A region at the center of the allograft. Bottom arrow: A region at the graft-host interface. A: High power magnification of region corresponding to top arrow. The allograft exhibits no cellular infiltration whatsoever in this region (200x, H&E stain). B: High power magnification of region corresponding to bottom arrow. A predominantly lymphocytic infiltration is seen in this area (200x, H&E stain).

Figure 9

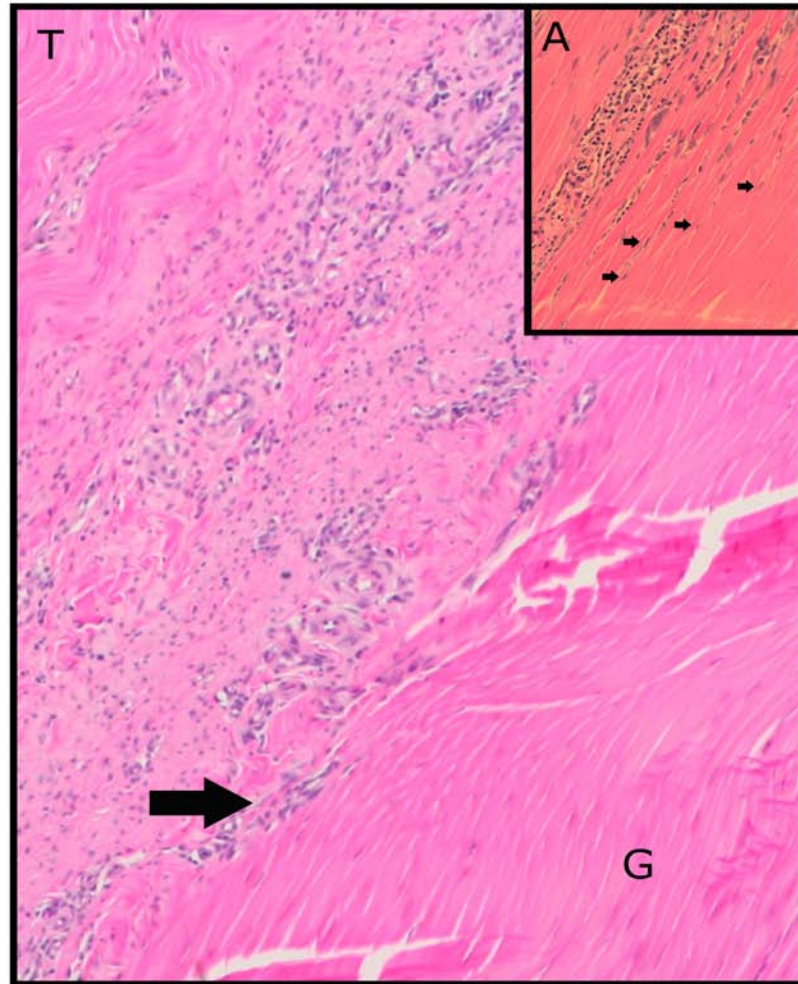

Figure 9: Photomicrograph of longitudinally sectioned FDS tendon taken from a site of type-2 allograft implantation. T: A region of host tendon. G: A region of type-2 autograft in situ. Arrow: A region of the graft-host interface. A: A high power magnification of region corresponding to the area indicated by the arrow. A predominantly lymphocytic reaction at graft-host interface is visible. The small arrows indicate cells with tenocytic morphology intimately associated with the allograft (200x, H&E).

CHAPTER V

CONCLUSIONS

5.1 Study limitations

This study has several limitations that prevent strong conclusions from being made from the available data. The ante-mortem effects of surgery, as well as allo and auto transplantation were assessed using the modality of ultrasound. Ultrasonographic examination is a clinically useful, non-invasive technique for assessment of tendon injury.[157,158] CSA is a key objective measurement made as part of a complete ultrasonographic examination.[159] Other potentially less objective measurements, such as fiber alignment score and degree of echogenicity, could have been utilized for a more complete ultrasonographic assessment. However these parameters have a higher degree of inherent subjectivity in comparison to CSA measurement.

Other ante-mortem analyses may have helped to characterize the host response to allotransplantation. In solid organ transplantation, measurable serum markers of rejection include: C-reactive protein, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6).[160] It is possible that transplantation of an inadequately decellularized allogenic tendon-derived biomaterial may have resulted in measurable serum increases in these markers. Similarly, where musculo-skeletal allografts have been implanted in an intra-articular environment, synovial fluid markers of inflammation have been measured.[139] In this study, the relatively small amount of tissue transplanted and the site of transplantation (non-articular) may have resulted in changes in these systemic parameters that were too small to measure. As outlined previously, the host immune response to musculoskeletal allotransplantation is believed to be regulated by specific phenotypes of T-lymphocytes.[147] These sub-populations are believed to determine graft acceptance or rejection and can be differentiated by the prevailing local cytokine profile and their surface antigens. Analysis of the mRNA profiles of lymphocytes and macrophages at the graft-host

interface could have been useful in characterization of the host response to the two allotransplantation protocols used in this study.

In this investigation, post-mortem evaluation of tendon tissue was limited to gross examination and histological analysis. Histology provides an effective evaluation of the host cellular and tissue architectural responses to surgery and/or transplantation. Subjective scoring systems of the type employed in this study have been utilized in previously published investigations of tendon healing.[161,162] While subjective scoring systems lack the rigor of objective numerical data, they represent an attempt to quantify otherwise subjective broad histological observations. For these reasons, two veterinary pathologists blinded to treatment groups were used in this study. As good correlation was achieved between observers, their scores were pooled for subsequent statistical analyses.

A further limitation to this study was its length. Clinical cases of FDS tendon injury may take 15 to 18 months to heal fully.[17] For this reason, it was possibly unreasonable to assess allograft incorporation after only 12 weeks. Ideally, the experimental subjects would have been maintained for at period of at least 15 months. However, the duration of this particular study is longer than other previously published investigations of tendon healing.[162,164-166]

5.2 Conclusions based on ultrasonographic data

The response of equine FDS tendon to surgical insult alone was a 10% increase in CSA at 12 weeks post-operatively. The tendon response to allo or auto transplantation however, varied significantly between the three treatment groups receiving implants. The type-1 allograft treated tendons continued to increase in CSA at every time-period and had not stabilized ultrasonographically by the conclusion of the study. A reduction in CSA would have been have been an *in vivo* indication of a healing and remodeling response to implantation. The hypoechoic

zone surrounding the type-1 allograft at 12 weeks combined with the observed increase in tendon CSA suggests delayed or impaired healing in response to the presence of the type-1 allograft.

In contrast, the CSA of the type-2 allograft and autograft treated tendons appeared to have stabilized by the conclusion of the study period. For both of these two groups, the ultrasonographic appearance of the engrafted tissue was one of incorporation into the host tendon. For the type-2 allograft, and the EDL autograft, the lack of a hypoechoic zone surrounding the implant and progressive reductions in CSA are *in vivo* indications of tendon remodeling and healing in the presence of the implant. The relatively hypoechoic appearance of the autograft within the host tendon at 12 weeks is a characteristic that correlates with the documented ultrasonographic appearance of immature reparative tendon tissue.[159] This ultrasonographic finding is suggestive of a more rapid remodeling of the autograft tissue in comparison to either the type-1 or type-2 allografts. Unfortunately, no previously published reports of the ultrasonographic evaluation of tendon allografts are available for comparison. While transplantation of fresh, autologous LDE tendon tissue has been evaluated as a potential treatment for FDS tendon injury, the ultrasonographic appearance of this transplanted tissue has not been documented.[167] Rapid remodeling of a tendon bioscaffold may result in a construct that is unable to withstand normal mechanical forces early in the healing process. This may explain why autograft facilitated repair of FDS injury failed to demonstrate clinical efficacy when evaluated by Grant *et al* (1982).[168]

5.2 Conclusions based on histological data

Surgical insult to the FDS tendon without implantation resulted in the loss of normal fascicular architecture, and a mild increase in vascularity and cellularity. In none of the treatment groups was normal tendon architecture restored by the end of the 12 week post-operative period. Histologically, fresh autograft EDL tendon was observed to lose almost all of its native fascicular

architecture when transplanted. The higher histological scores for macrophage and neutrophil densities in the autograft implanted tendons provides evidence for the ongoing and extensive nature of this remodeling process 12 weeks after implantation. Conventional paradigms concerning inflammation indicate that neutrophils are associated with an active, acute or persistent inflammatory response while macrophages are associated with sub-acute or chronic inflammation.[169] The degradation products of any biomaterial can function as chemoattractants for a variety of cell types.[152] In the absence of clinical, pathological and histological evidence of infection it is likely that the extensive degradation of unmodified autogenous tendon provides the bioactive constituent molecules for the cell population observed. An important clinical implication for this finding is that an implant derived from autogenous tendon, rapidly broken down *in vivo*, is unlikely to provide a scaffold for infiltration by fibroblastic cells.

In contrast to the autograft, both the type-1 and type-2 allografts sustained only limited remodeling by the conclusion of the 12 week study period. For both autologous biomaterials, the remodeling process was not histologically complete by the conclusion of the study. However, in each case, differences were observed in the character and extent of the cellular response at the graft host-interface. A greater degree of lymphocytic infiltration and a significantly greater width of reaction were observed at the graft-host interface in the type-1 allograft implanted group. An interpretation of this observation would be that the local cytokine profiles at the graft host interface differ between the two allograft treated groups. Similar studies investigating acellularized musculo-skeletal biomaterials have attributed a Th-1 cytokine response to the type of fibrous encapsulation and marked lymphocytic infiltration seen in the type-1 allograft treated group.[147]

The different protocols used in the production of the type-1 and type-2 allografts may, in part, explain the histological differences observed. In the evaluation of any novel biomaterial, the interpretation of an immunological response can be complicated by the presence other antigenic

stimuli such as: non-collagenous proteins, cells and cell-remnants.[126] It is possible that the protocol used to process the type-1 allograft was less effective in removing these types of residual donor antigens. If present in sufficient quantity, such antigens could provoke a spectrum of response from delayed incorporation to overt graft rejection.

As mentioned, PAA treatment of collagen is associated with the cross-linking of collagen molecules through the generation of oxidative hydroxyl radicals.[123] Excessive chemical cross-linking has been associated with adverse immunological responses to decellularized xenogenic and allogenic collagen derived biomaterials. In addition, chemical cross-linking associated with PAA treatment has been implicated in the delayed remodeling of BPTB allografts.[122] In this study, preparation of the type-2 allograft involved a prolonged exposure to PAA and additional washing steps relative to the type-1 allograft protocol. It is possible that these additional treatments were more effective in removal of antigenic material, and did not modify native collagen to an extent that would elicit an undesirable host response. Evidence of this is provided by the small number of fibroblastic cells intimately associated with the type-2 allograft that were not present within the type-1 allograft.

It is also possible that PAA-Triton X-100 residues in the type-1 allograft were responsible for the more extensive inflammatory reaction observed. As mentioned previously, PAA rapidly breaks down to acetic acid and liberates oxygen and heat.[120] In an *in vitro* study, treatment of tendon with 0.1% PAA for 3 hours followed by 3, fifteen minute washes with PBS did not elicit any cytotoxic response from human fibroblasts.[170] This finding is in agreement with *in vivo* studies of PAA treated heart valves and human skin allografts.[127] In theory, emulsification of cellular phospholipids by residual non-ionic surfactants could have led to cellular damage and subsequent release of inflammatory mediators. *In vitro* studies of Triton X-100 have detected cytotoxicity in human fibroblasts at concentrations of not more than 10^{-1}-10^{-2} g/L.[171] However, Triton X-100 has been evaluated in numerous decellularization protocols with tissue exposure

periods from several hours to up to 14 days with no deleterious *in vivo* effects being attributed to this chemical.[127]

5.2 Final remarks

This study has identified a physico-chemical process that may lead to the production of an "off-the shelf" biomaterial for use in equine orthopedic surgery. Additional studies are needed to further characterize the cellular response of horses to this type of biomaterial and evaluate its biomechanical properties. If effective, an allogenic biomaterial of this type may develop widespread clinical applications in the field of equine tendon and ligament repair.

CHAPTER VI

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Appendix

Subjective histological scoring system used for grading treated tendons

Variable	Score & criteria
Fibroblast density[1]	0 = normal amount for adult FDS tendon 1 = mild increase 2 = moderate increase 3 = severe increase
Neutrophil density[2]	0 = absent 1 = mild increase 2 = moderate increase 3 = severe increase
Macrophage density	0 = absent 1 = mild increase 2 = moderate increase 3 = severe increase
Lymphocyte density	0 = absent 1 = mild increase 2 = moderate increase 3 = severe increase
Neovascularization	0 = normal amount for adult FDS tendon 1 = mild increase 2 = moderate increase 3 = severe increase
Organization of fibrils at graft-host interface	1 = haphazard 2 = linear

1. For tendons receiving autograft or allograft treatments, fibroblast density scores were derived from observations across the entire visible grafted area. For sham operated tendons, fibroblast density scores were obtained from the center of the eac tendon section.

2. For tendons receiving allograft treatments, lymphocyte, macrophage and neutrophil density scores were obtained from observations at the graft-host interface. For sham operated or autograft treated tendons, these scores were derived from observations across each tendon section

Objective histological scoring system used for grading treated tendons

Variable	Score & criteria
Width of reaction at graft host interface:	average of 5 observations per slide

VITA

James Charles Albert Hart

Candidate for the Degree of

Master of Science

Thesis: AN *IN VIVO* EVALUATION OF A NATURALLY DERIVED CYTOCOMPATIBLE AND ARCHITECTURALLY OPTIMISED TENDON ALLOGRAFT IN THE HORSE

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Equine Registrar

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Pages in Study: 66 Candidate for the degree of: Master of Science

Major Field: Veterinary Biomedical Science

Scope and Method of Study:

In horses, debilitating tendon injuries can result from external trauma. Currently, the repair of tendon defects arising from trauma relies heavily upon de novo tissue regeneration. Endogenous repair and subsequent remodeling can take as long as 18 months. The biomechanical properties of healed tendon are considered inferior those of uninjured tendon. In this study, cadaveric equine tendon was processed using two variations of a previously published physico-chemical method of allograft processing. The resultant biomaterials were then implanted into the FDS tendons of eight normal horses. Sham operated and autograft implanted tendons served as controls. All tendons were examined ultrasonographically every 2 weeks post operatively. After sacrifice at 12 weeks, tendon tissue was harvested and assessed grossly and histologically.

Findings and Conclusions:

In tendons receiving type-1 allografts, ultrasonographic cross-sectional area (CSA) was observed to increase at every time-point. The type-1 allografts remained well demarcated and surrounded by a hypoechoic zone of variable width. While remaining visible in situ, type-2 allografts exhibited incorporation into host tendon. CSA measurements for the type-2 allograft treated tendons stabilized by 6 weeks post-operatively. Histologically, type-1 allografts elicited a significantly larger width of reaction and greater amount of fibrosis. A predominantly lymphocytic infiltration was observed at the graft-host interface. The graft itself remained largely acellular. The width of reaction surrounding type-2 allografts was significantly less than that surrounding the type-1 allografts. Type-2 allografts were also infiltrated with small numbers of cells exhibiting tenocytic morphology.

ADVISER'S APPROVAL: ______________________________

Henry Jann