NOVEL MODELS OF TENDON INJURY AND GAP JUNCTION MODULATION IN TENDON CELL AND TISSUE REPAIR

A DISSERTATION PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Injuries of energy-storing tendons such as the equine superficial digital flexor tendon and the human Achilles tendon are responsible for significant health and financial costs. A good model of acute tendinopathy of energy-storing tendons is required to better understand the changes that occur within the injured tendon and thereby to aid in the development of successful treatment regimens. Connexin43 plays an important role in wound healing and is involved with the spread of cell death signals following injury. Understanding the effect of Connexin43 modulation on tendon injury could improve tendon healing rate and quality.

Models of acute injury were developed in the pelvic limb superficial digital flexor tendon of sheep and adapted for the thoracic limb superficial digital flexor tendon in horses. The sheep injury model was used to investigate the effects of injury on Connexin43 expression and the effect of Connexin43 antisense oligodeoxynucleotides in the peracute (2 to 4 hours) post injury stage. The model in horses was monitored clinically and ultrasonographically and these findings were related to the gross and histological changes at post mortem after 4 days. A surgical model of acute tendon injury was successfully developed and applied to sheep and horse energy-storing tendons. This has potential for modelling tendon injury in horses and humans. Increased Connexin43 levels were measured at the injury site at 2-4 hours post injury. Antisense oligodeoxynucleotides did not significantly reduce Connexin43 levels in the injured tendons in the acute period.

Equine superficial digital flexor tendon-derived fibroblasts derived from different horses exhibit different cell growth rates that may be an indicator of a genetic ability to heal more effectively.

The new models of acute tendon injury may facilitate development of an accurate model of clinical tendon injury in energy storing tendons to improve our knowledge of the problem and our treatments.

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GLOSSARY

Accessory carpal bone	Carpal bone on the back of the thoracic limb of the horse.
Accessory ligament of the deep digital flexor	Ligament that connects the deep digital flexor tendon to the back of the proximal metacarpus/metatarsus.
Accessory ligament of the superficial digital flexor	Ligament that connects the superficial digital flexor tendon to the back of the carpus/tarsus.
Achilles tendon (human) or Calcaneal tendon	Tendon complex of the soleus and the gastrocnemius tendons that attaches these two muscles to the calcaneus or point of the heel.
Actin stress fibres	Fibres in non-muscle cells involved in cell movement
Acute phase proteins	Proteins whose plasma concentrations alter in response to inflammation
Adhesions	Fibrous bands that form between tissues and organs. A problem in tendon healing as they interfere with the tendon sliding motion.
Aminopropetides	Triple stranded structures at the amino end of procollagen that are cleaved when procollagen becomes collagen.
Angiogenesis	Development of new blood vessels
Anti-sense oligodeoxynucleotides	A complementary sequence to a specific mRNA blocks its expression
Apoptosis	Programmed cell death
Autologous conditioned serum	Serum conditioned to raise the amounts of anti-inflammatory cytokines
Autologous bone marrow	Bone marrow removed from the patient and returned at a distant site
Beta aminoproprionitrile fumerate	Interferes with collagen cross-link formation by inhibiting lysl oxidase
Biomechanical forces	Internal and external forces acting on the body
Bystander region	Region greater than or equal to 400 μm from the injury
Cartilage Oligomeric protein	A glycoprotein component of the extracellular matrix
Collagen	Structural protein family with about 20 members. Collagen I and Collagen III the most common in tendon.
Collagenase	Enzyme that breaks down collagen
Computed tomography	Imaging modality that uses radiographic images to produce cross-sectional slices through the area of interest.
Connective tissue	Supportive tissue that support, connects or separates other types of tissues and organs.
Connexin43	Gap junction protein found in most mammalian cells.
Connexin	Connexins are structurally conserved non-glycosylated integral membrane proteins in vertebrates. They consist of two extra-cellular facing loops, 4 trans-membrane domains, 1 intracellular

	loop and the amino and carboxyl domains on the cytoplasmic side.
Connexon or hemi-channel	Channel in the plasma membrane made up of 6 connexins in a circle around a central pore. Connects the intra and extracellular environments and can dock with another connexon on an adjacent plasma membrane to form a gap junction.
Coronal plane	Also known as frontal plane. Divides body or limb into ventral and dorsal sections.
Corticosteroid	Chemicals that include steroid hormones produced by the adrenal cortex and analogues of these.
Crimp	Planar undulations in the collagen fibres that allow flexibility and energy-storage
Cristae	Multiple inner folds of the mitochondrion that create a large surface area for reactions to occur on.
Cursorial	Adapted specifically to run
Cytokines	Cell signalling molecules
Cytoplasm	Liquid compartment of the cell
Degranulation	Cellular process that releases antimicrobial cytotoxic molecules from secretory vesicles called granules found inside some cells
Desmotomy	Cutting of a ligament
Dimethyl sulphoxide	An organosulphur compound that is a polar solvent.
Dystrophic calcification	Pathological accumulation of calcium salts in a tissue
Eccentric exercise	Lowering phase of an exercise
Elastic modulus	Mathematical description of a substances tendency to be deformed non-permanently when a force is applied to it.
Electrotherapy	Use of small electrical currents into the muscles and tendons as a medical therapy
Endoplasmic reticulum	Network of membrane vesicles involved with protein synthesis
Endothelial cells	Cells lining blood vessels
Energy-storing tendons	Tendons which store elastic energy by reversible stretching of collagen molecules when they are under load. This energy is released with the tendon acting like a spring to aid locomotion.
Epidemiological study	The investigation of the patterns, causes and effects of a condition in a defined population.
Excisional	Removal by surgery
Extra-cellular matrix	Extra-cellular part of animal tissues that provides structural support to the cells in addition to performing tissue specific roles.
Extracorporeal shock wave therapy	Therapy involving the use of high pressure sound waves.
Extrinsic	Extraneous, not part of

Ex vivo	Latin 'out of the living' refers to experiments done in or on tissue in an artificial environment not necessarily in culture.
Fibroblast	Most common cells of connective tissue. Responsible for producing the extra-cellular matrix.
Fibro-elastic	Composed of collagen and elastic fibres.
Gap junction	Intercellular junction made up when two hexameric hemi-channels/connexons on adjacent plasma membranes dock. Allow molecules of <1kDa to pass through.
Gap junction plaque	Collection of a few to 1000's of gap junctions in one area of the plasma membrane.
Glucagon	Peptide hormone secreted by the pancreas to raise blood glucose levels
Glyceryltrinitrile	Alternative name for nitroglycerin which is a nitric oxide generator resulting in relaxation
Granulation tissue	The perfused fibrous tissue that replaces the fibrin clot in a wound.
Haematoma	Localised collection of blood outside a blood vessel.
Haemostasis	Process which causes bleeding to stop.
H&E	Haematoxylin and Eosin
Halothane/Isoflurane	Inhalational anaesthetic drugs
Hierarchical structure	Arranged in several layers.
Histopathological	Microscopic changes in tissues that indicate the manifestation of disease
Hyalinisation	State of having become hyaline
Hyaluronic acid	Anionic non-sulphated glycosaminoglycan widely distributed in connective tissue.
Hydrotherapy	Part of medicine that uses water for pain relief and treatment. The temperature and pressure of the water are use to stimulating blood circulation, cool the area and provide resistance to movement.
Hygroscopic	Ability of a substance to attract and hold water.
Hypoxic	Reduced oxygen
Immunohistochemistry	The process of detecting antigens(proteins) in tissue sections using the principle of specific antibody-antigen binding.
Incidence	The number of new cases developing a specified condition within a specified period of time.
Insulin like growth factor -I	Or Somatamedin is a protein hormone similar to insulin important in growth.
Intercellular	Between cells
Intracellular	Within a cell

Intracytoplasmic	Within the cytoplasm of the cell
Intralesional	Introduced into or performed within a lesion
Intrinsic	Contained wholly within the organ on which it acts
Ischaemia	Greek – Restricted blood supply
Isometric contraction	Force generation within a muscle without a change in length
In vitro	Latin 'within the glass' refers to experiments in culture.
In vivo	Latin 'within the living' used to describe experiments involving a whole living organism.
Laminin	Trimeric cross-shaped protein in the base layer of tissues.
Lysl oxidase	Copper dependent enzyme that cross-links collagen and elastin.
Macrophages	Greek 'big-eaters' differentiation of monocytes in tissues responsible for engulfing apoptotic cells and pathogens.
Magnetic resonance imaging	Imaging modality that uses a powerful magnet to align protons within the body in one direction. When the magnet is switched off, the protons return to their normal alignment, releasing energy as they do so. This energy is interpreted by software to create an image.
Matric metalloproteinases	Enzymes that breakdown components of the extra-cellular matrix.
Mesoderm	Middle of the three germ layers in the early embryo
Metacarpal (horse)	Related to the metatarsal bone (cannon) which lies between the carpus and the metacarpophalangeal (fetlock) joints.
Metatarsus	Related to the metacarpal region that lies between the tarsus (hock) and the metatarsophalangeal (fetlock) joints.
Micro-damage	Sub-clinical injury
Mimetic peptides	Proteins that bind to the connexon/hemi-channel and block communication with the extracellular environment. At high concentrations can also uncouple gap junctions.
Mitochondrial	Relating to the mitochondrion, a membrane intra-cellular organelle responsible for energy production.
Morbidity	A diseased state or illness
Mucoid degeneration	Conversion of the cell or tissue substance into a glutinous substance like mucus.
Myofibroblast	Muscle connective tissue cell
Myotendinous Junction	Region of highly folded tissue between the muscle fibres and the tendon.
Necrosis	Greek 'death' Cell injury that results in premature cell death
Non-specific gap junction blockers	Substances that block gap junction communication in all types of gap junctions e.g. octanol, carbenoxolone and inhalation anaesthetics.New blood vessel ingrowth

Neovascularisation	
Neutrophils	The most abundant white blood cells in mammals. Important ion innate immune responses.
Palmar annular ligament	Fibrous band of tissue around the metacarpophalangeal joint in horses
PBS	Phosphate buffered saline
Plasma membrane	Lipid bilayer membrane around mammalian cells.
Platelet rich plasma	Blood plasma enriched with platelets. The idea being that they contain and release growth factors and cytokines beneficial in healing of bone and soft tissues.
Polymerase chain reaction	Molecular biology technology to amplify a single or a few copies of a piece of DNA across several orders of magnitude to improve detection.
polymorphonuclear cells	Another term for Granulocytes. White blood cells that contain granules in their cytoplasm and have varying shapes of nucleus.
Polysulphated glycosaminoglycans	Semi-synthetic glycosaminoglycan predominantly chondroitin sulphate.
Prevalence	The proportion of a given population found to have a specified condition at a given point in time.
ProstaglandinE1and E2	Locally acting messenger molecules derived from fatty acids
Proximally	Towards the trunk
Pyknotic	Appearance of irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis
Radiofrequency therapy	Therapy for pain that acts by interruption of nerve conduction on a semi-permanent basis.
Re-epithelialisation	Proliferation of epithelial cells that then migrate across the wound bed to heal the wound.
Regeneration	Restoration of the original tissue or organ to its normal state following injury.
Regional anaesthesia	Local anaesthesia affecting a significant part of the body for example half of a limb.
Risk factor	Variable associated with an increased risk of disease or infection.
mRNA	Messenger ribose nucleic acids that are responsible for carrying the code for protein synthesis
RTV	Silastic elastomer used to make stretchable culture dishes
Scarring	Areas of fibrous tissue that replace tissue following injury
Sclerotherapy	Injection of a substance to shrink blood vessels.
Sharpey's fibres	Collagenous fibres that connect the tendon to the collagen in the bone.
Sonogram	A diagnostic image created by echoes of ultrasound waves.

Stem cells	Cells found in all multicellular organisms, which can divide and differentiate into diverse specialized cell types.
Subcutaneous	Under the skin
Superficial digital flexor tendon (equine/ovine)	Tendon of insertion of the superficial digital flexor muscle. In the thoracic limb, the muscle originates on the humerus and caudal radius and inserts on the distal first phalanx and proximal second phalanx. Flexes the carpus and distal limb joints and supports the limb in the stance phase. In the pelvic limb originates on the femur and acts to extend the tarsus and flex the distal limb joints. Forms part of the 'reciprocal apparatus'.
Tenascin-C	Extra-cellular matrix glycoprotein important in development and repair
Tendinitis	Inflammation of tendon
Tendinopathy	Pathology/disease of tendon
Tendinosis	Chronic degeneration of tendon
Tendon	Collagenous structure that transmit tensile force from muscle to bone.
Tendolipomatosis	Accumulation of lipid cells between tendon fibres
Tenoscopic	Endoscopy of tendon sheaths in horses.
Tensile force	Resistance of a material to the force trying to pull it apart.
Transection	Division of a structure by cutting transversely
Transforming growth factor β-I	Polypeptide member of the growth f actor beta superfamily of cytokines
Tuber calcaneus	Point of the tarsus where the gastrocnemius attaches
Ultrasonography	Imaging modality that uses the reflection pattern of high frequency sound waves from tissues to create an image.
Vacuoles	Enclosed membrane compartment containing inorganic and organic molecules
Vasoconstriction	Narrowing of blood vessels due to contraction of the muscular wall of the vessel
Vinculum	Bond or tie, in horses it is the attachment of the superficial digital flexor tendon to the digital flexor tendon sheath wall.

CHAPTER 1

OVERVIEW OF THESIS

1.0 PROBLEM STATEMENT

The superficial digital flexor tendon is the most commonly injured tendon in racehorses internationally (Genovese et al., 1990). Tendon injuries are also the cause of morbidity in sport and working environments in humans (Sharma and Maffulli, 2006). The human Achilles tendon and equine superficial digital flexor tendon are functionally equivalent in that they store and release elastic energy to increase the efficiency of high-speed locomotion (Williams et al., 2001, Alexander, 1988).

No treatment of tendinopathy is guaranteed to return a horse to competition level and prevent re-injury (Firth, 2000). A good model of acute tendinopathy of energy-storing tendons is required to better understand the changes that occur within the injured tendon and thereby to aid in the development of successful treatment regimens (Dirks and Warden, 2011).

Connexin43 is a type of gap junction protein that is present in many tissues including mammalian tendons (McNeilly et al., 1996), where it plays an important role in wound healing (Coutinho et al., 2003) and is also involved in the spread of cell death signals

following injury (Contreras et al., 2004) . Modulation of Connexin43 has been shown to improve the rate and quality of wound healing in skin (Qiu et al., 2003, Mori et al., 2006) and cornea (Grupcheva et al., 2012, Ormonde et al., 2012), and to reduce swelling and cell death in spinal cord (O'Carroll et al., 2008). Investigation of the effect of Connexin43 modulation on tendon injury is important to determine if such targeted therapy could improve tendon healing rate and quality.

1.1 RESEARCH AIMS

To develop an appropriate model of acute tendon injury in energy-storing tendons in sheep, to adapt the model for horses and use it to investigate acute changes in tendon injury.

To investigate the effects of gap junction modulation on wounded tendons *in vivo* and scrape-wounded tendon-derived fibroblasts *in vitro*. This will allow research into the role gap junction modulation may play in improving healing in energy-storing tendons.

1.2 ETHICS

The Massey University Animal Ethics Committee approved all procedures in this thesis involving live animal studies.

1.3 OVERVIEW OF THESIS STRUCTURE

Due to the wide span of the study, from the *in vitro* to *in vivo* environment and across species, the methodologies are many and varied. Each of the studies is therefore presented in an individual chapter to assist flow and understanding. Also all reagent information is provided in the table of reagents (Appendix 1). To avoid repetition some references to methods in other chapters are present. The general discussion brings

together the overall results and limitations to better explain the path to the future directions.

CHAPTER 2

SUMMARY AND CRITICAL REVIEW OF LITERATURE

2.0 INTRODUCTION

2.0.1 RATIONALE FOR RESEARCH ON TENDON INJURIES

Tendon injuries are the cause of morbidity in sport and working environments in many species, particularly horses and humans (Sharma and Maffulli, 2006, Firth, 2000). The equine superficial digital flexor tendon (Figure 1) and Achilles tendon are functionally equivalent in that they store and release elastic energy to increase the efficiency of high speed locomotion (Alexander, 1988). They are therefore termed energy-storing tendons. The many protocols used to treat energy-storing tendon injuries are an indication of a lack of a standardised therapy guaranteed to be successful long term (Firth, 2000, Alfredson and Cook, 2007). Whilst the majority of affected equine and human athletes are able to return to competition following such injuries, recurrence rates are high and permanent retirement from competition is often the final outcome.

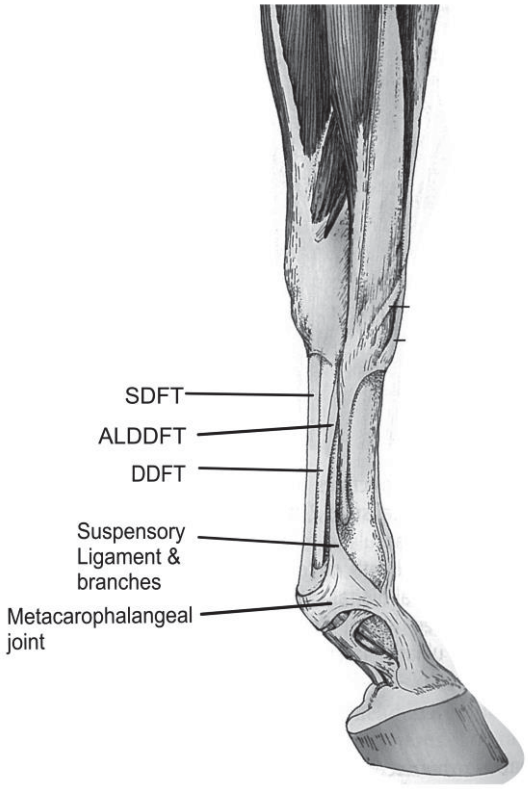


Figure 1 - Distal thoracic limb of the horse
This image was published in Veterinary Anatomy, Dyce, Sack and Wensing. - 4th Edition, Pg. 600. Copyright Elsevier, 2010
SDFT – Superficial Digital Flexor Tendon
ALDFT – Accessory Ligament of the Deep Digital Flexor Tendon
DDFT – Deep Digital Flexor Tendon

2.0.2 WHY FOCUS ON THE FORELIMB SUPERFICIAL DIGITAL FLEXOR TENDON?

Superficial digital flexor tendon injury affects all breeds of horses, in particular high-level performance horses (Dyson, 2004, Murray et al., 2006, Singer et al., 2007). It is also a major problem in racing thoroughbreds and standardbreds internationally (Genovese et al., 1990, Hawkins and Ross, 1995). Injury to the superficial digital flexor tendon is an important reason for retirement of competition horses. Tendon breakdown injuries are sudden and dramatic and may be observed by many spectators (Peloso et al., 1994). Such situations have a significant negative impact on issues associated with animal welfare, jockey safety, economic viability and the public perception of racing

and other equine sporting events (Bailey et al., 1998, Bourzac et al., 2009). A return to competition is achievable in many cases but the high incidence of re-injury may result in destruction of the affected horse (Williams et al., 2001, Dowling et al., 2000).

2.1 PREVALENCE AND INCIDENCE OF SUPERFICIAL DIGITAL FLEXOR TENDON INJURY IN RACING HORSES.

Epidemiological studies performed in many countries largely concentrate on thoroughbred race horses and have identified similar age, exercise and gender-related risk factors for equine tendon injury (Perkins et al., 2005, Kasashima et al., 2004, Williams et al., 2001, Lam et al., 2007). Approximately 11% of racing thoroughbreds sustained superficial digital flexor tendon injuries in training and racing in Japan in 1999 (Kasashima et al., 2004). In Hong Kong superficial digital flexor tendon injury was the most common reason for retirement of racehorses and accounted for approximately 14 % of all retirements in a study spanning 12 years (Lam et al., 2007). Injuries occurred in nearly 7/1000 starts in national hunt racing in the United Kingdom during 2000 and 2001, of these 90% were due to superficial digital flexor tendon injury (Pinchbeck et al., 2004). These figures show superficial digital flexor tendon injury is a significant cause of wastage within the racing industry with an overall incidence of 8-30% in racing thoroughbreds around the world (Palmer et al., 1994, Goodship et al., 1994, Williams et al., 2001).

Superficial digital flexor tendon injury is also one of the top ten most common injuries of standardbred racehorses but is less common than in thoroughbred race horses (Ross and Dyson, 2003). Epidemiological studies are sparse but once injured the issues limiting return to racing and the high recurrence rates are similar across the two types of racing (Reef et al., 1990).

The true numbers of superficial digital flexor tendon injuries affecting racing horses are likely to be underestimated in studies concentrating on racing injuries alone because many cases occur during training (Lam et al., 2007, Kasashima et al., 2004, Perkins et al., 2005). In addition race-related conditions may not become clinically apparent for several hours after racing and deterioration and euthanasia that occur away from the racetrack will not have been recorded in most studies (Williams et al., 2001).

2.1.1 OCCURRENCE IN OTHER COMPETITION HORSES

In 2002, in the United Kingdom, 17% of injuries recorded in event horses competing in cross country competitions were tendon injuries. There is also evidence of superficial digital flexor tendon injury in elite eventers; elite show jumpers, (Murray et al., 2006) and dressage horses (Dyson, 2004).

2.1.2 FINANCIAL IMPLICATIONS OF EQUINE TENDON INJURY

Career earnings in flat racing thoroughbreds in Hong Kong that were retired due to tendon injury were less than half of those in the population retired for other reasons. Other costs incurred are the veterinary surgeons fees for diagnosis, treatment and monitoring of the condition and such costs can go well into the thousands of dollars. Most moderate to severe tendon injuries require a period of approximately one year out of training during that time no earnings will made to cover feeding, and management costs. After all this time and expense even carefully managed horses can undergo re-injury of the tendon when hard exercise is resumed (Marr et al., 1993a). Even considering the lowest figures, superficial digital flexor tendon injury is responsible for significant financial and welfare costs in the horse industry.

2.2 AETIOLOGY

2.2.1 THE INFLUENCE OF AGE

An increase in superficial digital flexor tendon injury rate with an increase in age over all race types has been shown in the United States of America, United Kingdom, New Zealand and Japan (Mohammed et al., 1992, Williams et al., 2001, Kasashima et al., 2004, Perkins et al., 2005). Higher proportions of Hong Kong racehorses retired due to tendon injury at 3 (19.7%) and 4 years (17.4%) compared with 2 year olds (5.6%) or > 5yr (11.1%) (Lam et al., 2007). New Zealand thoroughbred racehorses of more than 5 years of age were 15.26 times more likely to incur injury to the superficial digital flexor tendon compared with 2 year olds (Perkins et al., 2005). The increase in injury rate with an increase in age can be explained by the accumulation of micro-damage (Patterson-Kane and Firth, 2009), this will be discussed in more detail in a later section (2.6 Tendon Pathology and Degeneration).

Older racehorses are likely to have been exposed to a greater amount of exercise and potentially more races. During maximal load the superficial digital flexor tendon operates close to its failure limit and may incur subclinical damage, therefore cumulative high-speed exercise is likely to predispose to injury (Cohen et al., 2000).

2.2.2 INFLUENCE OF LIMB

Superficial digital flexor tendon injuries resulting in retirement were more likely to have occurred in the thoracic limbs than pelvic limbs (Kasashima et al., 2004, Perkins et al., 2005, Lam et al., 2007, Williams et al., 2001). When racing in a clockwise direction the risk of retirement due to superficial digital flexor tendon injury was 1.75 times greater in the right thoracic limb compared with the left (Lam et al., 2007). This increased risk

is not simply due to increased load as the non-lead, or left leg in this direction, has a greater or equal ground reaction force depending on the speed (Witte et al., 2004).

2.2.3 OTHER FACTORS E.G. TRACK SURFACE, TRACK TYPE

Track surface has been shown to have a significant effect on severe injuries in thoroughbred racehorses with firm turf having a lower risk of injury than dirt (Mohammed et al., 1992). More specifically however, firm going has been shown to be a risk factor for superficial digital flexor tendon injury (Perkins et al., 2005). The overall rate of clinical conditions and fatalities was higher when horses raced over obstacles compared to flat racing in the UK and Australia (Bailey et al., 1998, Williams et al., 2001). The study in Hong Kong, in which 14.2% of all retirements were due to superficial digital flexor tendon injury, was carried out entirely on flat-racing horses of which 87% ran on sand based turf (Lam et al., 2007). Physical predispositions such as fatigue, poor conformation, incoordination and excess biomechanical force are other factors cited to be involved in tendon injury (Silver, 1983).

2.3 HUMAN ACHILLES TENDON

The human Achilles tendon or calcaneal tendon is a compound tendon that attaches the soleus, gastrocnemius and plantaris muscles to the tuber calcaneus (Figure 2).

Figure 2 - Human Achilles tendon

Tendinopathy of the human Achilles is a common sporting injury and also occurs in the general population (Jarvinen et al., 2005). Achilles tendinopathy accounts for 6-50% of human sports injuries; it is the most commonly ruptured tendon in humans (Mazzone and McCue, 2002). Achilles tendon injuries rank within the top three sports-related injuries of the human foot and ankle and 18% of all running injuries involve this tendon (Werd, 2007). A poor response to even prolonged conservative therapy for Achilles tendinopathy means that surgery is frequently required (Alfredson and Cook, 2007).

2.3.1 SIMILARITIES OF EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON AND HUMAN ACHILLES TENDON

The human Achilles tendon and equine superficial digital flexor tendon are functionally equivalent in that they store and release elastic energy to increase the efficiency of high-speed locomotion by reducing muscular work (Williams et al., 2001). Exercise related tendon injury is more common in the mid-portion of both the Achilles (Alfredson and Cook, 2007) and the equine superficial digital flexor tendon (Webbon, 1977) and factors such as age, exercise and gender have been shown to influence its occurrence

(Lam et al., 2007, Perkins et al., 2005, Houshian et al., 1998). Increasing age is associated with increasing risk of injury to the superficial digital flexor tendon (Perkins et al., 2005). In humans there is a bimodal distribution of age and Achilles tendon injury with peaks at 30-39 years and 80 years of age (Houshian et al., 1998).

2.4 TENDONS – STRUCTURE AND FUNCTION

2.4.1 GENERAL STRUCTURE

Tendons are collagenous structures that transmit tensile force from muscle to bone. Particular adaptations of this enable the upright stance of bipeds and locomotion of vertebrates. Tendons show variation in structure and physiology to optimise function. Some of these functional adaptations whilst ensuring the efficiency of the tendon may contribute to their eventual injury.

2.4.2 SECTIONS OF THE TENDON

Tendons do not have a uniform structure and distinct areas are apparent within all tendons. Tendons and muscles develop independently in the mesoderm and link at the myotendinous junction (OBrien, 2008). Collagen fibres from the tendon connect to the perimysium to provide the mechanical link so the muscle can transmit force to its tendon. Laminin, Tenascin-C and Collagen type IV are thought to play important roles in this junctional region (Pedrosa-Domellof et al., 2000).

The mid-body of the tendon consists of collagenous fibres in a hierarchical structure with tendon fibroblasts along the fibres. The tendon fibroblasts in this region in adults are long and thin with processes that connect cells to each other along the same fibre and also to cells along adjacent fibres. The blood supply is lower in this region than

elsewhere in the tendon; it is extrinsic and enters the tendon via the paratenon and associated structures. The tendon attaches to the bone via Sharpey's fibres, collagenous fibres that are contiguous with the collagen in the tendon and the collagen in the bone.

2.4.3 FUNCTION

Tendons are fibro-elastic in nature and are resistant to mechanical loads. The size, shape and attachment to the bone vary depending on the function of the muscle to which they are attached (Kannus, 2000).

2.4.3.1 ENERGY-STORING TENDONS

The maximum tensile stress that a tendon can withstand depends on its cross-sectional area relative to that of the muscle fascicles. Tendons exposed to the highest stresses are those that act as springs, to store energy during loading and release it by elastic recoil as the limb is unloaded by retraction from the ground (Alexander, 1988, Alexander, 2002). Such tendons improve the efficiency of locomotion by reducing the metabolic expenditure of the muscles. The most important examples of these are the distal limb flexors and extensors of large mammals (Ker, 2002).

Large cursorial (adapted to or specialised for running) mammals have lengthened distal limbs (Alexander, 1977). Long muscle fibres store and return elastic energy during the stance phase and the short fibres reduce the energetic cost of force generation (Minetti et al., 1999). The equine thoracic limb is a series of two compressive springs in series, the upper spring from scapula to elbow and the lower spring distal to the elbow (McGuigan and Wilson, 2003). Optimisation of the musculoskeletal system for locomotion would suggest a length change in the distal spring and that proximal muscles would be isometric during stance. Experimental data showed a tenfold greater

change in length in the distal compared to the proximal spring (McGuigan and Wilson, 2003). Equine flexor tendons act to support and resist hyper-extension of the metacarpophalangeal joint and are antagonised by the extensors that act synergistically to provide support for the limb (Parry et al., 1978, Goodship et al., 1994).

The superficial digital flexor tendon produces high force to resist bodyweight on the limb during the stance phase (Dyce, 1987)) and flexes the digit during the swing phase of locomotion (Batson et al., 2003). It is an energy-storing structure that significantly improves locomotor efficiency at high-speed gaits due to the expendable elastic crimp within the collagen fascicles (Biewener, 1998). Elastic recoil contributes up to 36 % of the work required for galloping (Biewener, 1998). Approximately 93% of energy stored is released in elastic recoil whilst the remainder is lost as heat (Alexander, 2002). Stiffness is load per unit area required to deform a structure. Superficial digital flexor tendon needs to have low stiffness (i.e. be very elastic) to store the maximum amounts of energy (Batson et al., 2003). Energy-storing tendons in humans (Cillan-Garcia et al., 2013) and horses (Batson et al., 2003) have a much lower elastic modulus than the positional tendons and this makes them prone to increased strain.

Strain is the elongation of a structure shown as a percentage of its initial length. Energy-storing tendons need to experience high levels of strain to store sufficient amounts of energy and as a result of their conflicting need for strength, often have narrow mechanical safety margins (Riemersma et al., 1996, Stephens et al., 1989, Wilson et al., 2001). The *in vivo* superficial digital flexor tendon strain at gallop has been measured at 11-16 %; *in vitro* strain at rupture of the equine superficial digital flexor tendon has been measured at 12-21%. This suggests that at maximum exertion the equine superficial digital flexor tendon operates close to physiological safety limits (Riemersma and Schamhardt, 1985, Stephens et al., 1989).

2.4.4 TENDON FAILURE

Tendon is a cable made up of crimped collagen microfibrils where stress behavior is determined by properties of individual fibrils and fascicles and depends on the age of animal. Normal tendon does not fail in response to strain; the muscle-tendon/ tendon-bone/bone interface fails. The maximum tensile stress which a tendon can withstand depends on its cross-sectional area relative to that of the muscle fascicles. The maximum isometric force of muscle is approximately 1/3 of the tendon failure point however repetitive submaximal loads and fatigue can lead to failure. The safety factor is the difference between tendon stress and breaking point and is reduced in muscles that supply elastic recoil during locomotion. The tensile strength of a tendon reaches a plateau after collagen matures and then declines with an increase in age (Kirkendall and Garrett, 1997).

Tendons demonstrate characteristic sigmoidal stress-strain curves (Figure 3) when stretched to failure *in vitro*. The tangent to the curve is the elastic modulus and the area under the curve represents strain energy per unit volume (Sharma and Maffulli, 2006).

Area 1 is the 'toe' or area of high compliance. The ‘toe’ of the curve is caused by straightening of the crimp within the collagen along the line of stress (Kirkendall and Garrett, 1997) .Crimp is a planar zigzag waveform of the collagen fibrils in the tendon (PattersonKane et al., 1997b). Crimp morphology is important for mechanical behaviour of tendon and the straightening of the crimp or wave pattern is reversible if the strain is not greater than 4% (Patterson-Kane et al., 1998b, Diamant et al., 1972). New fibres in healed wounds have shorter crimp than in uninjured fibres. Crimp length is therefore not only dependent on the biological age of the animal and length of the tendon but also on the physical age of the fibre and the effects of injury.

The linear elastic region of the curve corresponds to when the crimp angle is zero and the fibrils are stretched (Ker, 2002, Wilmink et al., 1992). The amount of stretch is affected by the stiffness of the tendon and if stretched more than 8-10 % the wave form does not reappear (Wilmink et al., 1992). If the load is removed energy is generated by elastic recoil. The elastic recoverability is less than 100% with the remainder lost as heat that may contribute to tendon damage (Ker, 2002). Temperatures of as much as 45°C have been measured in the core of the equine superficial digital flexor tendon in a galloping horse and despite tendon fibroblasts exhibiting the ability to withstand high temperatures, repetitive hyperthermia is likely to be a causative factor in tendon degeneration (Wilmink et al., 1992).

If the tendon strain is above 8-10 % the curve passes the yield point at which irreversible damage occurs and the tendon undergoes plastic deformation, crimp will no longer return if the load is then released (wilson and Goodship, 1994). The failure point of the tendon is when the fibrils rupture and the stress-strain curve rapidly falls to zero. This results in clinical signs of tendon injury.

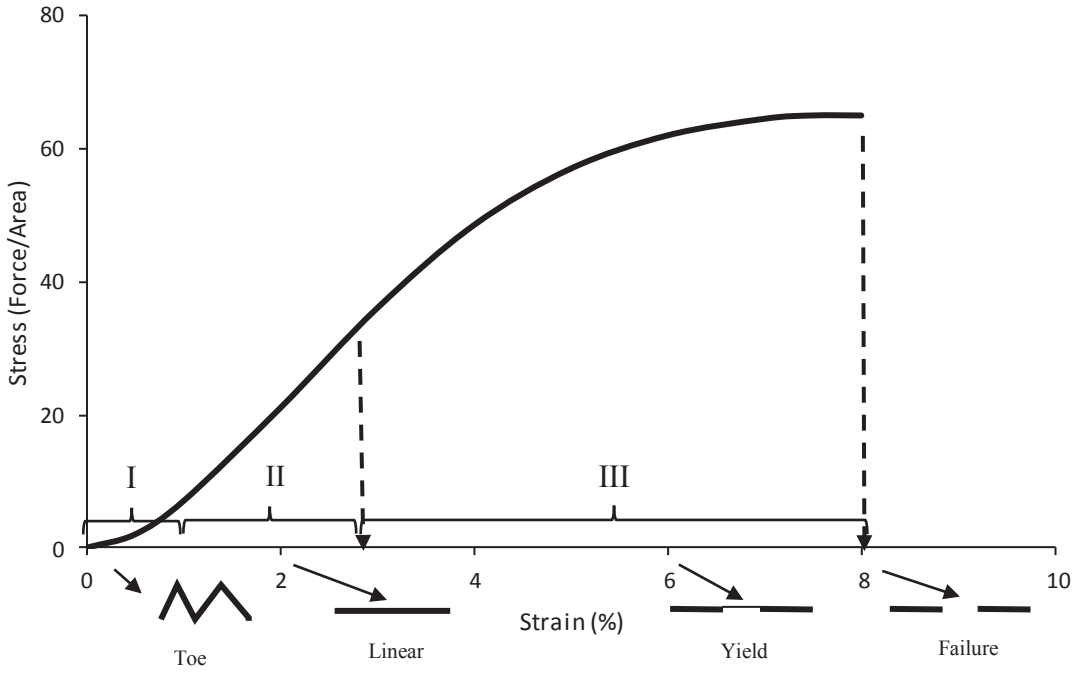


Figure 3 - Stress-strain curve demonstrating the basic physical properties of a tendon.

2.5 TENDON INJURY

Tendon injury is often described as tendinopathy from tendino = tendon, pathy = disease, tendinosis (tendino = tendon, osis = degeneration) or tendinitis (tendino = tendon and itis = inflammation), the latter is less accurate as the condition is not predominantly a result of inflammation.

2.5.1 CLINICAL SIGNS OF TENDON INJURY

The clinical signs of tendon injury include heat, swelling, pain on palpation and loss of function. The mid-metacarpal region is the most common site for the superficial digital flexor tendon to be injured (Gaughan, 1994) and such injuries often produce a classic bow of the tendon (Figure 4). In moderate to severe cases, the whole palmar/plantar metacarpus/metatarsus is swollen and pitting oedema is present, in this case the horse will be visibly lame and have a dropped metacarpo-/metatarso- phalangeal joint (fetlock). In mild cases where sufficient peripheral fibres remain intact, only minimal

changes may be apparent with subtle swelling visible due to damaged core fibres. Lesions in thoroughbred racehorses are mainly in the core of the tendon but occasionally involve the periphery. In a study by Webbon (1977) the most frequent site of injury was also the site where normal tendon has the smallest cross-sectional area.

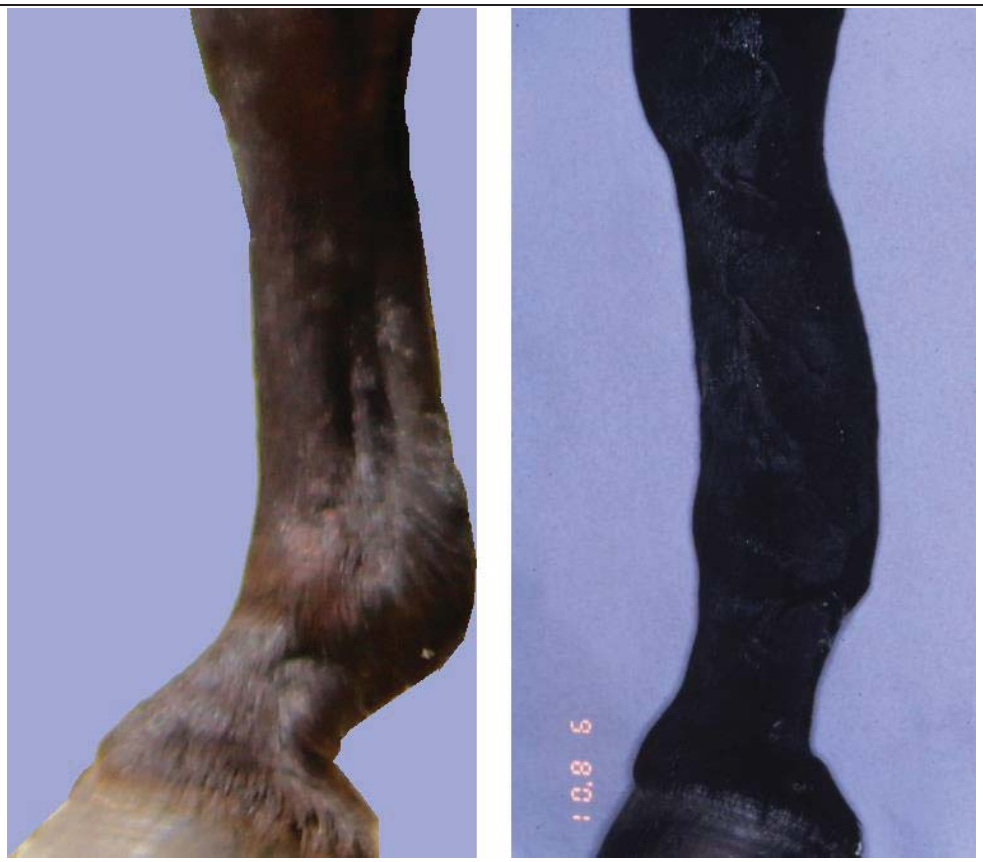


Figure 4 - Normal equine superficial digital flexor tendon on left and 'Bowed' tendon on right showing classic banana shape to the back of the limb.

2.5.2 HISTOPATHOLOGY

Degenerative tendinopathy is the most common histopathological finding in spontaneous tendon rupture. Tendon degeneration may lead to reduced tensile strength and predisposition to rupture (Sharma and Maffulli, 2006). In humans, degenerate tendons had a higher proportion of apoptotic cells than normal tendons and more apoptosis was seen with increasing age (Yuan et al., 2003).

Post mortem studies identified discoloured regions within tendons of asymptomatic horses and these areas were histologically abnormal with increased collagen type III levels consistent with fibrous scar formation (Webbon, 1977).

In samples taken at the time of repair of spontaneously ruptured Achilles tendons in human, evidence of pre-existing degeneration was present in 97% compared with 26% of the normal population (Kannus and Jozsa, 1991). The changes consisted of hypoxic, degenerative tendinopathy, mucoid degeneration, tendolipomatosis, and calcifying tendinopathy or a combination of all these changes (Kannus and Jozsa, 1991). To my knowledge, no similar study has been published on the histopathological appearance of acute tendinopathy of equine superficial digital flexor tendons however the findings are likely to be similar based on the gross post mortem study.

2.5.3 ULTRASONOGRAPHY

Ultrasonography is the most common imaging modality used to evaluate tendons. A consistent relationship between ultrasound and histopathology has been demonstrated (Marr et al., 1993b) and ultrasonographic changes correlate more closely with the severity of injury than palpable enlargement of the tendon and degree of pain (Henninger, 1992). Whilst very useful, ultrasound is less sensitive at identifying changes at the periphery of lesions. Ultrasonographic tissue characterisation is a method of using a computer program to detect changes in tendon scans to try to detect more subtle injuries. This technique has not been directly compared with histological appearance although it has been used to monitor repair of surgically created tendon lesions (Bosch et al., 2011).

The cross-sectional area of the damaged tendon on ultrasound images will usually be enlarged in comparison to published normal values and when compared to the

contralateral limb. Longitudinal scans enable evaluation of the tendon fibres and a subjective score is often used to describe the percentage of normal fibres (Marr et al., 1993b, Reef et al., 1989). Quantification of ultrasound changes is used to confirm the diagnosis and detect the severity of damage. Repeated ultrasonographic examination is used to monitor repair using measurements of tendon and lesion cross-sectional areas (Reef et al., 1989) as well as evaluation of fibre alignment. A maintained integrity of fibre alignment is believed to have the most correlation with successful outcome (Dowling et al., 2000). The peri-tendinous tissues can be evaluated for oedema and swelling although ultrasonography is less consistent for assessment of these tissues (Marr, 1992) .

Other methods for diagnosing tendon injuries are magnetic resonance imaging and computed tomography. Magnetic resonance imaging is a more sensitive tool for detecting tendon lesions than ultrasound and is particularly useful for imaging within the hoof. It is normally not required, too costly and too time-consuming, for uncomplicated mid-metacarpal lesions. Computed tomography with contrast enhancement may also be used to detect tendon enlargement and neovascularisation but requires a general anaesthetic so is again usually reserved for taxing cases or imaging within the hoof where ultrasound is less useful.

2.6 TENDON PATHOLOGY AND DEGENERATION

It was initially thought that exercise-induced tendon injury was the result of one supra-physiological event but it is now largely accepted that an accumulation of submaximal damage culminates in the final mechanical failure (Patterson-Kane et al., 2012). Epidemiological data have shown an association between age and exercise and strain-induced tendinopathy in humans and horses (Gibbon et al., 1999, Houshian et al., 1998,

Riley et al., 1998). Tendon degeneration may lead to reduced tensile strength and predisposition to rupture (Sharma and Maffulli, 2006). It is likely that the causes are multifactorial. During cyclic loading, ischaemia is associated with the period of maximum tensile load (Goodship et al., 1994). Superficial digital flexor tendon injury often occurs towards the end of a race and may be when fast deep digital flexor tendon fibres are fatigued therefore reducing support of fetlock and transferring load to the superficial digital flexor tendon (Butcher et al., 2007).

Asymptomatic injury is routine in conditions that arise in life and therefore repair must be ongoing (Ker, 2002). Micro-damage such as collagen disruption and micro-tears accumulate in the matrix and are not repaired by the resident tendon fibroblasts. This may weaken the tendon sufficiently for rupture to occur, in some cases during normal activity (Riemersma and Schamhardt, 1985, Stephens et al., 1989). This pre-existing degeneration has been implicated as a risk factor for acute rupture in both humans and animals (Sharma and Maffulli, 2006, Werd, 2007). Tendon degeneration has been detected *post-mortem* in 25-30% of horses and humans with no overt signs of clinical injury (Kannus and Jozsa, 1991, Webbon, 1977). Such changes are common in people over 35 and are associated with spontaneous rupture (Kannus and Jozsa, 1991).

The altered collagen content in ruptured and degenerate tendons indicates that the mature collagen network is partially removed by proteinases and this predisposes the tendon to rupture (Riley et al., 2002). Combined with the narrow mechanical safety limit within which energy-storing tendons operate this makes them increasingly liable to clinical injury or failure.

Possible reasons for failure of the cellular reparative response include low cell numbers (Birch et al., 2008, Batson et al., 2003), low activity of the superficial digital flexor

tendon fibroblasts(Birch et al., 1997), low matrix metabolism (Birch et al., 2008), insufficient recovery times and hyperthermia (wilson and Goodship, 1994).

Mean peak temperature in central core of tendon in galloping horses can rise as high as 45 degrees Celsius and may contribute to cell death (wilson and Goodship, 1994). Heat stress in cultured equine tendon fibroblasts resulted in increased apoptosis and reduced cell survival. Gap junction blockade was suggested to reduce these effects (Burrows S, 2009).

2.6.1 CHANGES IN COLLAGEN FIBRES

Collagen crimp characteristics are altered by age and exercise with a reduction in the crimp angle in the core of older or exercised horses compared with the periphery (PattersonKane et al., 1997b). The mean average diameter of the tendon fibrils is reduced in the core of older and exercised horses indicating a reduction in tensile strength (PattersonKane et al., 1997b). These changes are likely to contribute to the development of clinical tendon injury. There also an increase in stiffness of the tendon due to the increased collagen cross-linking in older tendons. Aldimine and ketamine cross-links are replace by covalent cross-links that increase the stability of the tendon and reduce fibre slippage. The reduction of slippage of fibres increases the stiffness of the tendon but reduces the elasticity of the tendon and increases the risk of tendon injury.

Injured tendon heals slowly and with a fibrous scar rather than regeneration of normal tendon tissue. This results in greater load transmission to adjacent tendon and a high risk of re-injury. Numerous treatment options are available, predominantly because the ideal treatment has not yet been identified.

2.7 TREATMENT OF TENDON INJURY

Tendon injury due to overuse is common among elite and recreational equine athletes and can be difficult to treat successfully (Dowling et al., 2000, Firth, 2000). Treatments for tendon injuries are multi-factorial, prolonged and the wide variety of therapies used in humans and animals is a clear indication that long term success is not guaranteed (Alfredson and Cook 2007, Firth 2000). Even in human medicine the ideal treatment for tendinopathy, particularly of energy-storing tendons remains elusive (Alfredson and Cook, 2007).

There are sparse scientific data for most treatments, although, some randomised trials exist for the use of non-steroidal anti-inflammatory drugs, extracorporeal shockwave therapy, glyceryltrinitrile, sclerosing injections, electrotherapy and eccentric exercise (Andres and Murrell, 2008, Alfredson and Cook, 2007, Waguespack et al., 2011,Kersh, 2006 #617) . Inconsistent results are achieved with extra corporeal shock wave therapy, therapeutic ultrasound and low-level laser. Current data support the use of eccentric strengthening protocols, sclerotherapy and nitric oxide patches in human Achilles tendinopathy but larger, multicentre trials are needed to confirm the long-term response to such treatments (Chester et al., 2008,Alfredson, 2007 #155). At sites of tendon injury neovascularisation is associated with ingrowth of nerve fibres and the development of pain. Sclerosing agents are thought to affect pain causing nerves as well as blood vessels resulting in short term pain relief (Maffulli et al., 2010). Long term effectiveness has not been demonstrated. The areas of the tendon commonly injured in both horses and people are those with a lower blood flow (Jarvinen et al., 2005, wilson and Goodship, 1994). Nitric oxide may be useful to increase blood flow to the injured areas and has been shown to improve subjective and objective measures of recovery (ref Murrell 2007). Similarly, preliminary work with growth factors (Thomopoulos et al.,

2010), platelet rich plasma (Nguyen et al., 2011) and stem cells (Fortier and Travis, 2011, Ahmad et al., 2012) is promising, but further study is required in these fields. Surgery remains an option and often has a positive outcome in people although this is dependent on age, concurrent disease and the reasons why the condition has proven refractory to conservative therapy. Surgical treatment in horses has a higher success rate in standardbred racehorses than thoroughbreds (Hogan and Bramlage, 1995) although is still not a guaranteed success.

2.7.1 CONSERVATIVE THERAPY

Conservative therapy is the initial approach in both horses and humans. A review of non-surgical therapy for Achilles tendinopathy found that eccentric exercises had the most evidence of effectiveness in treatment of mid-portion Achilles tendinopathy (Kramer et al., 2010).

Human athletes undergo a period of rest or altered mobility following Achilles tendon injury and as with horses have a personalised physiotherapy protocol developed. Strength, stretching and mobility exercises and orthotic treatments are more practical in human athletes (Alfredson and Cook, 2007) and this may be why the re-injury rate is lower. Eccentric exercise has been proven successful in 90% of mid body Achilles tendon injury in the first year post injury (Alfredson and Cook, 2007). This therapy requires the patient to exercise despite pain and would be hard to measure and perform ethically in horses. A review of prospective randomised controlled trials determined that eccentric exercise was beneficial although the variety of protocols and evaluation systems prevented recommendations for dose and duration of therapy (Kramer et al., 2010).

Horses with tendon injury are confined to achieve rest and reduce the expansion of lesions. They may be bandaged and a recent study has shown immobilisation using a cast for 10 days reduced lesion propagation in a surgical model in horses (David et al., 2012) compared with bandaging alone. This is followed by a gradually increasing exercise program usually monitored by both clinical examination and ultrasonography. . At walk the accessory ligament of the deep digital flexor tendon and suspensory ligaments are loaded more than the superficial digital flexor tendon so the injured tendon is supported during rehabilitation (Riemersma et al., 1996).

2.7.2 TOPICAL TREATMENTS

Application of ice and cold water to reduce inflammation immediately after injury is a mainstay of treatment of human and equine injury. The aim is to reduce fluid, protein and inflammatory mediator leakage from damaged vasculature via vasoconstriction to reduce expansion of the lesion.

Extra corporeal shock wave therapy increased neovascularisation in a horse superficial digital flexor tendon collagenase model but did not alter the ultrasound appearance of the lesions (Kersh et al., 2006) and no significant effect was reported after using extra corporeal shock wave therapy on a collagenase accessory ligament of the deep digital flexor tendon injury model (Waguespack et al., 2011). Overall, the evidence for using extra corporeal shock wave therapy particularly in energy-storing tendons is poor (Al-Abbad and Simon, 2013).

2.7.3 INTRALESIONAL TREATMENTS

Intralesional injections of anabolic growth factors such as insulin-like growth factor–1 may improve cellular response to injury by modulating gene expression within the cell

(Dahlgren et al., 2006). In a collagenase model insulin-like growth factor–1 resulted in better ultrasound appearance and more type I collagen than controls (Dahlgren et al., 2002). Transforming growth factor β-1 stimulated the production of type I collagen (Murphy et al., 1994) and cartilage oligomeric matrix protein production and cell replication in equine tenocytes (Goodman et al., 2004).

Corticosteroid treatment of tendon injury is a controversial subject in human and veterinary fields. Intralesional injections are deleterious resulting in collagen necrosis and hyalinisation (Pool et al., 1980). Also collagen fibre necrosis and dystrophic calcification with decreased tendon strength can occur for up to 1 year after treatment (Henninger, 1992).

Intralesional hyaluronate treatment of tendon injury has also produced conflicting results. The ultrasound appearance of tendon of lesions was reported to be subjectively better following intralesional hyaluronate (Spurlock et al., 1989) and reduced adhesions developed on damaged intrasynovial tendons (Gaughan et al., 1991,Oryan, 2012 #1199). However, clinically hyaluronic acid produced no benefit over controlled exercise alone (Dyson, 2004).

Beta aminoproprionitrile fumerate binds lysyl oxidase and inhibits cross-link formation (Stryer, 1988).Treatment of superficial digital flexor lesions with Beta aminoproprionitrile fumerate significantly reduced re-injury in the treated limb in comparison to conservative management alone or in combination with intralesional hyaluronic acid, intralesional polysulphated glycosaminoglycans or systemic polysulphated glycosaminoglycans (Dyson, 2004). The initial results for this treatment were positive but longer term outcomes were less so, notably some horses injured the contralateral limb (Reef et al., 1996).

There are conflicting opinions on the benefit of using polysulphated glycosaminoglycans for the treatment of tendinopathy. Contradictory effects on the healing of collagenase induced superficial digital flexor tendon lesions are reported (Redding et al., 1999, Marxen et al., 2004). Also non-blinded subjective assessment suggested a positive effect following intra-muscular injections of polysulphated glycosaminoglycans in horses with clinical superficial digital flexor tendinopathy (Dow et al., 1996). In contrast, Dyson (2004) found no benefit over controlled exercise alone with the use of intralesional or intramuscular polysulphated glycosaminoglycans clinically. Also although Marr (1993a) reported no significant difference in return to racing, compared with conservative therapy, a higher re-injury rate occurred in national hunt horses treated with polysulphated glycosaminoglycans. .

A review of stem cell therapy in equine tendon injury reported that bone marrow and adipose derived stem cells engraft into tendon and improve tendon architecture (Reed and Leahy, 2013). Stem cells are also reported to have a positive effect on re-injury rates (Smith and Webbon, 2005) although no published controlled trials exist to confirm this (Taylor et al., 2007). Also, they do not decrease recovery period and the resulting healed tendon tissue is still inferior to native tendon (Reed and Leahy, 2013). Intralesional injection of autologous bone marrow derived mesenchymal stem cells had no measurable effect on the fibril diameter of collagen in healing tissue in the superficial digital flexor tendon of an experimental model 16 weeks after injury (Caniglia et al., 2012). Umbilical cord blood-derived stem cells are more naïve and are proposed as an alternate source of stem cells that could promote improved regeneration of tendon tissue (Reed and Leahy, 2013).

Neovascularisation is thought to be one of the early signs of tendon injury and associated with tendon pain (Kristoffersen et al., 2005). Tarantula venom extract

(Theranekon ®), a sclerosing agent, injected over a repaired tendon transection model in rabbits resulted in reduced inflammation and strongly ameliorated clinical symptoms, structural organization and biomechanical properties (Oryan et al., 2012).

2.7.4 SYSTEMIC TREATMENTS

A review of non-steroidal effects on healing in humans did not detect deleterious effects on soft tissue injuries and therefore short-term low dose administration is considered acceptable (Chen and Dragoo, 2013). Corticosteroids are the most potent anti-inflammatories and are often given to horses with acute tendon injury within the first 24 hours to reduce inflammation (Dowling et al., 2000).

2.7.5 SURGICAL TREATMENTS

Desmotomy of the accessory ligament of the superficial digital flexor tendon increases involvement of the superficial digital flexor muscle and therefore reduces the peak load on the superficial digital flexor tendon (Henninger et al., 1991). Studies showed a range of 52-85% of horses treated by this returned to racing (Hogan and Bramlage, 1995, Fulton et al., 1994, Gibson et al., 1997) and 27/38 of horses that returned to racing did not have a recurrence in 5 races and started in a median of 18 races following recovery from surgery (Hawkins and Ross, 1995). Although horses treated in this manner are 1.3x more likely to race on 5< occasions they are also 5.5x more likely to get a suspensory ligament injury (Gibson et al., 1997). In a standardbred study 82% raced and 69% had 5 or more starts following desmotomy of the accessory ligament of the superficial digital flexor tendon and the surgery appears to lower recurrence in standardbred racehorses (Fulton et al., 1994, Hogan and Bramlage, 1995).

Carbon fibre implants induced persistent abnormalities in collagen and the return to racing was the same as other treatments (Goodship et al., 1980).Whilst initially the tendon cells appeared to line up along carbon fibres at the periphery close to the carbon fibre the tissue was disorganised and had infrequent fibroblasts (Nixon et al., 1984).

Firing, the burning of tendon using heated needles was popular in the past as it caused increased fibrous scarring and was thought therefore to make the tendon stronger. The need for strength and elasticity in the energy-storing tendons intuitively goes against the use of this practice and yet it is still widely performed in the UK. A study in a collagenase model showed no direct benefit on healing, type I collagen production or performance and identified the disadvantage of increased adhesion formation (silver 1983).

Tendon splitting is used in acute severe cases to drain the haematoma in the core of the damaged tendon. Ultrasound showed that collagenase induced lesions with a cross sectional area bigger than 30% showed significant decrease in the cross sectional area in the split compared with the controls at days 15, 30 and 60 after splitting(Alves et al., 2002). There were more mature repaired tissues in the split tendons at 30 and 60 days after splitting. The split technique used in acute large core lesions is reported to have reduced the time of wound healing and improved the scar formation (Alves et al., 2002).

2.7.6 PROBLEMS WITH TREATMENT IN HORSES

As discussed in the previous sections energy-storing tendons are special cases and therefore in general have a poorer response to treatment and a greater demand for high quality healing. Treatments are aimed at reducing the initial expansion of the lesion followed by rehabilitation of the tendon and attempts to promote healing by regeneration more than scarring. Currently there are no scientifically proven treatments

giving consistently good results in returning horses with SDF tendon injury to optimal athletic ability (Dowling et al., 2000, Taylor et al., 2007). Improving healing of superficial digital flexor tendon lesions is therefore important for improving welfare and reducing financial impacts of tendon injury on the racing and sport horse industry. A good model of tendon injury is required to investigate the suitability of the multitude of therapies available.

2.8 MODELS OF TENDON INJURY

2.8.1 WHY WE USE MODELS IN RESEARCH

Investigation of the aetiology, pathogenesis and treatment of clinical conditions requires a standard expression of the condition to reduce individual variation and thereby improve the detection of significant differences. Models of disease at the cellular, organ and whole animal levels have been developed for numerous conditions and are responsible for many of the medical achievements in the last century.

2.8.2 WHY AN APPROPRIATE TENDINOPATHY MODEL IS ESSENTIAL

There is increasing clinical interest in tendinopathy and a corresponding increase in the research using animal models to study this condition (Warden, 2009, Huang et al., 2004). No good clinical model of acute tendinopathy exists in elastic, energy-storing tendons. There is a need for repeatable, well-characterised animal models of tendinopathy for the investigation of both human and animal tendinopathies (Dirks and Warden, 2011, Warden, 2007). Elucidating the early pathologic changes in tendon injury may enable development of preventative tactics and effective treatments.

2.8.3 *IN VITRO* MODELS

2.8.3.1 *IN VITRO* CELL MODELS

In vitro cell models use at one extreme immortalised cell lines to reduce cellular variation to a minimum (Freshney et al., 2007) and at the other extreme primary cell lines to try and mimic results of the variability within a single cell type *in vivo* (Freshney et al., 2007). They are used to provide a proof of principle for the action of materials or methods on a cell type before organ and *in vivo* experiments.

Advantages of *in vitro* models include the ethical and financial advantages of reducing animal usage; the ability to perform very specific and subtle manipulations; examination of the responses of a single cell type and the ability for direct measurements to be made more easily in comparison with whole animals (Freshney et al., 2007).

The alteration in the environment of the cells results in selection for those cells more suited to surviving under cell culture conditions; this may differ significantly from the conditions found *in vivo*. Most cultures consist of single cell types so also do not explore the influence and effects of other cell types on them. Cells are traditionally cultured in a monolayer although the use of three dimensional substrates and whole organ cultures aim to address some of the disadvantages of the cell monolayer models (Freshney et al., 2007).

Whole organ cultures obviously involve more than one cell type and therefore are likely to be more representative of the organ *in vitro*. They are however more difficult to maintain for long periods of investigation, have a higher welfare cost and are not exposed to the normal circulation so still suffer limitations (Freshney et al., 2007).

2.8.3.2 *Ex vivo* Tendon Models

Ex vivo tendon models involve whole tendons maintained outside the animal. They are often used to investigate loading and how this influences tendon behaviour using mechanised means to apply consistent and measureable loads (Dirks and Warden, 2011). The advantage over cell culture models is that the extracellular matrix effects and responses can be measured and the mixed cell population in the tissue contributes to the effects so more closely approximates the *in vivo* responses. The lack of vascular and systemic responses remains a limitation (Dirks and Warden, 2011) and such cultures are also difficult to maintain over a long period of time.

2.8.3.3 Choice of Species

Rodent, chickens and rabbits have been traditionally used as a source of tissue for extraction of cells for tendon cell experimentation (Tsuzaki et al., 2000, Bernard-Beaubois et al., 1998, Waggett et al., 2006, Albrechtsen and Harvey, 1982). Species and tendon differences have become better recognised more recently and source tissue more closely related to the target cell population is increasingly used. The proportion of studies involving horse superficial digital flexor tendon derived fibroblasts serves to further indicate the clinical impact of damage to this tendon (Yoon et al., 2004, Goodman et al., 2004, Hosaka et al., 2006, Murray et al., 2010). In humans, tissue collected at post mortem or as biopsy during surgery, such as the human Achilles, is a popular choice for tendon experimentation (Tsuzaki et al., 2005).

2.8.4 *In vivo* Models

Animals used for *in vivo* and *ex vivo* models may exhibit the condition being investigated, be genetically modified to do so or have medical or surgical procedures to produce a similar presentation. In all cases, the welfare and financial costs are

significant and such costs are weighed against suitability of the model and the potential benefits. This is obviously a more accurate method of measuring the likely response in that animal and in some cases related species, though this is not guaranteed. Manipulations and measurements can be more difficult and often require sacrifice of the test animal, again increasing the welfare cost.

2.8.5 *IN VIVO* TENDON MODELS

2.8.5.1 CHOICE OF SPECIES

Rabbit and rodents have been chosen more commonly for development of models of tendon injury due to financial considerations, short gestational period, rapid growth rates and short life span (Warden, 2007). Large animals such as horses, goats and dogs are recognised as better models of tendinopathy because they have naturally occurring tendinopathies (Warden, 2007, Patterson-Kane et al., 2012). Sheep are well recognised as models of human orthopaedic disease including tendinopathy (Crovace et al., 2008, Ismail et al., 2008, Turner, 2007). Sheep are easy to obtain and house, tractable, trainable and acceptable to the public as research animals so are potentially good candidates for the development of a sheep tendon injury model (Turner, 2007). The equine superficial digital flexor tendon is clinically and functionally similar to the human Achilles tendon so is a potential model tendon for human disease (Patterson-Kane et al., 2012).

2.8.5.2 PHYSICAL MODELS

Models of tendon injury can be created by physical or chemical methods. Physical disruption creates histological changes that should be more similar to the clinical condition that is caused by recurrent mechanical overload.

2.8.5.3 OVER USE

Methods described for creating *in vivo* mechanical overload injuries include using forced treadmill running, loading via artificial muscle stimulation and direct tendon stretching (Warden, 2007, Banes et al., 1999a, Dirks and Warden, 2011, Silva et al., 2011, Messner et al., 1999). Intuitively, overload via treadmill running should be a good model however it has been difficult to ethically induce consistent overuse injuries with running on the flat as many of the species used for research are habitual runners (Warden, 2007). Including a slope has improved this with uphill running proving to be successful in the induction of Achilles tendinopathy and downhill running for supraspinatus tendon changes (Soslowsky et al., 2000, Glazebrook et al., 2008). Specific models therefore have to be designed for the different anatomical site.

2.8.5.4 SURGICAL MODELS

Surgical models may use excisional and or transectional methods. These models involve sharp injury and both extrinsic and intrinsic repair mechanisms (Iwuagwu and McGrouther, 1998, Little and Smith, 2007, Bruns et al., 2000, Crovace et al., 2008). Tendinopathy is due to acute tearing of the tendon fibres due to chronic micro-damage accumulation within the tendon and predominantly involves intrinsic repair so such models may be less than ideal (Little and Smith, 2007).

Recent models include; needle disruption of the murine Achilles tendon (O'Brien, 2007), partial transection of the sheep infraspinatus tendon (Little and Smith, 2007) and use of an arthroscopy burr to create a lesion in equine superficial digital flexor tendon (Schramme et al., 2010). The murine model does aim to create intrinsic injury similar to the clinical presentation. The entry to the tendon was not sealed so some extrinsic repair may have occurred. The mineralisation that developed in this model makes it less

appropriate as a model for equine superficial digital flexor tendon injury (O'Brien, 2007). The sheep partial transection model involves both intrinsic and extrinsic repair so is again of limited use for equine superficial digital flexor tendon injury. The horse superficial digital flexor tendon model (Schramme et al., 2010) achieves intrinsic repair by closure of the arthroscopy portal. A limitation of this model is that the torn fibres and damaged cells are removed from site.

2.8.5.5 CHEMICAL MODELS

Collagenase, ProstaglandinE1, ProstaglandinE2, corticosteroids and cytokines are examples of substances injected into tendons to create a model by chemical damage (Warden, 2007). Most knowledge of tendon healing in horses is based on the collagenase model, this involves chemical damage to the tendon and results in marked inflammation, vascular damage and haemorrhage followed by fibrosis (Dahlgren et al., 2002). The use of collagenase models to investigate mechanical overload injury to energy-storing tendons has been increasingly questioned (Patterson-Kane and Firth, 2009, Stone et al., 1999). In particular the model results in marked peri-tendinous inflammation which is thought to be due to leakage of the collagenase from the injections or erosion from the core to the paratenon (Schramme et al., 2010). This model therefore does not create a true model of a core lesion surrounded by a region of intact tendon so treatments developed using this model may also be less than ideal.

Tendons injected with a species-specific cytokine preparation showed changes consistent with a mild reversible tendon injury with no matrix damage or collagen degeneration (Stone et al., 1999). The cytokine preparation did not alter the cross-sectional area or structural properties at 4 weeks (Stone et al., 1999) so is probably too mild to act as a model for superficial digital flexor tendon and Achilles tendon injury.

2.8.6 OVERALL LIMITATIONS OF CURRENT MODELS

There are a number of limitations of the current models used to investigate tendon injury and these include the following:

- It is difficult to induce consistent overuse injuries ethically and specific models have to be developed for each anatomical site.
- Surgical models are generally excisional and therefore involve extrinsic healing which is not consistent with the majority of clinical tendinopathy cases.
- The arthroscopic method only involves intrinsic repair but removes tissue debris and cellular components from the core lesion that is inconsistent with clinical tendinopathy.
- The chemical collagenase model is widely used to investigate equine tendon injury but produces a marked inflammatory response inconsistent with the clinical injury.

Considering the limitations of the current models of acute injury to energy-storing tendons the development of a more appropriate *in-vivo model* was required to test the interventions.

2.9 CONNEXINS, CONNEXONS, HEMI-CHANNELS AND GAP JUNCTIONS

2.9.1 CONNEXINS

Connexins are structurally conserved non-glycosylated integral membrane proteins in vertebrates that assemble to form connexons/hemi-channels and therefore gap junctions

(Falk, 2000a). The protein structure consists of two extra-cellular facing loops, 4 trans-membrane domains, 1 intracellular loop and the amino and carboxyl domains on the cytoplasmic side (Goodenough and Paul, 2009). Connexins are involved with a multitude of functions other than gap junction assembly that have been reviewed elsewhere (Dbouk et al., 2009, Jiang and Gu, 2005).

2.9.2 HEMI-CHANNELS AND CONNEXONS

Connexin subunits assemble to form a hexameric connexon or hemi-channel. Connexin subunits do not assemble randomly, only homo and specific hetero-oligomeric connexons form (Falk, 2000c). Connexin composition, stoichiometry and organization within connexons determine properties of the hemi-channels and gap junctions (Benedetti et al., 2000). Connexons align and dock with connexons in adjacent plasma membranes to form gap junctions. Un-docked connexons are known as hemi-channels and allow communication between the intra-cellular and extra-cellular environments.

2.9.3 GAP JUNCTIONS

Gap junctions are dodecameric complexes within the plasma membrane made up of a hexameric connexon in one plasma membrane docking to a connexon in an adjacent plasma membrane (Falk, 2000a, Oviedo-Orta et al., 2002). A complete double membrane intercellular channel is formed by the inter-digitation of the 12 extra-cellular loops present in each connexon/ hemi-channel to form 2 concentric barrel-like structures with a central channel (Evans and Boitano, 2001). .

Gap junctions allow the transfer of molecules, including ions; metabolites and messengers, less than 1 kDa in size between cells by diffusion (Kumar and Gilula, 1996,

Banes et al., 1999b). Direct cell-to-cell communication enables cells to respond to changes in their environment in a co-ordinated manner (Banes et al., 1999b).

Gap junction plaques are aggregates of less than a hundred to thousands of gap junctions and act as the 'functional organelles' of cell-to-cell communication (Segretain and Falk, 2004). These plaques are often irregular in shape with wavy or jagged edges and can contain areas lacking gap junctions within them (Falk, 2000b).

2.9.3.1 GAP JUNCTIONS IN TENDONS

Connexin32 and Connexin43 have different but often overlapping distributions in multiple organs (Beyer et al., 1989) and have been identified as the major gap junction proteins in tendons (Ralphs et al., 1998, McNeilly et al., 1996) but are not expressed in same connexon. Connexin32 and Connexin43 link tenocytes longitudinally but Connexin43 also makes lateral connections between tenocytes in different rows (Ralphs et al., 1998, McNeilly et al., 1996). Tendons undergo tensile loading and therefore their cell-cell junctions must be able to withstand some elongation and recovery of the associated extra-cellular matrix (Ralphs et al., 2002). Mechanotransduction mechanisms must be present to generate signals in the first place (Ralphs et al., 2002). Tendon cells *in vivo* joined along their cell rows by longitudinally organised actin stress fibres linked end to end by adherens junctions. Both stress fibre and cell junction formation are mechanically regulated (Ralphs et al., 2002). Connexin43 expression is up-regulated in tendon cells *in vitro* in response to mechanical loading (Banes et al., 1999a). A generalised gap junction blocker, octanol, reduces DNA or collagen production in response to mechanical load i.e. gap junction communication appears to be necessary for load-induced collagen synthesis (Banes et al., 1999a, Waggett et al., 2006). Tendon cells need to be able to signal via gap junction to process and respond to mechanical

load signals to increase cell division and matrix production (Banes et al., 1999b). Selective down-regulation of Connexin32 and Connexin43 expression by antisense oligodeoxynucleotide treatment has shown that Connexin32 and Connexin43 have short-term opposing effects on tenocyte collagen synthesis (Waggett et al., 2006).

2.9.3.2 GAP JUNCTIONS IN EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON AND HUMAN ACHILLES TENDON

Connexin32 and Connexin43 are present in equine superficial digital flexor tendons and human Achilles tendon (Young et al., 2009, Wall et al., 2007). Expression of Connexin32 and Connexin43 are higher per tendon cell and tendon cell numbers are also higher in fetal tendons compared with foals, young horses and old horses (Stanley et al., 2007). In developing chick embryos the importance of spatial and temporal variations in GJ expression in pattern formation have been demonstrated (Becker et al., 1999). The higher levels in developing foals may correspond to increased requirements for communication driven responses to mechanical load.

Human tendon cells *in vitro* express Connexin43 gap junctions and these are co-localised with actin (Wall et al., 2007). Cell to cell junctions are less obvious in human than in avian tendon cells and human cells have long processes with Connexin43 gap junctions at the tips (Wall et al., 2007). The numbers of cells with Connexin43 and actin co-localisation were reported to increase in response to mechanical load (Wall et al., 2007). Whilst primary tendon fibroblasts differ in size and morphology *in vitro*, Connexin43 and its importance within these cells appears to be consistent in horses, humans, birds and rodents (Burrows S, 2009, Wall et al., 2007, McNeilly et al., 1996).

2.9.4 FUNCTION OF GAP JUNCTIONS AND HEMI-CHANNELS

In the 1970's it was recognised that ionic and metabolic coupling between cells was related to the presence of gap junctions (Gilula et al., 1972). Gap junctions are now known to allow direct passage of small metabolites between cells (Oviedo-Orta et al., 2002) and to interact with other cell adhesion molecules and proteins (Musil et al., 1990) Gap junctions therefore provide direct cell-cell communication (Falk, 2000c) including propagation of calcium fluxes between cells during mechanical stimulation (Ralphs et al., 1998) and spread of cell death signals from injured cells to healthy neighbouring cells (Cusato et al., 2003, Burrows S, 2009). Gap junctions are crucial in the development and differentiation of tissues and organs (Falk, 2000c) due to their involvement in the spatial and temporal control of embryogenesis (Becker et al., 1999). They are also involved in normal and delayed wound healing (Wang et al., 2007, Saitoh et al., 1997, Brandner et al., 2007). Gap junctions are highly mobile and dynamic (Falk et al., 2009) with a half-life of individual connexons as low as 30 minutes enabling rapid responses to changes in load or environment.

2.9.4.1 LOAD AND GAP JUNCTIONS

Connexin43 expression was up-regulated in cultured avian tendon cells exposed to tensile load but had an inhibitory effect on collagen production (Waggett et al., 2006). Connexin32 had a stimulatory effect on collagen production and on basal levels of collagen synthesis (Waggett et al., 2006). The opposite effects of Connexin32 and Connexin43 on collagen production in response to load indicate cells integrate mechanotransduction signals through the gap junction network to create final load responses (Waggett et al., 2006).

Cyclic loading of whole avian *digital flexor profundus* tendons stimulated deoxynucleic acid and collagen synthesis which was blocked by octanol, a reversible gap junction blocker (Banes et al., 1999a).Within the group of responders it was noticeable that not all responded, this suggests some were inhibited from entering cell division (Banes et al., 1999b). Gap junction communication is one way this may have been regulated and therefore may be important in wound healing in tendons.

2.9.4.2 WHAT DO GAP JUNCTIONS DO IN HEALING ?

Cutaneous wound healing is a complex orchestrated event of a number of overlapping stages to repair tissue discontinuity. The process involves haemostasis, inflammation; granulation and tissue remodeling and re-epithelialisation regulated by a balance of cell proliferation and programmed cell death (Coutinho et al., 2003). Cell death is a normal part of the wound healing process and gap junctions mediate the spread of cell death following injury (Coutinho et al., 2005). Gap junctions are thought to co-ordinate cells and events of wound healing by intercellular communication (Coutinho et al., 2003, Goliger and Paul, 1995). Connexin43 was expressed in multiple cell types in skin and is involved in many stages of tissue repair. The events following wounding of skin are accompanied by a change in the distribution of connexin expression (Brandner et al., 2004, Coutinho et al., 2003) with connexin expression decreased at wound edges and increased at adjacent unwounded areas (Brandner et al., 2004, Matic et al., 1997, Saitoh et al., 1997). Within 8-24 hours of wounding Connexin43 is decreased in the wound bed and all layers adjacent to the wound. This is seen in human and rodent models and in cutaneous, tongue and corneal wounds (Brandner et al., 2004, Coutinho et al., 2003, Matic, 1997 #77). Connexin43 expression persists in the wound margins in most non-healing wounds in an *ex vivo* study of healing and non-healing wounds in humans

(Brandner et al., 2004). Disruption of the healing processes results in delayed healing and excessive scarring.

There is an association of contractile apparatus and gap junctions in epidermal cells growing over an open wound which suggest synchronised locomotion (Gabbiani et al., 1978). It is possible that Connexin43 down-regulation in response to wounding is due to the migratory state of cells. The elimination of cell-cell communication during migration may allow the co-ordination of spatiotemporal fluctuation of second messengers to be more specific in every cell (Matic et al., 1997).

2.9.5 GAP JUNCTION MODULATION IN GENERAL

Gene knockout mice were originally used to determine the importance of individual gap junctions however many of the resulting conditions were so severe the mice did not survive. Non-specific gap junction blockers such as octanol, carbenoxolone and halothane have been widely used to investigate gap junction function and subsequently more specific gap junction blockers have been developed (Bodendiek and Raman, 2010).

Antisense oligodeoxynucleotides can specifically block mRNA translation and protein expression. They have been used to investigate gap junction functions in many tissues including responses to loading and wounding (Waggett et al., 2006, Mori et al., 2006). Mimetic peptides are proteins that bind to the extracellular domain of the connexon/hemi-channel and block communication with the extracellular environment (Boitano and Evans, 2001, Evans and Boitano, 2001). At high doses, mimetic peptides also cause gap junction uncoupling (O'Carroll et al., 2008).

2.9.6 GAP JUNCTION MODULATION IN WOUND HEALING

2.9.6.1 ANTISENSE OLIGODEOXYNUCLEOTIDES

Down-regulation of Connexin43 by topical application of antisense oligodeoxynucleotides in pluronic gel has been shown to dramatically accelerate and improve incisional and excisional cutaneous wound healing, with reduced scar formation in neonatal mouse skin wound and burn models (Brandner et al., 2004, Coutinho et al., 2005, Qiu et al., 2003, Ghatnekar et al., 2009). A single application of antisense oligodeoxynucleotides transiently down-regulated Connexin43 at the wound edge for 24 hours (Qiu et al., 2003). Cell death is a normal part of the wound healing process and gap junctions mediate the spread of cell death signals following injury (Coutinho et al., 2003). Expression of TGF-β1, collagen α1 and total collagen was increased in a fibroblast wound-healing model following down-regulation of Connexin43 expression (Mori et al., 2006). In a rat skin model, down-regulation of Connexin43 resulted in wounds with enhanced granulation tissue; rapid angiogenesis; myofibroblast differentiation and wound contraction. These wounds exhibited less inflammatory infiltrate and closed more quickly than untreated controls (Mori et al., 2006). In a neonatal mouse burn model antisense oligodeoxynucleotide treated wounds had increased rate of closure at all times compared with untreated controls (Coutinho et al., 2005). Acute knockdown of Connexin43 at the time of wounding resulted in a marked reduction of neutrophils and subsequently macrophages (Mori et al., 2006). It is possible to regulate specific Connexin43 expression with high spatial and temporal resolution using application in a pluronic gel (Becker, 1999). Pluronic F-127 gel is a weak surfactant and at 30% w/v sets as a hygroscopic gel at physiological temperatures. It has been used as a reservoir of antisense oligodeoxynucleotides that are continuously

released as the gel dissolves. This overcomes a major disadvantage of antisense oligodeoxynucleotide use *in vivo*, which is their rapid breakdown in serum (Law et al., 2006).

2.9.6.2 MIMETIC PEPTIDES

The mimetic peptide Gap27 corresponds to the amino acid sequence in the second extracellular loop of Connexin43 and inhibits gap junction intercellular communication. Both Connexin32 and Connexin43 are inhibited by Gap27 (Evans and Boitano, 2001) and Gap27 improves fibroblast migration in monolayer cell culture (Wright et al., 2012). Another mimetic peptide, Peptide5 reduced swelling in a sheep *ex vivo* spinal cord injury model more than Gap27 (O'Carroll et al., 2008).

The rate and quality of wound healing in a number of tissues has reportedly improved in response to gap junction modulation (Mori et al., 2006, Qiu et al., 2003, Coutinho et al., 2005). Gap junctions and modulation of them has also been shown to influence collagen production in tendon cells (Waggett et al., 2006). Together these suggest that gap junction modulation may have a positive influence on tendon healing. In order to investigate this further an appropriate model of acute tendon injury is needed. This project aimed to create such a model, describe the gap junction expression in injured tendon and look at the effects of Connexin43 modulation on *in vivo* and *in vitro* on tendon injury.

2.10 HYPOTHESIS

The main hypothesis to be tested was that development of models of acute tendon injury *in vivo* and *in vitro* would enable testing of novel therapies involving gap junction modulation to improve tendon healing.

The specific hypotheses to be tested within this were:

- Connexin43 antisense oligodeoxynucleotides would alter Connexin43 gap junction expression in a novel ovine model of acute tendon injury.
- The reduction of Connexin43 expression in a novel ovine tendon injury model would increase wound healing by reducing inflammation and the spread of the lesion.
- A core lesion could be created in the mid metacarpal region of the equine superficial digital flexor tendon that would clinically, ultrasonographically and histologically mimic a core lesion in acute tendon injury.

2.11 AIMS

- Create an appropriate ovine model of acute tendon injury
- Determine Connexin43 gap junction expression in normal and injured ovine tendons
- Measure the level of Connexin43 gap junctions in response to treatment with oligodeoxynucleotides.
- Using the ovine injury model created, develop a model of an acute tendon lesion in the core of the equine superficial digital flexor tendon that did not have the limitations of the current published tendon injury models
- Determine the clinical, ultrasonographic and histological changes created by the lesion to determine if they were consistent with changes in clinical injury.

- Compare clinical, ultrasonographic and histopathological changes in response to the acute core lesion to enhance understanding of the acute post-injury phase.
- Determine the effect of modulation of gap junction communication on superficial digital flexor tendon-derived fibroblasts *in vitro*.
- Parameters to be studied included
    - Rate of closure of a scrape-wound as a measure of healing potential.
    - The type of collagen produced as an indicator of biomechanical strength.
- Develop a method to reproduce uniaxial load in cell culture that mimicked the *in vivo* situation.

CHAPTER 3

CONNEXINS AND ANTISENSE OLIGODEOXYNUCLEOTIDES IN A NOVEL OVINE ACUTE TENDON INJURY MODEL

3.0 INTRODUCTION

It is well established that changes in gap junction expression occur in wounds and wound healing (Saitoh et al., 1997, Coutinho et al., 2003, Matic et al., 1997, Wang et al., 2007). In general, a rapid decline in connexins occurs at the wound edge within 6-8 hours of wounding (Saitoh et al., 1997, Coutinho et al., 2003). Following this, the expression of each of the connexins present in the tissue follows an independent path to return to normal levels (Saitoh et al., 1997, Coutinho et al.).

Tendon lesions enlarge following the initial insult (Bosch et al., 2010a). This is also true of lesions in the eye and spinal cord (Cusato et al., 2003,O'Carroll, 2008 #377). Gap junctions allow death signals to pass from dying cells to adjacent cells and are thought to be responsible for this effect (Cusato et al., 2003). Anti-sense oligodeoxynucleotides targeting Connexin43 have previously been used to enhance the down-regulation to facilitate wound healing and reduce inflammation in rabbit corneas, rodent skin and human skin explants (Brandner et al., 2004, Coutinho et al., 2005, Qiu et al., 2003, Mori

et al., 2006). In part, this may be due to a reduction in lesion expansion and if this were the case in tendons would reflect a benefit of modulating gap junction expression in this tissue.

An appropriate model of acute injury of energy-storing tendons is yet to be described (Patterson-Kane and Firth, 2009). Development of such a model is essential for measurement of gap junction levels in response to injury in this tissue and for testing of any treatment regimen. An appropriate model would include tearing rather than cutting of tendon fibres; involve only intrinsic healing; be applicable to energy-storing tendons and produce histological changes consistent with acute injury.

The hypothesis to be tested was that Connexin43 antisense oligodeoxynucleotides would alter Connexin43 gap junction expression in a novel ovine model of acute tendon injury. More specifically the reduction of Connexin43 expression would potentially increase the rate of wound healing by reducing inflammation and the spread of the lesion as it has been shown to in other tissues.

Aims:-

- Create an appropriate ovine model of acute tendon injury Determine Connexin43 gap junction expression in normal and injured ovine tendons
- Measure the level of Connexin43 gap junctions in response to treatment with oligodeoxynucleotides.

A novel ovine model of acute superficial digital flexor tendon injury was therefore developed as the basis of an experimental system that could ultimately be used to study the pathogenesis of acute tendon rupture and healing in animals.

Once the model was developed Connexin43 levels were measured in the acutely injured tendon as an indication of the success of the model in causing tendon injury. Connexin43 was chosen as a marker of injury and repair as it is predominant in gap junctions linking tendon fibroblasts and in wound healing (Brandner et al., 2007, McNeilly et al., 1996).

This ovine model was used to assess the effects of Connexin43 antisense oligodeoxynucleotides on Connexin43 gap junction levels. If the use of antisense oligodeoxynucleotides in the model system indicated a more effective or rapid rate of repair, then therapeutic use of antisense oligodeoxynucleotides in tendon injury would need to be evaluated. Antisense oligodeoxynucleotides are broken down by serum (Law et al., 2006) therefore would require a delivery vehicle. Pluronic gel has previously been identified as an inert, nontoxic polymer for delivery of the unmodified antisense oligodeoxynucleotides (Becker, 1999). It provides sustained release to overcome the short half-life of antisense oligodeoxynucleotides in the presence of serum (Law et al., 2006). Therefore, pluronic gel was tested for suitability as a delivery vehicle in tendon.

3.1 MATERIALS AND METHODS

3.1.1 ANIMALS

Cadaver, cross-bred sheep (*Ovis Aries)* were used in the first instance to develop a technique for creating a model of acute tendon injury in the pelvic limb superficial digital flexor tendon.

The *in vivo* model studies were then performed over a 3-month period in 73 Romney crossbred sheep anaesthetised for non-recovery abdominal or urogenital surgery that

was unrelated to the current study. Thirty–four male and forty female sheep, aged between 6-9 months, were used (Appendix 2).

Control pelvic limb superficial digital flexor tendons were collected from eight sheep undergoing the same non-recovery abdominal surgery; four sheep were subjected to euthanasia at 2 h and four at 4 h. Additional tendons were collected, within 30 min of slaughter, from five cross-bred sheep of a similar age at a local abattoir**.**

3.1.2 MODEL DEVELOPMENT IN CADAVERS

Cadaver sheep were used to develop a model of acute tearing of the core of the sheep pelvic limb superficial digital flexor tendon. The skin was removed from over the pelvic limb calcaneal tendon and the individual tendons identified. The superficial digital flexor tendon was located (Figure 5) and measurements were taken to determine at what level the stab incision should be made to ensure that the lesion would remain within the tendinous tissue and would also avoid the flattened area over the tuber calcaneus. The superficial digital flexor tendon was excised in four sheep and the cross-sectional area measured so that a lesion approximately 40% of the diameter of the tendon could be made.

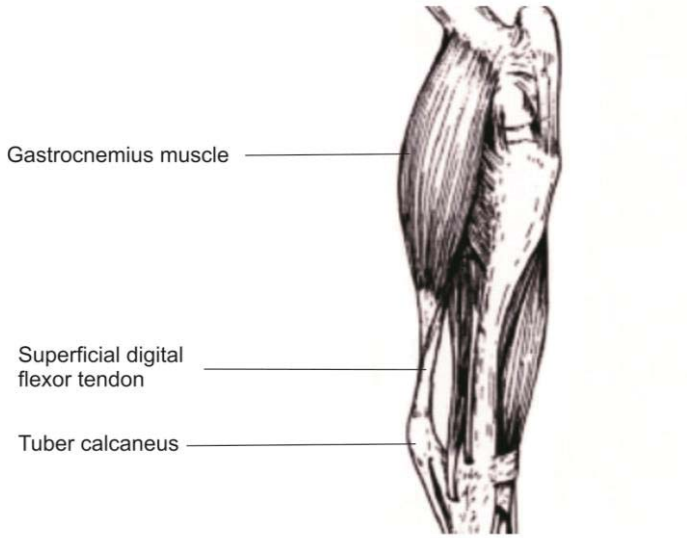


Figure 5 - Medial view of sheep pelvic limb
Modified from Anatomie compare des mammiferes domestiques by Barone, Tome 2 (1980), Editions Vigot.

3.1.3 ANAESTHESIA FOR *IN VIVO* STUDY

General anaesthesia was induced using intravenous ketamine hydrochloride 10 mg/kg and diazepam 0.5 mg/kg. Isoflurane, in 10% oxygen delivered through a circle anaesthetic circuit, was used for maintenance (Figure 6). A Diplomat of the American College of Veterinary Anaesthetists, unaware of the treatment groups, decided which sheep were to be euthanized at 2 h post intervention, and which could undergo anaesthesia for a further 2 h (total 4 h). The decision was based on how well each individual animal was tolerating the anaesthesia.

Figure 6 - Sheep under anaesthesia.
Delivered via a circle system and with an oesophageal stethoscope in position for monitoring purposes.

3.1.4 MODEL TECHNIQUE *IN VIVO*

The crura of both pelvic limbs of the sheep were prepared for aseptic surgery. A 1 cm long stab incision was made through skin, sub cutis and paratenon on the medial aspect of the superficial digital flexor tendon on the caudo-medial aspect of the limb 2 cm proximal to the tuber calcaneus and using a number 10 scalpel blade. A number 11 scalpel blade was then used to make a stab incision, 1 mm deep and 2 mm long, into the superficial digital flexor tendon core. A 1 mm barb was created at the tip of a 14 G catheter stylet by bending with haemostats. The barbed catheter stylet was introduced through the incision, directed proximally along the long axis of the tendon and thrust backwards and forwards 1 cm. This tore the tendon fibres to create a mechanical core lesion. The incisions in the tendon and the skin were sealed with cyanoacrylate glue (Figure 7 a-d). The sheep were maintained under anaesthesia for a total of 2 or 4 h post-intervention and then underwent euthanasia by intravenous injection of 10 mL of pentobarbitone sodium. A record was made as to whether subcutaneous haemorrhage occurred at the time of surgery and at tendon collection (Appendix 2). During each sheep surgery session a sheet was used to record sheep identification, breed, age, sex, time of induction, time of euthanasia, time of wound creation, treatment applied and time of collection of the left and right superficial digital flexor tendons (Appendix 4).

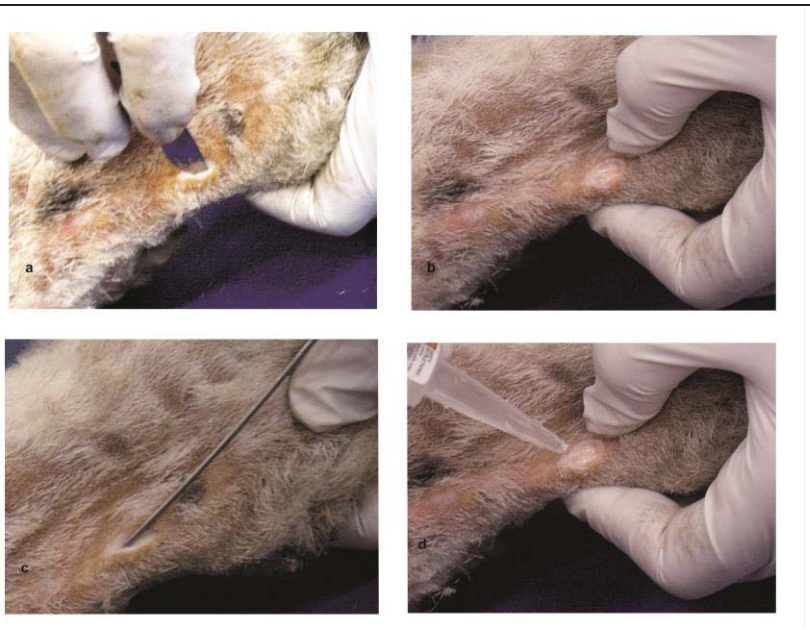


Figure 7 - Lesion creation in cadaver.
a) A stab incision is made 1cm proximal to the tuber calcaneus and over the medial aspect of the superficial digital flexor tendon.
b) A stab incision is made into the superficial digital flexor tendon.
c) The barbed catheter stylet is inserted via the stab incision, directed proximally along the tendon and thrust forwards and backwards 15 times.
d) The stab incision in the tendon is closed with cyanoacrylate glue.

3.1.5 CONTROL TENDONS

Initially, control tendons had been collected from abattoir sheep following euthanasia with a captive bolt and exsanguination. The tendons were collected within 10 min of death and transported on ice prior to being processed; all tendons were processed within 1 h of collection. When re-evaluating the protocols it was apparent that it was important to control for the effects of the anaesthesia, the concurrent procedures that the sheep were exposed to and pentobarbitone euthanasia. Additional tendons were therefore collected from the sheep undergoing general anaesthesia and the non-recovery surgery without surgical intervention in the pelvic limb superficial digital flexor tendons. The general anaesthesia control and all treatment group tendons were collected within 10 min of euthanasia using a pentobarbitone overdose and kept on ice until processed. All tendons were processed within 1 h of collection.

3.1.6 PILOT *IN VIVO* STUDIES

Initial studies *in vivo* were required to test the reproducibility and consistency of the acute injury model in anaesthetised sheep. These studies were also important to determine the best technique for the main *in vivo* study and to test the feasibility of use of pluronic gel as a delivery agent for antisense oligodeoxynucleotides in the model.

The first study used 10 thrusts of a barbed 14G x 8 cm stylet in 16 tendons in eight sheep; including six sheep euthanized at 2 h and two at 4 h. A second study compared 10 with 15 thrusts of the barbed 14G stylet. This study used 50 tendons in 25 sheep with 10 thrusts in the left pelvic limb and 15 in the right. Nineteen of these sheep were euthanized at 2 h post intervention and six sheep at 4 h.

3.1.6.1 INSTILLATION OF PLURONIC GEL

A 24% solution of pluronic gel was prepared (Appendix 3) and agitated overnight at 4°C. Prior to the experiment 0.2 mL of pluronic gel was drawn up into a 1 mL syringe, the syringe was capped and stored on ice until immediately prior to instillation into the injury cavity via the catheter stylet. In 16 tendons in 8 sheep the 15 thrust model was used to create a lesion in both limbs and the pluronic gel was instilled prior to stylet removal in one randomly assigned limb per sheep. India ink was added to the Milli-Q water in six pluronic gel samples to see if the extent of the gel penetration within the lesion could be visualised.

3.1.7 MODULATION OF CONNEXIN EXPRESSION

The murine antisense Connexin43 oligodeoxynucleotides (Table 1) and the murine sense Connexin43 oligodeoxynucleotides (Table 1) as used by Qui (Qiu et al., 2003) were checked using the Basic Local Alignment Search Tool (BLAST,

http://blast.ncbi.nlm.nih.gov/Blast.cgi, Pub Med) and the murine and ovine sequences were found to be identical.

Table 1 - Sequence of Oligodeoxynucleotides

Murine antisense Connexin43 oligodeoxynucleotides	5' GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC 3'
Murine sense Connexin43 oligodeoxynucleotides	5' GAC AGA AAC AAT TCC TCC TGC CGC AAT TTA C-3'

The oligodeoxynucleotides were made up in sterile PBS. For the surgery experiment 0.2 mL of pluronic gel without oligodeoxynucleotides or containing 50 μM Connexin43 sense oligodeoxynucleotide or 50 μM Connexin43 antisense oligodeoxynucleotides were drawn up into a 1 mL syringe, the syringes were capped, labelled and stored on ice until immediately prior to instillation.

Sixty-four tendons were randomly assigned to one of four treatment groups and to 2 or 4hr duration of exposure within each treatment. The groups were: Group M the injury only model (n=20), Group P –the injury model with instillation of 0.2 mL of the pluronic gel vehicle (n= 13). Group S (n=19) and AS (n=22) comprised the injury model tendons treated with 50 μM sense oligodeoxynucleotide or antisense oligodeoxynucleotides, respectively, each delivered in 0.2 mL of pluronic gel before stylet removal. The tendons used in the pluronic gel study (n=8) were evaluated as part of this study bringing the total number of tendons in the four treatment groups up to 82 (Table 2). The two control groups were the anaesthetised controls (GA) 18 tendons and the abattoir controls (n=10).

Table 2 - Table of the number of tendons imaged by confocal laser scanning microscope each of the *in vivo* treatment groups

Group	2 hrs	4 hrs
M	8*	11
P	8*	12
S	10	9
AS	12	10
GA	8	10
	Time 0	
A	10	

* 1 sample lost

Legend:
M = Model (15 thrusts)
P = Model (15 thrusts) and pluronic gel
S = Model (15 thrusts) and pluronic gel with 50µM sense oligodeoxynucleotide
AS = Model (15 thrusts) and pluronic gel with 50µM antisense oligodeoxynucleotide
GA = Control anaesthetised sheep
A = Control Abattoir sheep

3.1.8 SAMPLE COLLECTION AND PROCESSING

Each pelvic limb superficial digital flexor tendon was dissected from the surrounding tissue and transected at 1.5 cm and 4 cm proximal to the tuber calcaneus (Figure 8). The surface of the superficial digital flexor tendon was examined under bright light for gross evidence of the stab incision, discolouration or haemorrhage. The 2.5 cm long section of superficial digital flexor tendon was examined for evidence of fibre disruption, haemorrhage or discolouration in the core, and then placed in 2% paraformaldehyde.

Within 2 hours of collection the tendon tissue was removed from the paraformaldehyde fixative and washed with phosphate-buffered saline (PBS). It was then split lengthways into quarters. One quarter was snap frozen in *n*-hexane chilled in dry ice and stored at -80ºC. The second quarter was immersed in 10% formol saline overnight, then paraffin

embedded and sectioned for Haematoxylin and Eosin (H&E) staining the following day or mounted in a cryomould in optimal cutting temperature compound™, snap-frozen and then stored at -80°C.

Care was taken to orientate the tendon section with the injured surface facing upwards in the cryomould. The third quarter was divided and a 0.5 cm length of tendon tissue was removed from the centre region, conserved in RNA Later™ and stored at -80°C**.** The remaining part of the third quarter and the final quarter were stored in 10% formol saline. All were labelled with sheep identification, time of euthanasia, time of collection and which limb the tendon came from (Appendix 5).

Figure 8 - Collection of the pelvic limb superficial digital flexor tendon following euthanasia.
a) An incision was made over the pelvic limb superficial digital flexor tendon from 1 to 3 cm proximal to the tuber calcaneus and the tendon is identified.
b) The tendon was separated from the adjacent deep digital flexor and gastrocnemius tendons then transected.
c) The tendon is divided lengthwise showing the core lesion.

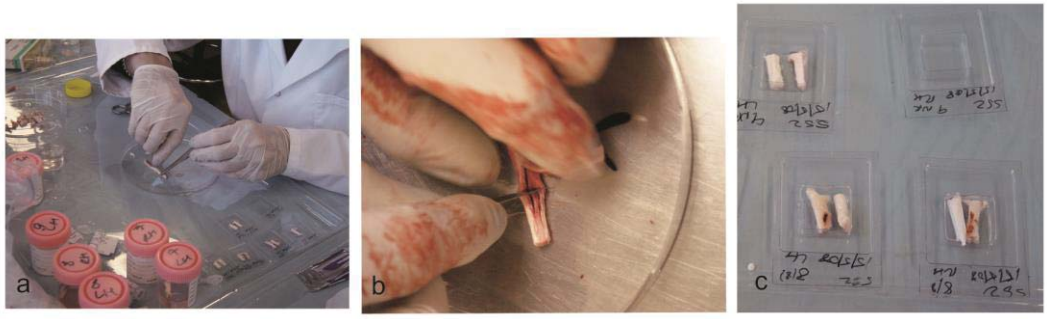


Figure 9 - Tendon processing
a. The tendon was removed from the paraformaldehyde and split lengthwise.
b. A 0.5 cm section as removed and place in RNA Later™ overnight.
c. One half of the tendon was snap frozen in Optimum Cutting Temperature™ in cryomoulds and stored at minus 80° C prior to cryo-sectioning.

3.1.9 HISTOLOGY CUTTING STUDY

The aim of this study was to determine the best method of cutting the tendon samples. Ten tendon samples, taken from cadavers used to develop the method of injury, were soaked in 10% formol saline overnight, then paraffin embedded and sectioned for H&E staining the following day. A further 10 cadaver tendons were mounted in a cryomoulds in OCT™, snap-frozen and then stored at minus 80^{o}C prior to cryo-sectioning.

3.1.10 HISTOLOGY

Longitudinal cryo-sections (16 μm thick) were cut (Reichert-Jung Cryocut 1800, Leica Microsystems, New Zealand) and adjacent sections stained with H&E (Appendix 6) and immunofluorescent labelling respectively. Haematoxylin and Eosin sections were examined and photographed by standard, bright field microscopy (Olympus BX51 and Olympus XC50 camera, Olympus). Images were processed using version 5.0 software (Soft Imaging Solutions, Olympus). Ten randomly selected sections were also stained using Masson's trichrome stain for connective tissues and were compared to adjacent H&E sections to determine if the special stain provided extra information (Appendix 7).

3.1.11 IMMUNOFLUORESCENT LABELLING FOR CONNEXIN43

The cryo-sections were air dried and stored at -80^{0}C. For immunofluorescence, sections were thawed, washed 3 x 10 minutes with PBS and blocked for 1 h with 10% normal goat serum and 0.1% Triton X-100 in PBS in a humidity chamber. Slides were rinsed once with PBS and air-dried. Rabbit polyclonal Connexin43 antibody diluted 1:2,000 in PBS, was applied to the slides; these were incubated overnight at 4^{o}C in a humidity chamber. The following day, the slides were washed 3 x 15 minutes with PBS. The secondary antibody AlexaFluor 568 goat anti-rabbit diluted 1:1000 in PBS was added

and the slides incubated in the dark at room temperature for 2 h. Slides were rinsed and washed 3 times for 15 minutes with PBS then air-dried. Nuclei were stained using mounting medium Prolong Gold™ with 4', 6-diamidino-2-phenylindole (DAPI) and slides sealed with clear nail varnish.

3.1.12 QUANTITATIVE CONFOCAL LASER SCANNING MICROSCOPY

Immuno-stained tendon sections were examined using a confocal laser scanning microscope (CLSM, Leica SP5 DM600B) and images captured using Leica software (Leica Microsystems, New Zealand).

The CLSM was set up for dual channel fluorescence using DAPI and AlexaFluor 568 filters with the laser settings at 405 nm and 561 nm respectively. A region of normal-appearing tendon was located on the section by identifying the presence of long, thin tendon fibroblast nuclei at a point at least 400 μm from the injury. This was termed the 'bystander' area as it was within the same section as the injury, appeared normal in terms of nuclear size and shape and had normal tendon fibre alignment (Figure 15b).

Images were collected using 63x1.4 oil-immersion HCX PL APO UV objective lens. Optical sections were scanned in xyz-mode, with an 8-bit resolution, and a pinhole of 3.76 Airy units (359.3 μM). A unidirectional scan at 400 Hz and line averaging of 2 was used to scan a format of 1024x1024 pixels. The confocal gain and offset settings were optimised for the bystander region then used for all images for that slide (Appendix 8).

The Leica Z-stack software was used to collect vertical stacks of images (Z-stacks) comprising 6 x 0.5 μm adjacent slices collected at 3 sites where inflammation and tendon fibre disruption was evident. Care was taken to avoid inter-fascicular

(endotendon) regions and blood vessels. Within the control tendons, Z-stacks were collected from three randomly selected areas (Appendix 9).

Concatenated images of each of the Z-stacks of immune-stained Connexin43 gap junction plaques and DAPI stained nuclei were exported. The images were imported into image analysis software (ImageJ software, National institute of Health, USA) and converted into digital 8-bit images with manual threshold applied at 50% (suggested by Professor Colin Green). Automated counting was achieved by selecting an appropriate size range for nuclei and for connexin respectively. This was used to make counts and measure the total gap junction plaque area in each image. The procedure was written into a user-designed plug-in (kind gift from Sarah Burrows, RVC, UK) to ensure standardisation of the protocol (Appendix 10). The results were imported into Microsoft Excel and expressed as counts and area per 100000 μm^3 prior to statistical analysis.

3.1.13 STATISTICS

The numbers of Connexin43 gap junction plaques in the treatment groups did not fall into a normal distribution and, therefore, were log-transformed for statistical analysis. The statistical model used to analyse the log-transformed count of Connexin43 gap junction plaques included treatment, time and area as fixed effects while their interactions, as well as tendon within time and treatment were classed as random effects (Proc Mixed, SAS 9.3 Software). A result was deemed significant if $P<0.05$ using analysis of variance.

3.2 RESULTS AND DISCUSSION

3.2.1 SELECTION OF SHEEP AS THE MODEL SPECIES

The aim of this part of the thesis was to develop a sheep model of tendon injury based upon the previously reported murine needle injury transection model (OBrien, 2008). The larger size of the sheep superficial digital flexor tendon made injury to the core, rather than an excisional or transectional injury, physically practical and facilitated sealing of the tendon with cyanoacrylate glue. The use of the cyanoacrylate glue to seal the entry to the lesion prevents extrinsic repair of the defect.

Sheep are well recognised as models of human orthopaedic disease, including tendinopathies (Ismail et al., 2008, Crovace et al., 2008, Turner, 2007), and are easy to obtain and house, tractable, trainable and acceptable to the public as research animals (Turner, 2007). The sheep pelvic-limb superficial digital flexor tendon is of the same type as the human Achilles and the equine superficial digital flexor tendon, is part of the Achilles complex and is an energy-storing tendon.

Mineralisation of the Achilles tendon had been reported in the murine model (Shoemaker et al., 2006) which would make it less appropriate for modelling equine superficial digital flexor tendon injury as mineralisation is not common in clinical tendinopathy unless intra-tendinous steroids have been administered as part of a treatment regimen. Mineralisation is not a reported finding in other sheep tendinopathy models (Bruns et al., 2000, Gerber et al., 2004) therefore was unlikely to be of concern for the model development in the current study. The short duration of this acute injury model would not have stimulated mineralisation; however it was important to develop the model in an appropriate species that could potentially be followed through all the

stages of healing. The sheep pelvic-limb superficial digital flexor tendon proved to be an appropriate tendon for the development of this acute injury model.

3.2.2 INJURY MODEL

3.2.2.1 OPTIMISATION OF THE INJURY TOOL

Early attempts at making the lesion involved the use of a variety of sizes of hypodermic needles 16-20 gauge to damage the core of the sheep pelvic-limb superficial digital flexor tendon. Histological examination showed that a smooth needle produced only slight separation of the fibres and some cutting by the needle bevel. Subsequently, using haemostats, a barb was created on a 16 gauge needle to tear the tendon fibres as well as cut them. The introduction of the barb resulted in grossly visible, tearing of the fibres. The barb was then created on a 14 gauge catheter stylet to enable a larger gauge needle with sufficient length to be used. A barb of 1 mm plus the diameter of the 14 gauge stylet, when rotated, was calculated to produce a lesion of approximately 5 mm diameter (Figure 10) with an approximate cross-sectional area of 19.65 mm^2 (given by πr^2 with r = 2.5).

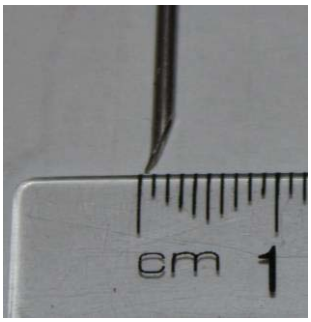


Figure 10 - Barbed 14 gauge intravenous catheter stylet against a ruler.
Shows approximate width of needle and barb is 2.5 mm.

The barbed 14 gauge catheter stylet was chosen for the *in vivo* studies because the diameter of injury created would be approximately 40% of the average cross-section of the pelvic limb superficial digital flexor tendon at this site (insert ref to result disc 3.2.3)

and was therefore more consistent with the size of moderate clinical tendon injury in horses (Marr et al., 1993a). As the long term aim would be to use the model to study acute tendon injury in humans and horses, and to create the injury in horses, a comparable lesion size was an important aspect of model development.

3.2.2.2 COMPARISON OF MODELS TESTED

The tearing by the barb in the model more closely reproduced what is seen in clinical damage in comparison with just the cutting action of the needle bevel. This is similar to the model described by Schramme (2010) in which an arthroscopic burr was used to tear the tendon fibres in the core of the equine superficial digital flexor tendon. One advantage of the model developed in the current study over that of the Schramme model (2010) is that the damaged fibres and haemorrhage as well as any fluid exudates remain within the lesion as they would in the clinical situation. In the model described by Schramme (2010) the tenoscopic technique flushes the debrided tendon and any fluids from the cavity. The method of injury creation in the model described in the current study did not extract the damaged fibres and debris.

Tendinopathy is due to chronic tearing of tendon fibres and predominantly involves intrinsic repair so many surgical models may be less than ideal (Little and Smith, 2007, De Grandis et al., 2012) as they use excisional and transectional methods. These models involve sharp injury and both extrinsic and intrinsic repair mechanisms (Little and Smith, 2007, Iwuagwu and McGrouther, 1998, Bruns et al., 2000, De Grandis et al., 2012). Schramme addresses this by entering the tendon via the palmar annular ligament and the vinculum and suturing the palmar annular ligament at the end of the surgery (Schramme et al., 2010). In the model developed in the current study the stab incision into the core allowed the formation of intrinsic damage. Sealing of the stab incision with

the cyanoacrylate prevented extrinsic factors influencing lesion development and healing. The persistent adherence of the glue to the tendon throughout the anaesthesia, collection and fixation would suggest it prevented any extrinsic effects on the injury model. Whether it would adhere when under load and throughout healing was not tested in this model. Cyanoacrylate glue is used to augment tendon repair (Oztuna et al., 2005) and results in superior mechanical strength compared with suturing alone. This suggests that cyanoacrylate glue will remain adherent under load. This procedure makes the current model appropriate for investigating acute tendinopathy.

The mean age of the sheep used in the current study was 7.5 months ranging from 6-9 months. These sheep were chosen for the study as they were immature and minimally exercised thus unlikely to have underlying chronic degeneration due to age or exercise-related micro damage (PattersonKane et al., 1997a, PattersonKane et al., 1997b). Acute damage in response to the injury was therefore more easily detected and characterised.

3.2.2.3 ANAESTHETIC PROTOCOL AND POTENTIAL EFFECTS ON GAP JUNCTIONS

The anaesthesia protocol was a standard teaching protocol for practical aspects of surgery and was tolerated well by most of the sheep. The induction drugs, injectable ketamine and diazepam, do not alter gap junction permeability (Mantz et al., 1993). However, volatile anaesthetics like isoflurane, are recognised as generalised gap junction channel blockers causing gap junction uncoupling and reduction of intercellular communication *in vitro* (Mantz et al., 1993, Wentlandt et al., 2006). The amount and duration of uncoupling appears to be dose-dependent and compound-dependent with halothane having a more profound effect than isoflurane (Mantz et al., 1993). There is also lack of agreement as to whether or not a significant uncoupling is achieved at clinical dose rates (Burt and Spray, 1989, Wentlandt et al., 2005, Mantz et

al., 1993). The aim in the current study was to measure amounts of gap junction rather than gap junction communication. There has been no study reported that shows gap junction levels are altered by isoflurane, although anaesthetic use may have an effect in lesion expansion. Lesions in the eye, spinal cord and tendon enlarge following the initial insult (Bosch et al., 2010a, O'Carroll et al., 2008, Cusato et al., 2003). Gap junctions are thought to be partly responsible for this effect by allow death signals to pass from dying cells to adjacent cells and gap junction modulation has been shown to reduce lesion spread in the central nervous system (O'Carroll et al., 2008). Blocking gap junction communication by isoflurane may therefore have reduced the lesion expansion in these sheep. If this was the case any reduction of lesion expansion as a result of treatment with the antisense oligodeoxynucleotides could have been less apparent. As all the sheep underwent the same protocol, the GA controls should have corrected for the other effects of the anaesthesia.

3.2.2.4 OPTIMISATION OF LESION FORMATION

The mean time of exposure to treatment (time from lesion creation to collection) across all the sheep in the 2 hour group was 114 ± 18 minutes. All except two tendons were within two standard deviations of the mean. The mean time of exposure to treatment across all the sheep in the 4 hour group was 227 ± 14 minutes. All except three tendons were within two standard deviations of the mean. Although there was a relatively wide range of times of exposure to treatment this was not related to connexin levels (r=0.13) (Figure 11 and Appendix 2).

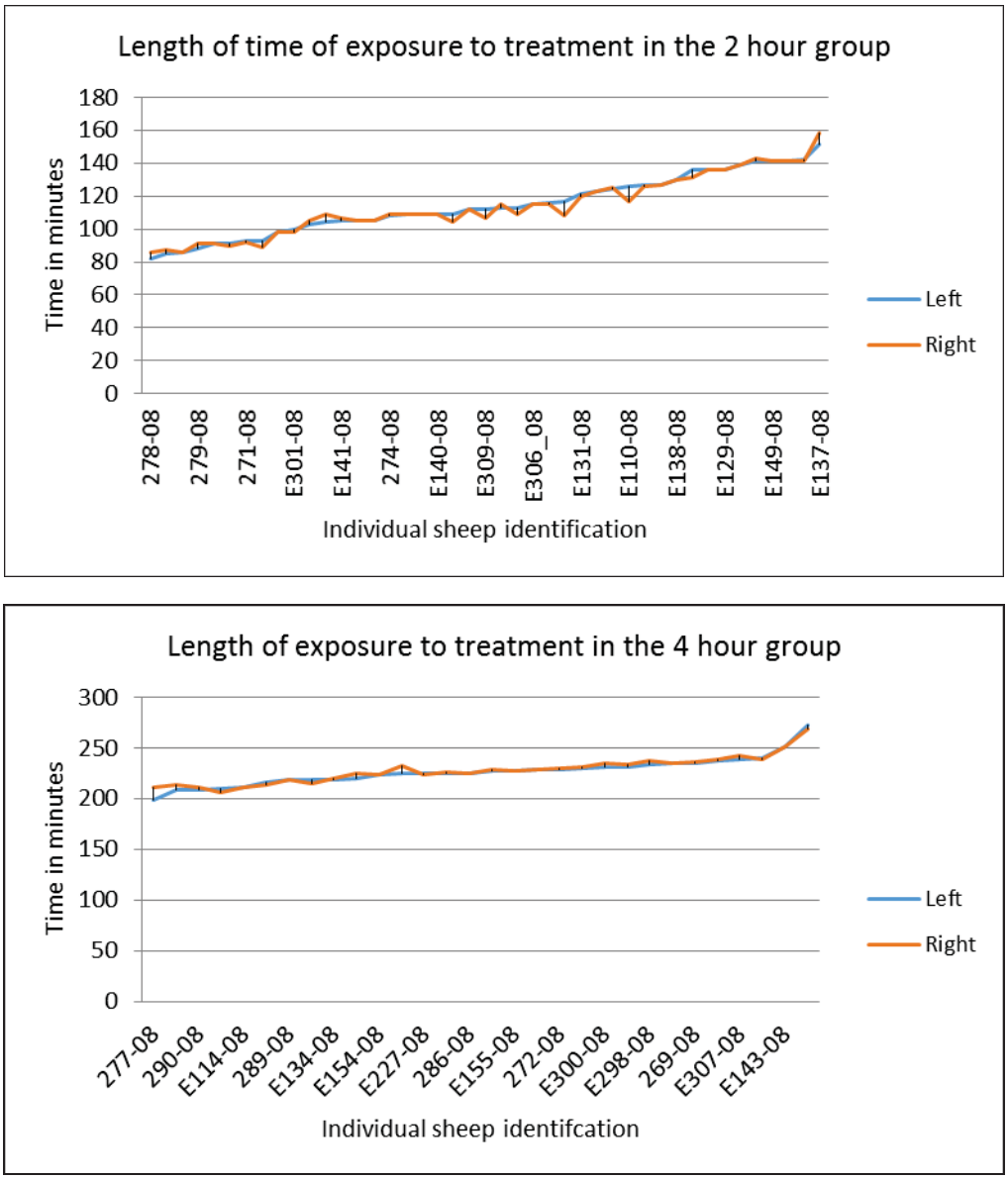


Figure 11 - Graphs of the times of exposure for individual tendons to the treatments in each of the two time groups.
Time of exposure is from application of treatment to collection of tendon following euthansia. Differences between left and right limbs within the same individual are due to the time taken to collect the tendons.
The mean in the 2 hour group was 114 ± 18 minutes. The mean in the 4 hour group was 227 ± 14 minutes.

3.2.2.5 CHOICE OF INJURY TECHNIQUE

In the first study using 16 tendons the lesions created by 10 thrusts of the barbed stylet were not easily visible by eye and histologically only 12/16 of the tendons had changes indicating inflammation (section 3.1.6). In the comparative study of 10 and 15 thrusts (section 3.1.6) the 15 thrusts of the barbed stylet resulted in gross haemorrhage and fibre disruption in 17/25 tendons and all tendons were positive for histological signs of

inflammation (Table 3; Figure 12). In comparison when 10 thrusts were used few tendons had grossly visible lesions and only 6/25 had histological evidence of inflammation. The 15 thrusts injury model was therefore used for the remainder of the study. The pilot study also identified subcutaneous haemorrhage as a potential complication of the technique and in the main study, skin and subcutaneous haemorrhage occurred in 16/146 (11%) of the sheep legs during lesion formation but only impeded visualisation of the tendon in 2/146 (0.1%) of the wounds (Appendix 2).

Tendon tissue is robust consistent with its biomechanically demanding role particularly in energy-storing tendons that operate close to their mechanical failure limits (Stephens et al., 1989). It is unsurprising that it took more than ten thrusts to create gross and histologically visible damage. Clinical tendon injury is visible grossly and therefore a comparable model needed to produce grossly visible lesions. The grossly visible lesion, in the divided tendon, was useful for correct orientation of the lesion within the cryo-moulds to optimise section creation.

Creation of the model required a reproducible injury that would not be so vast as to be painful and mechanically unsound in conscious animals. The increase in the number of thrusts allowed for more reproducible gross and histological signs with no evidence of loss of tendon integrity. The sheep were anaesthetized and therefore non-weight-bearing so the degree of discomfort and any reduction in the mechanical properties of the tendon could not be measured. From the gross comparison of the appearance with clinical lesions in other species it is likely that the sheep, when conscious and standing, would have felt a degree of discomfort that could have been easily controlled by routine analgesia.

3.2.3 MEASUREMENTS OF SHEEP SUPERFICIAL DIGITAL FLEXOR TENDONS

The mean diameter of the eight tendons measured was 8.88 mm (standard deviation 0.64 mm) and therefore the cross-sectional area at the site of injury was 61.9 mm^2 (given by πr^2 with r = 4.44). The stylet therefore should have made a lesion of approximately 30% of the cross-sectional area consistent with a mild to moderate clinical tendon injury.

Table 3 - Effects of number of thrusts on gross and histological signs of injury

	Gross positive	Gross negative	Histology positive	Histology negative	Total
Initial 10	2	14	12	4	16
Comparative 10	4	21	6	19	25
Comparative 15	17	8	25	0	25

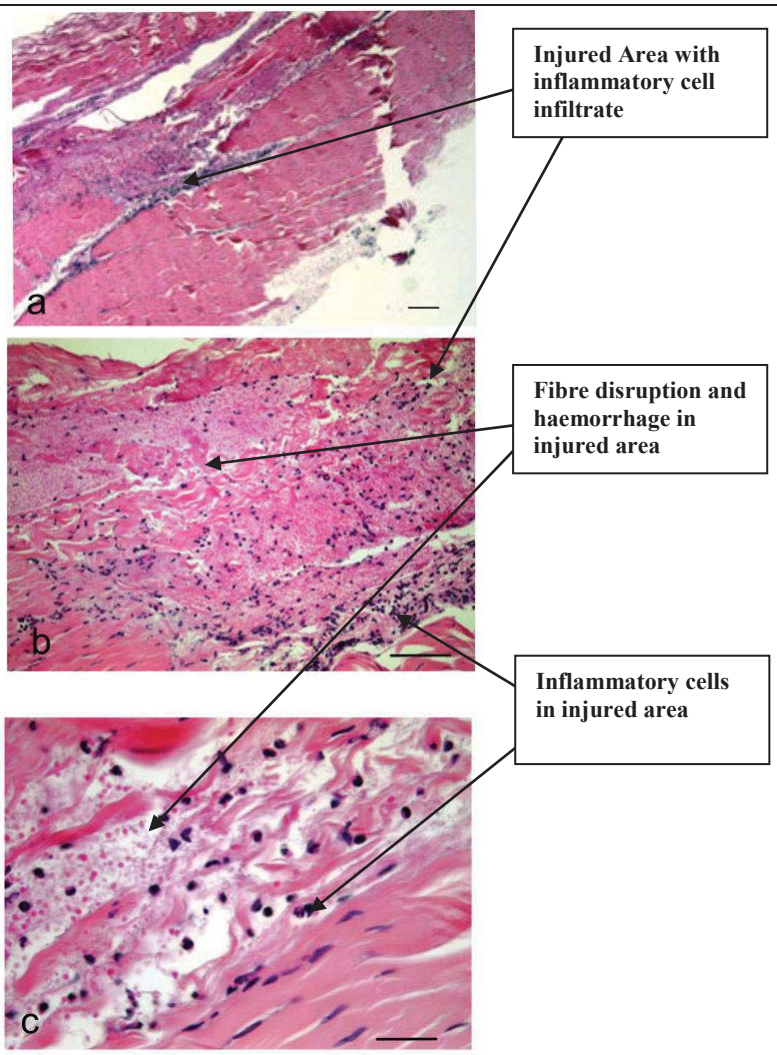


Figure 12 - Haematoxylin and Eosin stained sections of injured area of tendon.
Shows haemorrhage, inflammatory cell infiltrate, loss of tissue architecture and fibre disruption in a tendon from the injury (I) group.
Bright field microscopy of injured sheep tendon at different magnifications. For images a and b image bar = 200 µm for image c image bar = 50 µm.

3.2.4 MODEL TECHNIQUE *IN VIVO* - POST MORTEM FINDINGS

At post-mortem examination, red discolouration at the site of the stab incision was grossly visible in all tendons. When the tendons were divided longitudinally, red discolouration and linear disruption of the fibres in the core were also apparent. The discolouration and damaged fibres extended 1 cm proximally from the stab incision site with a width of approximately 1 mm. The cyanoacrylate glue was apparent on the surface of the tendon directly over the stab incision and the stab incision into the fibres could not be manually parted. When sectioning through this area grittiness could be felt.

Tendon degeneration , a component of clinical tendon injury, (Cetti et al., 2003) would normally not be apparent for at least four hours after the insult and was not detected in the tendons from this sheep model. The development of subcutaneous haemorrhage was an unforeseen, albeit mild, complication of the technique. The bleeding was from subcutaneous vessels and it was important to remove fluids from the surface of the tendon surface prior to sealing of the stab incision so the haemorrhage made this a little more challenging. Sealing of the tendon prevented any extrinsic effect of blood on the tendon injury model. Application of a tourniquet during model creation would easily address the problem of subcutaneous haemorrhage. Tourniquets are often applied during distal limb surgery due to subcutaneous haemorrhage and in the treatment of distal limb wounds in standing patients and are well tolerated.

The duration of the injury or exposure to a treatment was based on time of injury and time of euthanasia of the sheep. Injury creation time was affected by onset of anaesthesia for the teaching surgery and some students were slower than others at anaesthetising the sheep. Euthanasia and therefore collection times were also influenced by the student teaching environment. It was important to remove tendons and place them into paraformaldehyde quickly after euthanasia therefore tendons were collected immediately after euthanasia.

The time points of 2 and 4 hours were selected for this study as they would be consistent with the time when veterinary intervention for an acute injury could be sought and a point when an intervention may be applied. This period was considered important as Connexin43 has a half-life of 1.5- 2 hours (Gaietta et al., 2002) and, based on other studies, expression was expected to alter rapidly in response to injury. A single application of antisense oligodeoxynucleotides in pluronic gel has been shown to significantly reduce Connexin43 expression within the spinal cord by 4-8 hours and for

up to 48h (Cronin et al., 2006). The time scale was slightly earlier than most other experiments focussed on gap junctions. The main focus of the current study was to evaluate connexin expression in tendon tissue affected by injury and interventions in the acute period.

3.2.4.1 HISTOLOGY CUTTING STUDY

Normal tendon showed marked artifactual fibre separation when processed using formalin fixation and paraffin embedding. There was less artifactual separation and maintenance of normal tendon architecture by cryo-sectioning.

3.2.4.2 HISTOLOGY

Uninjured tendons were characterised by the presence of regular wavy (crimped) collagen fibres with rows of oval to spindle-shaped tendon fibroblast nuclei along the fibres (Figure 13).

Endothelial cells could be identified lining blood vessels. There was no inflammatory cell infiltrate. Occasional artifactual separation of the tendon fibres without associated cellular infiltration was apparent. (Groups A [abattoir controls] and GA [general anaesthetic]).

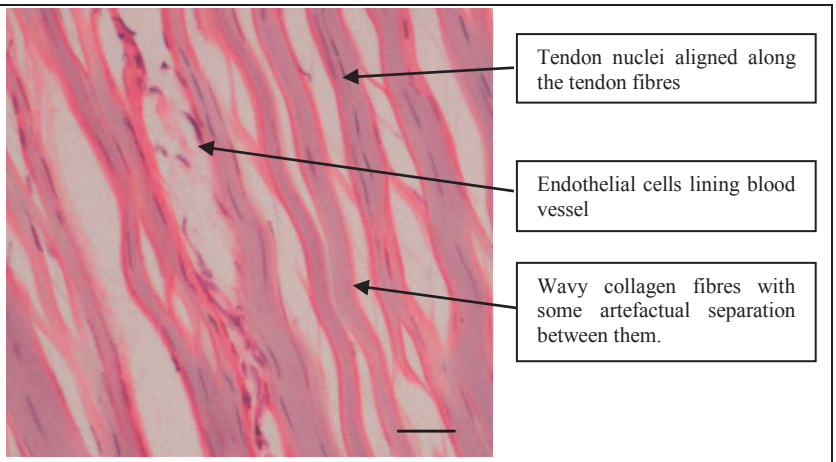


Figure 13 - Haematoxylin and Eosin staining of normal ovine superficial digital flexor tendon.
Bright field microscopy 200x magnification (bar = 50 μm)

All sections of the injured tendon had disruption of collagen fibrils with both fibre separation and tearing of the fibres. For a section to be considered to be injured and inflamed the site of fibre separation had to be associated with polymorphonuclear cell (neutrophil) infiltration (Figure 12).

The cyanoacrylate glue used to seal the tendon was apparent in 13% (19/146) of the sections and appeared as a highly refractive area on the surface of the tendon. The cyanoacrylate glue was applied only at the edge of the tendon at the site of the stab incision and this would not necessarily have been on the section for histology in all cases. It may have been better to section through this site routinely to confirm persistence of the glue after processing although the glue was clearly visible on the edge of the tendons at the time of collection and could be felt when sectioning through the tendons prior to embedding. The glue prevented parting of the fibres at the site of the stab incision and no subcutaneous tissue had become adherent to the external surface of the glue. The purpose of the cyanoacrylate glue was to prevent extrinsic involvement in the tendon response to injury. The continued presence and adherence of the glue at 2 and 4 hours suggested that the aim to prevent extrinsic tissue involvement was achieved. In the tendons that had pluronic gel stained with India ink instilled there was no evidence of blue staining outside the tendons at collection again suggesting a good seal had been created.

Studies of longer duration would be required to determine if the glue persists and retains its adherence over a prolonged period in tendon. Cyanoacrylate glues have been used to augment suturing of tendon lacerations in sheep tendon explants and were found to be superior compared to suturing alone (Oztuna et al., 2005). Due to the longitudinal orientation of the stab incision in the tendon the closure in the current study would not be under large tensile stress and therefore the glue should be potentially strong enough

to retain adhesion for longer duration studies in load-bearing tendons. The tendon should begin to seal itself from the external environment within 4 to 6 hours, again reducing the involvement of extrinsic aspects of repair.

No India ink staining was apparent when the tendons were split during collection or in the histological sections from the stained pluronic gel treated tendons. This is likely to be due to removal of the ink during the decolourisation and dehydration steps of the staining process. Although this prevented a visual evaluation of the extent of gel penetration within the lesion the 0.2 mL of gel instilled was 40% of the maximum of the predicted volume the lesion could be (based on a volume of $\pi r^2 L$ where r = the width of the stylet and barb and L the 1 cm the stylet was thrust back and forth). This maximum assumes complete disruption of the tissue in that volume and removal of the torn fibres from the lesion, which would not be the case in this model. It is therefore reasonable to expect that the majority of the injured tendon would have been exposed to the gel. Also the pressure effect of instilling that volume of gel would have been significant as a cavity, as such, was not created because the fibres were torn but not removed.

Characterisation of acute tendon injury is based on histopathological evidence of fibre disruption with associated inflammation and is largely performed on H&E sections. Masson's Trichrome clarified that the tissue was collagen but tendon is not difficult to tell apart from other tissues and this more complex special stain did not add any information over that provided by H&E staining. It was interesting that the mature collagen in the areas of uninjured tendon remained red, as the differentiating stain could not penetrate into the dense tissue.

3.2.4.3 LIMITATIONS OF THE MODEL

A limitation of the model is that only one aspect of clinical tendinopathy could be tested. Acute tendinopathy usually occurs during normal activity following weakening of the tendon, thought to be caused by accumulated micro-damage due to inadequate repair of subclinical injury (Jarvinen et al., 2001). The occurrence of subclinical damage is evidenced by post-mortem lesions being identified in the superficial digital flexor tendon of horses (Webbon, 1977) and the human Achilles tendon in individuals with no history of clinical signs (Leppilahti et al., 1996).

Sheep have been trained to work on high speed treadmills (Pedley, 2010) thus a reproducible regimen to create chronic tendon changes could be developed in sheep using this equipment. The subsequent application of the acute model described, to the chronically degenerate tendons in the exercised sheep, would then provide a more accurate clinical model for human and horse tendinopathy in energy-storing tendons.

3.2.4.4 USE OF PLURONIC GEL TO DELIVER OLIGODEOXYNUCLEOTIDES

Pluronic gel is a weak surfactant and at 24% w/v sets as a hydroscopic gel at physiological temperatures and allows the reservoir of antisense oligodeoxynucleotides to be continuously released as gel dissolves (Law et al., 2006). Pluronic gel was tested as a delivery vehicle to provide sustained delivery of the antisense oligodeoxynucleotides and overcome the breakdown of the antisense oligodeoxynucleotides by serum. Tendons often produce a marked inflammatory response to relatively small volumes of injected foreign material. It was therefore also important to determine if pluronic gel was inert and had any effect on tendon tissue when injected. The pluronic gel was easy to instil into the tract left by the catheter stylet and did not harden in the syringes during administration. No gel exuded from the tendon

after withdrawal of the stylet and cyanoacrylate glue closure of the tendon was not impeded in the gel-injected tendons.

Of the 8 tendons (four collected at each of 2 and 4 hours of anaesthesia) with the 15 thrusts only model, 1 sample was lost, 1/3 showed positive inflammation on histological examination at 2 hours and 2/4 positive for inflammation on histological examination at 4 hours. Of the 8 tendons with the model including gel, histological indicators of inflammation were present in all four tendons collected at 2 hours and in 3 out of 4 collected at 4 hours (Table 4, Appendix 2). Instillation of the pluronic gel with or without oligodeoxynucleotides may have caused an inflammatory response in the sheep tendons.

Table 4 - Table of gross and histological signs of inflammation in pluronic gel study

	Gross +ve	Gross -ve	Histo +ve	Histo -ve	Totals
Model 2hrs	1	2	1*	2	3
Model 4 hrs	0	4	2	2	4
Model + Pluronic gel 2 hrs	1	3	4	0	4
Model + Pluronic gel 4 hrs	0	4	3	1	4

* 1 sample lost

The effect of pluronic gel was also determined by assessing its effect on Connexin43 expression. When all groups (GA, M, P, S and AS) were included in the statistical model, pluronic gel alone did not have a statistically significant effect on Connexin43 gap junction plaque count or area. Nonetheless, all the gel containing groups (P,S,AS) had a four to five fold higher gap junction plaque count in the bystander area compared with the bystander area in group M (injury only). This was statistically significant in the sense-treated group only (Table 5).

3.2.4.5 POSSIBLE CAUSES OF INFLAMMATION RELATED TO PLURONIC GEL

The inflammatory response to the pluronic gel could be a result of pressure generated within the tendon during instillation of the gel, the volume instilled or due to the composition of the gel itself. Healthy tendons are dense fibrous tissues and firm pressure is needed when injections are made into them, whilst injured areas of tendon are easy to inject (Dyson, 2004). Pressure necrosis is a potential cause of the inflammation and could have caused increased Connexin43 gap junction plaque counts. The gel was injected into the tract created by the stylet as the stylet was pulled back to avoid this; no resistance to injection was detected during instillation of the gel. Histologically, necrosis due to pressure would not have been visible due to the short time scale of the study so this cannot be confirmed. As the pluronic gel solidifies at body temperatures (Becker, 1999) it may have expanded resulting in locally increased pressure within the lesion. The 0.2 mL of pluronic gel instilled into the lesion was approximately 40% of the lesion size so the relative amount may also have been a contributory factor. Pluronic gel is an inert polymer that has been shown to be non-inflammatory in HET-CAM (INVITTOX PROTOCOL Number 47 (1990), ISSN #0960-2194) a pharmacy test used to identify inflammation in drug carriers (Colin Green personal communication) and has also resulted in an anti-inflammatory effect in spinal cord segments *ex vivo* (Zhang et al., 2010). The composition of the pluronic gel itself was therefore unlikely to be the cause of the inflammation. Based on these findings injection of pluronic gel, or any vehicle, would need further investigation before it could be recommended as a delivery technique to tendons.

Table 5 - Connexin counts and area in response to treatment group

Test	*Parameter*	*Overall significance*	*LsMean (log_{10})*		*Actual Number*	*Standard Error*
Effect of anaesthesia = Group A compared with GA	**GJ Count**	**P <0.0103**	A GA 2hrs GA 4hrs	3.308_a 2.374_b $2.758_{a,b}$	2031.42 236.54 572.80	0.252 0.282 0.282
	Total GJ Area	**P <0.0074**	A GA 2hrs GA 4hrs	-3.135_a -4.297_b -3.993_b	0.0007 0.00005 0.0001	0.252 0.282 0.282
Effect of injuring the tendon = Group M comparing I and B	**GJ Count**	**Region P < 0.0007** **Region x time P<0.0352**	B I B 2hrs B 4hrs I 2hrs I 4hrs	2.374_a 3.309_b 2.084_a $2.663_{a,b}$ 3.574_b $3.042_{a,b}$	236.59 1909.85 121.34 460.26 3749.73 1101.54	0.319 0.266 0.432 0.468 0.365 0.386
	Total GJ Area	**Region P < 0.0077**	B I	-4.129 -3.315	0.00007 0.00048	0.249 0.150
Effect of experimental conditions on bystander region = Groups M,P,S,As and GA region B	**GJ Count**	**TX P< 0.0455**	M P S As GA	2.458_a $3.100_{a,b}$ 3.180_b $3.095_{a,b}$ 2.566_a	287.08 1258.93 1513.16 1244.51 368.13	0.259 0.277 0.224 0.230 0.148
	Total GJ Area	**Not significant**	Treatment Time	P<0.964 P<0.114		
Effect of treatment = Comparing each of the groups M,P,S,As and GA and the regions I and B within the groups with each other.	**GJ Count**	**Tx P < 0.1128** **Region P < 0.0001** **TX*Region P<0.0530**	M P S As B I M x I P x I S x I As x I	2.827_a $3.831_{a,b}$ $3.310_{a,b}$ 3.752_b 2.895_a 3.716_b 3.337_a 3.534_a 3.591_a 4.401_b	671.43 6776.42 2043.15 5649.40 785.24 5199.96 2171.20 3419.79 3897.62 25159.38	0.263 0.332 0.250 0.261 0.164 0.139 0.265 0.332 0.250 0.261
	Total GJ Area	**Region P < 0.0001**	B I	4.097_a -3.296_b	0.00008 0.00051	0.174 0.139

Double lines delineate separate tests. Within individual tests groups with different subscripts are significantly different from each other.

GJ = Gap Junction

M = Model (15 thrusts) P = Model (15 thrusts) and pluronic gel

S = Model (15 thrusts) and pluronic gel with 50 μM sense oligodeoxynucleotide

AS = Model (15 thrusts) and pluronic gel with 50 μM antisense oligodeoxynucleotide

GA = Control anaesthetised sheep A = Control Abattoir sheep

B = Bystander Region I = Injured Region

3.2.5 GAP JUNCTION LEVELS MEASURED USING QUANTITATIVE CONFOCAL SCANNING LASER MICROSCOPY

The large sheep to sheep variation in gap junction counts in this short time course study resulted in a wide range of values within each group and may have overshadowed smaller variations between groups. In this study, the age of the sheep increased from 6-9 months as the trial progressed. Fetal, immature and young adult horses have been shown to have higher gap junction plaque counts than mature adults (Young et al., 2009, Stanley et al., 2007). Thus age-related changes in Connexin43 gap junction levels may have contributed to the variation. Stress may alter gap junction expression (Oviedo-Orta et al., 2002) and could have contributed to the variation between the sheep. The sheep in all the groups, apart from the abattoir control group, were from the same flock and were subject to the same management therefore exercise and stressors were likely to have been similar, apart from individual variation. Treatments were carried out in different sheep and individual variation would significantly affect the range of gap junction levels in the treated groups (Appendix 11).

3.2.5.1 REGIONS EXAMINED ON CONFOCAL SCANNING LASER MICROSCOPE

Using the confocal scanning laser microscope the region of injury was identified by the presence of tendon fibre disruption, rounded reactive tendon fibroblast nuclei, and the segmented inflammatory cell nuclei of neutrophils (Figure 14a). The bystander region was identified by the presence of spindle-shaped tendon fibroblast nuclei on regular undamaged tendon fibres. Neither inflammatory cellular infiltrate nor tearing of fibres was present although occasional artefactual fibre separation was seen (Figure 14b). The bystander region was defined as being greater than or equal to 400 μm from the injury site in the same section. Gap junctions are associated with lesion spread in a number of

tissues (Asklund et al., 2003, Cusato et al., 2003, Rodriguez-Sinovas et al., 2007) and the area of tendon injury expands following the initial insult (Bosch et al., 2010a). Gap junction plaque count and total gap junction area were measured in both the injury and bystander areas in all treatment groups. These two measurements were made because gap junction plaques fragment into smaller gap junction plaques during down-regulation of expression before decreasing in number (Cronin et al., 2006). The total number of gap junction plaques may therefore lag behind total gap junction area in the early period of down-regulation after injury or down-regulation.

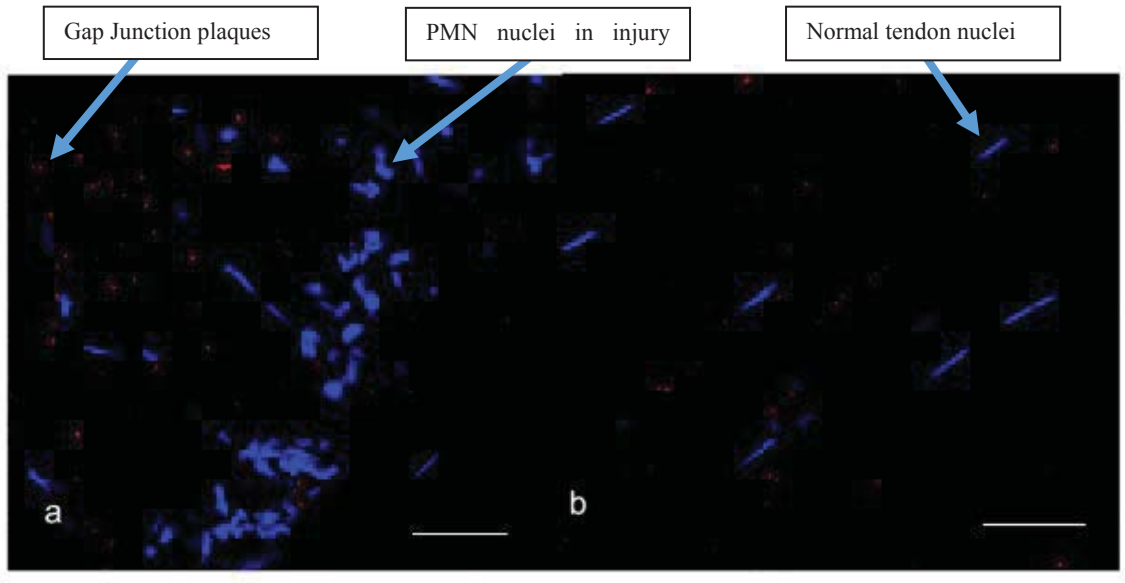


Figure 14 - Connexin expression in sheep tendon
Confocal microscopy using x63 magnification (bar = 25 µm)
Injured area of sheep superficial digital flexor tendon with DAPI stained nuclei (blue) and Immunofluorescent staining of Connexin43 gap junctions using rabbit polyclonal Connexin43 antibody (red).
Bystander area of sheep superficial digital flexor tendon stained as for a)

3.2.5.2 CONNEXIN43 PLAQUE LEVELS IN THE CONTROL OVINE TENDONS

The Connexin43 gap junction plaque count and area were significantly higher in tendons from the abattoir sheep compared with the GA controls overall ($P<0.01$; $P<0.007$, respectively). There was a nearly 10-fold difference in gap junction plaque count and area between the abattoir and GA control groups at 2 h. There was no significant difference by 4 h. There was a trend of increasing gap junction plaque count

in the GA control tendons from 2 to 4 h but this was not statistically significant (Table 5).

The higher Connexin43 gap junction plaque count in the tendons from abattoir-killed sheep compared with anaesthetised controls was an unexpected finding. Whilst of roughly the same age, the abattoir sheep were from a different flock and may have been exposed to different management and environmental factors that potentially could have altered gap junction expression in tendon. Cytosolic stress can result in increased gap junction expression by reducing degradation of biotinylated gap junctions and by reducing lysosomal degradation of gap junctions and this increases the gap junctions recycled to the cell surface (VanSlyke and Musil, 2005). Systemic stress is transmitted via humoral factors in the circulatory system such as acute phase proteins, cytokines, cortisol and glucagon. Functional gap junction communication is required for the uptake and spread of such factors into the tissues (Lin et al., 1998, Ramachandran et al., 2007) therefore, stress due to travelling and containment of the abattoir sheep may have contributed to increased tendon gap junction plaque levels.

The inhalational anaesthetic, isoflurane, is a non-specific gap junction blocker (Evans and Boitano, 2001) and may have modulated gap junction communication in the GA sheep (Waggett et al., 2006). This fact necessitated the use of the GA control tendons as the control group for this study. The GA sheep were housed in a loose-box prior to surgery resulting in a period of relative immobilisation that may have reduced gap junction expression (Banes et al., 1997). The environment would also have been warmer than the sheep would normally be used to and heat stress down-regulates Connexin43 expression by tendon fibroblasts from horses (Burrows S, 2008). However, heat stress of cultured tenocytes is reported to result in higher pro-inflammatory cytokines and (Hosaka et al., 2006) so could have been expected to increase connexin expression.

Abdominal surgery results in alterations in levels of pro and anti-inflammatory cytokines IL-1, 1L-6, 1L-10 and this response can be influenced by the anaesthetic used, with isoflurane having less anti-inflammatory effect than propofol (Gilliland et al., 1997). In conclusion, the complex interaction of all the stages of anaesthesia and surgery on cytokine release and signalling and consequently gap junction expression may explain why the GA controls had lower numbers of gap junctions than the abattoir sheep. This response requires further investigation and highlights the importance of selecting appropriate controls.

3.2.5.3 CONNEXIN43 PLAQUE LEVELS IN UN-INJURED AND INJURED OVINE TENDONS

The Connexin43 gap junction plaque count and area were significantly higher ($P< 0.0007$; $P< 0.008$) in the injured region of the tendons compared with the bystander region. Gap junction communication is involved in inflammation (Oviedo-Orta et al., 2002), differentiation (Kumar and Gilula, 1996), proliferation (Lucke et al., 1999), cell migration (Oviedo-Orta et al., 2002) and tissue contraction (Ehrlich and Rittenberg, 2000), all important events for successful wound healing.

Wounding causes haemorrhage that involves blood vessel constriction and the formation of a fibrin clot, this releases cytokines that generate an inflammatory response. Wounding also alters gap junction expression via the release of pro-inflammatory cytokines and chemokines (Coutinho et al., 2003).

The increase in Connexin43 gap junction levels in the injured region of the tendon in the injury group, compared to the controls and bystander regions, was consistent with the recruitment of inflammatory cells to an injured area. Inflammatory cells have high numbers of gap junction plaques (Oviedo-Orta et al., 2002) and areas of injury were identified based on the presence of damaged fibres and polymorphonuclear cells. Cell

counts were not measured as overlap of the nuclei in the injury regions made accurate identification of the cell numbers and type from nuclear morphology, unrealistic. Most neutrophil-derived markers are not cell-specific markers although a marker for neutrophils in broncholavage fluids appears to have good specificity (Metso et al., 2002). It may have been useful to perform immunohistochemistry for CD18 on neutrophils (Marr et al., 1997) and determine co-localisation of gap junctions and neutrophils. Neutrophils are easy to recognise and with the significant overlap of the cells in the injury area immunohistochemistry was unlikely to have provided additional information. At the times assessed, 2-4 hours post-injury, the Connexin43 gap junction levels in the bystander region were not significantly different from the GA control (Table 5). This apparent lack of a bystander effect in the injured tendons was interesting as tendon injuries are known to expand for hours to several days, after the initial insult (Reef et al., 1990). There was a significant, statistical interaction between time and region (P<0.0352). An increase in the Connexin43 gap junction plaque count was observed in the bystander regions of injured tendons with a concurrent decrease in the inflamed regions of injured tendons from 2 to 4 h; no such changes were noted in total gap junction plaque areas (Table 5). Effects within the bystander regions follow the initial insult within 1-72 hours depending on the tissue (Cusato et al., 2003, Asklund et al., 2003). In an *in vitro*, traumatic brain injury model, connexins accentuated cell death and connexin blockade reduced cell death within 24 hours (Frantseva et al., 2002). Effects on the bystander region in the current model may have become more apparent with an increased time course. This is supported by trends of a decrease in Connexin43 gap junction plaques in the injured region, and an increase in the adjacent bystander region, from 2-4 hours in the injury group. The gap junction plaque levels decrease in

areas where cells are dead or dying and increase in the injury zone spreading out from the initial site (Coutinho et al., 2005, Cronin et al., 2006).

Non-specific gap junction blockers, such as octanol and carbenoxolone, have altered the extension and appearance of necrotic lesions and apoptosis by limiting the effect on cells in the bystander region in spinal cord and cornea (Contreras et al., 2004, Cusato et al., 2003). Volatile anaesthetics have also been shown to uncouple gap junctions in hippocampal slices (Wentlandt et al., 2006) and the isoflurane anaesthetic agent could have blocked gap junction intercellular communication and affected lesion expansion in the current study. Other factors including the short duration of the study, overall increased cellularity in the immature tendons and the variation between sheep may also have precluded detection of a subtle bystander effect.

3.2.5.4 ANTISENSE OLIGODEOXYNUCLEOTIDE MODULATION OF CONNEXIN EXPRESSION

There was no significant overall effect of the treatment on Connexin43 gap junction plaque count or total Connexin43 gap junction plaque area in the bystander region (Table 5). Plaque counts in the injured region of the antisense oligodeoxynucleotides treated tendons were significantly higher compared with group M (P=0.0066), group P (P=0.0526) and Group S (P=0.0339) (Table 5, Figure 15). The Connexin43 gap junction plaque total area was not significantly different. The finding may be due to fragmented gap junction plaques resulting in a larger number of small plaques but no difference in total Connexin43 gap junction plaque areas, compared with the other treatment groups.

This lack of reduction in Connexin43 gap junction plaque number was unexpected and contradicts the published data on antisense oligodeoxynucleotides treatment (Becker et al., 1999, Qiu et al., 2003, Mori et al., 2006, Cronin et al., 2006). The previous studies using Connexin43 antisense oligodeoxynucleotides were performed in rodents and on

porcine and human cells. Whilst the sequence of the murine antisense oligodeoxynucleotides is the same for sheep and horses it is possible that the tertiary folding structure is different between species and coded for at a different site, and that this influenced the effectiveness of the antisense oligodeoxynucleotides.

Gap junction protein knockdown begins with a reduction in size of gap junction plaques followed by a reduction in number of plaques as cytosolic stores are exhausted (Cronin et al., 2006). This and the Connexin43 half-life of 1.5-2 hours may explain why the first time points at which gap junction counts are significantly reduced in published data are within 4-24 hours (Cronin et al., 2006, Law et al., 2002, Mori et al., 2006, Brandner et al., 2004) and are dependent on tissue type (Becker et al., 1999). The time points of this study (2 – 4 hours) may therefore represent an early effect of antisense oligodeoxynucleotide treatment on connexin gap junction plaque morphology within the lesion site itself that has not yet been reported. Reverse transcriptase polymerase chain reaction to confirm down-regulation of expression and following some animals for a longer time period would enable further investigation of this.

Following wounding whilst gap junction cell-cell coupling is reduced, surface hemi-channel levels are increased in both cardiac muscle and astrocytes (Smyth et al., 2010). These are new data reported following the completion of data collection and processing in the current study. Differentiation between gap junctions and hemi channels was not attempted. Intuitively the hemi channels are likely to be smaller than the gap junctions and this could be another explanation for the increased number of smaller areas of staining as hemi channels move to the membrane before expression is down-regulated.

In this model the antisense oligodeoxynucleotides were delivered in pluronic gel to act as a slow release reservoir to overcome the effect of serum breakdown. The rate of

dissolution of the pluronic gel is related to the concentration of the pluronic in the gel. The rate and duration of exposure of the tissue to the antisense oligodeoxynucleotides and the antisense oligodeoxynucleotides to serum breakdown is in turn related to the dissolution of the pluronic gel. The knockdown of Connexin43 expression is achieved by sustained exposure of the tissue to the antisense oligodeoxynucleotides. The 24% gel has been a successful delivery vehicle for antisense oligodeoxynucleotides in other tissues (Cronin et al., 2006) It is possible that this percentage was not ideal in tendon tissue due to high serum concentration in response to injury. A higher concentration gel may be required for effective delivery to tendon tissue.

A further reason that the gap junction plaque count remained high in the tendons treated with antisense oligodeoxynucleotides may have been that the Connexin43 antisense oligodeoxynucleotides may have promoted increased survival of tendon and inflammatory cells in the injured area due to a reduction in bystander effect. Tendon fibroblasts and inflammatory cells were the predominant cell types present in the sections and both express Connexin43 with inflammatory cells having prominent Connexin43 levels (Zahler et al., 2003). The overlap of the cells made individual cell identification and counts impractical and no cell-specific labelling was performed to differentiate between the cell types.

In summary, the factors that could have potentially contributed to the apparent failure of antisense oligodeoxynucleotides to decrease Connexin43 gap junction plaque levels include; a) the potential inflammatory effect of injecting a volume of material (pluronic gel) into the tendon b) the antisense oligodeoxynucleotide dose and product used, c) increased survival of cells, and d) the presence of serum in the lesion. Further studies would be required to address these questions.

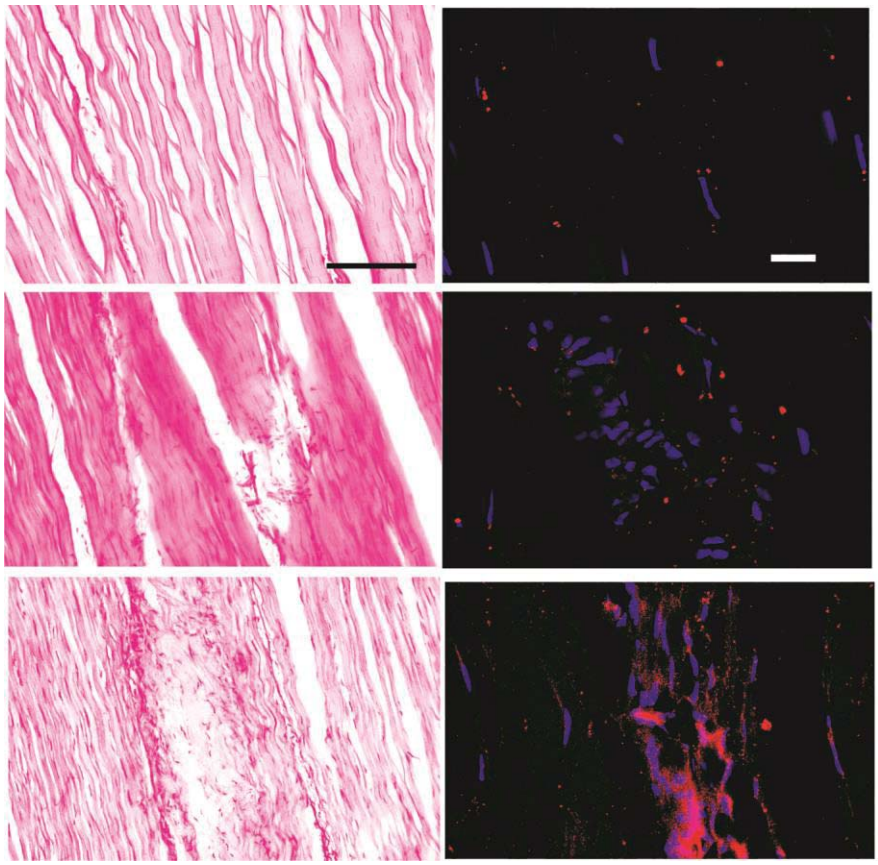


Figure 15 - Haematoxylin and Eosin and confocal images

a) Haematoxylin and Eosin stain of tendon from GA control sheep 5. (Bar = 200 µm)
b) Confocal image of tendon from GA control sheep 5 nuclei stained with DAPI (blue) and connexins with Alexa 568 (red). (Bar = 25 µm)
c) Haematoxylin and Eosin stain of tendon from sheep 273 (injury model)
d) Confocal image of tendon from sheep 273 (injury model) nuclei stained with DAPI (blue) and connexins with Alexa 568 (red)
e) Haematoxylin and Eosin stain of antisense treated tendon from sheep 298
f) Confocal image of antisense treated tendon from sheep 298 nuclei stained with DAPI (blue) and connexins with Alexa 568 (red)

3.2.6 LIMITATIONS

External factors prevented follow up of the sheep for a longer time, which would have been preferable as most of the data on down-regulation of connexins by antisense oligodeoxynucleotides shows significant down-regulation at 6-8 hours. Also, the sheep were not recovered from the anaesthetic so the injured tendons were not subjected to weight-bearing and loading. This was not ideal as loading is thought to affect lesion expansion (van Schie et al., 2009). A progression of this study would ideally allow postural loading of the tendons after recovery from anaesthesia and examination of the tendons at later time points, for example at 24 h, 5 and 7 days post injury.

External factors also prevented ultrasound imaging of the lesions, the common clinical method of monitoring tendon lesions. This is less of an issue in this acute injury period as significant changes in mild lesions can take a week or more to be visible (De Grandis et al., 2012).

The large sheep to sheep variation in gap junction counts in this short time-course study resulted in a wide range of values within each group and may have overshadowed smaller variations between groups (Table 5). A larger sample size, intra-animal controls and a longer time course may alleviate this problem. The use of a pressure gauge to measure the force of the thrusts of the stylet would standardise the creation of the lesions and help reduce the sheep to sheep variation further.

Quantification of the effect of anti-sense oligodeoxynucleotides on the spread of the lesion could indicate the likely efficacy as a therapeutic agent. Prognosis of recovery following tendon injury is very dependent on the original lesion size and therefore anything that can block lesion spread will potentially improve healing. The inhalational anaesthetic may have had an influence on lesion spread due to its gap junction blockade effects so monitoring for longer and post recovery would again have been ideal.

Sectioning the tendons through the cyanoacrylate glue and submitting samples for electron microscopy to determine if a good seal had been achieved would have confirmed that only intrinsic repair would occur in this model. Injecting into the tendon lesion with a dye to see if egress occurred at the stab incision would also confirm a seal had been created.

3.2.7 CONCLUSIONS

- A mechanical model of acute tendon injury in the core of the ovine superficial digital flexor tendon was successfully developed and used to determine histological and Connexin43 changes in response to injury.

- Connexin43 levels in uninjured tendons were higher in abattoir animals compared with anaesthetised controls and confirmed the need for the appropriate specific controls for future studies.

- The increase in Connexin43 levels in response to injury indicates the model's suitability for studying the effects of transient modulation of Connexin43 expression, or function, on tendon injury repair.

- Injection of pluronic gel, with or without oligodeoxynucleotides, into the tendon resulted in increased gap junction plaque counts.

- The present study is the first to report the effect of Connexin43 antisense oligodeoxynucleotides in injured ovine superficial digital flexor tendon, in the first four hours after injury.

- Adding Connexin43 antisense oligodeoxynucleotides to injured tendon did not reduce Connexin43 expressions in the peracute period. However, the Connexin43 gap junction plaques in the injury area itself appeared to be fragmented resulting in a larger number of small plaques, with no difference in total Connexin43 gap junction plaque area, compared with the other treatment groups. This is consistent with early gap junction protein knockdown, which begins with a reduction in size of gap junction plaques followed by a reduction in number, as cytosolic stores are exhausted (Jaderstad et al., 2010).

CHAPTER 4

CREATION OF A CORE LESION IN THE EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON AS A MODEL OF ACUTE TENDON INJURY.

4.0 INTRODUCTION

The most commonly injured tendon in horses is the superficial digital flexor tendon (Dowling et al., 2000, Genovese et al., 1990). The most significant injury of this tendon involves the central core of the mid-metacarpal region of the tendon (Wilmink et al., 1992, Firth, 2000). Acute injury occurs because of incomplete healing of subclinical micro-damage causing accumulated damage within the tendon and therefore weakening it (Birch et al., 1998, Patterson-Kane and Firth, 2009). The core fibres then fail acutely and, except in complete rupture, the injury and repair mechanisms are via intrinsic processes. An ideal model of acute tendon injury in energy-storing tendons such as the equine superficial digital flexor tendon has still not been identified (Schramme et al., 2010).

Clinically, tendon injury is diagnosed from history as well as clinical and ultrasonographic signs of tendon damage (Ross and Dyson, 2003, Dowling et al., 2000). Clinical signs of tendon injury consist of lameness associated with swelling of the

tendon and surrounding tissues. The tendon is often painful on palpation and signs of mechanical failure may be present. Affected horses are usually lame although the severity of the lameness is often variable. Ultrasound examination for evidence of tendon swelling, loss of tendon tissue architecture, core lesions, fibre tearing and subcutaneous oedema is the main diagnostic and prognostic tool used in clinical practice (Dowling et al., 2000). Ultrasound is known to be insensitive to subtle tendon lesions (Dyson, 2004). Comparison of ultrasound findings with gross and histopathology techniques would enable better characterisation of lesions identified as well as those missed by ultrasound examination.

Extrapolating from the ovine acute tendon injury model described in Chapter 3, an operative method to create a lesion in the core of the equine thoracic limb superficial digital flexor tendon was developed. This was tested *in vivo* to determine how effectively it would mimic the acute stage of tendinopathy. Such a core lesion could be used to accurately mimic acute changes in clinical over-load tendon injury.

The hypothesis was that a core lesion could be created in the mid metacarpal region of the equine superficial digital flexor tendon that would clinically, ultrasonographically and histologically mimic a core lesion in acute tendon injury.

The aims of the study were to

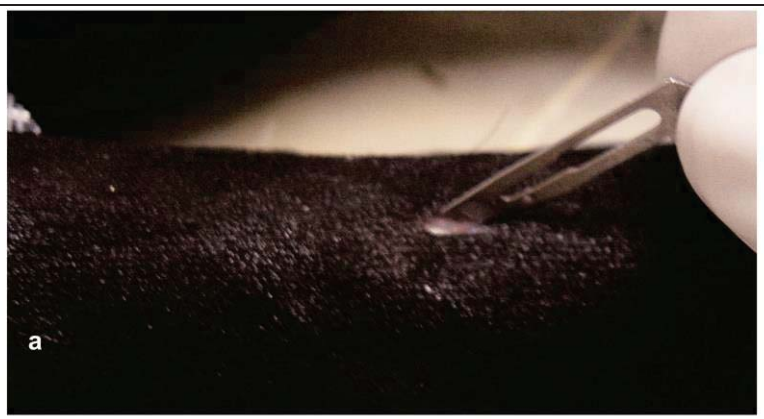
- Extrapolate from the ovine injury model described in Chapter 3 and develop a model of an acute tendon lesion in the core of the equine superficial digital flexor tendon that did not have the limitations of the current published tendon injury models. The technique to create the lesion was first developed in cadavers.

- Adapt the procedure to create the model in standing horses.
- Determine the clinical, ultrasonographic and histological changes created by the lesion to determine if they were consistent with changes in clinical injury.
- Compare clinical, ultrasonographic and histopathological changes in response to the acute core lesion to enhance understanding of the acute post-injury phase as demonstrated by these parameters.

4.1 MATERIALS AND METHODS

4.1.1 MODEL DEVELOPMENT IN CADAVERS

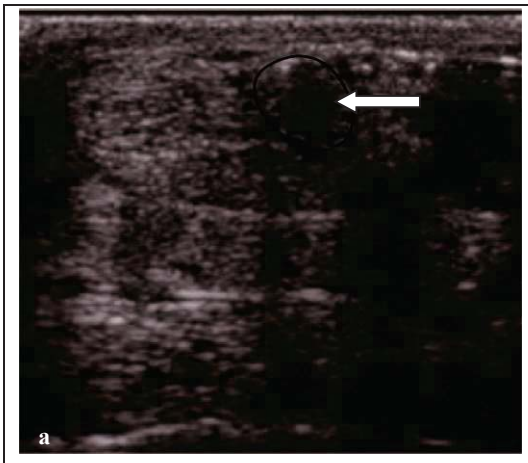
Cadaver thoracic limbs of 4 horses (*Equus caballus caballus)* undergoing euthanasia for reasons unrelated to thoracic limb lameness were used to perfect the creation of a lesion in the core of the equine superficial digital flexor tendon, prior to *in vivo* application. Skin incisions were made over the palmar aspect of the mid-metacarpal region of the superficial digital flexor tendon. A stab incision was made into the tendon and a 12 gauge catheter stylet with a barb inserted and directed proximally and thrust back and forth 1 cm to tear the tendon fibres (Figure 16). Water was injected into the lesion created prior to removing the stylet. Ultrasound examination was performed to determine if the fluid-filled lesion was detectable ultrasonographically (Figure 17).



a) A number 11 scalpel blade was used to make a stab incision through the skin and then into the core of the superficial digital flexor tendon.

b) The barbed catheter stylet was inserted into the core of the tendon and directed proximally along the long axis of the tendon and pushed and pulled back and forth.

Figure 16 - Creating the superficial digital flexor tendon core lesion in a cadaver limb.



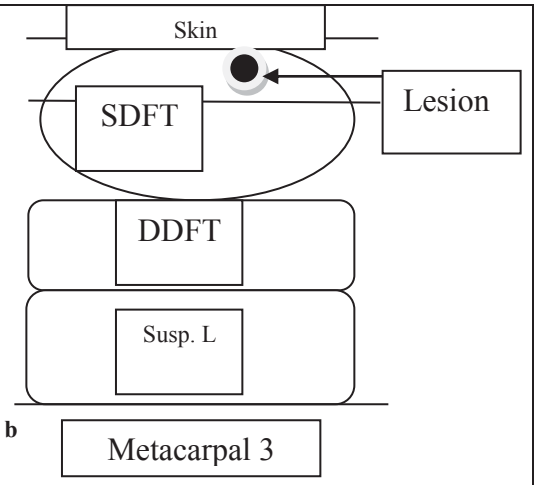


Figure 17 – Cadaver lesion on ultrasound.
a) Ultrasound of the cross section through the mid-metacarpus with a cadaver lesion distended with water (White arrow points to lesion). b) Schematic of anatomy.
SDFT – Superficial digital flexor tendon; DDFT – Deep digital flexor tendon; Susp. L – Suspensory ligament; Metacarpal 3 – palmar aspect of the 3rd Metacarpal bone. Dark dot on figure indicates the lesion created in the tendon.

4.1.2 SOURCE OF HORSES

Four thoroughbred horses (*Equus caballus caballus*); one mare and three geldings that were 4, 5, 7 and 11 years old and donated for reasons other than tendon injury were used for the study.

4.1.3 PRE-OPERATIVE EXAMINATION

All horses' limbs were examined clinically for evidence of superficial digital flexor tendon injury and lameness. All clinical findings were noted irrespective of perceived significance (Appendix 12).

Horses were sedated with 10-20 µg/kg detomidine and 10 µg/kg butorphanol administered intravenously. The hair from the palmar aspect of the thoracic limbs was removed using clippers and the skin cleaned with chlorhexidine scrub then wiped with methylated spirit soaked swabs before the application of acoustic coupling gel. Routine bilateral ultrasonographic examination of the thoracic limb superficial digital flexor tendon was performed. Ultrasound images were collected using a Phillips HDI3000 ultrasound machine (Phillips, New Zealand) and a 16MHz linear array transducer with a Phillips Tl12-5 50 standoff (901805, Veterinary sales and service, Florida USA) (Figure 18 a and b). The standoff was used to enable full examination of the superficial digital flexor tendon and the subcutaneous tissues around it (Reef, 2001). Transverse images were collected at 4 cm intervals distal to a mark near the accessory carpal bone, skin markings with white correction fluid were made at the level of the top of the standoff at the first examination. At subsequent examinations, the standoff and transducer were aligned with these skin markings to ensure consistency (Figure 18c). Three longitudinal images were collected encompassing all the transverse regions.

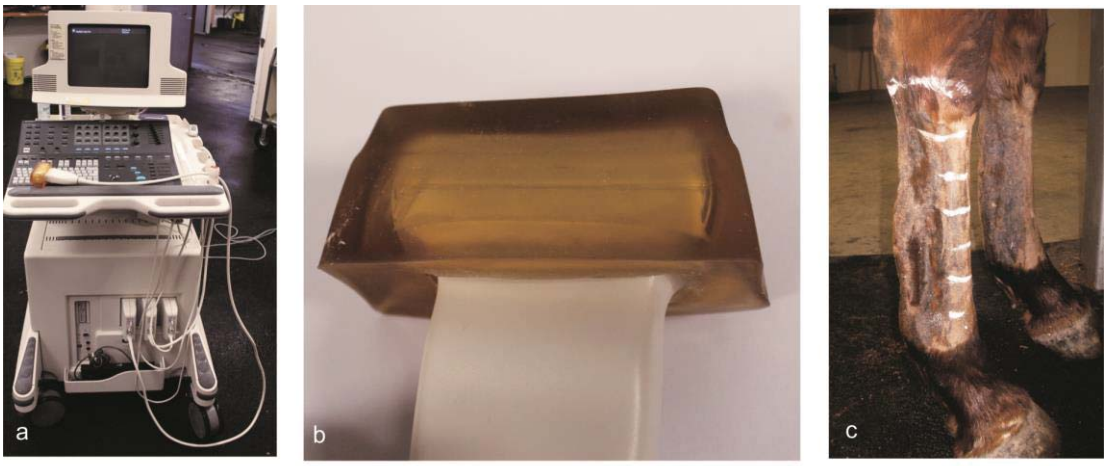


Figure 18 - Ultrasound Technique
a) Ultrasound machine
b) Linear transducer with gel standoff in situ
c) Skin markings for consistent positioning of standoff and transducer in repeat scans

4.1.4 OPERATIVE MODEL CREATION *IN VIVO*

(Appendix 13 for checklist of equipment and drugs). Horses were sedated with 20 µg/kg detomidine and 10 µg/kg butorphanol administered intravenously. Local anaesthesia of the palmar metacarpal region was achieved by performing regional anaesthesia of the deep and superficial branches of the ulnar nerve (Ross and Dyson, 2003). This was achieved by injection of 10 mL of mepivicaine via a 20 gauge, 1 inch needle inserted perpendicular to the limb, between *ulnaris lateralis* and *flexor carpi ulnaris* at a level 10 cm proximal to the accessory carpal bone (Figure 19).

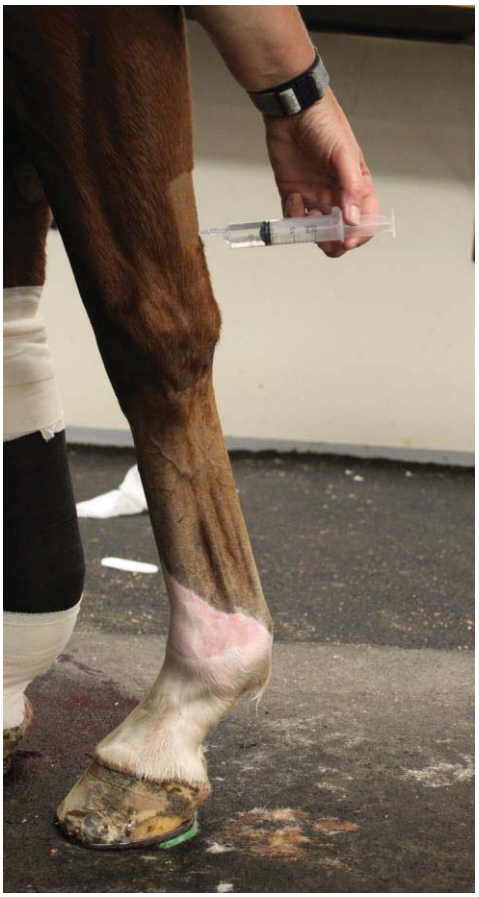


Figure 19 - Positioning of the needle for ulnar nerve block.

A tourniquet was applied proximal to the carpus and the distal limb was prepared for aseptic surgery (Hague et al., 1997).The limb was held up by an assistant and the carpus was flexed. The skin was rolled slightly medially prior to a 1 cm stab incision being made through the skin on the palmar aspect of the limb at 8 cm distal to the accessory carpal bone using a number 11 scalpel blade. This was so the incision would not interfere with subsequent ultrasound examinations. Using a fresh number 11 scalpel blade a stab incision was made into the core of the superficial digital flexor tendon and the tendon fibres parted to allow a sterile barbed 12 gauge catheter stylet to be inserted through the stab incision and directed proximally. The catheter stylet had been barbed by bending the tip using artery forceps to produce a 1 mm barb giving an overall lesion of 3 mm diameter when the stylet was rotated.

The stylet was pushed and pulled back and forth 15 times for a distance of 10 mm, the stylet had been marked previously so the length of the lesion could be controlled. The stylet was withdrawn and the stab into the tendon was closed using cyanoacrylate glue. The cyanoacrylate glue was allowed to dry before the skin incision was closed using a skin staple. The procedure was repeated at 12 and 16 cm distal to the accessory carpal bone. The limb was then bandaged routinely and the tourniquet removed. One limb in each horse was randomly chosen for creation of a single lesion at 12 cm distal to the accessory carpal bone and the other limb had lesions created at 8, 12 and 16 cm distal to the accessory carpal bone. The skin incisions were made at 8, 12 and 16 cm in both limbs.

4.1.5 POST-OPERATIVE CARE AND EVALUATION

The horses were confined to the stable for the duration of the study. Daily heart rate, respiratory rate, appetite and demeanour were recorded. Daily clinical examination of the limbs, lameness and ultrasonographic evaluations were performed. Lameness was evaluated using a scale out of 10 where 0 is sound and 10 non-weight bearing. Non-steroidal anti-inflammatory drugs were administered if indicated by clinical parameters such as increase in lameness of more than 25%, more than 50% increase in diameter of the metacarpal region from swelling or an increase in heart or respiratory rate of more than 25%. Bandages were reset daily when ultrasound examination was performed.

4.1.5.1 ULTRASONOGRAPHY

Horses were examined ultrasonographically prior to lesion creation, within 4-6 hours post-operatively and then daily until euthanasia. Identical depth, frequency, focus, gain and magnification settings were used for each evaluation and the skin markings were used to ensure consistent transducer positioning. Images were collected and stored using

Merge Fusion PACS™ software and viewed using a standard PC computer running eFilm™ software (Merge Healthcare, Hartland, Wisconsin, USA). Images were examined in a blinded fashion by two board-certified equine surgeons (Michael Archer and Frederick Pauwels) and scored with a rubric of: Lesion – yes /no; If yes – mild (1), moderate (2) or severe (3) (Figure 6). The ultrasound scores were compared with the expected scores based on the gross physical appearance of the lesions. Images included normal and injured areas of tendon.

Table 6 - Ultrasound lesion scores with description

Score	Description
0	No lesion
1	Lesion involving less than or equal to 25% of the tendon cross-sectional area
2	Lesion involving 25% to 50% of the tendon cross-sectional area
3	Lesion involving more than 50% of the tendon cross-sectional area

4.2 SAMPLE COLLECTION AND PROCESSING

4.2.1 TISSUE COLLECTION

On day 4 post lesion creation, the horses underwent euthanasia by captive bolt and exsanguination. The thoracic limbs were disarticulated at the mid radius. The thoracic limb was held in extension whilst the palmar skin was reflected to expose the superficial digital flexor tendon. The superficial digital flexor tendon was marked to correspond with the marks on the skin used for ultrasonography. The tendon was held using a cutting board and sectioned in the coronal plane using a Feather™ cryotome blade then

sectioned transversely at a level proximal to the 4 cm distal accessory carpal bone mark and distal to the 20 cm mark. The dorsal and palmar halves were reflected and photographs used to record gross visual changes. The dorsal ½ was pinned to a tongue depressor and frozen in a single piece in *n*-hexane over dry ice. The palmar half was sectioned at 8, 12 and 16 cm distal accessory carpal bone and each section was then divided into 1 cm sections. Most of the sections were mounted in a cryomould in optimal cutting temperature compound, snap-frozen and then stored at minus 80^{o}C. One section per lesion was placed in RNA later and then stored at minus 80^{o}C for subsequent RNA extraction and polymerase chain reaction if required.

4.2.2 HISTOPATHOLOGICAL EXAMINATION

Longitudinal cryo-sections (16 μm thick) were cut (Reichert-Jung Cryocut 1800, Leica Microsystems, Germany) and 64 adjacent sections stained with Haematoxylin and Eosin (H&E) and Herovici stains (Appendix 14). Seventeen of the sections underwent immunohistochemical staining for collagen propeptides (Appendix 15). The amount of fluorescence was subjectively scored relative to the positive, horse skin wound, and negative, mouse brain, controls. Zero was negative or equal to background, + above background, ++ equal to positive control and +++ greater than positive control. Thirteen sections were also stained with Masson's trichrome staining protocol (Appendix 7) to compare with the H&E staining. The mouse brain was sourced from a mouse undergoing euthanasia for another reason and snap frozen in OCT within 1 hour of euthanasia. The horse skin wound tissue was collected from a horse undergoing euthanasia for another reason and was snap frozen in OCT within 1 hour of euthanasia using pentobarbitone overdose.

4.2.2.1 HAEMATOXYLIN AND EOSIN STAINING

H&E stained sections were examined for evidence of acute inflammation. The sections were examined in a blinded fashion by an American board-certified pathologist (Cameron Knight) and assigned a score as determined by the examination rubric; acute injury – yes /no. Herovici, H &E and Masson's stained sections were examined using standard, bright field microscopy (Zeiss Axiophot, Carl Zeiss, Auckland, New Zealand). Images were captured with Olympus D72 Camera, x20 objective) and processed using CellSens™ Dimension 1.5 software (Olympus, Adelaide, Australia). Images were stored and exported as .TIF files.

4.2.2.2 HEROVICI STAINING FOR COLLAGEN

The Herovici polypichrome stain was used to determine if detectable new collagen had been deposited at the injury site within 4 days (Appendix 14).

4.2.2.3 IMMUNOHISTOCHEMICAL PROTOCOL FOR COLLAGEN I AND III

Tendon sections were stained for aminopropeptides representing collagen type I and III as described by Young, Becker, Fleck, Goodship, & Patterson-Kane, (2009) (Appendix 15). Fluorescently stained sections were examined on the Zeiss Axiophot (details in section above) using the blue G365 dichroic mirror – 395 Emission – 460/50 to illuminate the DAPI; the green – 470/40 dichroic mirror – 510 Emission – 535/50 for the Alexa 488 bound to procollagen I and the red – 564/12 dichroic mirror – 580 Emission – 590 for the Alexa 568 bound to the procollagen III. Positive control tissue was a healing equine skin wound and negative control sections were from mouse brain and also tendons without primary antibodies added.

The 10x objective was used to scan the section for evidence of tendon injury such as fibre disruption, necrosis and inflammatory cell infiltrate. When the area of injury had

been determined, an image of the DAPI staining was collected at 20x magnification with a manual exposure time of 250 milliseconds. The immunofluorescence of the procollagen I and III were collected at the same site using 800 milliseconds exposure and the appropriate fluorescence cubes.

Results were scored as 0 = background, + = above background, ++ = level with positive control and +++ = above positive control

4.3 STATISTICS

The sensitivity and specificity of the ultrasound scores as determined by Surgeons A and B compared with the gross appearance of the lesion were calculated (Dohoo et al., 2003) (Appendix4.5 and 4.6).

Cohen's kappa was used to measure agreement of the ultrasound and gross examination of the tendons for each surgeon and the agreement of the surgeons with each other. The analysis was conducted in STATA 12 (StataCorp, TX, USA).

4.4 RESULTS AND DISCUSSION

Lesions were created in the core region of the superficial digital flexor tendon of horses using a barbed catheter stylet. The lesions were technically easy to create in standing horses and the procedure was well tolerated. Horses with the lesions had clinical signs of subcutaneous and tendon swelling consistent with acute tendon injury. The lesions created had histological changes consistent with acute inflammation although these changes were more discrete than the gross changes would have suggested. Less than 50% of the lesions were detectable ultrasonographically although when correctly detected the lesion was given an accurate score 81-90 % of times. Therefore, this core lesion would be a good model for subtle acute injury to the core of the equine

superficial digital tendon. Although the ultrasound detection was below 95%, the overall clinical detection would have been higher when all parameters were considered.

4.4.1 LESION DEVELOPMENT IN CADAVERS

The core lesion was technically easy to create in cadaver limbs. Dissection of the tendon allowed direct visualisation of the amount of fibre damage created in the tendon and therefore enabled development of the ideal technique prior to whole animal studies. Ultrasonography in the cadavers was limited by the inability to get the superficial digital flexor tendon under load, this would only have been possible if the complete limb had been removed from the trunk and held in a loaded position and this was not practical in the source material available. Due to the absence of haemorrhage, the lesion required distension with fluid to be visible ultrasonographically. Cadaver lesion creation was a useful means to practise and perfect the technique. *In vivo* lesion creation was necessary to determine lesion progression and the clinical effects of the core lesion.

4.4.2 LESION DEVELOPMENT *IN VIVO*

4.4.2.1 SOURCE OF HORSES

The horses used had no clinical or ultrasonographic indications of tendon injury detected at the onset of the study. Provided two experienced personnel were available the lesions could be created in the horses using standing sedation and regional local anaesthetic blocks. The horses were all thoroughbreds consistent with one of the main populations susceptible to exercise-induced acute tendon injury (Williams et al., 2001, Dyson, 2004). The ages of the horses (4, 5, 7 and 11) meant that sub clinical pre-existing chronic tendon changes could potentially exist in these horses. It has been reported that there is an age-related alteration in fibre sizes and the crimp angle of the

core collagen fibre in untrained horses more than 10 years of age similar to that in younger horses that had been exposed to exercise (PattersonKane et al., 1997a).

If pre-existing chronic tendon degeneration existed in any of the horses used it should not have been detrimental to the study. Chronic degeneration results in alterations of fibre size and crimp angle and alterations of the tendon cells, some areas becoming acellular and others containing cells with rounded nuclei (Webbon, 1978, PattersonKane et al., 1997b). Acute injury results in fibre disruption and tearing with haemorrhage and infiltration of polymorphonuclear cells and macrophages therefore would be easily differentiated from chronic injury (Patterson-Kane and Firth, 2009).

4.4.2.2 PRE- AND POST-OPERATIVE CLINICAL EXAMINATIONS

All basic parameters of the horses were within normal limits prior to lesion creation. No palpable abnormalities of the tendons of the distal thoracic limbs were apparent in the horses prior to lesion creation. No lameness was detected at walk apart from a slight stiffness in the left pelvic limb in horse C. The horses were unshod and were short-striding at trot consistent with this. A mild lameness was detectable at trot in 3 out of the 4 horses but these were considered subtle enough that they would not have impeded detection of pain in response to model creation (Appendix 12 a-d).

The horses were all unshod throughout the study. Generally, the use of unshod horses for lameness investigations can be problematic as they can develop lameness issues such as foot soreness in response to being exercised on a firm surface, which is required for accurate lameness evaluation. Fortunately, this was not of concern in this study as the horses were not exercised prior to lesion creation. Only five lameness evaluations were required and the horses were managed on box rest following lesion creation. Initial therapy for an acute tendon injury involves a period of box rest followed by an

ascending exercise program. In future studies the horses should be trained prior to injury to create the underlying chronic changes usually pre-existing in acutely injured tendons. The horses should be followed for several weeks post lesion creation and they should be shod.

All horses had mild swelling on the palmar aspect of the distal thoracic limb over the superficial digital flexor tendon within 12 hours of lesion creation. Post-operative pain was minimal. One of the four horses, Horse C, had marked distal limb swelling of the right thoracic limb 12 hours post operatively and this was due to subcutaneous haemorrhage at the time of surgery. The horse was 1/10 lame at the walk and had a 20% increase in heart rate and a 30% increase in respiratory rate (Table 7). This horse responded to a single dose of non-steroidal anti-inflammatory medication (2.2 mg/kg phenylbutazone intravenously) and cold hydrotherapy (10 minutes twice daily for one day). This produced a reduction in swelling and lameness, and a return of the heart and respiratory rates to pre-operative levels.

An increase in heart rate is caused by a multitude of physiological states and pathological conditions including exercise, fear, cardiac disease, hypovolaemia, anaemia, infection and pain. Similarly increased respiratory rates occur in response to exercise, fear, respiratory and cardiac compromise, infection and pain. Horse C showed no other evidence of cardiac or respiratory compromise on auscultation, her temperature had remained within normal limits and she was being rested. Pain due to lesion creation was therefore the most likely cause of the changes in her basic parameters and consistent with the clinical findings of limb swelling and lameness. Following lesion creation all other horses were sound at walk and mildly bilaterally lame resulting in a short striding gait at trot in a straight line.

Table 7 - Daily parameters monitored

HORSE	DATE	HEART RATE	RESPIRATORY RATE	SWELLING OF METACARPUS	LAMENESS AT WALK
A	19/08/09 pre surgery	40	14	NONE	SOUND
	19/08/09 post surgery	40	12	SLIGHT	SOUND
	20/08/09	36	12	SLIGHT	SOUND
	21/08/09	36	12	SLIGHT	SOUND
	22/08/09	36	14	SLIGHT	SOUND
B	08/09/09 pre surgery	44	16	NONE	SOUND
	09/09/09 post surgery	40	14	SLIGHT	SOUND
	10/09/09	44	16	SLIGHT	SOUND
	11/09/09	28	12	SLIGHT	SOUND
	12/09/09	24	12	SLIGHT	SOUND
C	08/09/09 pre surgery	40	12	NONE	STIFF left pelvic
	09/09/0 post surgery	48	16	MARKED RF	Right thoracic limb*
	10/09/09	40	12	MODERATE	SLIGHT RF
	11/09/09	36	12	MODERATE	SOUND
	12/09/09	36	12	MODERATE	SOUND
D	19/08/09 pre surgery	36	12	NONE	SOUND
	20/08/09 post surgery	36	14	SLIGHT	SOUND
	21/08/09	36	12	SLIGHT	SOUND
	22/08/09	36	12	SLIGHT	SOUND
	23/08/09	36	14	SLIGHT	SOUND
	24/08/09+	36	14	SLIGHT	SOUND

***** Tourniquet not well applied on the right thoracic limb and significant subcutaneous haemorrhage during lesion creation. Horse responded well to hydrotherapy and phenylbutazone 2.2mg/kg for 24 hours. Following treatment, the lameness resolved and the swelling decreased although remained present.
+ Due to external factors, Horse D did not undergo euthanasia until 1 day later than intended.

4.4.2.3 LESION COULD BE CREATED IN STANDING HORSES

The regional anaesthesia was effective in all cases. The superficial digital flexor tendon in the thoracic limb is supplied by the lateral and medial palmar nerves. The ulnar nerve block desensitises the ulnar nerve just above the carpus, from which the palmar branches of the caudal cutaneous antebrachial nerve and lateral and palmar nerves branch. Therefore, a successful nerve block would have prevented the horse feeling any pain during the procedure.

The tourniquet provided effective haemostasis and enabled good visualisation of the tendon and subsequent stab incision. Creation of lesions of a fixed length was achievable using the mark on the stylet and it would have been possible to extend the length of the lesion by thrusting the stylet in for a greater distance. Larger skin incisions would have enabled easier visualisation of the tendon and stab incision.

It was difficult to make a lesion at an exactly defined distance from the distal to the accessory carpal bone with the carpus in the flexed position due to skin movement relative to the tendon. At post-mortem, it was easy to determine the exact distance of the lesion from the distal to the accessory carpal bone skin markers. Due to interference of the flexed limb, the most proximal lesion could only be practically created at 11 or more centimetres distal to the accessory carpal bone. Most overload lesions are a single lesion with the worst damage in the core of the mid-metacarpal region (Wilmink et al., 1992, Firth, 2000). A single longer lesion in each superficial digital flexor tendon in the mid-metacarpal region warrants further investigation. Historically the prognosis for effective healing of tendon injuries was largely based on the volume of the lesion therefore a single larger lesion would be the next progression. Historically the prognosis for effective healing of the lesion was based on the original size of the lesion and a subjective fibre score. There are data suggesting this alone is not an accurate measure for prognostication (Aro et al., 2012).

The lack of subcutaneous haemorrhage (in all except horse C) enabled effective drying of the tendon prior to sealing with cyanoacrylate glue. This is important to potentiate the effectiveness of the glue at closing the defect and thereby constraining the lesion development and healing to intrinsic processes. The cyanoacrylate glue dried quickly and was allowed to set prior to skin closure so no adherence between tendon and subcutaneous tissues could occur. Cyanoacrylate glue has been shown to strengthen

traditional suturing of tendon defects (Oztuna et al., 2005) and therefore was expected to maintain integrity with loading of the tendons.

During creation of the lesion in the right thoracic limb of horse C subcutaneous haemorrhage occurred from damage to a vessel during the skin incision. Better application of the tourniquet would prevent this minor complication.

4.4.3 ULTRASONOGRAPHIC CHANGES

No ultrasonographic abnormalities were detected in the pre-operative ultrasound scans of any of the horses. Post operatively there was minimal interference of the staples with the ultrasound due to the skin incisions being created off the palmar midline. Cyanoacrylate glue could have been used in place of staples but would not have changed that the skin incision needed to be off midline as disruption of the skin and the glue itself would attenuate the beam and compromise ultrasound interpretation.

4.4.3.1 ULTRASOUND LESION SCORES

Ultrasound examination had an overall detection rate of 48% for the presence of lesions without significant differences between surgeons (A 173/360 and B 172/360) (Figure 8). This is not significantly different to the probability of detecting the lesion correctly by chance alone. The severity of the accurately identified lesions was correctly identified by the surgeons in 81-90% (surgeon A identified 81%, and surgeon B identified 90 %) of the scans.

Surgeon A and Surgeon B agreement with each other on if a lesion was present or not was fair (k 0.2856). Surgeon A had fair agreement with gross examination on the presence of the lesion on ultrasound (A=*k* 0.0461). Surgeon B had poor agreement with gross examination on the presence of the lesion on ultrasound (B=-0.1161). Surgeon A

had slight agreement with gross examination on the score of the lesion on ultrasound (*k* =0.1813). Surgeon B had slight agreement with gross examination on the score of the lesion on ultrasound (*k*= 0.0393).

Table 8 - Ultrasound findings compared with gross lesions

Surgeon A

Gross lesion y/n	0	1	Total
0	87	146	**232**
1	41	87	**128**
Total	**127**	**233**	**360**

Surgeon B

Gross lesion y/n	0	1	Total
0	132	100	**232**
1	88	40	**128**
Total	**220**	**140**	**360**

Surgeon A sensitivity = 87/232 = 0.375 and specificity = 87/128= 0.68

Surgeon B sensitivity = 132/232= 0.569 and specificity = 40/128 = 0.31

Therefore, Surgeon A was less likely to correctly detect that a tendon did not have a lesion than he was to detect a lesion was present. Surgeon B was more likely to correctly determine that a lesion was not present than to correctly detect a lesion.

More severe lesions were more consistently identified correctly (Appendices 16 and 17). These data confirm the clinical knowledge of the relative insensitivity of ultrasonography alone for the detection of subtle lesions.

Ultrasound examination has been the current main clinical tool for imaging tendon injuries since it was first described in 1986 (Yoo et al., 2012). Computer assisted

tomography and magnetic resonance imaging are becoming more popular but are not mobile or cost effective for general clinical practice. Clinical examination of tendon injuries involves ultrasound examination and determination of size and echogenicity changes within the tendon (Reef, 2001, Marr et al., 1993b, Marr, 1992). Acute tendon injury results in haemorrhage and serum exudation into the injured area and therefore swelling of the tendon. This presents on the ultrasound scan as an increase in tendon diameter and reduced echogenicity of the injured area. It is well recognised that ultrasound examination of tendons is a relatively insensitive tool (van Schie et al., 2009), particularly in mild lesions. In the clinical situation, these surgeons would have been expected to have a higher lesion detection rate, as they would use history, clinical examination, lameness investigation and ultrasound examination together to come to a diagnosis, particularly in subtle cases. In a clinical ultrasound examination, the surgeon can view the skin and subcutaneous tissues as well as the tendons and this can assist with lesion detection. In this study as the presence of subcutaneous haemorrhage from the skin incisions, and the variation in degree of this haemorrhage between horses, was thought likely to bias the examination of the images. The surgeons were therefore only provided with the section of the image that showed the superficial digital flexor tendon.

Video ultrasound images of the whole length of the tendon would have enabled construction of a 3 dimensional sonogram of the tendon (Wood et al., 1994). A 3-dimensional image may have improved the detection rate of the lesions but this has not been fully validated. Ultrasonographic tissue characterisation has also been recommended for lesion monitoring (Bosch et al., 2011) although this method cannot be used for determining prognosis and imaged lesions were not compared with gross *ex vivo* and histological findings. On the video sonograms it would also have been harder

to correlate the sites of lesions on ultrasound with the lesions on the skin and therefore with histopathology.

4.4.4 SAMPLE COLLECTION TECHNIQUES

Tendon is very fibrous and hard to cut without tearing. Collection and processing of both thoracic-limb superficial digital flexor tendons takes 4-6 hours. In all limbs there was significant discolouration and haemorrhage apparent when the skin was removed (Figure 20a). Once the subcutaneous tissues were dissected the sites of stab incisions into the tendon could be visualised and although fibrin was adherent to them no subcutaneous tissues were adherent (Figure 20b). Once the superficial and deep digital flexor tendons were separated, it was practically easier to divide the tendon in a coronal plane using the cutting board and a Feather® trimming blade (No.130 type S) (Figure 20c). Most but not all lesions were exactly in the centre of the tendon (Figure 20d and e) therefore it might have been better if the tendon had been divided into smaller lengths before splitting.

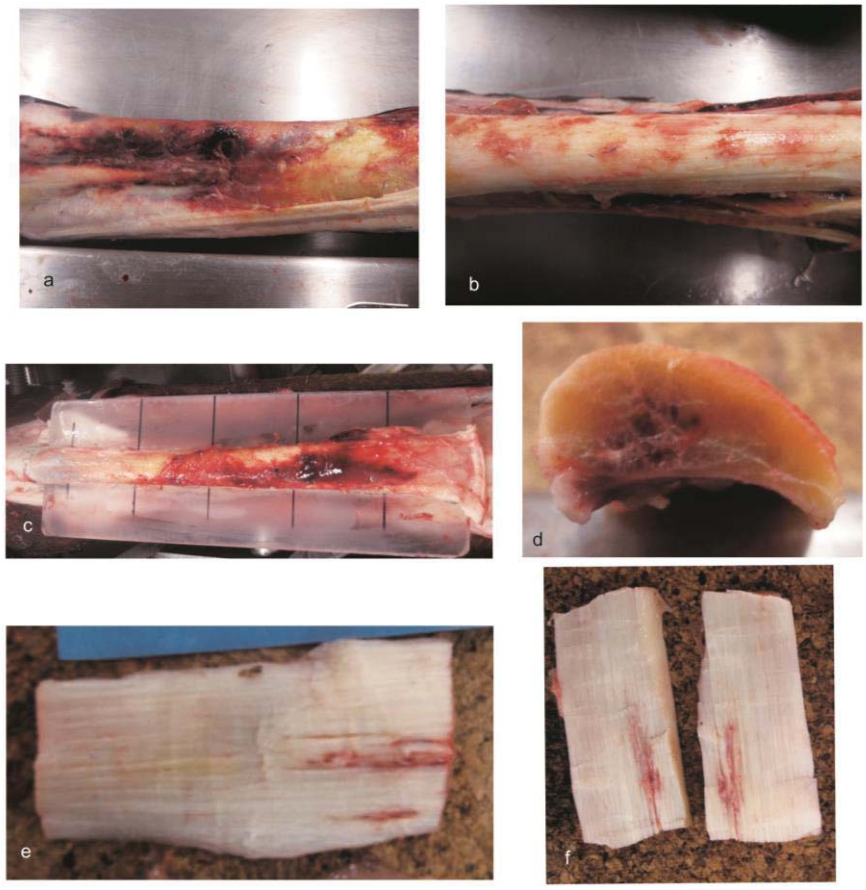


Figure 20 - Gross appearance of tendons

a) Thoracic limb with skin reflected to expose the tendons
b) Thoracic limb with subcutaneous tissue removed and stab incisions into tendon visible
c) Cutting board used to hold tendon prior to coronal sectioning
d) End on view of tendon section to show offset lesion
e) Lengthwise view of 4cm section of tendon following coronal sectioning showing lesion offset from centre
f) Tendon with discolouration proximal and distal to stylet insertion and fibre disruption proximal to stylet insertion. Proximal on the horse is towards the top of the page and section is 4cm long.

4.4.4.1 COMPARISON OF NORMAL AND INJURY MODEL TENDONS

Discolouration extended both proximally and distally from the site of stylet insertion (Figure 20f). As the stylet was only directed proximally, the distal discolouration was thought to be dependent haemorrhage. The dependent haemorrhage had not been apparent in the sheep tendons this could have been due to a number of factors. The sheep were anaesthetised so potentially had a lower relative blood pressure compared with the conscious horses. This may have resulted in reduced haemorrhage at the surgery site, however no tourniquet was in place for the sheep surgery and subcutaneous haemorrhage was apparent. The sheep being recumbent throughout not only reduced the

effects of gravity on any exudate or haemorrhage produced but also abolished any effects of tendon loading and movement on lesion expansion or movement of the haemorrhage. These coupled with the shorter duration of the sheep model are likely to be responsible for the differences noticed.

Fibre disruption was also apparent proximal to stylet insertion. No necrosis was apparent in the lesions at this time. Uninjured tendon was white with regular fibre orientation and no discolouration. There were no discoloured areas in the uninjured tendon sections in the study tendons.

4.4.4.2 COMPARISON OF HISTOLOGICAL SECTIONS WITH CLINICAL HISTOLOGY

Of the 64 sections that underwent histological examination, inflammation was not clearly apparent in 22 although fibre disruption was visible. This is likely to be due to the relatively avascular nature of the tissue resulting in limited and localised response to injury. It was apparent in some sections (Figure 21a-d) that there had been significant injury to the tendon and neutrophils, macrophages with engulfed material, fibrin and fibre disruption were apparent. It was notable that normal tendon with no indication of damage existed within 100 μm of the injury site. In other sections, macrophages and neutrophils were apparent without an obvious injury area in the H&E section (Figure 21e and f) and the fibre disruption was apparent on the adjacent Herovici section (Figure 21g and h). These findings show that it is technically difficult histologically to determine injury and inflammation in tendon tissue unless the section is directly at the site of the lesion. In subsequent experiments, a single longer and wider lesion may therefore be indicated.

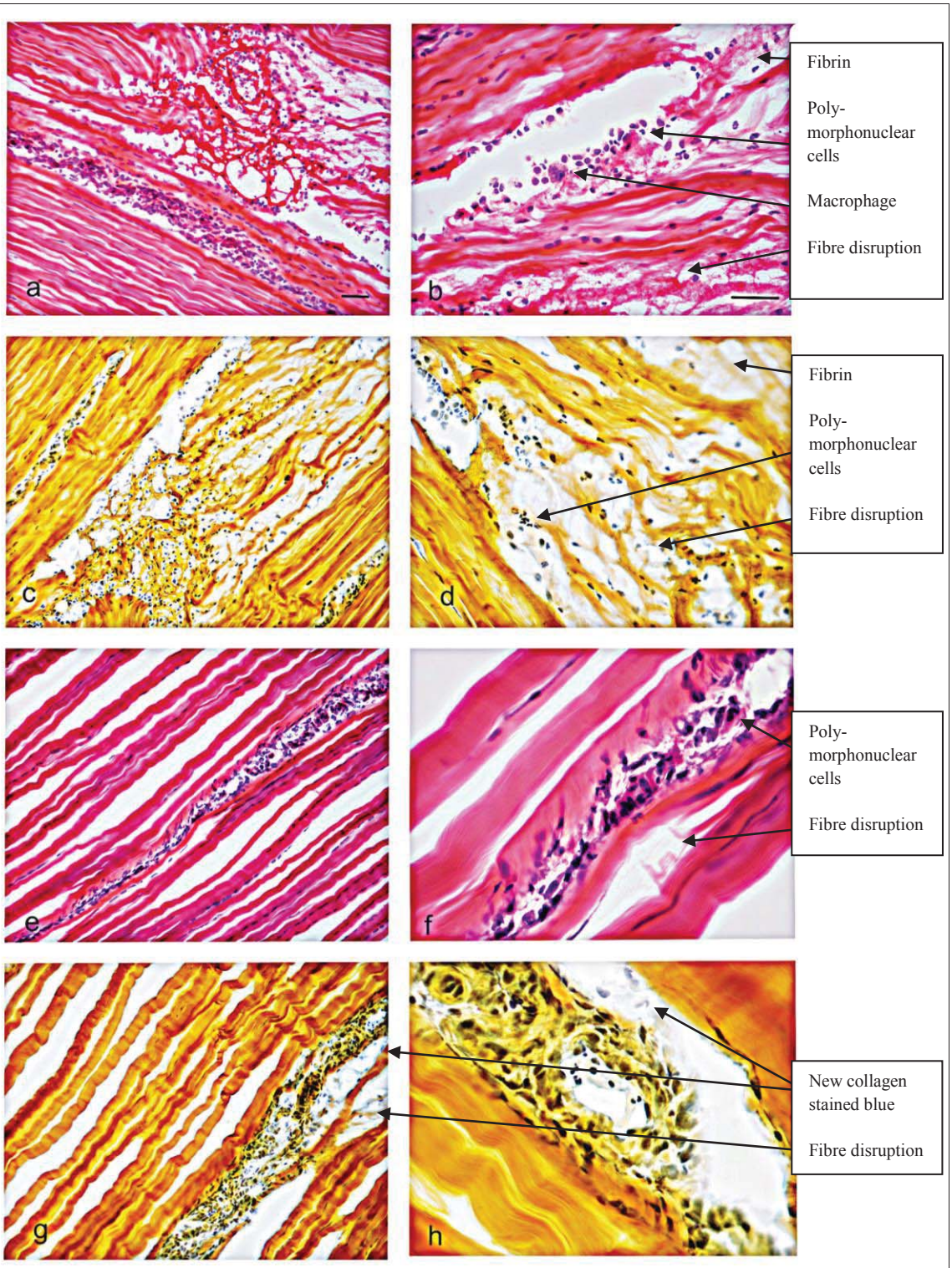


Figure 21 - Haematoxylin & Eosin (pink) and Herovici (yellow) stains.
Scale bars for a, c, e and g = 200 μm and for b, d, f and h = 100 μm

a) H&E showing fibre disruption, polymorphonuclear cells, macrophages and fibrin.
b) Same as a) with increased magnification shows mixed inflammatory cell infiltrate.
c) Herovici stain showing fibre disruption, polymorphonuclear cells, macrophages and fibrin.
d) Same as c) with increased magnification shows fibre disruption and cell infiltrate.
e) H&E x 10 magnification showing inflammatory cells but no obvious fibre disruption.
f) Same as e) with increased magnification.
g) Herovici stain of section adjacent to e) with fibre disruption now apparent as well as the inflammatory cells and some new collagen (pale blue).
h) Same as g) with increased magnification.

4.4.4.3 IMMUNOHISTOCHEMICAL STAINING FOR PROCOLLAGEN I AND III

All the sections stained well with DAPI and the majority had some procollagen I and procollagen III staining (Figure 22) although procollagen III was predominant. Sections in areas of no grossly visible lesions had either some staining for both procollagen I and III (5/7) or no staining for procollagen I (2/7) (Table 9). This could suggest either that low levels of procollagen are produced in un-injured adult horse tendon or that the grossly non-injured areas were responding at a cellular level to injury elsewhere in the tendon. Based on the minimal histological findings even close to injury areas the latter is less likely. Methods to increase procollagen I production, especially in response to injury, require investigation as a means to promote improved quality of tendon healing.

Table 9 - Detection of procollagen types I and III in tendon sections

Identification	Lesion Y or N	Fluorescence of 488 procollagen I	Fluorescence of 568 procollagen III	Fluorescence of DAPI stained nuclei
ALFPa 12-16 4	N	+	++	+
ALFPa 16-20 1	Y	+	+	+
ARFPa 8-12 4	Y	++	++	+
ARFPa 12-16 2	N	0	++	+
BLFPa8-13 3	N	+	+	+
BLFPa 8-13 5	Y	+	+	+
BLFPa 13-20 9	Y	+	++	+
BRFPa 11-16 1	N	+	+	+
BRFPA 11-16 4	Y	+	+	+
CLFPa 6-12 2	N	+	+	+
CLFPa 12-16 5	Y	+	+	+
CLFPa 16-20 3	Y	+	+	+
DLFPa 4-8 4	N	0	+	+
DLFPa 8-12 1	Y	+	+	+
DLFPa 8-12 2	Y	+	++	+
DRFPa 8-12 4	Y	0	0	+
DRFPa 16-20 2	N	+	++	+

0 = fluorescence less than or equal to negative control, + = fluorescence more than negative control and less than positive control and ++ fluorescence equal to positive control.

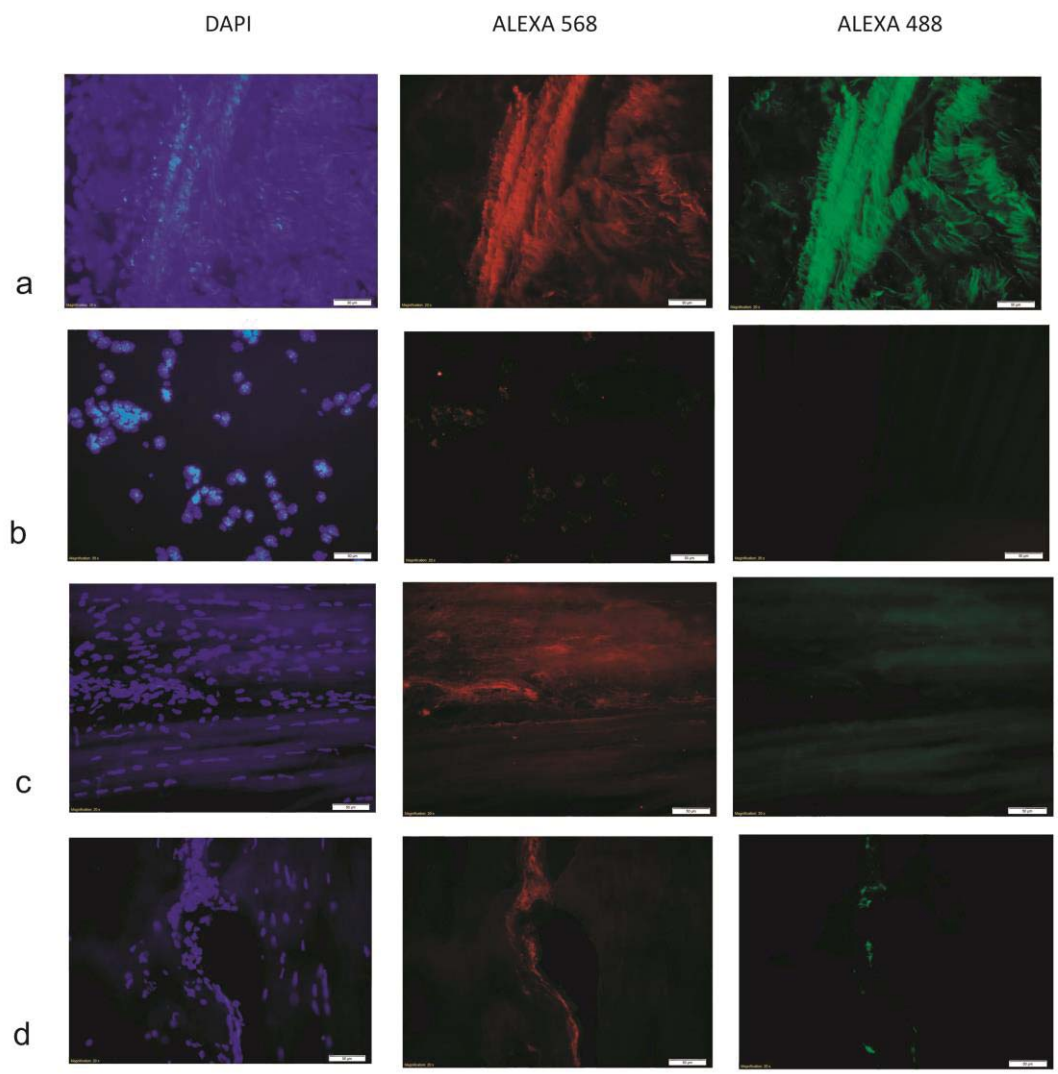


Figure 22 - Examples of immunofluorescent staining for procollagen
DAPI stained nuclei (blue); procollagen III with Alexa 568 secondary (red) and procollagen I with Alexa 488 secondary (green). Bar on images = 50 µm
a) Positive control horse skin wound – the staining shows cell nuclei and procollagen I and III are present in the wound.
b) Negative controls of mouse brain – cell nuclei are present but no procollagen I and III.
c) Example of positive staining for nuclei; some staining for procollagen III and minimal staining for procollagen I.
d) Example of sparse but positive staining for procollagen I and III.

4.4.5 LIMITATIONS

The horses in this study had a relatively wide age range and therefore this could have increased the inter-horse variation. Horse B was 11 years old and would therefore be expected to have some underlying chronic micro trauma within the tendon although these were not detectable on clinical or ultrasonographic examination nor on histological sections. Ideally, young untrained horses would have been used to minimise the risk of chronic micro trauma. The horses were also unshod making accurate

detection of lameness challenging and this would not have been ideal had the horses been followed for a longer period.

Ultrasonography was used as a screening tool to confirm no significant underlying pathology existed in the tendons although as the study showed, this is a relatively insensitive tool. Computer assisted tomography may have been more sensitive but required general anaesthesia and would have been significantly more costly.

The short study period allowed investigation of the response to acute tearing injury in the core of the superficial digital flexor tendon. It would have been ideal to have sufficient horses to follow some out to a longer time point to determine if the lesion healed in a similar fashion to clinical injury.

4.4.6 CONCLUSION

- The aim of this study to create a lesion in the core of the equine SDFT that would mimic the acute phase of tendon injury was achieved.
- Furthermore, the lesion proved technically easy to create in standing sedated horses and resulted in minimal post-operative discomfort.
- The study showed that even in the hands of experienced equine surgeons grossly apparent tendon lesions could prove difficult to detect on ultrasound examination and to confirm histologically.
- New collagen was detectable in lesions less than or equal to four days old and type III collagen was the predominant procollagen detected in these lesions.

The use of this model has the potential to improve the understanding and investigation of tendon injury and healing.

CHAPTER 5

EVALUATING THE ROLE OF CONNEXIN43 IN WOUND HEALING USING A CELL CULTURE SYSTEM.

5.0 INTRODUCTION

The equine superficial digital flexor tendon is an energy-storing tendon that is commonly injured in equine athletes and has similarities to the human Achilles tendon (Firth, 2000). The major problems arising from tendon injury are the slow rate of tendon healing and poor biomechanical properties of the healed tendon (Birch et al., 2008). Tendon fibroblasts are responsible for collagen and extra-cellular matrix production and therefore are largely responsible for tendon healing. The low tendon fibroblast numbers in adult tendon and specialisation for anaerobic metabolism and therefore for efficient locomotion limit the ability of these cells to effectively respond to injury and therefore the ability of tendon to heal (Stanley et al., 2007). In horses, healing is further complicated as they remain standing most of the time (David et al., 2012), thus their injured tendons are under constant and varying load.

Tendon fibroblasts produce different types of collagen. The mechanically superior collagen type I is produced in normal healthy tendon and in injured areas of tendon that have undergone effective remodelling. Collagen type III is produced during repair in

injured tendons and if it is not remodelled persists as a fibrous scar (Smith et al., 2002). The reduced ratio of type I to type III collagen in healed tendon is thought to be a considerable component of the predisposition of tendons to re-injury (Kannus et al., 1997).

Connexin43 gap junctions are one of the two types of gap junction linking fibroblasts in the same tendon fibril and also fibroblasts on different fibrils (Ralphs et al., 1998). Gap junctions aid intercellular communication including the spread of cell death signals in injured tissue (Cusato et al., 2003). Uncoupling the gap junctions in injured tissue may reduce this effect and also aid in cell migration to close the defect (Wright et al., 2009), thus modulation of gap junction expression may enhance tissue repair.

Cell culture models have been established to investigate the effect of modifying connexion 43 gap junctions on healing cells. Modulation of connexion 43 gap junction action, by mimetic peptides for example, has been shown to improve the rate of scrape-wound healing in cultured human dermal fibroblasts (Wright et al., 2009). Consequently cell culture studies were undertaken to evaluate the following hypothesis: Modulation of gap junction communication in cultured equine superficial digital flexor tendon-derived fibroblasts would improve the rate of scrape-wound healing and would increase the ratio of production of collagen type I to collagen type III.

The aim of these studies was to:

- Determine the effect of modulation of gap junction communication on superficial digital flexor tendon-derived fibroblasts *in vitro*.
- Determine the effects of modulation of gap junction communication on rate of closure of a scrape-wound as a measure of healing potential.

- Determine the effects of modulation of gap junction communication on the type of collagen produced as an indicator of biomechanical strength.
- Measure collagen production and secretion into the media and deposited on the cell monolayer using the Sircol™ assay.
- This assay requires low serum media that does not support good tendon fibroblast growth therefore cell doubling rates in three specialised low serum media were therefore measured to determine the best media to use in the scrape-wound experiment.
- Develop a method to reproduce uniaxial load in cell culture that mimicked the *in vivo* situation.

5.1 MATERIALS AND METHODS

5.1.1 SOURCE OF TENDONS FOR CELL EXTRACTION

The thoracic limb superficial digital flexor tendons of thoroughbred horses 2-5 years old were used as a source of tendon fibroblasts. Preparative studies were performed on cells obtained from thoroughbred horses of any age undergoing euthanasia at Massey University Veterinary Teaching Hospital (Palmerston North, New Zealand) or the local hunt clubs for a reason unrelated to the thoracic limb superficial digital flexor tendon. The cells for the data generating studies were from thoroughbred horses between 2-5 years old, with no history of tendon injury, undergoing humane slaughter for human consumption at an abattoir in the South Island of New Zealand. Thoracic limb superficial digital flexor tendon tissue was collected and cells extracted as described (Appendices 18 to 20).

5.1.2 CELL CULTURE

Unless stated otherwise all cells were grown in DMEM with 4.5 mg/mL glucose containing 100 U/mL penicillin/100 μg/mL streptomycin and 10% FBS and incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Cells were passaged when 70-90% confluent using the technique described in Appendix 21. Once the initial extracted cell stock had expanded to fill 4 x 175 mL tissue culture flasks they were prepared for freezing as described in Appendix 22 and then placed in a commercial isopropyl alcohol freezing container (Mr Frosty 5100 freezing container, Thermo scientific, NY, USA), transferred to a -80ºC freezer for 24 hours prior to storage either in the same freezer or in liquid nitrogen. The freezing container cooled the cells at -1 ºC per minute to optimise cell survival.

5.1.3 METHOD DEVELOPMENT FOR CELL CULTURE - STATIC STUDIES

5.1.3.1 EVALUATING DIFFERENT SUBSTRATES FOR CELL CULTURE

Equine superficial digital flexor tendon-derived fibroblasts were seeded onto glass coverslips, plastic coverslips or tissue culture plastic at a density of 70 x 10^4 cells/mL of medium. Cell culture plastic was used as the positive control when comparing cell adhesion and growth on commercial and laboratory made collagen gels. Uncoated glass coverslips were used as a negative control. A silastic elastomer (RTV) had been used to create a dish for loading cells in culture. The intention was to use RTV dishes in the loading experiments. Fibroblast growth on RTV was an unknown, although previous studies (Neidlingerwilke et al., 1994) on the same material had used collagen-coating. The cells were examined subjectively at 24 and 48 hours. As collagen gel coating is

recommended for growth of tendon fibroblasts on glass coverslips (Burrows, 2009) methods to prepare and evaluate fibroblast growth on collagen were developed.

5.1.3.2 EXTRACTING COLLAGEN FROM RAT AND HORSE TENDONS.

Collagen for coating coverslips, prepared using an adaptation of the method described by Bornstein (Bornstein, 1958), can be purchased commercially. Collagen is freeze dried and shipped in aliquots as a powder requiring reconstitution (Appendices 23 and 24).

A protocol for making collagen gel from rat tail tendons was adapted from the methods described by Ehrmann and Gey, and Goslin (Goslin, 1991, Ehrmann and Gey, 1956). The full method is provided in Appendix 25**.**

An experiment was performed to determine if collagen gel could be made from horse limb (Appendix 26**)** or tail tendon (Appendix 27). The major adaptations to the rat-tail method were the increased amount of time required for the horse tail tendon to soften and an increased concentration of acetic acid solution required to soften the tendon. It was not possible to extract collagen from horse superficial digital flexor tendon using this method.

5.1.3.3 COLLAGEN GEL COMPARISONS

A comparison of commercial rat tail collagen (6-10 μg/cm^2), laboratory-made rat tail collagen gel and laboratory-made horse tail collagen gel was undertaken to determine which of these collagen substrates were preferred by the equine tendon fibroblasts. The laboratory-made collagen was gelled using riboflavin at 1:2 and 1:8 ratios of riboflavin: collagen and these were also compared for cell growth. Growth on tissue culture plastic was a positive control while glass was used as a negative control.

5.1.3.4 FINAL COLLAGEN COATING PROCEDURE

Laboratory-made rat tail collagen was used for most of the pilot studies until it stopped gelling at approximately 18 months post extraction. No reason was identified for this although a problem with the riboflavin was excluded by replacing it with fresh stock. After this time, commercial collagen was used at 20 µL per 13 mm coverslip and the gel was reconstituted and gelled as per the manufacturers' recommendations for the main studies (Appendix 25).

5.1.3.5 DETECTION OF COLLAGEN

5.1.3.5.1 Herovici Stain for Collagen

Herovici polypichrome stain was used as described (Herovici, 1963) to stain confluent monolayers of cells on collagen-coated glass coverslips and collagen-coated RTV (see Dynamic Studies 5.1.4) and also used to stain scrape-wounded cell monolayers. With Herovici stain, the new collagen appears blue and mature collagen red. The stain was tested to determine whether new collagen deposited along the scrape-wound during repair could be detected.

Table 10 - Dilutions of primary antibodies evaluated to identify optimal titres for immunofluorescent staining of procollagen I and procollagen III.

Cells present	Scrape Wound	Dilution of primary antibody to aminopropeptide for collagen type I	Dilution of primary antibody to aminopropeptide for collagen III	Fluorescent Staining		
				COLL 1	COLL 3	DAPI
NO	NO	1:20	1:40	-ve	-ve	-ve
NO	NO	1:80	1:120	-ve	-ve	-ve
YES	NO	1:20	1:40	+ve o/e	+ve	+ve
YES	NO	1:80	1:120	+ve	+ve	+ve
YES	NO	NO	NO	Broken		
YES	YES	1:20	1:40	+ve o/e	+ve o/e	+ve o/e
YES	YES	1:80	1:120	+ve	+ve	+ve o/e
YES	YES	No primary antibody	No primary antibody	-ve	-ve	+ve
YES	YES	1:20	1:40	+ve o/e	+ve	+ve o/e
YES	YES	1:80	1:120	+ve	+ve	+ve o/e
YES	YES	1:20	NO	+ve	-ve	+ve
YES	YES	1:20	1:40	+ve	+ve	-ve*
YES	YES	1:80	1:120	Broken		
Tissue						
Mouse brain	N/A	1:20	1:40	-ve	-ve	+ve o/e
Mouse brain	N/A	1:80	1:120	-ve	-ve	+ve o/e
Skin wound	N/A	1:20	1:40	+ve	+ve	+ve
Skin wound	N/A	1:80	1:120	+ve	+ve	+ve o/e

Staining of some scrape wounded cultures was performed to determine if antibody titres needed to be different in these cultures compared with unscraped cultures.
o/e = over exposed, * = incorrect mounting, +ve = positive, -ve = negative and N/A = not applicable.
Control tissues below double line.

5.1.3.5.2 Procollagen Antibodies

Immunofluorescence (Appendix 15) was used to determine the presence of collagen types I and III in cell culture using a method adapted from Stanley (Stanley et al., 2007). The controls for collagen I and III propeptides had been identified during sheep and horse immunohistochemistry (Chapters 3 and 4). The horse skin wound was a positive control for both collagens I and III propeptides and mouse brain was a negative control. A series of dilutions of the antibodies were tested (Table 10) and the final dilutions and antibodies used are shown (Table 11). Unseeded, collagen-coated coverslips were stained to check for cross-reactivity of the antibodies to the collagen gel coating. Coverslips without cells were stained to check for cross-reactivity to the collagen gel coating. Once confluent the cell monolayers were scraped on day 1, 2 or 3 and all were fixed and stained on day 4.

Secondary antibodies were used at 1:500 dilutions. No cell, mouse brain and no primary negative controls and horse skin wound positive control.

Table 11 - Immunofluorescent antibodies

	Primary	**Secondary**
Coll1	Monoclonal mouse anti –procollagen Type I amino-propeptide (M38 National Hybridoma Bank, Iowa,USA)1:80	AlexaFluor488 conjugated goat anti-mouse (A11001, Invitrogen, NZ)
Coll3	Polyclonal rabbit anti-procollagen Type 3 amino-propeptide (Novotec, France)1:120	Alexa 568 Goat anti-rabbit (A 21069, Invitrogen, NZ)
Connexin43 monoclonal	Anti-Connexin43 mouse monoclonal (MAB3068, Chemicon) 1:125 dilution in 5% Normal goat serum (633827A Invitrogen, NZ)	AlexaFluor488 conjugated goat anti-mouse (A11001 Invitrogen, NZ)

Nuclei were stained using mounting medium Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI P36935, Invitrogen, New Zealand) unless otherwise stated. Superficial digital tendon derived fibroblasts nuclei were stained with 0.1% propidium iodide when staining fixed cells for Connexin43.

5.1.3.5.3 Sircol™ Assay for Collagen

A commercial collagen assay (Sircol™, LabTek, Australia) was performed as per the manufacturer's instructions (Appendices 28 and 29) to determine the total collagen released into the media by confluent equine superficial digital flexor tendon-derived fibroblasts and those that were scrape-wounded.

The optimal collagen concentration range that can be detected using this method is 5-25 µg/mL. Samples with ≤ 5 µg/mL of collagen in the media required an overnight concentration step to optimise detection of the collagen, see Appendix 28.

5.1.3.5.4 Effect of Media Serum Concentrations and Substrate

Experiments were performed to determine if it was possible to detect collagen from collagen-coated RTV dishes (see Section 5.3.3.3) or collagen coated tissue culture plastic as this could cause experimental error in the Sircol™ assay. The effect of using a medium containing phenol red and of serum concentrations was also assessed. The medium used was DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and the appropriate FBS concentration and was compared with water with or without FBS. The media were collected after 24 hours of contact with either collagen-coated tissue culture plastic or collagen-coated silastic/RTV for the pilot (Appendix 30).

5.1.3.5.5 Effect of Specialised Media and Time of Exposure on Collagen Detection

In initial studies, cells that had been scrape-wounded and exposed to mimetic peptides in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS exhibited more cell death than expected. Media containing less than or equal to 5% FBS were required if the total collagen in the media from the experiments were to be measured using the Sircol™ assay (Appendix 28). Thus, specialised media aimed at supporting cells in a low serum environment were assessed for suitability for use in the Sircol™ assay. Advanced™ DMEM and Opti-MEM™ each containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS were compared with DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS. Media were collected from confluent cell monolayers grown on tissue culture plastic at 18, 24, 36 and 72 intervals hours and frozen until assayed (Appendix 31).

5.1.3.6 MEASUREMENT OF CELL GROWTH

Cells in these media were also assessed for cell growth using the xCelligence system (Roche Diagnostics NZ Ltd, Auckland). The xCelligence system measures electrical impedance using specialised 96 well plates. Impedance is increased by an increase in cell adhesion or cell spreading and can be used to monitor cell viability, cell morphology, cell numbers and adhesion (Limame et al., 2012). This study measured cell numbers over time to compare the proliferation of the cells from the 9 tendons in three different media. Only 9 of the 10 cell populations grown from the tendons could be used due to plate constraints. The culture with the slowest growth at this point was omitted.

Equine superficial digital flexor tendon-derived fibroblasts from each of 9 tendons were re-suspended in either Opti-MEM™ containing 100 U/mL penicillin/100 µg/mL

streptomycin and 5% FBS, Advanced™ DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS or DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS. Because of space limitations, cells from one tendon only, were also suspended in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS to evaluate response to the standard concentration of FBS used for growing the fibroblasts. To set the baseline, 100 µL of medium was added to each well of the specialised 96 well plate, placed into the reader and the software protocol run according to manufacturer's instructions. The cells were added as a suspension in 100 µL of medium to achieve a final density of 2 x 10^4 cells/ mL. The plate was then placed in the xCelligence reader and incubated for 72 hours at 37ºC with 5% CO_2. Specialised software measured cell doubling rates and generated plots representing cell expansion.

5.1.3.7 DEVELOPMENT OF THE SCRAPE-WOUNDING TECHNIQUE

5.1.3.7.1 Final Scrape-Wounding Technique

Following pilot studies (Appendix 32), sterile P10 pipette tips were used to create a scrape-wound in the centre of a monolayer of equine superficial digital flexor tendon-derived fibroblasts grown on 13 mm, collagen-coated glass coverslips in the well of a 24 well plate.

5.1.3.7.2 Identification of Sampling Intervals for Scrape-Wounds

Scrape-wounding experiments for measurement of wound closure and cell migration rates were based on adaptations to a previous method described by Wright. (Wright et al., 2008). Initial studies were performed to determine the rate of wound closure and appropriate time points for measurement in equine superficial digital flexor tendon-derived fibroblasts.

5.1.3.7.3 Final Sampling Interval

No significant changes were detected in the first 12 hours in the first scrape-wounding experiment with the addition of mimetic peptides. Images were therefore collected at 12, 36, 54 and 72 hours (T_{12}, T_{36}, T_{54} and T_{72}) aiming to reduce the negative effects that time out of the incubator had on the scrape-wound closure.

5.1.3.7.4 Imaging Scrape-Wounds

It was noted during the pilot scrape-wound experiments that it was difficult to ensure consistency of the site of the width measurements, particularly with the dishes being moved between incubator and microscope and with the growth and migration of the cells altering the appearance of the wound. To address this multiple images were collected at 20x magnification using a Leica DMIL microscope, with Leica DFC290 camera (Leica, NZ). These covered the entire scrape-wound and were combined in Xara (Xara Extreme Pro, Xara Group Ltd, Hemel Hempstead, UK) to create a single image of the wound. The area of the wound was then masked in black and exported as a .TIFF file. A purpose written plug-in to detect the black area was used in ImageJ **(**ImageJ software, National institute of Health, Maryland, USA) to measure the wound area (Figure 23, Figure 24, Figure 25 and Appendix 33).

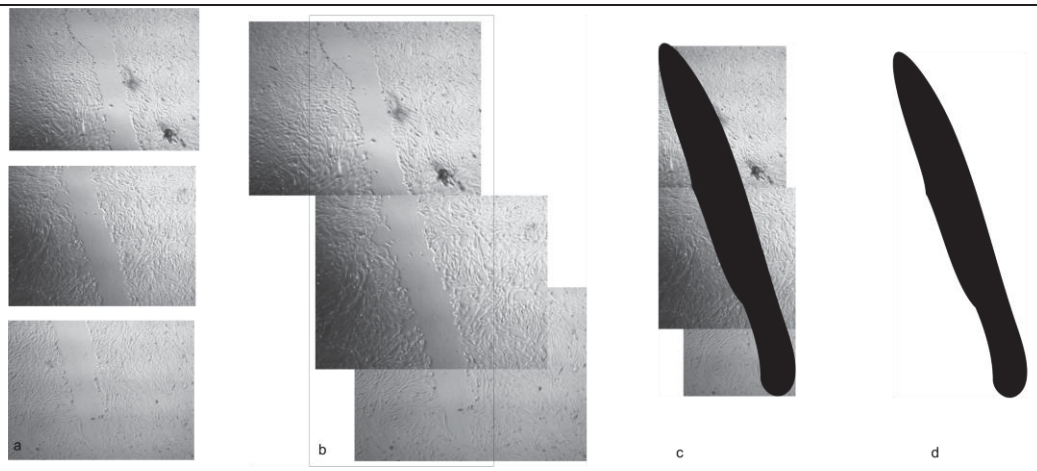


Figure 23 - Pictorial representation of the processing of the scrape-wound images.
a) Individual photographs were combined to create whole scrape-wounds at each time point
b) The wound margins were traced and the area of the wound masked.
c) The masked wound area was enclosed in a rectangle (same size for that well across time)
d) The cell background was removed and the rectangle containing the masked wound area was exported as a .TIF file

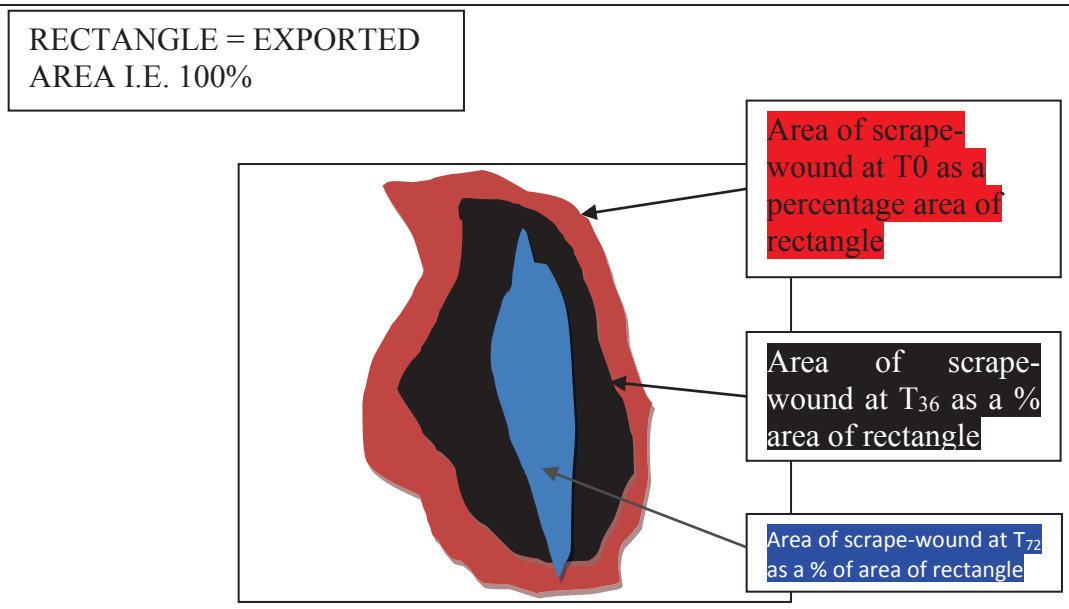


Figure 24 - Method for measuring scrape-wound closure.
Percentage changes in area from time 0 to 36 hours given by (area at time 0- area at time 36 hours/area at time 0) X 100/1
Percentage changes in area from time 0 to 72 hours given by (area at time 0- area at time 72 hours/area at time 0) X 100/1

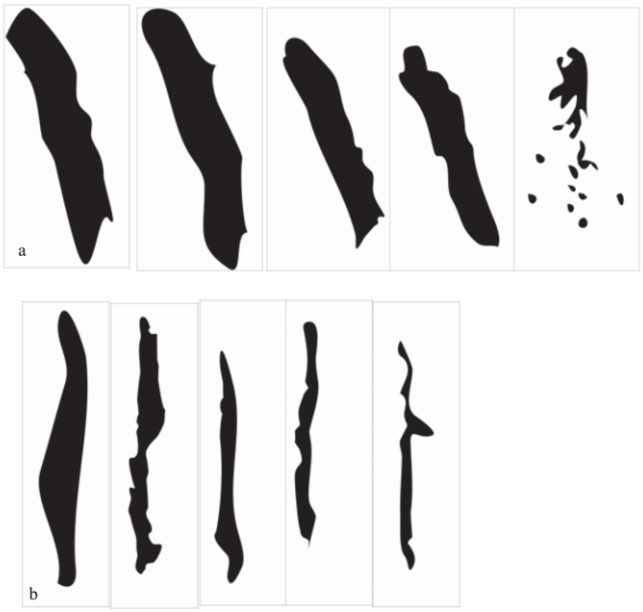


Figure 25 - Pictorial representation of scrape-wound closure in a monolayer of fibroblasts.
Images from left to right were collected at times 0, 12, 36, 54 and 72 hours. Series a) and b) are examples from two different horses.
a) Well 1A2
b) Well 6A4

5.1.3.8 CONNEXIN43 EXPRESSION/COMMUNICATION IN CULTURE

The immunofluorescent staining method for Connexin43 in equine tendon fibroblasts was adapted from Stanley (Stanley et al., 2007) and the slides were viewed using a confocal laser scanning microscope (CLSM Leica SP5 DM600B, Leica Microsystems, Germany) (Appendices 3.7 and 3.8). See Appendix 15 for the protocol and Table 11 for details of antibodies. The effects on Connexin43 expression by Connexin43 antisense oligodeoxynucleotides, sense oligodeoxynucleotides or Connexin43 mimetic peptides were assessed in cell culture.

5.1.3.9 MODULATION OF CONNEXIN43 IN CULTURE

Two methods were used to modulate Connexin43 in cell culture:

1. Antisense oligodeoxynucleotides to block expression

2. Mimetic peptide to block function

5.1.3.9.1 Antisense Oligodeoxynucleotides

Once the equine superficial digital flexor tendon derived fibroblasts were confluent, the media were removed and cells washed with DMEM containing no serum. Antisense oligodeoxynucleotides or sense oligodeoxynucleotides at final concentrations of 2 or 10 μM / mL, in DMEM with no serum, were incubated with the cells for 2 hours after which time the cells were fixed and stained for Connexin43 using immunofluorescent-labelled antibodies.

5.1.3.9.2 Determination of Solubility of Pluronic Gel in Tissue Culture Medium.

As pluronic gel is a recognised delivery vehicle for sense or antisense oligodeoxynucleotides in tissues, an experiment was performed to determine if pluronic gel dissolved in media or if the concentration of FBS in the media altered solubility. The pluronic gel was prepared as described in Appendix 3. Three x 2 mm diameter spots of pluronic gel were placed in each of 6 x 35 mm petri dishes and media added as indicated in Table 12.

Table 12 - Pluronic gel in media.

DISH	MEDIA	FBS	VOLUME	RESULT
1	NO	NO	0	INTACT
2	NO	NO	0	INTACT
3	CMEM	NO	0.5	DISSOLVED
4	CMEM	NO	1.5	DISSOLVED
5	CMEM	10%	0.5	DISSOLVED
6	CMEM	10%	1.5	DISSOLVED

CMEM= DMEM 100 U/mL penicillin/100 μg/mL streptomycin FBS = Fetal calf serum

5.1.3.9.3 Mimetic Peptides

Peptide5 (H-Val-Asp-Cys-Phe-Leu-Ser-Arg-Pro-Thr-Glu-Lys-Thr OH VDCPLSRPTEKT a kind gift from Prof Colin Green, Auckland) corresponding to an extracellular loop of Connexin43 was used at 10 and 500 µM / mL.

5.1.3.9.4 Mimetic Peptide Efficacy Assays

The blockade of gap junction communication by 500 µM / mL mimetic peptide was assessed using the Lucifer Yellow scrape loading method (Becker) (Appendix 34).

The ability of 10 µM / mL mimetic peptide to block hemi-channels was assessed using the low calcium propidium iodide influx assay (O'Carroll et al., 2008) (Appendix 35).

5.1.3.9.5 Final Scrape-Wounding and Mimetic Peptide Protocol

Cells, at passage 3-4, were plated at a density of 50 $x10^4$ cells/ mL onto collagen-coated (20 µL) 13 mm glass coverslips and grown to 80% confluence in DMEM containing 10% FBS. Media were removed, the monolayer washed with DMEM containing100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS and replaced with 200 µL of DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS with or without mimetic peptides at either 10 or 500 µM / mL concentrations. A minimum of 4 coverslips/ treatment group/ horse were prepared. At 90 minutes after addition of the mimetic peptides, a scrape-wound was made in the centre of all the coverslips using a P10 pipette tip. Images of the wound at 10x magnification were taken immediately after wounding and at 12, 36, 54 and 72 hours post-wounding. The media were replaced every 18 hours. A single image encompassing the whole wound area was created for each coverslip at each time point and the area measured as described in final imaging analysis section (5.2.5.4). The media on the cells were collected immediately prior to wounding and at 18, 36 and 54 hours following wounding and stored at -20ºC

for subsequent measurement of total collagen content. At the completion of the experiment the coverslips were fixed in paraformaldehyde prior to fluorescent antibody staining.

5.1.3.9.6 Statistics for Cell Wounding Assay

Repeated measures ANOVA (Dohoo et al., 2003) were used to determine if there was a significant effect of treatment on wound closure. This statistical test was selected as being appropriate for analysing data collected on the same unit over time.

5.1.4 DYNAMIC STUDIES

With the assistance of Mr Bruce Cann (Technical Facilitator, IVABS, Massey University, New Zealand) and the staff of the Mechanical Workshop of Institute of Fundamental Science (IFS, Massey University, New Zealand), a method was developed to apply uniaxial load to a tissue culture dish at strains and rates comparable with those measured in horses superficial digital flexor tendon at all gaits (Appendix 36). A silastic elastomer (RTV) dish was designed modifying a method described by Neidlinger-Wilke & Claes (1994). The final mould produced a dish with 0.75 mm thick sides and 1.3 mm thick ends. A mechanism was designed to apply a uniaxial load to the RTV dish. The mould was attached to the loading rig at each end using magnets. The loading rig could generate strains of 0-20% and was connected to a 12 V, 1.8 degree stepper motor to enable application of the load at speeds of 1-5 Hz. Once dishes of similar appearance could be made consistently, three were used for materials testing. The dishes underwent 2100 cycles at 10% strain and 1 Hz and the lengths of the dishes at cycle 0 and cycle 2100 were compared to determine if creep occurred with repetitive loading. The dish length was measured with a digital vernier calliper and the mean of three measurements recorded for each dish.

To determine if ‘waisting’ occurred and if there was a difference between the strain on different parts of the dish, precision lines were drawn on three of the dishes using a 0.5 mm roller ball and a vertical milling machine (Model 2VS, Maximart, Taiwan) with digital readout (Model NV300M, Fagor, NZ Figure 26). Video recordings were made of the three dishes under cyclical strain using the stepper motor with a 13.48 mm dot adjacent to the disc for scaling. Image capture was used to capture frames at the maximal and minimal strains. Image J (ImageJ software, National institute of Health, USA) was used to put a profile across the images and the distances between the peaks were measured to determine the size of the gaps between lines. This was used to determine if there was a change in distance between the lines in response to strain and if this was equal along and across the dish. Materials testing of the developed culture dish identified significant dish to dish variation in properties therefore this study was discontinued pending further development.

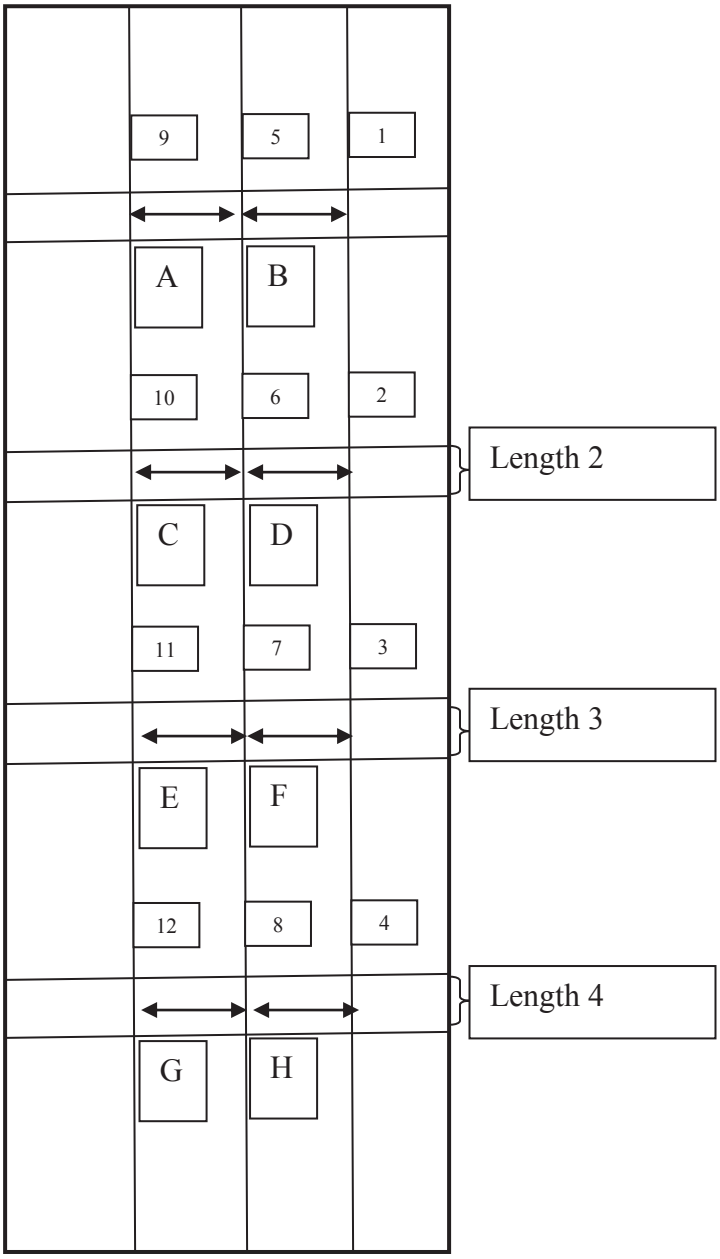


Figure 26 - Diagram of RTV dish and measurement positions.

5.2 RESULTS AND DISCUSSION

Protocols were developed to investigate the response of scrape-wounded equine superficial digital flexor tendon-derived fibroblasts in culture to modulation of Connexin43 gap junction communication. Cell doubling comparisons in specialised media containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS determined that DMEM containing 4.5mg/mL of glucose was the preferred growth

medium and identified cells from two out of ten tendons that grew more slowly than the others tested. Commercial and laboratory-made collagen gels were identified as the preferred substrates for tendon fibroblast growth. Consistent scrape-wounds were made using P10 pipette tips and image collection every 12 hours was optimum to measure closure rates without having a negative effect on cell survival. Anti-sense oligodeoxynucleotides did not alter Connexin43 gap junction numbers when added to equine superficial digital flexor tendon-derived fibroblast cultures. Connexin43 mimetic peptides affected hemi-channel opening and gap junction communication in tendon fibroblasts but did not alter fibroblast migration nor cell death. Multiple techniques to determine collagen levels were investigated but did not prove quantifiable in this system. An assembly was created to apply load to tendon fibroblasts in culture and the deformable RTV dish was tested for material properties.

5.2.1 DEVELOPMENT OF PROTOCOLS FOR STATIC STUDIES

5.2.1.1 DIFFERENT SUBSTRATES FOR CELL CULTURE

There was subjectively more growth of tendon fibroblasts on any surface coated with collagen gel. Cells grew on the horse and rat tail collagen gels, reaching confluence in 48 hours. Subjectively, there was no difference in cell density on rat tail collagen gel when using the 1:8 or the 1:2 ratio of riboflavin to collagen. In the partially coated RTV and culture dishes, there was clear demarcation of cell growth between the coated and uncoated surfaces. In all cases, the coated substrate area had obviously more growth than the uncoated area (Figure 27).

These results confirmed that equine superficial digital flexor tendon derived fibroblasts preferred collagen gel as a substrate on which to grow in comparison with glass, tissue

culture plastic or RTV. Of all collagen substrates tested, the laboratory-made rat tail collagen was the most effective at supporting cell growth. It is possible that the 'toughness' of the equine tendon did not allow sufficient collagen to be released into the acid solution to make a suitable gel. Due to the high concentration of acetic acid required to extract any collagen from the horse tendon there may have been a residual acetic acid contamination in the gel that made it less compatible with cell growth. The differences between the commercial and laboratory-made rat tail collagen gels tested were subjectively assessed and not markedly different so were thought to be of little consequence. Commercial collagen was used once the laboratory-made collagen stopped gelling, approximately 18 months after preparation. All final experimental results reported were from cells grown on the commercial rat tail collagen-coated glass coverslips.

As early as the 1900s glass was considered unsatisfactory as a substrate for tendon fibroblast growth (Ehrmann and Gey, 1956). Collagen fibres had been used as a cell culture substrate but were limiting as the cells had to grow along the fibres, this prompted the investigation of collagen gel as a cell culture substrate (Ehrmann and Gey, 1956). This early study reported that all of the cell types tested, apart from cells from frog skin, had improved growth on the collagen gel in comparison to glass and is consistent with the current study. It was interesting that of the three cell types with the most apparent improvement in growth, two were fibroblast cell lines and the third were HeLa cells. Uncoated or collagen-coated tissue culture plastic as well as collagen-coated glass have been used to grow equine superficial digital flexor tendon-derived fibroblasts (Burrows S, 2008), which is consistent with the current study. There was little difference between commercially prepared rat-tail collagen gel and that prepared in the laboratory. There are no reports of collagen from horse tendons being used as a

potential substrate despite the intuitive idea that horse fibroblasts may prefer a substrate from their own species. Alternative extraction and purification steps may improve the suitability of the horse tail collagen gel as a tissue culture substrate although availability and cost may prevent common use.

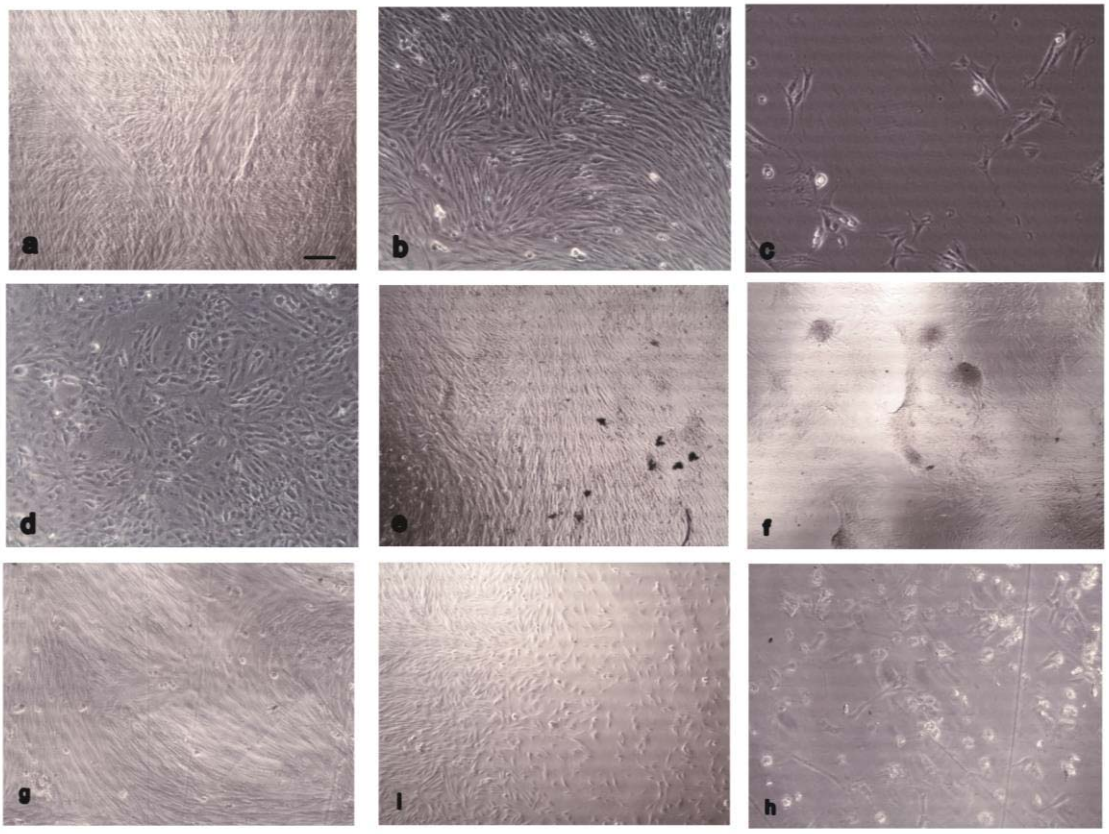


Figure 27 - Growth of equine superficial digital flexor tendon derived fibroblasts on different substrates.
48 hours post seeding at 3.6 x 105 cells/500 µL media. (Bar = 250 µm)

a) Rat tail collagen-coated glass coverslip with many fibroblasts attached
b) Horse tail collagen-coated glass coverslip with moderate fibroblast attachment
c) Glass with poor attachment and growth
d) Tissue Culture plastic with many fibroblasts attached
e) Commercial collagen-coated glass coverslip with many fibroblasts attached
f) Commercial collagen with fibroblasts stacking
g) Coated RTV
h) Uncoated RTV
i) Demarcation between many fibroblasts on the collagen-coated RTV on the right and the few fibroblasts on the uncoated RTV on the left.

5.2.1.2 SCRAPE-WOUND TECHNIQUES

5.2.1.2.1 Scrape-Wounding and Imaging

Scoring of the underlying culture substrate during scrape-wounding could influence the ability of the tendon fibroblasts to close the wound. Therefore, it was important to determine which method of scrape wounding was preferable. Pipette tips proved to be the best means to make the scrape-wound and this is consistent with reported data (Wright et al., 2009, Maffulli et al., 2000). The ideal sized tip depended on the size of the wound required and in this study when photographing the scrape-wounds at 20x magnification the P10 pipette tip produced a wound with a width less than the visual field of the microscope. It was therefore easy to image the entire wound over 2-4 visual fields and combine the images to create a single collated image encompassing the complete scrape-wound. This was important as the appearance of the wounds changed rapidly and when using width measurements the determination of the exact site of previous measurements was problematic.

5.2.1.2.2 Identification of Sampling Intervals for Scrape-Wounds

A repeated measures general linear model with a Bonferroni post hoc test identified the appropriate sampling intervals from the second pilot study on scrape wounding. The times with significant changes in wound width were approximately 3, 9, 22 and 47 hours to the nearest hour (Appendix 32).

In the final experiment a minimum of 12 hours between imaging reduced the time the culture dishes were out of the incubator. This was important because the incubator environment maintains the cells at 37^{o}C, 5% CO_2 in a humidified atmosphere and the microscope used for imaging did not have an environmental chamber to replicate those conditions. Therefore, during imaging the tendon fibroblasts were exposed to a

temperature of 20^{o}C for 30-40 minutes each time the plate was removed from the incubator. Cells will tolerate a reduction in temperature better than an increase; however, the reduction of temperature of the media also results in a reduction in pH. In this situation the buffers in the media should have compensated for this (Kramer et al., 2010). Most normal fibroblasts grow well between pH 7.4 and 7.7 and buffers in the culture medium equilibrate with atmospheric CO_2 such that an increase in CO_2 results in a lower pH (Freshney et al., 2007). Room air contains less CO_2 (approximately 394 ppm) than the culture incubator (50,000 ppm) and the phenol red in the media changes colour as the pH alters. Phenol red in DMEM is purple at pH 7.8, pink at 7.6, red at 7.4, orange at 7.0 and yellow at 6.5. The cultures changed from orange to pink the more time they were out of the incubator.

Cells in culture prefer low oxygen tensions and this is partially recreated with the addition of CO_2 to the air in the incubator. Obviously the addition of 5% CO_2 is only going to drop the percentage of oxygen by 5% but this is nearly 25% of the total oxygen (21%) in average room air (Kramer et al., 2010). In room air, the oxygen tension is higher and may result in more free radical damage to the cells. Oxygen tolerance may be provided by serum and control of oxygen tension is more critical when low or serum free media are used (Freshney et al., 2007). Tendon cells normally exist in a hypoxic environment and the fewer capillaries and thicker endothelial walls present at sites prone to injury in adult people are thought to be a contributory factor to hypoxic damage (Tuite et al., 1997). This is supported by histological evidence that hypoxic damage is a major component of tendon rupture in people (Jozsa and Kannus, 1997). Culturing of tendon cells in a more hypoxic environment may have made them more tolerant to other manipulations. The ideal oxygen and other gas tensions for tendon fibroblast growth is an area of tendon culture that has not been reported and

warrants investigation. Cell culture conditions that mimic *in vivo* conditions closely must always be the gold standard.

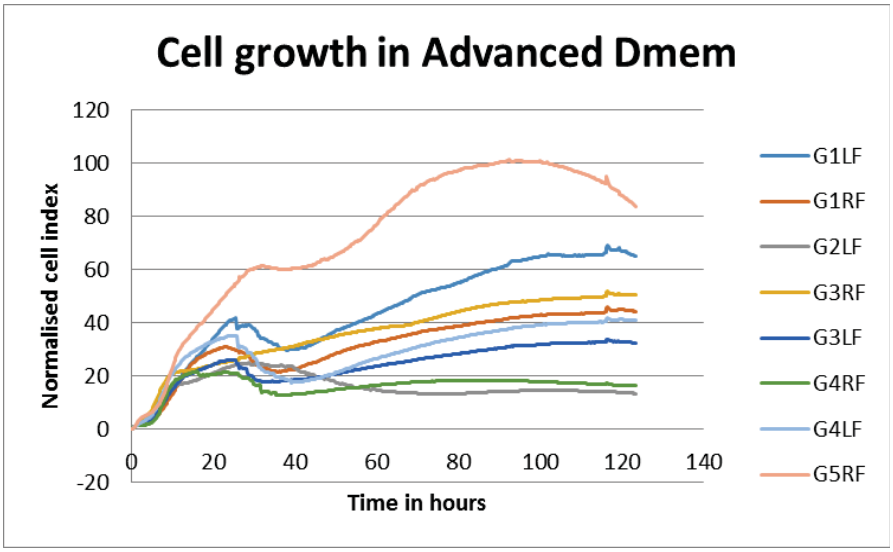
The use of DMEM supplemented with only 5% FBS was required for the Sircol™ assay for total collagen measurement. This low serum media changed the response of the tendon fibroblasts considerably with many lifting off and dying after 5 days rather than closing the scrape-wound. This is inconsistent with data reported by (Wright et al., 2012).

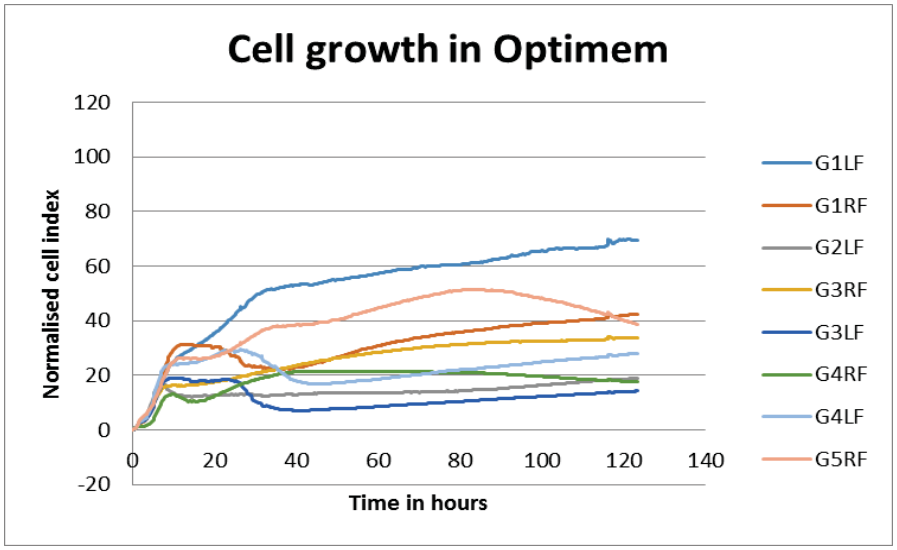
Oxygen tolerance is influenced by serum and therefore control of oxygen tension is likely to be more critical in low serum media (Freshney et al., 2007). Changes in oxygen tension during imaging and the metabolic demands of dividing in a low serum environment may well have been factors contributing to the cell death (Freshney et al., 2007) experienced in the initial mimetic peptide experiment. This prompted the investigation to identify the optimum low serum media to support cell growth and proliferation for these studies. Advanced™ DMEM and Opti-MEM™ both containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS are specialised media designed for low serum situations (Web Link 2). Cell growth in these media were compared to cell growth in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS using the xCelligence™ instrument and software (Appendix 37).

5.2.1.3 MEASUREMENT OF CELL GROWTH IN DIFFERENT MEDIA

Data obtained from the cell growth experiment showed a difference in doubling rates between equine superficial digital flexor tendon-derived fibroblasts grown in different media with DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS giving more uniform growth than the specialised media. Whilst the media had an

effect, a greater difference was apparent between the fibroblasts from different tendons. Fibroblasts from the left thoracic limb superficial digital flexor tendon from horse G2 (G2LF) and left thoracic limb superficial digital flexor tendon from horse G3 (G3LF) showed much poorer growth in all of the media tested in comparison to the fibroblasts from the other tendons (Figure 28, Appendix 38).





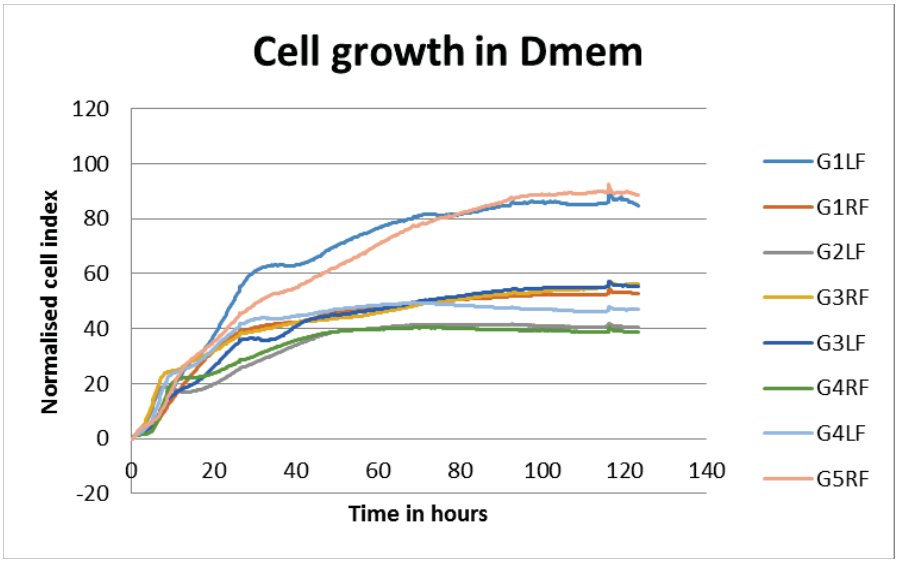


Figure 28 - Graphs of comparison of cell growth from different tendons in each of the three media

Cells were extracted from the left and right superficial digital flexor tendons of each of the five horses. Of the 10 tendons cultured, the nine chosen for the cell growth study were those with better growth at the time of seeding. The fibroblasts from G2LF were not included in the cell growth study as they had the poorest growth at the time of seeding. Interestingly G2RF exhibited the poorest doubling rate of the cells tested. This suggests an *in vivo* cause. Within horse variations were apparent as well as between horse variations. The superficial digital flexor tendon fibroblasts from different tendons in the same horse G3LF and G3RF had different growth rates. This is likely to be a result of *ex vivo* and *in vitro* factors whereas between horse variations could be a component of *in vivo* differences as well.

Potential *in vivo* causes of differences in cell proliferation include genetic background, developmental factors such as diet and training and the presence of existing lesions within the tendon. *Ex vivo* influences would include collection technique and extraction protocol whilst *in vitro* factors such as expansion and cell culture environment are cell specific and fundamental for good cell survival.

Although the 2 year old colts were New Zealand thoroughbreds, the specific breeding was not available as they were unbranded. There is a reported gender predilection for superficial digital flexor tendon injury in racehorses in Japan with entire males being at greater risk than geldings or mares (Kasashima et al., 2004). In Hungary, blood group type O was found to be more highly represented in people with tendon ruptures than in the general population (Jozsa et al., 1989). There are over 30 blood groups in horses although only 8 are major systems. The most important are A and Q with approximately 85% of thoroughbreds being positive for Aa antigens and 61% for Qa antigens. No association between blood groups and tendon injury have been identified in horses,

however the high risk groups such as racehorses are likely to have the same blood group.

Sequence variants of the gene for Tenascin and the collagen V α 1 are associated with Achilles tendinopathies in people (Mokone et al., 2005, Mokone et al., 2006). A number of genetically determined factors have been identified as affecting the occurrence of Achilles tendon injury (September et al., 2006). It is therefore reasonable to expect that other, as yet unidentified, genetic factors closely involved with tendon structure and function also have an effect on the susceptibility of tendons to be damaged as well as their ability to repair. This is an area warranting further investigation in horses although genetic testing of affected individuals may be controversial.

The two year-old colts from which the superficial digital flexor tendon-derived fibroblasts used for these cell culture experiments were extracted had been raised under the same conditions and had similar stature and body condition at euthanasia with no gross evidence of undernourishment or developmental abnormalities. They were unhandled and in New Zealand such young thoroughbreds would have most likely have been managed at pasture (Morel et al., 2007). As the horses were young and unbroken, subclinical degeneration due to exercise was unlikely to be present. Ideally, clinical palpation and ultrasound examination of the tendons would have been performed prior to collection but this was not practicable.

The tendon collection technique was identical and performed under the same conditions for all tendons retrieved from the abattoir. The prolonged time between tendon collection and cell extraction would have resulted in survival of the more robust cells. This is discussed further in the section on collection and would have been similar for all the tendon derived cells. The main difference between individual tendons was time from

collection to extraction (between 12-15 hours), as the tendon collection technique was faster than the extraction technique. This did not appear to affect cell doubling time based on the results of the cell growth study using the xCelligence system (see Section 5.1.3.6). Tendons were processed in order of collection and there were no trends observed between cell growth and collection time. Tendon fibroblasts that grew well when seeded continued to grow well throughout expansion and after cryopreservation.

A reduction in superficial digital flexor tendon cellularity, collagen production and gap junction quantities in tendon has been identified by comparing tendons from foals (1d-1 month) and those for young adult horses (2-5 years) (Young et al., 2009). The range of cell counts between the individuals within the young adult group in this study was narrow so the tendons collected would be expected to have similar cell numbers and be comparable to young adults.

The cell growth experiment used media containing 5% FBS to be consistent with conditions appropriate to the Sircol™ assay for the determination of total collagen content of the media. The cell growth rates were therefore not directly comparable to the final mimetic peptide and scrape-wound protocol in which DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS was used. Variations in cell doubling rates were observed between the tendon-derived fibroblasts from different horses as well as in different media. The fibroblasts from all tendons grew more quickly in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS than in either of the specialist low serum media tested. The xCelligence system has reportedly less intra-assay variability and is more sensitive than the conventional colorimetric 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide XTT assay in the evaluation of cell viability (Karaca et al., 2012). Electrical impedance is measured using microelectrodes integrated into the

bottom of specially engineered tissue culture plastic. This provides quantitative information about the status of cells, including cell number, cell adhesion, cytotoxicity, cell viability, and cell morphology (Web Link 1).

Whilst superficial digital flexor tendon derived fibroblasts grew faster in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS compared to DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 5% FBS in general culture, cells from one tendon only (G5LF) were cultured with 10% FBS in the cell growth experiment so no meaningful comparisons can be made. The reduced serum concentration in the media was clearly important but not the sole reason for altered fibroblast growth rates.

5.2.2 MODULATION OF CONNEXIN43 IN CULTURE

Modulation of Connexin43 gap junction communication has been shown to improve healing in a number of tissues (Mori et al., 2006, Qiu et al., 2003) using either down-regulation of Connexin43 gap junction expression or functional blockade of the gap junctions. Down-regulation of Connexin43 expression using Connexin43 antisense oligodeoxynucleotides has been successfully used in a number of wound models *in vivo* (Coutinho et al., 2005, Qiu et al., 2003, Mori et al., 2006). The Connexin43 antisense oligodeoxynucleotides are rapidly broken down by serum and therefore they are administered in tissues within a pluronic gel which breaks down in a concentration-dependent rate (Becker, 1999) and therefore prolongs exposure. In this study the amount of serum in the media did not affect the dissolution of the pluronic gel and as expected the pluronic gel dissolved over a 1 hour period.

5.2.2.1 USE OF ANTISENSE OLIGODEOXYNUCLEOTIDES TO REDUCE CONNEXIN43 EXPRESSION

Equine superficial digital flexor tendon derived fibroblasts exposed to Connexin43 antisense oligodeoxynucleotides were expected to show reduced expression of Connexin43 gap junction proteins. There was no difference in Connexin43 staining between the antisense oligodeoxynucleotide-treated fibroblasts and the sense oligodeoxynucleotides-treated controls (Figure 29). This suggested that unmodified antisense oligodeoxynucleotides were not effective at reducing Connexin43 expression in superficial digital flexor tendon fibroblasts when directly added to media. Therefore, antisense oligodeoxynucleotides were not effective in this system.

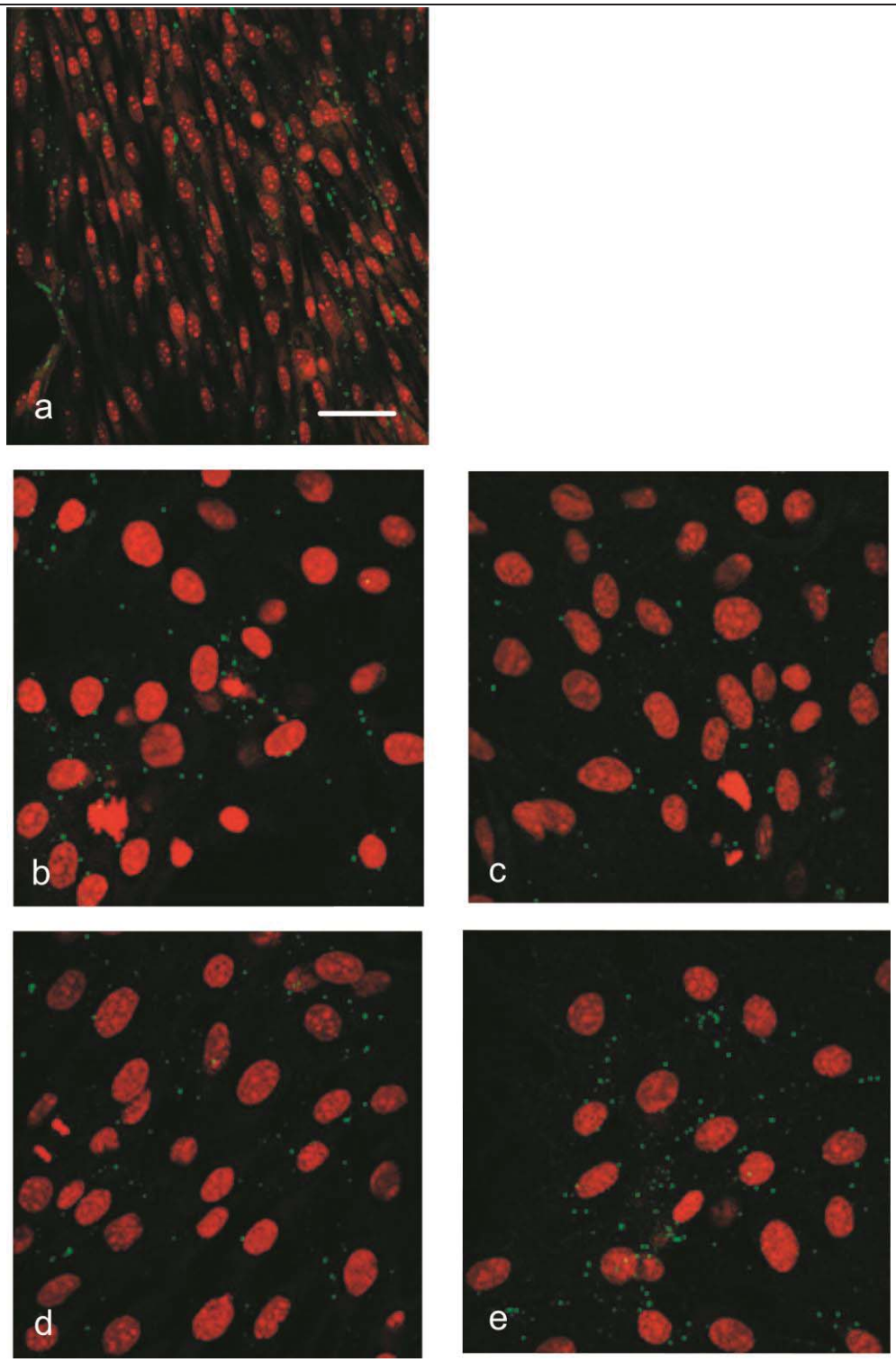


Figure 29 - Response of equine superficial digital flexor tendon-derived fibroblast to antisense oligodeoxynucleotides.

Fibroblasts stained with monoclonal antibody for Connexin43 and detected using Alexa 488 goat anti-mouse secondary antibody (Green). Cell nuclei counter-stained with propidium iodide (Red). Cultures were examined and imaged using confocal scanning laser microscopy. (Image bar a = 100 μm and b-e = 25 μm)

a) Untreated
b) Exposed to 2 μM sense oligodeoxynucleotides for 2 hours immediately prior to staining.
c) Exposed to 2 μM antisense oligodeoxynucleotides for 2 hours immediately prior to staining
d) Exposed to 10 μM / mL sense oligodeoxynucleotides for 2 hours immediately prior to staining
e) Exposed to 10 μM / mL antisense oligodeoxynucleotides for 2 hours immediately prior to staining

The effect of serum on antisense oligodeoxynucleotides is of particular importance when considering the use of antisense oligodeoxynucleotides as a treatment in tendon injury. A component of tendon injury is haemorrhage and serum exudation into the area of injury where the antisense oligodeoxynucleotides would need to be injected (Ross and Dyson, 2003). Pluronic gel has been described as a delivery mechanism for unmodified antisense oligodeoxynucleotides to combat the problems of stability and means of delivery (Cronin et al., 2006) and was therefore tested in the cell culture system used here.

The 24% pluronic gel tested had dissolved within 1 hour in all of the dishes containing media but not in the dishes without media. There was no apparent difference in response to the inclusion of FBS in the media. The lower volume (0.5 mL) of media did not fully cover the pluronic gel spots whereas the 1 mL was satisfactory (Table 12). This confirmed that the pluronic gel dissolves slowly in the face of culture media as has been reported previously. If the gel was to be used as a vehicle for treatment a sustained release action could be achieved over the time the gel took to dissolve and this could be tailored to the specific requirement by altering the gel concentration (Cronin et al., 2006). Results reported in Chapter 3 suggesting that the injection of pluronic gel in tendons may be inflammatory indicate that this delivery mechanism would require further investigation prior to use *in vivo*.

Waggett (2006) had shown successful action of antisense oligodeoxynucleotides on tendon fibroblasts in culture (Waggett et al., 2006) but as this required the use of a transfection agent it would not be clinically practical. These combined findings indicated that the use of antisense oligodeoxynucleotides for gap junction modulation might not be ideal in injured equine tendons and other methods of gap junction

modulation required investigation. These data led to experiments on the effect of mimetic peptides on superficial digital flexor tendon derived fibroblasts.

5.2.2.2 USE OF CONNEXIN43 MIMETIC PEPTIDES TO ALTER HEMI-CHANNEL AND GAP JUNCTION FUNCTION

Mimetic peptides block gap junction communication by binding to specific extra-cellular domains and either interfering with assembly of functional gap junctions or by having a blocking effect on the channel (Evans and Boitano, 2001). Wright (2008) showed an improvement in scrape-wound closure and increased cell migration of human dermal fibroblasts exposed to Connexin43 mimetic peptides *in vitro* using Gap27 mimetic peptide. As tendon cells are fibroblasts they have the potential to respond in a similar manner. Also, previous studies on spinal cord injury in sheep have shown a response to Connexin43 mimetic peptide peptide5 at doses from 5 to 500 μM/mL. The lower doses prevented Connexin43 hemi-channel opening without interfering with intercellular gap junction communication and higher doses blocked gap junction communication (O'Carroll et al., 2008). Spinal cord injury shows spread of the injury following the initial trauma, as does tendon. In addition peptide5 reduced cell death and swelling (O'Carroll et al., 2008) so implied peptide5 could be useful for tendon fibroblasts. Functional blockade of Connexin43 gap junction communication using Connexin43 mimetic peptides was therefore explored as another potential method for modulation of Connexin43 gap junction effects on tendon fibroblast healing.

5.2.2.3 MIMETIC PEPTIDE EFFICACY ASSAYS

Peptide efficacy tests confirmed that the Connexin43 mimetic peptide used, peptide5, functioned as expected; with the 10 μM/ mL dose blocking hemi-channels and the 500 μM / mL dose uncoupling gap junctions (Figure 30 and Figure 31). Propidium iodide

penetrates only dead cells and once in the cell stains nuclear proteins and does not pass through gap junctions. Propidium iodide can pass into the cytosol, and then into the nucleus, of living cells via open hemi-channels which can be induced to open in response to low calcium (O'Carroll et al., 2008). In cells exposed to low calcium and propidium iodide all the cells should take up the propidium iodide. In cells exposed to low calcium and propidium iodide treated with 10 μM/ mL Connexin43 mimetic peptides only a few dead cells take up the stain as the low dose of mimetic peptides blocked the hemi-channels.

There was minimal PI staining of the nuclei of tendon fibroblasts in a low calcium influx study treated with 10 μM / mL peptide5 whereas the positive control tendon fibroblasts were all stained with PI. This confirmed the action of 10 μM / mL peptide5 to block hemi-channel opening in calcium depleted fibroblasts and is consistent with other research (O'Carroll et al., 2008) (Figure 30).

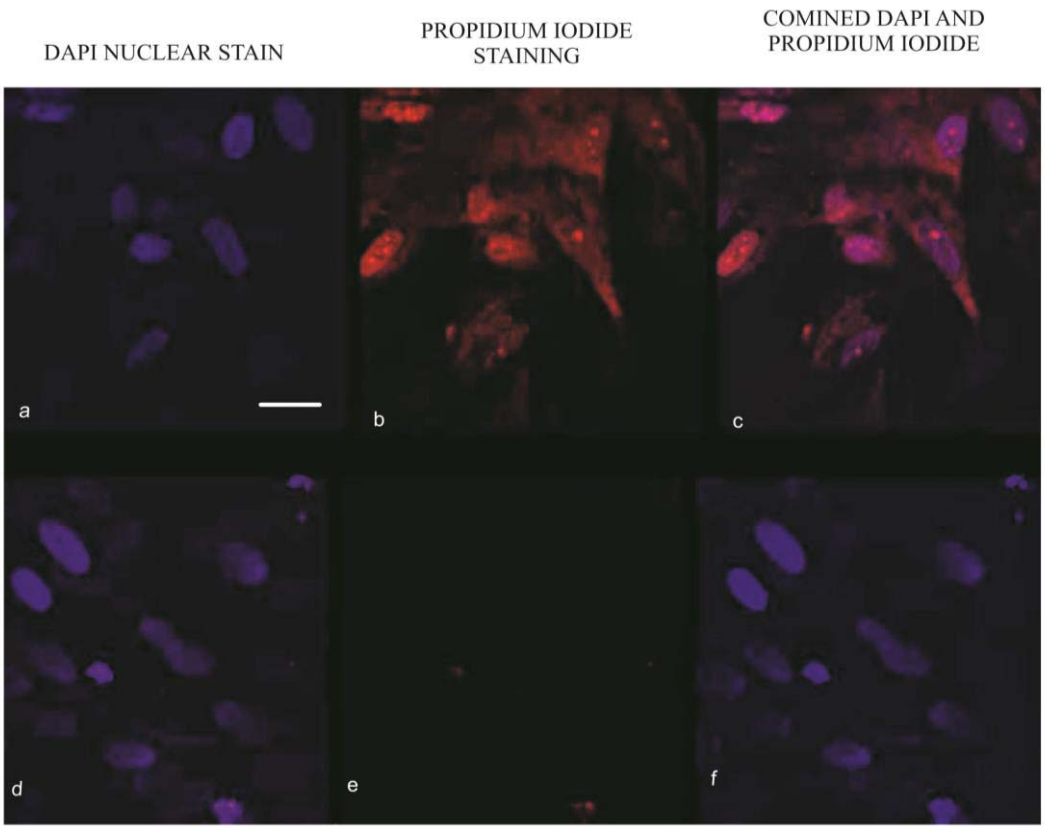


Figure 30 - PI influx into equine superficial digital flexor tendon-derived fibroblasts.
Under low calcium conditions without mimetic peptides, 0.1% propidium iodide (PI) enters living cells via open hemi-channels and stains the inside of the cells red. The nuclei are counter-stained blue with DAPI. Images a-c are of cells exposed to PI under low calcium conditions and without mimetic peptides. Images d-f are of cells are exposed to 10μM Connexin43 mimetic peptide for 30 minutes prior to exposure to the PI under low calcium conditions. These cells show no PI staining as expected as PI should not enter the living cells when the hemi-channels are closed. Imaged using confocal scanning laser microscopy and a 63 times air objective. (Bar = 25 μm)

The stain Lucifer Yellow cannot pass through intact cell membranes but once inside a cell can pass between cells via gap junctions. In the technique called scrape loading, a needle is used to damage a small group of cells in a monolayer culture in the presence of Lucifer Yellow and propidium iodide. The Lucifer Yellow enters the damaged dying cells and passes rapidly through the gap junctions from these cells to the other cells in the culture (Becker). When gap junction communication is blocked, for example by anaesthetics, the Lucifer Yellow is confined to the damaged, propidium iodide stained cells and those immediately adjacent to them (Mantz et al., 1993).

The transfer of Lucifer Yellow from scrape loaded cells was blocked by the 500 μM / mL dose of peptide5, but not by the 10 μM / mL dose (Figure 31). This result confirmed

that peptide5 at 500 μM / mL prevented intercellular Connexin43 gap junction communications; consistent with previous findings (O'Carroll et al., 2008).Therefore the experimental system appeared to be suitable for the assessment of mimetic peptides on wound closure.

Ideally, the control assays should have been carried out at the same time as the experiments in which the effect of mimetic peptides on wound closure was being assessed. As the timeline in Appendix 39 shows, it was not possible for one person to carry out the scrape-wound protocol and the efficacy assays simultaneously. These efficacy assays were carried out a week prior to the scrape-wound study, used the same tendon fibroblasts, albeit one passage earlier, and the same batch of peptide5 mimetic peptide. To carry out the assay simultaneously with the scrape-wound experiment would have required multiple personnel introducing inter-observer variation.

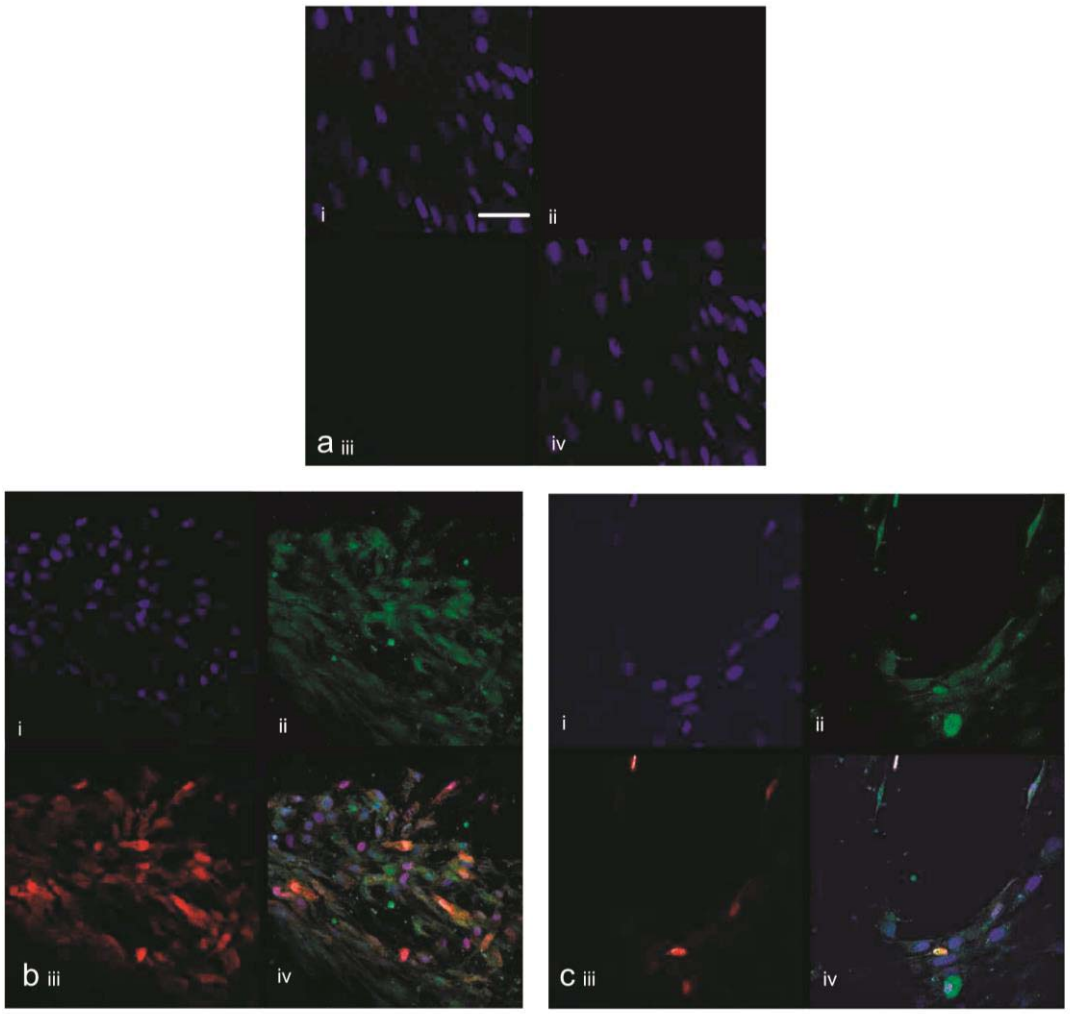


Figure 31 - Lucifer Yellow/Propidium Iodide scrape loading and response to mimetic peptides.
Imaged using confocal scanning laser microscopy and a 63 times air objective. (Bar = 25 µm)
(i) DAPI (blue), (ii) Lucifer Yellow (green), (iii) Propidium iodide (red), and (iv) Merged.
a) Control cells stained with DAPI and not scrape loaded.
b) 10 µM / mL mimetic peptide treatment and scrape loading shows dispersion of Lucifer Yellow throughout cells.
c) 500 µM / mL mimetic peptide treatment and scrape loading shows Lucifer Yellow restricted to scrape loaded and immediately adjacent cells.

5.2.2.4 FINAL SCRAPE-WOUNDING AND MIMETIC PEPTIDE PROTOCOL

The results show that the scrape-wounds closed in all cultures regardless of treatment or not with peptide5 (Figure 31 and Appendix 40). Either the lack of effect of treatment could have been due to the mimetic peptide not functioning as predicted, or that peptide5 at the doses used did not improve scrape-wound healing in this cell type. Fibroblasts from different source tendons responded differently to the treatments, cells from G1LF and G3LF showing inconsistent results between wells (Appendices 41 and 42).

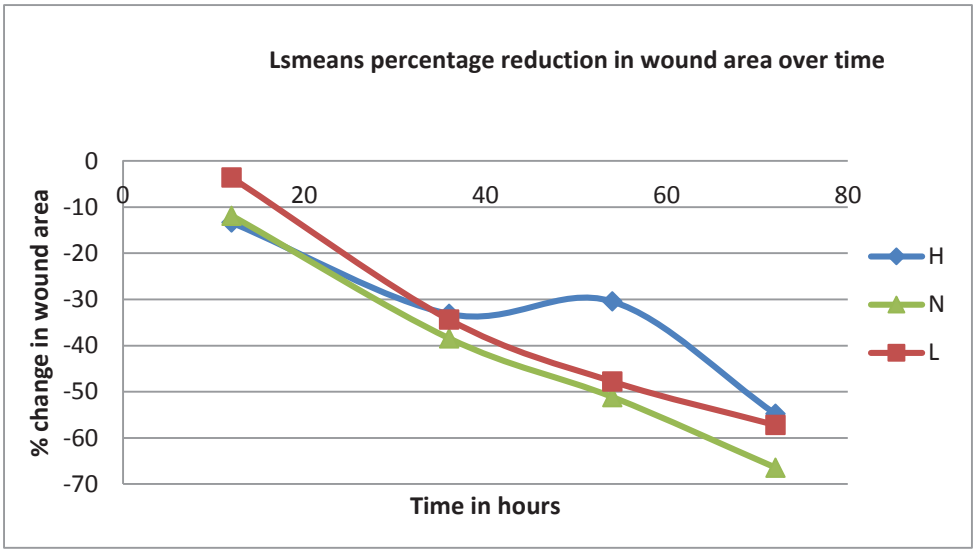


Figure 32 - Reduction in the size of the scrape-wound area.
Over a total of 72 hours with 54 hours of exposure to H (high 500µM), L (low 10 µM / mL) and N (no mimetic peptides). Each data point is the mean of three wells. (Lsmeans = Least squared means).

Peptide5 did not have an obvious effect on cell migration in equine superficial digital flexor tendon-derived fibroblasts. This may be explained by recent work suggesting the effects Connexin43 gap junctions have on cell migration are via the cytoskeleton rather than the cell membrane as was originally thought (personal communication David Becker, University College London.). Mimetic peptides are directed against the membrane or extracellular domains of the Connexin43 gap junction (Figure 33) so if taken up by the cell would be unlikely to have an effect as they would not encounter appropriate binding sites.

The previously reported positive effects of mimetic peptide on dermal fibroblast migration involved Gap27, which contains amino acids LFII from the membrane regions (Wright et al., 2009). Gap27 and peptide5 share the SRPTEKT sequence with peptide5 having the extra-cellular loop amino acids VDCFL in the place of the membrane region amino acids (Table 13).

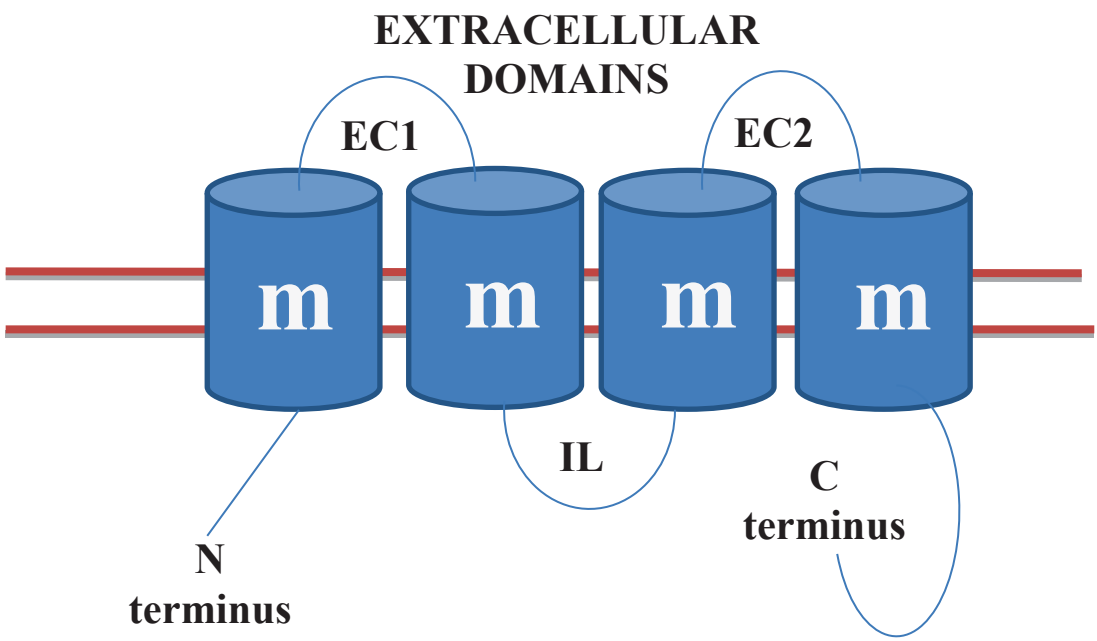


Figure 33 - Schematic of a connexin molecule with the N terminal region.
The membrane spanning domains (m); the extracellular domains (EC1 and EC2) the intracellular domain (IL) and the C terminus. Adapted from (O'Carroll et al., 2008).

Table 13 - Mimetic peptide sequences and the areas of the connexin protein with which they correspond.

EC1	LGTAV**ESAWGDEQSFRCNTQQPGCENVCYDKSFPISHVR**FWVLQ
EC2	FEVA**LLIQWYIYGFSLSAVYTCKRDPCPHQVDCFLSRPTEKT**LFII
Peptide 5	**VDCFLSRPTEKT**
Gap27	**SRPTEKT**LFII

Amino acids in the extracellular loops are in **bold** and those in the membrane domains are underlined. (O'Carroll et al., 2008)

Peptide5 had been shown to be more effective than Gap27 in reducing inflammation in wounded sheep spinal cord *ex vivo* (O'Carroll et al., 2008) and therefore was chosen for the current study as it was known to be effective in sheep. The positive effects on the sheep spinal cord may have been due to an anti-inflammatory effect (O'Carroll et al., 2008) and the prevention of vascular leakage, which could not have occurred in this scrape-wound study carried out on cultured cells. Goodenough (1996) stated that mimetic peptides including membrane domains inhibited gap junctions more effectively

and this may have applied to superficial digital flexor tendon fibroblasts and therefore Gap27 may have been a better choice of mimetic peptide to improve cell migration.

One oversight was the omission of a scrambled peptide control in the current study. The control used simply omitted peptides, but ideally, scrambled peptide should be used to control for the effects of adding a peptide to the media. The untreated control tendon fibroblasts were not significantly different from the treated tendon fibroblasts, therefore simply adding peptide to the media did not appear to have a measureable effect. A possible choice for a scrambled peptide is the peptide 40Gap27 SRPTEKNVFIV which does not alter Connexin43 gap junction coupling in rat aortic endothelial cells (Martin et al., 2005) or peptide8 GDEQSAFRCNTQQ did not alter spinal cord swelling in the *ex vivo* sheep model (O'Carroll et al., 2008). Scrambled peptides may contain shared sequences with active peptides without the appropriate binding domains or may be very different (Martin et al., 2005).

5.2.3 COLLAGEN DETECTION

5.2.3.1 HEROVICI

Herovici is a polypichrome stain technique that colours mature collagen red and newly formed collagen blue (Lillie et al., 1980, Herovici, 1963). The positive controls (Figure 34g-h) for chronic tendon injury showed red staining of the mature collagen and blue staining in the injury area consistent with new collagen production. There was no obvious staining of the commercial rat tail collagen gel substrate by this method; whilst the laboratory made rat tail collagen stained blue/red. Only very fine strands of blue could be seen in scrape-wounded cultures examined 4 days after injury. This staining was difficult to distinguish from the surrounding cells and therefore not quantifiable

(Figure 34a-f). Immunofluorescence techniques that result in a more distinct colour and can distinguish between collagen types were therefore preferred.

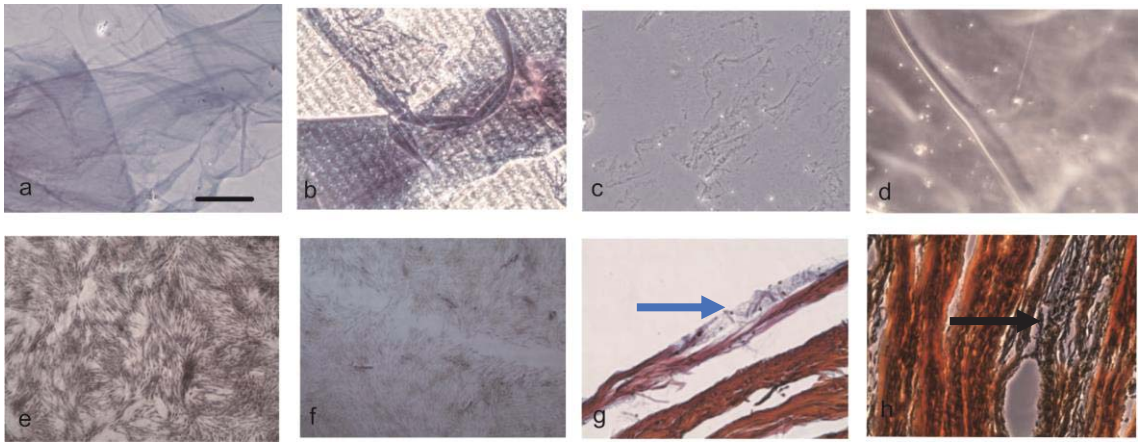


Figure 34 - Herovici staining of collagen substrate and equine SDFT derived fibroblasts (Bar = 250 µm).

a) Staining of laboratory-made rat tail collagen gel on a glass coverslip, not seeded with cells showing pale blue tinge to the substrate.
b) Staining of laboratory-made rat tail collagen gel on RTV elastomer, not seeded with cells. This highlights the refraction issues encountered when trying to image this material using transmitted light microscopy.
c) Commercial rat tail collagen gel on a glass coverslip, not seeded with cells had no blue uptake.
d) Commercial rat tail collagen gel on RTV, not seeded with cells. Further refraction issues demonstrated.
e) Equine SDFT derived fibroblasts seeded onto commercial rat tail collagen with no visible uptake of stain.
f) Scrape-wounded equine SDFT derived fibroblasts seeded onto commercial rat tail collagen with no visible uptake of stain.
g) Injured equine tendon 4 days post injury showing in blue new collagen on the edge of the tendon (blue arrow).
h) Staining of sheep tendon 4 hours post injury showing in blue new collagen between fibres (black arrow).

5.2.3.2 IMMUNOFLUORESCENT DETECTION OF COLLAGEN PROPEPTIDES

Collagen propeptides are cleaved to allow fibril formation and the levels of collagen type I and type III propeptides are likely to be indicative of the production of these types of collagen (Labens et al., 2007) . A number of control tissues were examined. There was minimal fluorescent staining of the collagen propeptides in the sections of adult horse tendon, consistent with mature collagen tissue not undergoing injury or repair. Staining of the neonatal tendon was visible although this was predominantly for the Type I propeptide. The skin wound stained for both collagen type I and type III propeptides and is consistent with normal wound healing (Figure 35). This wound was therefore a useful control for immune detection of type I and III collagen propeptides. Significant collagen production in cell culture was more likely to be difficult to detect

with immunofluorescence therefore cell culture positive controls had a risk of producing false negative results.

Collagen I and Collagen III propeptide staining was apparent in the cytoplasm of cultured cells adjacent to the scrape-wound but quantification would have been difficult using this method. Few obvious collagen fibres were detected in the cultures unlike in the skin wound. This is probably because 85% of the collagen produced is secreted into the media, which is replaced every 48 hours (Waggett et al., 2006).

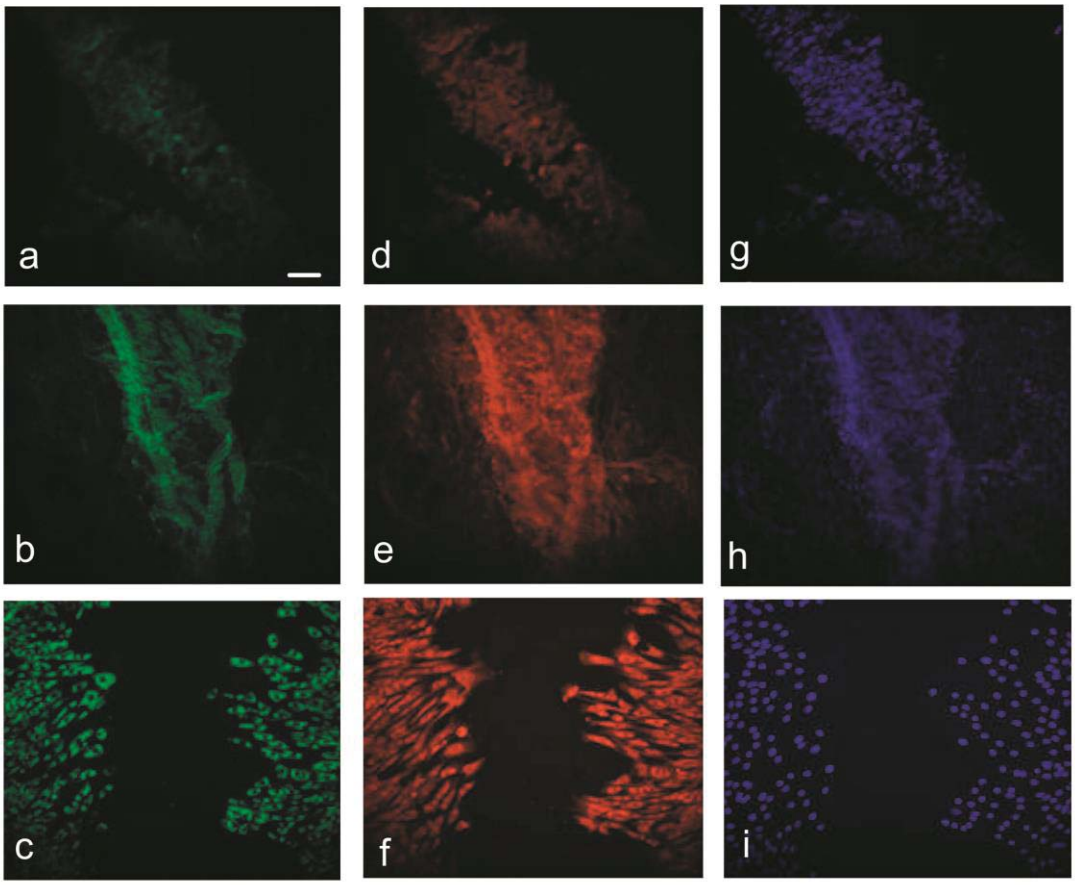


Figure 35 - Fluorescent detection of collagen type I and III propeptides.
Green is Alexa 488 goat anti-mouse conjugated to Monoclonal mouse anti –procollagen Type I amino-propeptide. Red is Alexa 568 goat anti-rabbit secondary conjugated to Polyclonal rabbit anti-procollagen Type 3 amino-propeptide. Blue is DAPI in mountant for live cell nuclear staining.
a, d and g mouse brain negative control, b, e and h skin wound positive control and c, f and i scrape-wound in monolayer of equine superficial digital flexor tendon derived fibroblasts. (Bar = 250 μm)

Quantitative reverse transcriptase polymerase chain reaction was considered as an alternative method for assessing collagen gene expression (Young et al., 2009). As expression would have altered in only a small proportion of cells, a change in the ratio of expression of collagen types I and III was considered likely to be below the levels of accurate detection.

Paraformaldehyde fixation of the scrape-wounded tendon fibroblasts in the mimetic peptide treated cultures resulted in a total absence of fluorescent staining. Previous cultures of equine superficial digital flexor tendon derived fibroblasts that had been scrape-wounded, such as above, had been fixed in cold methanol and stained immediately. The mimetic peptide-treated tendon fibroblasts could not be stained and viewed immediately due to closure of the microscopy suite over Christmas. It is possible that the paraformaldehyde cross-linked the terminal propeptides thereby interfering with antibody binding.

5.2.3.3 DETECTION OF TOTAL COLLAGEN USING THE SIRCOL™ ASSAY

Collagen secretion into the media is at a low level and close to the detection limit of the Sircol™ assay Appendix 28.

Table 14 - Sircol™ assay to determine effects of culture conditions on absorbance reading.

	FBS Concentration	Absorbance at 560nm	Collagen (µg/L)
Water	0	0	0
Water/RTV	0	0	0
DMEM	0	0	0
DMEM/RTV	0	0	0
WATER/SERUM	1	0	0
WATER/SERUM	2.5	0.57	3.68
WATER/SERUM	5	0.84	5.43
WATER/SERUM	10	1.80	11.69
RTV/DMEM/SERUM	1	0.42	2.69
RTV/DMEM/SERUM	2.5	0.86	5.59
RTV/DMEM/SERUM	5	1.45	9.36
RTV/DMEM/SERUM	10	3.55	23
DMEM/SERUM	1	0.1	0.64
DMEM/SERUM	2.5	0.38	2.44
DMEM/SERUM	5	1.35	8.77
DMEM/SERUM	10	3.49	22.57

Each reading is the mean of 4 different wells exposed to the same solutions.

Concentrations of serum above 1% in the water and serum groups gave absorbance's suggesting there was collagen in the media (Table 14). This is likely to be due to cross-reactivity of a serum component with the test and confirms the manufacturers' recommendation that low serum containing media should be used. The effects of the higher concentrations of serum were increased when combined with DMEM containing phenol red as the culture media. This clarified how much serum and phenol red would impact the detection of collagen in the culture media. Exposure of the water to the RTV did not appear to affect the assay.

Standard DMEM containing 1%penicillin/streptomycin and 5% FBS, Opti-MEM™ containing 1% penicillin/streptomycin and 5% FBS and Advanced DMEM containing 1%penicillin/streptomycin and 5% FBS were evaluated for use in the Sircol assay. Known concentrations of collagen were added to aliquots of each of the media, from which a standard curve was constructed and against which the amount of collagen in the samples could be assayed. Media were collected at 18 hour intervals as this was when the media would need to be refreshed in the mimetic peptide experiments. Even using an overnight concentration step, there was insufficient collagen in any media that had been exposed to the tendon fibroblasts for 18 h for detection using the Sircol™ assay (Table 15, Appendix 31). This suggested that the Sircol™ assay was not a useful means of measuring total collagen in the mimetic peptide experiment. Subsequently in this study, it was shown that insufficient collagen was released into the media during 18 hours of cell culture to be detected by the Sircol™ assay.

Table 15 - Collagen Concentration in Media from Equine Superficial Digital Flexor Tendon-derived fibroblasts.

Time (Hours)	Opti-MEM	Advanced DMEM	DMEM
18	0.03	0.24	0.04
24	0.08	0.41	0.03
36	0.04	0.15	0.15
72	0.04	0.08	0.03

Time indicates length of media exposure to cells. Collagen concentration measured by Sircol™ assay at 560nm. Each measurement is the average of four results. All media contained 5% FBS.

Waggett (2006) found that avian tendon fibroblasts release approximately 85% of the collagen produced into the media with the remainder being deposited in the cell layer. In a study of human tendon cells the concentration of collagen in the media was determined to be dependent on the duration the media remained in contact with the cells (Evans and Trail, 2001). Extrapolation of the graph of total collagen in the media from the human tendon cells suggested that a detectable amount of collagen would

potentially be released into the media between 18 and 24 hours although the levels would be close to the limits of the test. In the human flexor and extensor tendon cell study there was no significant difference between flexors and extensors with respect to total collagen production (Evans and Trail, 2001). Adult superficial digital flexor tendon fibroblasts, however have lower collagen synthesis than those fibroblasts from immature flexor tendons or from extensor tendons (Batson et al., 2003, Birch et al., 2008). Lower cell numbers and lower collagen synthetic activity may have contributed to the total collagen level being lower in the current study than in the human tendon fibroblast study.

Other techniques that could have been used to assess collagen secretion into the media include sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting or amino acid analysis. Dot Blot analysis (Bio-Rad, Hercules, California) and hydroxyproline determinations may also be used to determine collagen levels in the cells (Evans and Trail, 2001, Waggett et al., 2006).

5.2.4 SOURCE OF TENDONS FOR CELL EXTRACTION

It was difficult to source locally, young thoroughbred horses that were undergoing euthanasia. Only seven were sourced in 2 years and of those, only four resulted in viable cells, Appendix 43. This paucity was unexpected considering the high attrition rate of young thoroughbred horses (Rogers et al., 2007) but needs to be considered when designing protocols around such source material. The most successful source was an abattoir where horses were killed for human consumption. The increased value of young horses for this market resulted in horses with less risk of injury being available. Photography of the horses brands at the abattoir allowed for identification via the New Zealand Thoroughbred Racing Website form (Appendix 45) so racing history and

performance could be checked. This was important as tendons could have chronic injury due to age and or exercise.

For the first set of cultures, tissue was sourced from thoroughbred horses between 2 and 5 years old to reduce variation due to age and exercise related changes in the tendon cells and connexin expression. Immature equine digital tendons have a higher cellularity than mature digital tendons, but no significant difference has been reported between mature and aged tendons (Stanley et al., 2007). Connexin expression reduces markedly in early maturity (Stanley et al., 2007). Collecting cells from horses less than 5 years old reduced the chances of existing damage being present due to age or exercise effects on the tendons. Exercise and age-related micro-damage in the human Achilles tendon and equine superficial digital flexor tendon is thought to develop due to insufficient cellular reparative ability (Kannus and Jozsa, 1991, Patterson-Kane et al., 1998a). Degenerative and older tendons also have a higher proportion of apoptotic cells than normal tendon and would therefore have been likely to result in a lower yield of viable cells (Yuan et al., 2003).

Following a loss of frozen cell stocks a second collection of tendons was made at the abattoir. This proved ideal as the tendons were from 5 unbroken 2 year old thoroughbred colts from the same stud farm. This provided an internal control as these colts had been raised under the same husbandry and at the same location so would have been exposed to similar environmental influences. Being unbroken and with no visible signs of tendon injury these tendons were also unlikely to have age or exercise related subclinical micro-damage.

5.2.4.1 COLLECTION AND EXTRACTION

All limbs were collected within 30 minutes of slaughter and were processed for extraction within 15 hours of collection, the time required to return to the laboratory. Despite the relatively delayed processing time all abattoir collected tendons produced yields of cells that usually reached confluence in a T25 tissue culture flask within a week. The cooling of the transport media may have lowered the cell metabolism and reduced cell death during the transport period. There is a risk that only the most viable or strongest cells would have survived and therefore may have biased the cell population. However this should have been consistent between horses.

Extraction yields appeared to be affected by the method of euthanasia, with surprisingly low returns from tendons from horses undergoing euthanasia with pentobarbitone sodium compared with those killed by shooting or captive bolt and exsanguination. (Appendix 44). As a result, it became necessary to limit the source of tendons for tendon fibroblast extraction to horses killed by free bullet, or by captive bolt, and exsanguination. The effect of pentobarbitone sodium euthanasia on cell survival warrants further investigation as many tissues are collected following euthanasia by this method.

5.2.5 DYNAMIC STUDIES

Examination of the response of tendon fibroblasts under static and dynamic loading is important due to the nature of tendon tissue. Tendon fibroblasts have been shown to alter the genes that they express in response to load (Banes et al., 1999a) and gap junctions have been shown to regulate collagen expression of tendon cells in response to load (Banes et al., 1999b). It was therefore important to design a method for studying the response of loaded as well as static cultures to determine the likely efficacy of

modulating gap junction communication *in vivo*. Uniaxial load is the application of load to create strain in a single plane. Uniaxial strain can cause narrowing or contraction of the material in a perpendicular direction to the strain; the ratio of the two quantities is known as the Poisson ratio (Deodhar, 1923) . *In vivo*, tendons are fixed to the muscle at one end and insert on bone at the other end. In the superficial digital flexor tendon of horses the majority of the strain occurs between the proximal check ligament that attaches the tendon to the back of the carpus (Figure 1) and the insertion on the first and second phalanges. The system developed (the RTV dish and stepper motor, Appendix 46) was based on one described by Neidlinger-Wilke (1994) which produced axial and some constrictive transverse load on osteoblasts.

The apparatus and stepper motor enabled alteration of the amount of strain and the strain rate, and these were able to operate across the range of strains (5-20%) and speeds (0.5-4Hz) similar to those that have been measured previously in horse superficial digital flexor tendon (Stephens et al., 1989).

Materials testing was performed (Appendix 46) and determined there was significant effect of dish $P < 0.001$ on the material properties indicating a variation in material properties between the individual dishes. The lack of consistency in the material properties of the dishes delayed progression of this aspect of the study.

5.3 LIMITATIONS

Ideally, objective measurements of cell growth on the glass, tissue culture plastic, laboratory-made rat and horse tail collagen gel and the commercial collagen gel substrates would have been collected (5.2.3.2). Also cells should have been grown in the presence of pluronic gel, especially considering the results in chapter 3 (section 3.3.4.4) suggesting pluronic gel had an inflammatory effect in tendons. Vital staining

with PI and DAPI would have allowed identification of numbers and ratios of live: dead cells. Staining of the cells was possible, however mounting and viewing of cells grown on RTV proved problematic. The xylene based dehydration and mounting chemicals interacted with the RTV resulting in softening of the substrate. Mounting of the RTV resulted in an interface effect so that the cells could not be imaged. Mounting of the RTV cells upward with placement of a coverslip over the cells was attempted although sealing of the coverslip to prevent dehydration and loss of mountant was unsuccessful. Use of vital stains and a fluorescence microscope with a dipping objective may be a means to overcome this limitation.

Reverse transcriptase polymerase chain reaction was not performed on the cells exposed to antisense oligodeoxynucleotides to detect changes in Connexin43 expression (Section 5.2.7.1). Immunohistochemical staining is considered more sensitive for detection of small changes in Connexin43 gap junction numbers because the Connexin43 mRNA makes up such a small component of the expressed genome. Also the lack of differences between the treatment groups is consistent with the current published information on the response of cells in culture to unmodified antisense oligodeoxynucleotides without transfection agents.

Peptide5 did not appear to have an obvious effect on cell migration in equine superficial digital flexor tendon-derived fibroblasts. It was chosen as the mimetic peptide for this study due to previous positive effects *ex vivo* in sheep (O'Carroll et al., 2008). This may have been an incorrect choice of mimetic peptide as the previously reported positive effects of mimetic peptide on dermal fibroblast migration involved Gap27, which contains amino acids LFII from the membrane regions (Wright et al., 2009). Gap27 and peptide5 share the SRPTEKT sequence with peptide5 having the extra-cellular loop

amino acids VDCFL in the place of the membrane region amino acids and membrane amino acids may be more involved with migration.

Cells in culture are maintained at 5% carbon dioxide although may exist in much more hypoxic environments *in vivo,* particularly in a galloping horse. Investigation of the ideal culture conditions for equine superficial digital flexor tendon-derived fibroblasts is essential to ensure cells in culture behave as similarly to those in the live animal as possible. Use of a hypoxic chamber with exposure of cells to higher levels of carbon dioxide or more hypoxic conditions would be a means of investigating this further.

5.4 CONCLUSIONS

- Equine superficial digital flexor tendon-derived fibroblasts prefer collagen gel substrate for attachment and growth in comparison to glass and RTV silastic elastomer. In particular, they grow best on commercial or laboratory-made rat tail collagen.

- Scrape-wounds can be made in fibroblast monolayers growing on collagen-coated glass or tissue culture plastic using pipette tips. Scrape-wounds made with a P10 pipette tip close in 3-5 days if the fibroblasts are cultured in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS. The area of the scrape-wounds is best determined using by capturing images at set time points then masking and measuring the area of the wounds. Twelve hour intervals between image capture was sufficient to detect changes in wound area without compromising cell survival under the culture conditions used.

- Equine superficial digital flexor tendon-derived fibroblasts preferred high glucose DMEM over Advanced DMEM or Opti-MEM supplemented 100 U/mL

penicillin/100 µg/mL streptomycin supplemented with 5% FBS. High glucose DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and supplemented with 10% FBS was the optimum media of those tested. Cell doubling rates as determined using the xCelligence system confirmed within and between horse variations. This warrants more investigation as the differences could indicate a component of the variation in horses' ability to respond to injury and heal effectively.

- Unmodified Connexin43 antisense oligodeoxynucleotides do not down-regulate numbers of Connexin43 gap junctions in equine superficial digital flexor tendon derived fibroblasts detected by immunofluorescence when administered in culture media.

- Connexin43 gap junctions are present in equine superficial digital flexor tendon-derived fibroblasts in cell culture and transport Lucifer yellow between cells with transport being blocked by 500 µM / mL / mL mimetic peptide. Connexin43 hemi-channels are present in the membrane of equine superficial digital flexor tendon-derived fibroblasts and allow propidium iodide to enter living cells in a low calcium environment. This action is blocked by culturing with 10 µM / mL mimetic peptide.

- Peptide5 did not increase cell migration in equine superficial digital flexor tendon derived fibroblasts in this study.

- Herovici staining of new collagen in equine superficial digital flexor tendon fibroblasts in culture does not stain the new collagen strongly enough for quantification.

- Immunofluorescent staining of procollagen types I and III stains the cytoplasm of equine superficial digital flexor tendon-derived fibroblasts in monolayer cultures.

- Equine superficial digital flexor tendon-derived fibroblasts under the conditions in this study did not produce sufficient total collagen into culture media over 18 hours for detection using the Sircol™ ™ assay.

- A novel method to expose the fibroblasts to unilateral loading in culture was adapted from an existing method using loading of a RTV dish (Neidlingerwilke et al., 1994).

CHAPTER 6

GENERAL DISCUSSION

6.0 INTRODUCTION

Injury to energy storing tendons such as the equine superficial digital flexor tendon and the human Achilles tendon are responsible for significant welfare and financial costs. Clinically relevant models for the acute injury period in these tendons are lacking and this prompted investigation into the development of a more appropriate model.

Gap junctions and their effects on wound healing have become well recognised in only the last 15 years. The positive effects of modulation of gap junctions in the healing of challenging wound sites such as diabetic skin and the cornea are well described. This stimulated the investigation of the involvement of Connexin43 gap junctions in the injury and healing of energy –storing tendons.

The primary aims of the present study were to develop an appropriate model of acute tendon injury in sheep and to extrapolate this for horses, secondly this model was used to investigate Connexin43 expression and modulation on acute injury and healing, thus providing a basis for future studies applicable to energy storing tendons in both horses and humans.

6.1 INJURY MODEL IN SHEEP

A mechanical model of acute tendon injury in the core of the pelvic limb ovine superficial digital flexor tendon was developed as described in chapter 3. This confirmed that a barbed catheter stylet would create tendon fibre tearing and haemorrhage in the core of the tendon consistent with clinical injury (Patterson-Kane et al., 2012). Sealing of the incision with cyanoacrylate glue proved successful in the tendons of live animals and may be useful in other models to limit healing to intrinsic mechanisms. This tendon is an energy storing tendon and therefore the described model should prove relevant for acute injury in human Achilles and equine superficial digital flexor tendons injury.

6.1.1 CONNEXIN43 IN ACUTE TENDON INJURY

The present study is the first to report the effect of Connexin43 antisense oligodeoxynucleotides in injured ovine superficial digital flexor tendon, in the first four hours after injury. The increases in levels of Connexin43 in the injured areas of the tendons in this model are likely to be due to the inflammatory response. Inflammation is thought to be responsible for early progression of clinical tendon lesions. Current medical therapies in the acute stages of tendon injury are directed at reducing such inflammation (Smith and McIlwraith, 2012). This indicates the present model's suitability for studying the effects of transient modulation of Connexin43 expression, or function, on tendon injury repair.

6.1.2 ANAESTHETIC AND ABATTOIR CONTROLS

Connexin43 levels in uninjured tendons were higher in abattoir animals compared with controls that had been anaesthetised for 2 hours. This was an unexpected finding as the

induction drugs have not been reported to be associated with gap junction effects. Also, although the inhalational anaesthetic isoflurane is known to be a non-specific blocker of gap junction communication, there is no published evidence of it or any of the drugs administered reducing Connexin43 expression. The trend of increasing Connexin43 levels in the anaesthetised control sheep between two and four hours abolished the difference with the abattoir controls. Increasing Connexin43 levels in the anaesthetised control sheep could either indicate the effects of induction drugs wearing off or an increase due to systemic effects of the surgical procedures being performed.

The effects of travel, housing and stressors at the abattoir could have resulted in higher Connexin43 levels and this must be considered when using control tissue from such sources. The rapid half-life of Connexin43 enables cellular response to changes in the cellular environment however, the effects of environmental stress on gap junction communication has not been investigated.

6.1.3 PLURONIC GEL

Another unexpected finding was the apparent inflammatory response by the tendons to the instillation of pluronic gel. Tendons are known to be reactive to injection of even small amounts of foreign material, the response being either a consequence of the injection itself and/or the composition of the injected material. Therefore, despite pluronic gel passing pharmacy tests for detection of inflammatory effects this method of delivery requires further investigation before being used in tendons.

6.1.4 MODULATION OF CONNEXIN43 EXPRESSION IN TENDON

Adding Connexin43 antisense oligodeoxynucleotides to injured tendon did not reduce Connexin43 expression in the present study that covered the peracute injury period.

This could represent the peracute down-regulatory response that begins with fragmentation of gap junction plaques and reduced cell to cell coupling with increased hemi-channel levels. Immunofluorescence does not differentiate between these two states of Connexin43 and further studies are indicated to explore this.

6.2 INJURY MODEL IN HORSES

The ovine acute injury model from chapter 3 was successfully modified to create lesions in the forelimb superficial digital flexor tendon of horses. The aim of this study to create a lesion in the core of the equine SDFT that would mimic the acute phase of tendon injury was achieved. This energy-storing tendon is functionally similar to the human Achilles and therefore such a model would be appropriate for acute injury of the equine superficial digital flexor and human Achilles tendons.

Local anaesthetic techniques enabled lesion creation in standing horses and resulted in minimal post-operative discomfort. This makes the model attractive as a research tool.

6.2.1 ULTRASONOGRAPHY, GROSS AND HISTOLOGICAL EXAMINATIONS

Gross post mortem findings following the model creation included subcutaneous haemorrhage, swelling and oedema consistent with clinical tendon injury. Fibre tearing and haemorrhage was also visible within the tendon including dependent spread of haemorrhage distally. It was interesting that histological findings were very localised even at 4 days post injury and were relatively subtle compared with the gross appearance of the lesion.

It is recognised that a combination of history taking, clinical examination and ultrasonography are required to detect subtle tendon lesion in horses. The study of ultrasonographic detection of tendon lesions presented in chapter 4 showed that, even in

the hands of experienced equine surgeons, grossly apparent tendon lesions could prove difficult to detect on ultrasound examination alone.

Ultrasonography is used to monitor injury and healing of clinical tendon lesions in horses. The present study confirmed that this is a relatively insensitive tool when used in isolation. Whilst that is well recognised by equine practitioners, it has not been previously well documented. One study did determine that ultrasound tissue characterisation was a poor prognostic tool (Bosch et al., 2010b) but this technique has not been compared with *ex vivo* gross and histological findings.

6.2.2 COLLAGEN

Collagen types I and III propeptides were detectable, by immunofluorescence, in the injury model by 4 days post-wounding. Type III collagen was the predominant propeptide detected in these lesions as would be expected at this stage of tendon healing.

6.3 *IN VITRO* STUDIES

Testing treatments requires proof of principle data. Conditions are easier to control, manipulations more easy to make and samples easier to collect under cell culture conditions. This also results in less animal usage so should be part of any testing protocol. Sourcing ten tendons from unbroken 2 year old colts raised under the same conditions was fortuitous and should have kept the variation in environmental influences on the tendons, and therefore their cells, to a minimum.

6.3.1 TENDON FIBROBLAST CULTURE CONDITIONS

Equine superficial digital flexor tendon-derived fibroblasts demonstrated a preference for collagen gel substrate for attachment and growth. Whilst it was possible to make a collagen gel substrate from horse tail tendon this was not the preferred substrate when compared with rat tail derived collagen gel. Intuitively, collagen gel made from the same species would have been thought to be preferred, however, this was not found to be the case in the present study. The high concentration of acetic acid needed to soften the equine tendon tissue may have affected the collagen gel and reduced the subsequent attachment of cells.

Equine superficial digital flexor tendon-derived fibroblasts preferred DMEM with 4.5 mg/mL glucose over Advanced™ DMEM or Opti-MEM™ supplemented with 1% penicillin/streptomycin and 5% FBS. Cell doubling rates as determined by using the xCelligence electrical impedance system confirmed within and between horse variations. This warrants more investigation as the differences could indicate a component of the variation in the ability of different horses to respond to injury and heal effectively.

6.3.2 EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON DERIVED FIBROBLASTS HAVE FUNCTIONAL CONNEXIN43 GAP JUNCTIONS AND HEMI-CHANNELS.

Connexin43 gap junctions in equine superficial digital flexor tendon-derived fibroblasts in cell culture transported Lucifer yellow dye between cells and this transport was blocked by culturing with 500 μM / mL / mL mimetic peptide. Connexin43 hemi-channels in the membrane of equine superficial digital flexor tendon-derived fibroblasts

allowed propidium iodide to enter living cells in a low calcium environment. This action was blocked by culturing with 10 μM / mL mimetic peptide. This cell culture model is therefore appropriate for testing effects on Connexin43 gap junctions and hemi-channels in equine tendon fibroblasts.

6.3.3 GAP JUNCTION MODULATION IN CULTURE

Unmodified Connexin43 antisense oligodeoxynucleotides did not down-regulate numbers of Connexin43 gap junctions as detected by immunofluorescence staining of equine superficial digital flexor tendon-derived fibroblasts when administered in culture media. This may be due to poor uptake into the cells in the absence of a transfection agent. As pluronic gel is required as a reservoir to overcome the limitations of using antisense oligodeoxynucleotides in a serum containing environment, other means of altering connexin43 gap junction actions, such as mimetic peptides, are likely to be more clinically useful.

The mimetic peptide, Peptide5 did not alter the migration of superficial digital flexor tendon-derived cells to close a monolayer scrape wound. Peptide5 had been shown to be effective in reducing inflammation and cell death in an *ex vivo* sheep spinal cord model. This mimetic peptide may therefore be better as an *in vivo* treatment.

6.3.4 COLLAGEN DETECTION

Quantification of total collagen produced by scrape-wounded monolayer cultures of superficial digital flexor tendons was not achieved. Under the present study conditions equine superficial digital flexor tendon-derived fibroblasts did not produce sufficient total collagen into the culture media over 18 hours for detection using the Sircol™ assay.

Similarly Herovici staining of new collagen in equine superficial digital flexor tendon fibroblasts cultures did not produce a strong enough stain for quantification.

Immunofluorescent staining of procollagen types I and III successfully stained the cytoplasm of equine superficial digital flexor tendon-derived fibroblasts in monolayer cultures with and without scrape-wounds. Accurate comparison of the ratios of collagen types I and collagen type III with and without mimetic peptide treatment was not achieved in this study due to fixation problems in the final experiment.

6.3.5 RTV DISHES

A novel method to expose the superficial digital flexor tendon derived fibroblasts to unilateral loading in culture was developed from an existing method using loading of a silastic, RTV, dish (Neidlingerwilke et al., 1994). The dishes designed could withstand repetitive loads of up to 20% strain, and consistent with strains measured in galloping horses. Strain rates and speed of loading can be altered to closely mimic tendon loading *in vivo*. Injured horses remain standing most of the time and therefore their tendons remain under load. Most cell loading devices use radial load which is less consistent with clinical loading. The apparatus developed in the present study fixed the dish at one end and stretched from the other similar to the *in vivo* situation. A limitation of the apparatus was differences in material properties between the RTV dishes; this would have made interpretation of results of loading problematic, therefore dish construction must be standardised further before loading experiments can be undertaken.

6.4 LIMITATIONS

The major limitations of the study performed on the sheep were caused by external factors. The sheep were part of a surgery teaching practical and therefore the time they

were anaesthetised and when they underwent euthanasia were controlled by the speed at which the students worked. The situation exposed the sheep to the effects of the non-selective gap junction blocker isoflurane that may have influenced lesion expansion. It also prevented any of the sheep being recovered and the tendons subject to weight-bearing. The teaching situation prevented ultrasound imaging of the lesions although the early time course of the model made this less of a concern as lesion expansion would have continued for up to a week post injury. The sheep were initially just to be used to test the model and were ideal due to the low welfare cost. The technique and situation proved so successful that the treatment groups were subsequently included in the study. It was not possible to confirm perfect sealing of the tendon by the cyanoacrylate glue although it appeared well adhered at sample collection.

The lack of reduction of the Connexin43 levels in response to antisense oligodeoxynucleotides may be due to the short time period of the study or problems with the antisense oligodeoxynucleotide action in this site or species. Samples are stored for future reverse transcriptase polymerase chain reaction to determine if actual expression of Connexin43 was down-regulated by the treatment.

The wide age range of the horses used for the *in vivo* study described in chapter 4 is likely to have increased the inter-horse variation. Also as Horse B was 11 years old she would be expected to have some underlying chronic micro trauma within the tendon (PattersonKane et al., 1997a). Ideally, young untrained horses would have been used to minimise the risk of chronic micro trauma. The horses were also unshod making accurate detection of lameness challenging.

Ultrasonography was used as a screening tool to confirm no significant underlying pathology existed in the tendons although as the study has proven, ultrasonography

alone is a relatively insensitive tool. It would have been ideal to have sufficient horses to follow some out to a longer time point to determine if the lesion progressed and healed in a similar fashion to clinical injury.

In the *in vitro* study objective measurements of cell growth on the cell culture substrates should have been collected. Vital staining would have allowed identification of numbers and ratios of live: dead cells. Staining of the cells was possible, however mounting and viewing of cells grown on RTV proved problematic due to the interaction between the RTV and the xylene based dehydration and mounting chemicals. Use of vital stains and a fluorescence microscope with a dipping objective may be a means to overcome this limitation.

Quantitative reverse transcriptase polymerase chain reaction would have confirmed if the antisense oligodeoxynucleotides had any effects on Connexin43 expression in the cultured cells. However, there may have been no change if as suspected the oligodeoxynucleotides had not been taken up into the cell due to the absence of a transfection agent.

Peptide5 did not increase superficial digital flexor tendon derived fibroblast migration to close scrape wounds. Published data exists for the positive effect of Gap27 mimetic peptide on human skin fibroblast migration and this may have been a better choice of mimetic peptide despite peptide5 being known to be functional in sheep, which may be considered to be a more closely related species.

Cells in culture are maintained at 5% carbon dioxide although may exist in much more hypoxic environments *in vivo,* particularly in a galloping horse. Further investigation of the ideal culture conditions, over a range of oxygen concentrations, for equine

superficial digital flexor tendon-derived fibroblasts is needed to ensure these cells in culture behaved as similarly to those in the live animal as possible.

6.5 FUTURE DIRECTIONS

A progression of the sheep study would ideally allow postural loading of the tendons after recovery from anaesthesia and examination of the tendons at later time points, for example at 24 h, 5 and 7 days post injury. The isoflurane inhalational anaesthetic is a non-specific gap junction blocker so may have had an influence on lesion spread. Using a top up of the induction drugs (ketamine and diazepam) as a general anaesthetic would have been sufficient for lesion creation. It is also likely to be possible to create the lesion using sedation and regional local anaesthetic. Either of these techniques would avoid the use of isoflurane and the complicating factor of potential non-selective blockade.

A further progression would involve sheep undergoing physical training on a treadmill to create chronic micro-damage within the tendon. Computer assisted tomography and magnetic resonance imaging could be used to monitor the tendon changes. Changes would be confirmed by histology of tendons from sheep sacrificed at specific time points. Sheep have previously undergone treadmill training (Pedley, 2010)and exercise has been identified as an inciting factor in horses for alterations in tendon fibre properties (PattersonKane et al., 1997b). The acute model from the present study could be applied to these chronically altered tendons to provide the closest model to clinical disease to date. This would have significant value for the investigation of horse and human tendinopathy.

A similar study could then be performed starting with young horses with no pre-existing damage and again training them on the treadmill to try and get consistent chronic changes before creating the acute lesion.

Now the basic model technique has been determined it could be altered slightly to make a single larger lesion involving a greater length and cross-sectional area of the equine superficial digital flexor tendon that would mimic the common presentation of clinical injury. This would require minimal adaptation of the current protocol but may make ultrasonographic monitoring more accurate.

Horses with such lesions would be examined in the acute stages and also followed for longer periods of time to see how the lesions progressed initially and then how they healed.

Reverse transcriptase polymerase chain reaction to measure collagen expression coupled with immunofluorescence would also enable identification of alterations in collagen type I and III ratios.

Exposure of the sheep model to Peptide5 *in vivo* would be the next progression for examining the effect of mimetic peptides on tendon wound healing. The reported effects on the sheep spinal cord (O'Carroll et al., 2008) would support the use of this peptide in this species.

To further investigate effects of mimetic peptides on fibroblast migration the peptide Gap27 could be applied to tendon-derived fibroblasts *in vitro*. Both the peptides, peptide5 and Gap27, warrant further investigation as a combination therapy acting in different ways may give better results than one peptide alone.

Alternative methods of quantifying collagen expression in the media and cultured cells, such as SDS-PAGE and western blotting on media and reverse transcriptase polymerase chain reaction or, hydroxyproline determination or Dot Blot analysis in cells could be used to investigate this aspect of the response to Connexin43 modulation.

Finally, the variability of the material properties of the RTV dishes prevented use of this method to apply load to the cells. Further investigation into curing and quality control of the RTV dishes is needed to make use of the loading rig constructed for exposure of cells to uniaxial load. This would be warranted as the strain application mimics *in vivo* loading. This system would then enable investigation of the response of cells under load to closely mimic the *in vivo* situation.

6.6 SUMMARY

Injuries to equine and human energy storing tendons are common and result in significant financial and health costs. Good models for investigation of tendinopathy of the equine superficial digital flexor tendon and human Achilles tendon are essential for better understanding of tendinopathy in energy-storing tendons and for investigating new treatment options. The present study has identified good models of acute tendon injury and creating these models in tendons with evidence of age or exercise induced chronic changes would closely mimic clinical disease.

Connexin43 antisense oligodeoxynucleotides did not alter Connexin43 levels in the acute, 2 to 4 hour, post injury stage, in sheep tendon. Measurement of antisense oligodeoxynucleotide effects on expression of connexin43 in this period would confirm if Connexin43 down-regulation was beginning. Peptide5 did not alter tendon fibroblast migration *in vitro,* however based on results in spinal cord may have beneficial effects on tendon healing *in vivo* and this warrants further investigation.

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WEB LINKS

1. xCelligence System: https://www.roche-applied-science.com/sis/cellanalysis/index.jsp?id=cell_090303

2. Specialised Media: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Culture/Mammalian-Cell-Culture/Classical_Media/Advanced-D-MEM-and-MEM.html and Opti-MEM.html

APPENDICES

1 TABLE OF CONSUMABLES

Product	Catalogue number/name	Company	Country
Advanced™ DMEM	12491	Invitrogen	New Zealand
AlexaFluor488	AlexaFluor488 conjugated goat anti-mouse (A11001)	Invitrogen	New Zealand
AlexaFluor 568	Alexa 568 Goat anti-rabbit (A 21069)	Sigma Aldrich	New Zealand
Antisense and sense oligodeoxynuclotide	Desalted oligonucleotides	Sigma Aldrich	New Zealand
Butorphanol	Torbugesic 1%	Pfizer Animal Health	New Zealand
14-18g Catheters	14-18g Catheters	SVS Veterinary Supplies Limited	New Zealand
Cell strainer	BD falcon ref: 352350	Becton Dickinson	New Zealand
Chlorhexidene solution	Chlorhexidene solution	Amtech medical	New Zealand
Collagen	C7661	Sigma Aldrich	New Zealand
Collagen I amino propeptide	Monoclonal mouse anti – procollagen Type I amino-propeptide M38	National Hybridoma Bank	Iowa,USA
Collagen III amino propeptide	Polyclonal rabbit anti-procollagen Type 3 amino-propeptide	Novotec	France
Collagenase	Collagenase Type VIII C2139	Sigma-Aldrich	New Zealand
Coverslips	13mm diameter coverslips 174969 Menzel Glasser	Milton Adams	New Zealand
Cyanoacrylate glue	Dermabond	Smith & Nephew	New Zealand
Cryomoulds	4566 Cryomoulds	Siemens	New Zealand
Connexin43 Antisense	Oligonucleotide	Sigma Aldrich	New Zealand

Connexin43 Sense	Oligonucleotide	Sigma Aldrich	New Zealand
Detomidine	Domosedan	Zoetis NZ Limited	New Zealand
Diazepam	Pamlin	Parnell Laboratories	New Zealand
Dispase	Dispase II D4693	Sigma-Aldrich	New Zealand
DMEM	Dulbeccos modified eagles medium 4.5 mg/ml glucose	Sigma-Aldrich	New Zealand
Ethanol	Ethanol	BDH Merck Ltd	New Zealand
Feather® Blades	No.130 Type (S)	Feather®	Japan
FBS	Foetal Bovine Serum	Invitrogen NZ Sourced	New Zealand
Formol saline	HT501320 Neutral buffered 10% formalin	SIGMA	New Zealand
Hypodermic needles	Various sizes	SVS Veterinary Supplies Limited	New Zealand
India Ink		Fine Art Supplies	New Zealand
Isoflurane	Isorrane, 100% isoflurane USP	Baxter Healthcare	New Zealand
Ketamine hydrochloride	Ketamine hydrochloride 10%	Parnell Laboratories	New Zealand
Lucifer yellow	Lucifer Yellow CH dilithium salt	Sigma-Aldrich	New Zealand
Mepivicaine	PH034 - Scandonest Mepivicaine	Amtech New Zealand Limited	New Zealand
Methanol	Methanol anhydrous AR	Thermo Scientific	New Zealand
Milli-Q water	Home produced		
Normal goat serum	MM349882	Sigma-Aldrich	New Zealand
Peptide5	Mimetic peptide VDCPLSRPTEKT	Gift from Prof Colin Green, Auckland University	New Zealand
n-Hexane	n-Hexane	BDH Merck Ltd	United Kingdom

Optimal cutting temperature™ compound	O.C.T. (10.24% polyvinyl alcohol 4.26% polyethylene glycol 85.5% non-reactive ingredients)	Siemens	New Zealand
Opti-MEM™	11058	Invitrogen	New Zealand
Paraformaldehyde	P6148 Paraformaldehyde reagent grade	Sigma-Aldrich	New Zealand
Pen/strep	100 U/mL penicillin/100 µg/mL streptomycin 15140-122	Invitrogen	New Zealand
Pentobarbitone sodium	Pentobarbitone 500 mg/mL	Ethical Agents Ltd	New Zealand
Pipette tips	Corning Scientific multiple sizes	Invitro	New Zealand
Phosphate Buffered Saline	Phosphate Buffered Saline pH 7.4	Thermo Scientific	New Zealand
Plastic coverslips	Thermanox	Thermo Scientific	New Zealand
Pluronic gel	Pluronic F 127	Sigma Aldrich	New Zealand
polyclonal Cx43 antibody	C6219 polyclonal Cx43 antibody raised in rabbit	Sigma-Aldrich	New Zealand
Prolong Gold™ with DAPI	Prolong Gold™ with 4',6-diamidino-2-phenylindole (DAPI) (P36935)	Invitrogen	New Zealand
Propidium iodide	Propidium iodide ≥94% (HPLC) 81845-25MG	Sigma-Aldrich	New Zealand
Riboflavin	F2253Riboflavin 5′-monophosphate sodium salt dihydrate,	Sigma-Aldrich	New Zealand
RNA-Later	RNAlater AM72020	Invitrogen	New Zealand
RTV	RTV ME 601 A + B (9:1)	Wacker Chemicals	Munchen, Germany
Scalpel blades	Number 10, 11 and 15 scalpel blades	Becton Dickinson	New Zealand
Sircol™ Assay	Sircol™ Assay (With rat normal)	LabTek	Australia
Standoff	Phillips T L12-5 50 standoff 901805	Phillips Veterinary sales and service	Florida USA

Surgical staples	SSD Surgical stapler	SVS Veterinary Supplies Limited	New Zealand
Thermanox plastic coverslips	NUNC Thermanox 15mm diameter c/s 174969	Thermo Fischer	New Zealand
Tissue Culture Flasks	T25,T75,T125 Griener	Griener, Biolab	New Zealand

2 RESULTS FOR SHEEP SURGERY HISTOLOGY

See file 'APPENDIX 2 RESULTS FOR SHEEP SURGERY HISTOLOGY.xls' on disk.

3 PREPARATION OF PLURONIC GEL

Make up a solution of the flowing in milli-q water:-

	g/L
Potassium dihydrogen phosphate	0.15
Sodium phosphate dibasic heptahydrate	1.21

Sterile filter the solution and leave in fridge to cool.

Pluronic F 127 Sigma	30/100ml solution

Do this in a chiller and seal bottle with parafilm and place on rocker in chiller room overnight. Keep on ice until used to keep liquid.

4 SHEEP SURGERY PRACTICAL FORM

PROTOCOL NUMBER: **DATE:**

SHEEP ID	BREED	AGE	SEX	TIME GA	TIME EUTH	LEFT HIND			RIGHT HIND		
						TX	WOUND	COLLECT	TX	WOUND	COLLECT

5 SHEEP STUDY LABEL TEMPLATE

Protocol: Date:

Sheep ID: Age: Breed:

Model LH: Model RH:

Time Euthanised:

LH collected: RH collected:

6 AUTOMATED HAEMOTOXYLIN AND EOSIN STAIN USING LEICA AUTO-STAINER XL

	Minutes:secs
Wash	0:10
Haemotoxylin	4:00
Wash	1:00
Scotts Tap Water	0:30
Wash	1:00
Eosin	2:00
Water	0:30
70% Ethanol	0:30
95% Ethanol	0:30
100% Ethanol	0:30
100% Ethanol	1:00
Xylene	1:00
Xylene	1:00
Xylene	1:00

7 MASSON'S TRICHROME METHOD

Solutions

1. Bouin's Solution overnight
2. Mayer's Haematoxylin
3. Celestine Blue
4. Beibrich Scarlet-Acid Fuchsin Solution

Beibrich Scarlet, aqueous 1%	90 mL
Acid Fuchsin, aqueous 1%	10 mL
Glacial Acetic Acid	1 mL

5. 5% Phosphotungstic Acid, aqueous
6. 2% Light Green Solution

Light Green Yellowish	2.0gm
Distilled Water	98 mL
Glacial Acetic Acid	1.0 mL

7. 1% Glacial Acetic Acid

Staining Procedure

1. Deparafinize and hydrate to water
2. Mordant in Bouin's overnight at room temperature if formalin fixed
3. Wash in Running Tap Water (RTW) until yellow colour disappears.
4. Stain in Celestine Blue (Filter On) - 10 minutes
5. Rinse in RTW - 10 seconds
6. Stain in Mayer's Haematoxylin (Filter On) – 10 minutes
7. Rinse in RTW – 10 seconds
8. Place in Scott's Tap water – 2 minutes
9. Rinse in RTW – 2 minutes
10. Stain in Beibrich Scarlet-Acid Fuchsin (Filter On) – 2 minutes
11. Rise in RTW - 30 seconds
12. Cover in 5% Phosphotungstic Acid – 15 minutes
13. Rinse in RTW - 30 seconds
14. Stain with Light Green Solution (Filter On) – 1 minute
15. Rinse in RTW – 4 Dips
16. Blot dry with filter paper
17. Dehydrate in 95% Alcohol, Absolute Alcohol, and Xylene
18. Mount

Results

Nuclei – BLACK

Cytoplasm, keratin, muscle fibres, intercellular fibres – RED

Collagen – BLUE

Adapted from – "Manual of Histological Staining Methods of AFIP – 3rd Edition.

8 LEICA SETTINGS FOR SINGLE IMAGES

See file ‘APPENDIX 8 LEICA SETTINGS FOR SINGLE IMAGE.DOC’ on disk

9 LEICA SETTINGS FOR Z STACK

See file ‘APPENDIX 9 LEICA SETTINGS FOR Z STACK.DOC’ on disk

10 IMAGEJ CONNEXIN PLUG-IN

See folder ‘APPENDIX 10 PLUG IN’ on disk

11 CONNEXIN MEASUREMENTS

See file ‘APPENDIX 11 CONNEXIN MEASUREMENTS.XLS’ on disk

12 HORSE LAMENESS CHECKS

See folder ‘APPENDIX 12 LAMENESS CHECKS’ on disk

13 CHECKLIST FOR HORSE STUDY

Need for Horse Pilot	Sourced	Used	Repaid
Sedation – Detomidine 1 x10 mL			
Butorphanol 1x 10 mL			
Local anaesthetic			
Clippers			
Hibiscrub 500 mL			
Alcohol 1L			
No: 10 blades pkt			
No:11 blades pkt			
Sterile 14G barbed catheters 16			
Sterile gloves 16 pairs			
Superglue/dermabond			
Suture material			
Ruler			
Melolin 48			
Soffban 24			
Vetwrap16			
1 mL syringes 16			
Pluronic gel			

14 HEROVICI STAIN FOR COLLAGEN

Avoid oxidising agents in fixative.

Formalin:acetic:alcohol(10:5:85) recommended

If fixed in Bouins treat with a 5% solution of $Na_2S_2O_3$ for 5 minutes before staining.

Nuclear stains

A – Celestine Blue

- 0.25gm Celestine Blue (Lamb , London, Colour index 51050, batch 5041)
- 2.5 gm iron alum
- 50 mL distilled water
- Heat and allow to boil for 3 minutes
- Cool and add 10m modified MacLendrum's formula glycerol

B –

- 100 mL 5% aqeous aluminium sulphate($Al_2(SO_4)_3$.$18H_2O$, Ajax finechem PtyLtd, batch 0709199)
- Heat to boiling then slowly add 50 mL of 1% solution of alcoholic haematoxylin
- Boil for 3 minutes, allow to cool then add 100 mL distilled water, 10 mL 4%aqeous $FeCl_3$ and 1 mL concentrated HCl

Cytoplasmic stains

C- Metanil Yellow

- 0.25gm Metanil Yellow (Harleco, Philidelphia, item 266, colour index 13065)
- 60 mL distilled water
- 5 drops acetic acid

D –

- 60 mL distilled water and 5 drops acetic acid

E –

- 60 mL distilled water and 2 drops of saturated aqueous Li_2CO_3

Connective tissue stains

F – Picropolychrome mixture

- 1 = 0.05gm Methyl Blue (Gurr, London) and 50 mL distilled water
- 2 = 0.01gm Acid Fuchsin (BDH, Poole, UK. Product 34031, colour index 42685) and 50 mL of saturated aqeous picric acid

- Mix 1 and 2 and then add 10 mL glycerol and 0.5 mL of saturated aqueous Li_2CO_3.

Staining technique

1. Stain solution A 5 minutes
2. Wash in running water
3. Stain solution B 5 minutes
4. Wash for 15-30 minutes in running water
5. Stain solution C 2 minutes
6. Differentiate in solution D and rinse with water
7. Stain in solution E 2 minutes
8. Stain with solution F 2 minutes
9. 1% acetic acid for 2 minutes
10. Dehydrate
    a. Serial dilutions of xylene (get protocol 3 off machine)
11. Coverslip with Entellan

Results

Nuclei – black

Cytoplasm - green/yellow

Keratin, muscle and red blood cells - bright yellow

Mature collagen – red / purple

Hyaline cartilage – Blue-green non-fibrillar dots

Immature / Type III Collagen - blue

15 Indirect Immunofluorescence for Collagen Aminopropeptides and Nuclei in Tendon Cross Sections

PBS + 1% Tween 20 (PBST)

Phosphate buffered saline tablets (1 per 100 mL)	10
Distilled water	1 litre

Tween 20 1 mL

(very viscous, care with pipetting and wait until tablets dissolved)

Also, make up 100 mL PBS for solution/blocks

5% (v/v) NGS blocking solution

PBS 9.5 mL

Normal Goat serum (store in aliquots at -20) 500 µl

5% (v/v) NHS solution

PBS 9.5 mL

Normal Horse Serum (store in aliquots at -20) 500 µl

Coll1 Monoclonal mouse anti –procollagen Type I amino-propeptide

(M38 National Hybridoma Bank, Iowa,USA)1:80 in PBS 5% NGS

Secondary - AlexaFluor488 conjugated goat anti-mouse (A11001, Invitrogen, NZ) 1:500 in PBS 5% NHS

Coll3 Polyclonal rabbit anti-procollagen Type 3 amino-propeptide (Novotec, France) 1:120

Secondary - Alexa 568 Goat anti-rabbit (A 21069, Invitrogen, NZ) 1:500 PBS 5% NHS

Nuclei were stained using mounting medium Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI) (P36935, Invitrogen, New Zealand).

Controls

Positive – Horse skin wound
Negative – Mouse Brain and no primary controls

Method

1. Remove slides from freezer and bring to room temperature and allow condensation to dry off
2. Draw around section with resin pen, avoid OCT if possible

3. Rehydrate cells in PBST for 30min

4. Transfer slides to humid box (needs foil covered lid to protect from light)

5. Add 5% NGS blocking solution to slides so sections completely covered

6. Incubate for 30 minutes at room temp

a. Prepare primary antibodies now

7. Tip off excess blocking agent

8. Add primary antibodies to sections

a. For control omit antibody and use only blocking soln

9. Incubate for 2 hours at room temp

a. Prepare secondary antibody now

10. Wash in 3 changes of PBST over 10-15 minutes

11. Add secondary antibody to sections

12. Incubate for 2 hours at room temp

13. Wash in three changes of PBST over 10-15 mins

14. Drain slides on tissue to remove excess PBST

15. Apply one drop mounting medium Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI) (P36935, Invitrogen, New Zealand).

16. Allow to dry (keep protected from light) and store slides in slide trays wrapped in foil at 4C until required.

17. Slides can be kept for a max of 2-3 days at 4°C

Results

Green – Collagen I aminopropeptide

Red – Collagen III aminopropeptide

Blue – Nuclei

16 HORSE ULTRASOUND SPREADSHEET

See file ‘APPENDIX 16 HORSE ULTRASOUND SPREADSHEET.xlsx’ on disk

17 RESULTS OF ULTRASOUND EXAMINATION

	A ultrasound lesion	**ultrasound lesion**	
Gross lesion	0	1	
0	87	146	232
1	41	87	128
	127	233	360
	B ultrasound lesion	**ultrasound lesion**	
Gross lesion	0	1	
0	132	100	232
1	88	40	128
	220	140	360

	A lesion scores				
Gross lesion score	0	1	2	3	
0	86	105	38	2	231
1	25	46	5	0	76
2	16	28	8	0	52
3	0	0	0	0	0
	127	179	51	2	360

	B lesion scores				
Gross lesion score	0	1	2	3	
0	132	60	40	0	232
1	53	15	8	0	76
2	35	9	8	0	52
3	0	0	0	0	0
	220	84	56	0	360

18 COLLECTION AND EXTRACTION OF CELLS

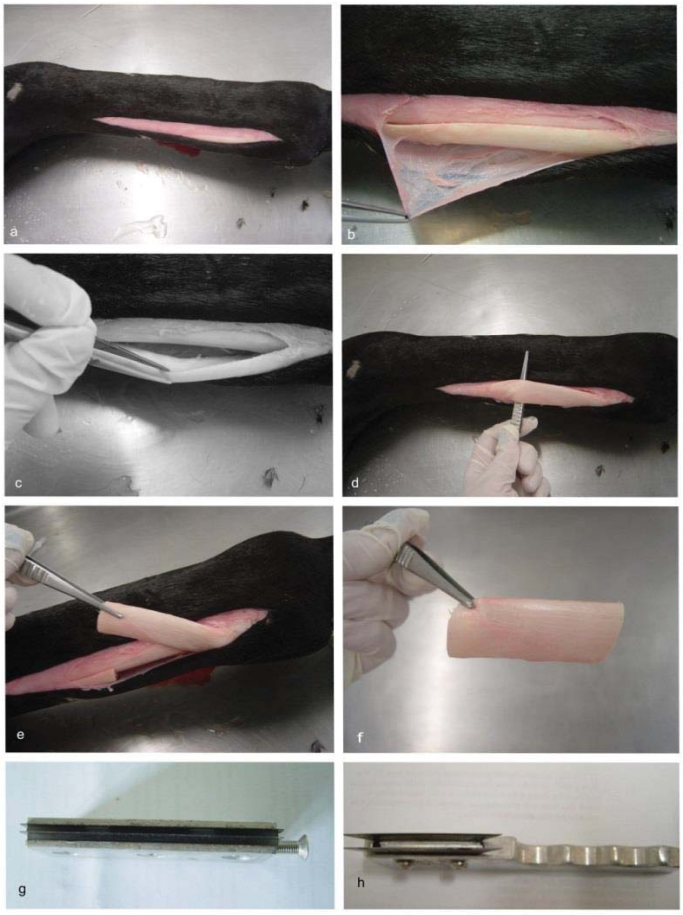


Figure A1 - Tendon collection protocol

Skin incision over lateral edge of the equine Superficial Digital Flexor Tendon (superficial digital flexor tendon)

Exposure of digital flexor tendons

Separation of superficial digital flexor tendon and Deep digital flexor tendon

Elevation of superficial digital flexor tendon

Transection of superficial digital flexor tendon at junction of proximal and middle thirds of the tendon

Middle one third of the superficial digital flexor tendon removed

Images g-h Cutting tools used to divide tendon after extraction

Cutting tool designed by John Wright (University of Queensland, Brisbane, Australia)

Massey cutting tool

Collection off site

All limbs were collected within 30 minutes of slaughter. All tendons were excised and immersed in cooled DMEM within 1 hour of collection. Cells grow optimally at a temperature similar to the body temperature of the animal from which they are sourced although reportedly can tolerate cooling to 4 °C for a number of days. As temperature changes will alter pH, a buffered medium is important (Kramer et al., 2010). The cooling of the cells lowers the metabolic rate therefore reducing energy demands, the chance of oxygen free radical damage and media exhaustion. Cooling the transportation media was reported to result in higher yields of viable cells from equine tendon (personal communication John Wright, University of Queensland, Brisbane).

Extraction

The tendons were transported on ice from the abattoir to the laboratory and the cells were extracted using a technique adapted from a method described by Burrows (S Burrows PhD thesis 2009) within 12 hours of death. In a laminar flow hood, the tendon was washed twice with warmed (37ºC) sterile phosphate buffered saline (PBS), to remove hairs and debris. Any loose connective tissue left at this point was stripped and the ends of the tendon segment were removed to leave a central portion of 2 cm in length. The tendon was then divided longitudinally and the peripheral regions discarded. This was initially done using the cutting tool designed by John Wright (Figure 5.1g) and subsequently one adapted from this by the engineering department at Massey University (Figure 5.11h). The Massey designed tool included a handle which enabled easier cutting of the stiff tendon tissue. A 5 mm^3 slice was snap frozen in n-Hexane over dry ice for histology. A further slice 5 mm x 2 mm was placed into "mRNA later" and was stored at -20ºC overnight and subsequently at –80ºC.

The remainder of the strips of the trimmed tendon were cut into 2 mm^3 pieces and placed in a 125 mL Erlenmeyer flask with 40 mL of DMEM per gram of tissue, 0.25 mg/mL of collagenase and 0.55 mg/mL of Dispase to digest the collagen matrix. The enzymes had been dissolved in DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and filter sterilized using a 0.22 µm syringe filter, prior to addition to the flask. The samples were then placed in an incubator at 37ºC with 5% CO_2 to digest for 8 hours. The samples were agitated gently every 4 hours. The supernatant was poured through a cell strainer (0.77 µm) to remove the tissue debris. The solution was centrifuged at 307 G (Eppendorf 5804-R, Becton Dickinson, NZ) for 5 minutes and the pellet re-suspended in 3.5 mL warmed DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 20% foetal bovine serum (FBS) , then transferred into a T25 tissue culture flask. The cells were incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Once the cells were confluent they were passaged and sub-cultured into T75 and then T125 culture flasks with DMEM containing 100 U/mL penicillin/100 µg/mL streptomycin and 10% FBS.

19 COLLECTION EQUIPMENT CHECKLIST

1. Item	Got	Packed
2. Small chilli bin	√	
Ice packs	√	
DMEM 700 mL	√	
Box Gloves	√	
Chlorhexidine 50 mL	√	
Alcohol 50 mL	√	
N0: 20 scalpel blades – I box	√	
Labels x 20	√	
50 mL centrifuge tubes x 14.	√	
Parafilm	√	
Pens	√	
Clipboard	√	
Laminated checklist	√	
Laminated horse collection Table	√	
Laminated telephone list	√	
Zip lock bags x 6	√	
Large bags x 18	√	
Plastic boxes for limbs		
Cable ties	√	
Labels for limbs	√	

20 HORSE IDENTIFICATION TABLE

Horse ID	Brand	Colour	Age	SDFT	Labelled	Killed	Collected	Process

21 PASSAGING CELLS

Equipment and reagents

Flow hood

Centrifuge

Inverted microscope

Phosphate buffered saline (PBS)

Dulbeccos modified Eagles medium high glucose (DMEM)

Trypsin 0.5 % in EDTA

Centrifuge tubes

Tissue Culture Flasks

Discard beaker

Pipettes

Method - Using Flow Hood

1. Remove media from cells and add 10 mL PBS and wash. (Serum inactivates Trypsin)
2. Add 3 mL/5 mL/7 mL (T25/75/175) Trypsin 0.5% in EDTA, return to incubator for 5 minutes
3. Check under microscope to see if rounding up. Bang side of culture flask sharply if any cells still adherent.
4. Add 2 mL DMEM supplemented with 10% FBS, to inactivate Trypsin. Transfer to 15 mL centrifuge tube and centrifuge at 1700 rpm for 5 minutes
5. Remove supernatant and resuspend in DMEM high glucose supplemented with 1% penicillin/streptomycin and 10%FCS. Either place into flask or divide between 2 flasks and add remainder of media to flask.
6. Count cells if required – see cell counting technique
7. Check cells are present in flask then return to incubator

22 CRYOPRESERVATION OF CELLS

Equipment and consumables

Cryo vials 10 per 175 flask

Pencil

50 mL Falcon tubes

FBS

Sterile Dimethyl sulphoxide (DMSO)

PBS

DMEM 10% FCS p/s

1) Defrost trypsin and FBS
2) Discard media from cells and wash with 1x PBS
3) Tripsinise cells 7 mL tripsin in 175 flask
4) Return to incubator for 5m
5) Label 10 cryo vials per 175 flask with horse ID/passage number/tendon/intials
6) Make up 10% DMSO in FCS = freezing solution (5 mL in 45 mL)
7) Check cells lifted off, add 5 mL DMEM 10%FCS to stop tripsin and centrifuge at 1700 rpm for 5m
8) Discard supernatant and resuspend in 10 mL/T175 or 5 mL /T75 freezing soln

9) 1 mL of this into each cryo tube, close and put in freezer

10) -80 overnight then into liquid nitrogen or leave in -80 until needed.

23 COMMERCIAL COLLAGEN INFORMATION

See file 'APPENDIX 23 COMMERCIAL COLLAGEN PRODUCT INFO.pdf' on disk

24 COMMERCIAL COLLAGEN C.O.A

See file 'APPENDIX 24 COA COMMERCIAL RAT TAIL COLLAGEN C7661.Mht'

on disk

25 COLLAGEN GEL FROM RAT TAIL TENDON EXPERIMENT

Aims:

To try protocol for making reconstituted collagen gel for tissue substrate from rat tail tendon.

Materials and Methods:

Materials:

- Tail tendon from freshly killed rats
- Sodium riboflavin-5-phosphate (Sigma-Aldrich R7774)
- Millipore filter 0.22u
- Glass coverslips 13 and 28mm

Methods:

1. Aseptically prepare tail for removal of tendon. Break up tail with ronguers, cut off tip and grasp 4 tail tendons using blunt artery forceps. Pull tendons from tail by winding artery forceps.
2. Wash with sterile PBS
3. Cut into strips approx 1-2mm in diameter and place in sterile distilled water to soften. Tease apart strands as tendon softens.
4. This takes 2 hrs in tail tendon.
5. Transfer to a sterile bottle containing 0.01% acetic acid.

6. Approx 100 mL per 2g tendon.
7. Place at 4 until tendon has become gelatinous, overnight.
8. Centrifuge at 2,300 rpm for 2 hours
9. Centrifuge at 1,800 rpm for 1 hour
10. Remove supernatant and store at 4 degreees for < 12months or freeze for indefinate use.

Reconstitution of collagen as a gel by dialysis

Equipment

- de-ionised distilled water
- dialysis tubing: visking 2-18/32" (medicell international)
- sterile forceps/scissors/dish/Al foil
- 50 mL centrifuge tubes
- sterile foil

1. Dialysis tubing boiled in distilled water for 5m, allowed to cool in sterile hood and covered with sterile foil.
2. Using sterile instruments a knot is tied in one end and placed into a bottle, keeping hold of the open end.
3. Fill tubing with tendon collagen to within 5 cm of top and leave tubing overlapping the rim of the bottle. Replace cap loosely and cover with sterile foil.
4. dialyse against distilled water for 48 hours at 4 degrees (increased viscosity apparent by retarded movement of air bubbles)
5. Change sterile water daily.
6. Remove from refrigerator and pour into sterile universals.
7. Sterility test by placing in DMEM 10% FCS in incubator and monitoring for 10 days.

Making collagen gel

Equipment

- 0.1% Sodium riboflavin-5-phosphate (Sigma-Aldrich R7774)
- 13mm coverslips, 35mm petri dishes or RTV dish

- culture medium

1. Turn off fluorescent lights
2. 1 mL riboflavin (flavin mononucleotide – 0.1%, Sigma F8383) with 7 mLs dialysed collagen
3. Put coverslips into wells of multi-well culture dish
4. Use 1 mL syringe to place a drop/20ul of mixture in centre of each coverslip
5. Use syringe plunger to spread across coverslip
6. Turn on UV lights and allow collagen to gel for 20 minutes
7. Remove any excess liquid
8. Place in fridge to dry overnight
9. Once gelled add "balancing solution I.e. culture medium" to cover coverslip and leave to equilibrate for 3 days

26 COLLAGEN GEL FROM HORSE SDFT TENDON EXPERIMENT

Aims:

To develop protocol for making reconstituted collagen gel for tissue substrate from equine limb tendon.

Materials and Methods:

Materials:

- SDFT from freshly killed horse
- Sodium riboflavin-5-phosphate (Sigma-Aldrich R7774)
- Millipore filter 0.22u
- Glass coverslips 13 and 28mm

Methods:

1. Aseptically remove tendon from limb or tail of horse.
2. Wash with sterile PBS
3. Cut into strips approximately 1-2mm in diameter and place in sterile distilled water to soften. Tease apart strands as tendon softens.

4. Transfer to a sterile bottle containing 0.01% acetic acid.
5. Approximately 100 mL per 2g tendon.
6. Place at 4 until tendon has become gelatinous.
7. Limb tendon not gelatinous at 3 days so increased acetic acid concentration to 0.2%
8. No response to increased acetic acid concentration so increased further to 1%

Result

Limb tendon did not become gelatinous despite being left in the acetic acid for 4 weeks. The acetic acid concentration was increased to 1% after the first week and this still did not produce any softening.

Conclusion

This technique is not suitable for collagen extraction from horse limb tendons.

27 COLLAGEN GEL FROM HORSE TAIL TENDON EXPERIMENT

Aims:

To develop protocol for making reconstituted collagen gel for tissue substrate from equine tail tendon.

Materials and Methods:

Materials:

- Tail from freshly killed horse
- Sodium riboflavin-5-phosphate (Sigma-Aldrich R7774)
- Millipore filter 0.22u
- Glass coverslips 13 and 28mm

Methods:

1. Aseptically remove tendon from tail of horse, very fiddly and only get about as much form one horse tail as from one rat tail!!
2. Wash with sterile PBS
3. Cut into strips approx 1-2mm in diameter and place in sterile distilled water to soften. Tease apart strands as tendon softens.
4. Tail tendon has softened overnight.
5. Transfer to a sterile bottle containing 0.01% acetic acid (100 mL per 2G of tendon) for 3 days.

6. Increase acetic acid conc to 0.2% for 2 days.
7. Centrifuge at 2,300 rpm for 2 hours
8. Centrifuge at 1,800 rpm for 1 hour
9. Remove supernatant and store at 4 degrees for < 12months or freeze for indefinite use.

Reconstitution of collagen as a gel by dialysis

Equipment

- de-ionised distilled water
- dialysis tubing: visking 2-18/32" (medicell international)
- sterile forceps/scissors/dish/Al foil
- 50 mL centrifuge tubes
- sterile foil

10. Dialysis tubing boiled in distilled water for 5m, allowed to cool in sterile hood and covered with sterile foil.
11. Using sterile instruments a knot is tied in one end and placed into a bottle, keeping hold of the open end.
12. Fill tubing with tendon collagen to within 5 cm of top and leave tubing overlapping the rim of the bottle. Replace cap loosely and cover with sterile foil.
13. dialyse against distilled water for 14 days at 4 degrees (increased viscosity apparent by retarded movement of air bubbles)
14. Change sterile water daily.
15. Remove from refrigerator and pour into sterile universals. 50 mL of diasylate from one tail.
16. Sterility test by adding to DMEM +10% FCS and placing in incubator for 10 days

Making collagen

Equipment

0.1% Sodium riboflavin-5-phosphate (Sigma-Aldrich R7774)

17. Turn off fluorescent lights
18. 1 mL riboflavin (flavin mononucleotide – 0.1%, Sigma F8383) with 7 mLs dialysed collagen
19. Place coverslips into wells of multi-well culture dish
20. Use 1 mL syringe to place a drop/20ul of mixture in centre of each coverslip

21. Use syringe plunger to spread across coverslip
22. Turn on UV lights and allow collagen:riboflavin to gel for 20 minutes
23. Remove any excess liquid
24. Place in fridge to dry overnight
25. Once gelled add “balancing solution ie culture medium” to cover coverslip and leave to equilibrate for 3 days

28 SIRCOL™ ASSAY INSTRUCTIONS

See file ‘APPENDIX 28 SIRCOL INFORMATION.pdf’ on disk

29 SIRCOL™ ASSAY PERMISSIONS

See file ‘APPENDIX 29 PERMISSION SIRCOL.pdf’ on disk

30 SIRCOL™ ASSAY ON BLANKS AND RTV

See file ‘APPENDIX 30 SIRCOL ON BLANKS AND RTV.xls’ on disk

31 SIRCOL™ ASSAY SPECIALISED MEDIA COMPARISON

See file ‘APPENDIX 31 SIRCOL SPECIALISED MEDIA COMPARISON.xlsx’ on disk

32 PILOT SCRAPE WOUND INFORMATION

See folder ‘APPENDIX 32 PILOT SCRAPE WOUND INFO’ on disk

33 IMAGEJ PLUG-IN FOR SCRAPE WOUND

Java Filter Plug-in Code:

```
import ij.*;
import ij.process.*;
import ij.gui.*;
import java.awt.*;
import ij.plugin.filter.*;

public class Filter_Plugin implements PlugInFilter {
  ImagePlus imp;
  public int setup(String arg, ImagePlus imp) {
    this.imp = imp;
    return DOES_ALL;
  }

  public void run(ImageProcessor ip) {
    int[] pixels = (int[])ip.getPixels();
    int width = ip.getWidth();
    int height = ip.getHeight();

    int threshold = 10;

    int offset, i, countOfPixels = 0;
    for(int y=0; y<height; y++){
      offset = y*width;
      for(int x=0; x<width; x++){
        i = x + offset;

        int red = (int)(pixels[i] & 0xff0000)>>16;
        int green = (int)(pixels[i] & 0x00ff00)>>8;
        int blue = (int)(pixels[i] & 0x0000ff);

        if(red < threshold && blue < threshold &&green < threshold){
          countOfPixels++;
          pixels[i] = (((255 - red) & 0xff)<<16)+(((255 - green) & 0xff)<<8) + ((255 -
blue) & 0xff);
        }
      }
    }
    int totalPixels = width*height;
    imp.updateAndDraw();
    IJ.showMessage("BlackArea", "Count = " + countOfPixels + " :: TOTAL = " +
totalPixels + " % Black = " + (((float)countOfPixels / (float)totalPixels) * (float)100));

  }
}
```

34 MIMETIC PEPTIDE EFFICACY LY/PI

Hypothesis:

Connexin43 mimetic peptides will block or uncouple gap junctions in a dose dependent manner.

Aims:

1. To confirm Connexin43 mimetic peptide at 500uM will uncouple gap junctions measured by reduced spread of scrape loaded of LY between cells

Potential outcomes:

1. PI adjacent to scrape and LY spreads across plate in a time dependent manner in control cells and those treated with 10uM Connexin43 mimetic peptide
2. PI/LY confined to cells immediately adjacent to scrape load in cells treated with 500Um Connexin43 mimetic peptide

Statistical models:

4 groups – Connexin43 mimetic peptide at 10Um and 500um, +ve control with no mimetic peptide and –ve control with no scrape.

Experimental protocols:

1. **<u>LY scrape wounding</u>**

- Coat RTV pieces with collagen:riboflavin 1:7
- Condition RTV dishes with 20%CMEM for 7 days in incubator
- Grow cells in T75 until confluent
- Passage and seed RTV pieces with $50X10^4$ cells/ mL
- Grow to 80% confluence
- Remove media and replace with
- DMEM no FBS no mp +ve control x 3
- DMEM no FBS 10uM Connexin43 mimetic peptide x 3 wells
- DMEM no FBS 500uM Connexin43 mimetic peptide x 3 wells
- No wound for the negative control x 3

- Apply for 30 minutes
- Add LY (0.05%) + PI (0.01%)
- Create scrape-loading wound on coverslip with P20
- Remove LY/PI media after 5m
- Rinse twice with PBS for 5min
- Fix in 2-4% fresh PFA 20min
- Mount and view using fluorescence microscope or confocal

Row	Horse 1	Horse 2	Horse 3	Horse 4
A	LYPI 10Um MP	LYPI 10Um MP	LYPI 10Um MP	LYPI 10Um MP
B	LYPI 500Um MP	LYPI 500Um MP	LYPI 50Um MP	LYPI 50Um MP
C	LYPI No MP	LYPI No MP	LYPI No MP	LYPI No MP
D	LYPI No scrape	LYPI No scrape	LYPI No scrape	LYPI No scrape

35 Low Calcium Influx Assay of Mimetic Peptide Efficacy

Hypothesis:

Connexin43 mimetic peptides will block or uncouple gap junctions in a dose dependent manner.

Aims:

To confirm Connexin43 mimetic peptide at 10uM will block gap junctions using low calcium PI uptake method

Potential outcomes:

PI not taken up by cells treated with mimetic peptides at 10 and 500uM

PI not taken up by cells in cations and no mimetic peptide

PI taken up by cells in no mimetic peptide/no cation

Statistical models:

4 groups –Connexin43 mimetic peptide at 10Um and 500um, +ve control with no cations or mimetic peptide and –ve control with HBSS with cations.

Experimental protocols:

Coat glass coverslips with collagen

Grow cells in T75 until confluent

Cells plated onto collagen-coated coverslips 50 x 10^4/ mL

Once cells 80% confluent

Cells rinsed with Ca^{2+} Mg^{2+} free HBSS containing HEPES Ph7.4 and EGTA 1um

Cells incubated in the same solution with

No mimetic peptides no cations – positive control

No cations and 10 uM Connexin43 mimetic peptide x 3 wells

No cations 500Um Connexin43 mimetic peptide x 3 wells

HBSS with cations x 3 wells – negative control

for 30 minutes.

Cells incubated in the same solution containing 2 um PI (0.1%) apart from negative control which has HBSS with cations and PI (0.1%)

Fix in 2-4% fresh PFA

Cells imaged on an inverted fluorescence microscope and images captured digitally using (need to check).

Images imported into Image J and number of cells containing PI counted.

36 DYNAMIC STUDIES

Construction of an RTV DISH

A silicon elastomer dish was designed modifying a method described by Neidlinger-Wilke & Claes (1994). The silicon rubber used was RTV a kind gift from the company (Wacker DL, Germany). The RTV was mixed according to the manufacturer's instructions in a 9:1 ratio of Part A to Part B and was found to gel overnight at room temperature.

A mould was constructed from Perspex, and used to cast RTV dishes that could be used for the loading experiments Figure a. A prototype RTV dish with 1.33 mm thick walls failed abruptly when stretched. Failure also propagated from any sites of imperfection within the dish. A second prototype with walls 0.75 mm thick was constructed and this failed at the ends.

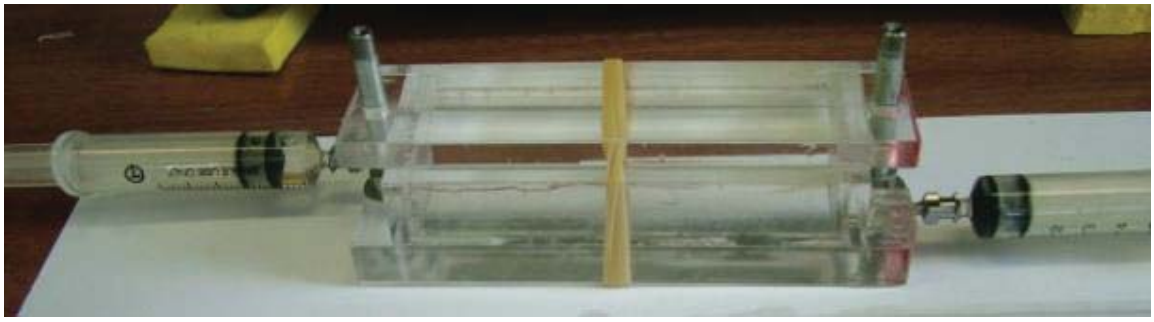


Figure A2 Mould for constructing silastic/RTV dishes

Curing problems

Persistent bubble formation in the elastomer resulted in further adaptations to the mould and the protocol. The elastomer is cured more quickly by heat so curing at approximately 70° C was trialled; this reduced the large bubbles in the base of the dish but there were still numerous bubbles in the sides (Figure b).

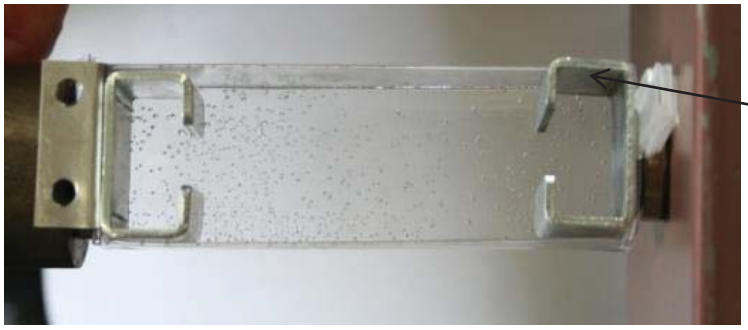


Figure A3 Bubbles visible in the base of the RTV/silastic dish

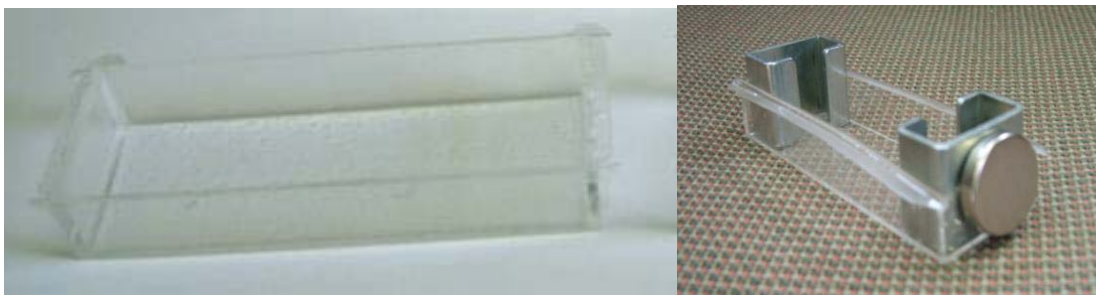


Figure A4 Final dish appearance with and without metallic inserts

Final protocol for making RTV dish to apply load to the fibroblasts

The final mould produced a dish with 0.75 mm thick sides and 1.3 mm thick ends Figure c.

The most effective techniques to avoid bubble formation were achieved when the two components were mixed together and allowed to stand for 20 minutes or until all bubbles were dispersed. The majority of the polymer was then poured into the base of the mould. The insert that casts the inside of the dish was gently attached to the mould, pressed down and secured in place with rubber bands. Inserted needles were used to inject elastomer at either end of the mould to ensure complete filling and to push any bubbles towards the top (Figure A2). The dish was left to cure for 24 hours and then the mould was carefully opened. The dishes were cleaned with detergent, washed and autoclaved as described (Neidlingerwilke et al., 1994). Alconox powdered precision cleaner (Alconox Inc., USA) was chosen as an equivalent detergent to the 7X-PF (MP Biologicals, Germany) used by Neidlinger-Wilke (Neidlingerwilke et al., 1994) , as both are anionic detergents.

Applying load to the RTV dish

A mechanism was designed to apply a uni-axial load to the RTV dish. The mould was attached to the loading rig at each end using magnets. The loading rig could generate strains of 0-20% and was connected to a 12 V, 1.8 degree stepper motor to enable application of the load at speeds of 1-5 Hz.

Preparing the RTV dish for cell growth

Checking cell toxicity of RTV

Glass coverslips were coated with RTV, washed with Alconox and conditioned in DMEM containing 100 U/ mL penicillin/100 µg/ mL streptomycin and 10% FBS for 7

days. The equine superficial digital flexor tendon-derived fibroblasts were seeded at 7 x 10^5 cells/ mL. Few cells adhered directly to the uncoated RTV and if they did attach, formed discrete islands and piled up on each other. Coating with commercial or laboratory-made collagen gel resulted in confluent growth of the cells on the collagen-coated area of the dish.

Determining the correct order of processing

The RTV dishes were collagen-coated and conditioned in DMEM containing 100 U/ mL penicillin/100 µg/ mL streptomycin and 20% FBS for one week prior to seeding. A pilot study determined that collagen coating should be applied prior to conditioning. Conditioning the RTV prior to coating with the collagen interfered with the collagen gelling onto the RTV. Collagen gel coating applied to the RTV prior to conditioning remained in place during the conditioning period and supported subsequent cell growth.

Using RTV discs in culture plates

RTV discs were cut using a punch (Figure d) then collagen-coated and conditioned for pilot studies on scrape wounding which would include the presence and absence of mimetic peptides.

Figure A5 Punch to make 13mm RTV discs

Initial studies were to be carried out on collagen-coated RTV discs to determine if the mimetic peptides proved to have an effect on equine superficial digital flexor tendon-derived fibroblasts. Persistent problems with cells dying and lifting off were due to the RTV discs floating on the surface of the media. This resulted in the collagen drying out during conditioning and being a less than ideal substrate for cell adherence. Also if cells did adhere to the RTV they rapidly dehydrated and died due to lack of nutrition. A further issue with using the RTV discs occurred when dehydrating and mounting. The discs were melted by the xylene dehydration step and it was difficult to view cells at

high power as a result of the multiple interfaces. It was therefore decided to use glass coverslips for the static studies.

Final protocol for preparing RTV dish for cell culture

The final protocol involved coating the base of the RTV dish with 70 µL of collagen prior to conditioning with DMEM containing 100 U/ mL penicillin/100 µg/ mL streptomycin and 20% FBS for seven days.

Materials testing of RTV dishes

Once dishes of similar appearance could be made consistently, three were used for materials testing. The dishes underwent 2100 cycles at 10% strain and 1 Hz and the lengths of the dishes at cycle 0 and cycle 2100 were compared to determine if creep occurred with repetitive loading. The dish length was measured with a digital vernier calliper and the mean of three measurements recorded for each dish.

To determine if 'waisting' occurred and if there was a difference between the strain on different parts of the dish, precision lines were drawn on three of the dishes using a 0.5 mm roller ball and a vertical milling machine (Model 2VS, Maximart, Taiwan) with digital readout (Model NV300M, Fagor, NZ).

Video recordings were made of the three dishes under cyclical strain using the stepper motor with a 13.48 mm dot adjacent to the disk for scaling. Image capture was used to capture frames at the maximal and minimal strains. Image J (ImageJ software, National institute of Health, USA) was used to put a profile across the images and the distances between the peaks were measured to determine the size of the gaps between lines. This was used to determine if there was a change in distance between the lines in response to strain and if this was equal along and across the dish.

Res and Disc

The apparatus and stepper motor enabled alteration of the amount of strain and the strain rate, and these were able to operate across the range of strains (5-20%) and speeds (0.5-4Hz) similar to those that have been measured previously in horse superficial digital flexor tendon (Stephens et al., 1989).

Studies of behaviour in domestic and feral horses indicate that if space is not limited, on average they walk at approximately 9 km/hr and for a total of 1-2 hrs/day spread over

19 hours (Kurvers et al., 2006). Horses also stand or graze for approximately 60% of the day (17 hrs). The strain in the superficial digital flexor tendon at walk is approximately 5% and the same leg is loaded once every second (Stephens et al., 1989). The walk therefore approximates to loading at 5% strain for 3600-7200 cycles (1-2 hours) at 1 Hz in 24 hours. This doesn't allow for the strain that the tendon is exposed to during standing. The intention was for the tendon fibroblasts for each experiment to have been subjected to 5% strain for 1000 cycles at 1 Hz (17 minutes) every hour for 24 hours to approximate to the loading which a tendon is exposed to in a horse undergoing confinement and controlled exercise, the recommended therapy for tendon injuries. See section below on materials testing for an explanation of why this aspect of the study was not continued.

RTV conditioning

The RTV dishes required conditioning to leach out unknown toxic chemicals that could diffuse into tissue culture media and kill the cells. The conditioning also allowed for serum proteins to adhere to the dish surface as a result of charge and protein binding. This could improve cell attachment to the dish, however, a collagen gel needed to be applied on top of the RTV to provide a good substrate for cell growth. Collagen coating the RTV dish after it had been conditioned resulted in poor adherence of the gel. It is possible that the protein in the serum binding to the RTV may have interfered with the collagen binding. It was also not possible to fully dry the surface of the RTV after removing the conditioning media which prevented good adhesion of the collagen gel. Consequently, the dishes were coated with collagen before conditioning and the collagen gel coating did not appear to degrade or lift off from the dish during conditioning.

Materials testing

Cells have been shown to adapt to strain by altering their orientation and mechanotransduction properties (Arnoczky et al., 2008). Creep in the dish could cause this to occur and therefore in a dynamic system it is important to avoid creep in the cell substrate. Creep is the plastic deformation of a material as a result of continuous or prolonged cyclic stress. The intended maximum strain was 20% as the high elasticity of the superficial digital flexor tendon permits it to undergo deformation of up to at least 10-16% in galloping horses (Trelstad and Hayashi, 1979). At 1 Hz, 2000 cycles approximate to 30 minutes of walking and this is a feasible time horses would be walked if they had a tendon injury. Materials testing used a strain rate higher than at walk as this would be most likely to detect any creep. With the digital vernier scale the mean RTV dish length was 100.63 mm prior to the strain experiment and 100.61 mm following repetitive loading. The LSmeans of the length measurements from the video detected no difference in length at 10 or 2000 cycles confirming the findings of the digital vernier scale. Therefore, no significant longitudinal creep was detected by either measurement method. There was significant effect of dish $P< 0.001$ and frame i.e. the degree of strain $P< 0.0048$ on the width of the dishes. LSmeans statistical assessment detected a Poisson effect on width with a smaller mean difference between the lines at 2000 (0.377) cycles than at 10 (0.462) cycles although this was not significant $P< 0.1896$). There was a significant effect of position $P <0.0010$ and dish $P<0.001$ on the length measurements with the dish nearest the load being stretched more than the fixed end of the dish. This position effect was expected as the load was applied at one end of the dish. The dish effect indicated a variation between the individual dishes. There is a nearly significant effect of dish x position interaction $P<0.0534$, this was likely to be due to the altered stiffness of the material between dishes. Also time x frame $P<0.0550$ which is logical as the frame number increases with increased time. The lack of consistency in the material properties of the dishes delayed progression of this aspect of the study. The dishes can be autoclaved so had the width results not indicated creep it would have been possible to use a single dish for multiple experiments to remove the dish effect.

37 xCELLIGENCE™ SOFTWARE MANUAL

See file ‘APPENDIX 37 XCELLIGENCE RTCA SOFTWARE MANUAL.pdf’ on disk

38 xCELLIGENCE™ DATA

See folder ‘APPENDIX 38 XCELLIGENCE NUMBER DATA’ on disk.

39 TIMING FOR SCRAPE WOUND EXPERIMENT AND LY/PI

T0 - Add mimetic peptides to cultures and freeze removed media for Sircol

LY/PI Scrape loading assay – rinse and add mimetic peptides

PI low calcium influx assay – rinse and add calcium free medium with mimetic peptides

T30 scrape wound and image wounds (takes 40minutes)

LY/PI Scrape loading assay – add LY/PI and scrape load

PI low calcium influx assay – add PI

T35 – imaging scrape wounds

LY/PI Scrape loading assay – Remove LY/PI, RINSE 2x 5 minutes

PI low calcium influx assay- fix and image immediately

T45 imaging scrape wounds

LY/PI Scrape loading assay – fix for 20 min

T65 imaging scrape wounds

LY/PI Scrape loading assay - image

T70 Scrape wound imaging complete

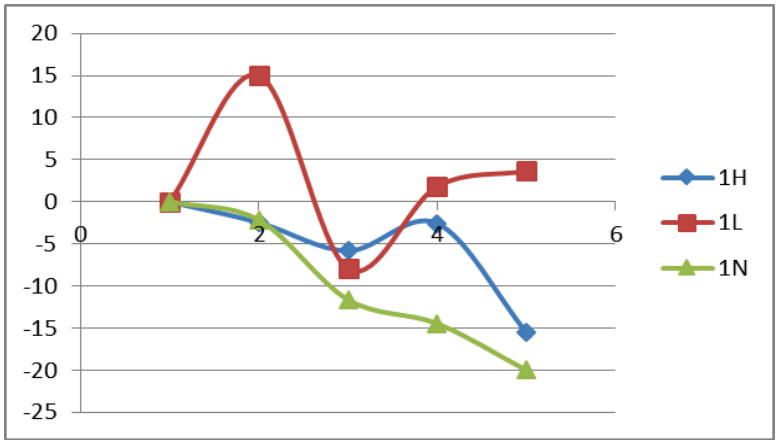
40 STATS OUTPUT DATA FOR MIMETIC PEPTIDE SCRAPE WOUND STUDY

See file ‘APPENDIX 40 OUTPUT FOR MP AND SCRAPE REPEATED MEASURES.lst’ on disk

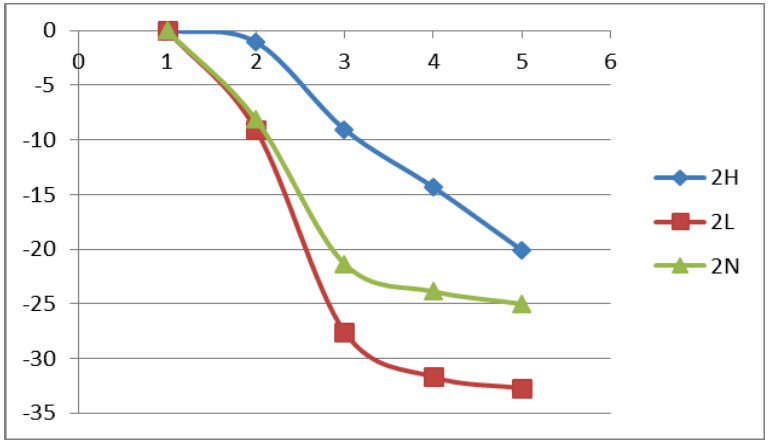
41 Spreadsheet For Mimetic Peptide Scrape Wound Study

See file ‘APPENDIX 41 C10 MIMETIC PEPTIDE WOUND SPREADSHEET AND GRAPHS.xlsx’ on disk

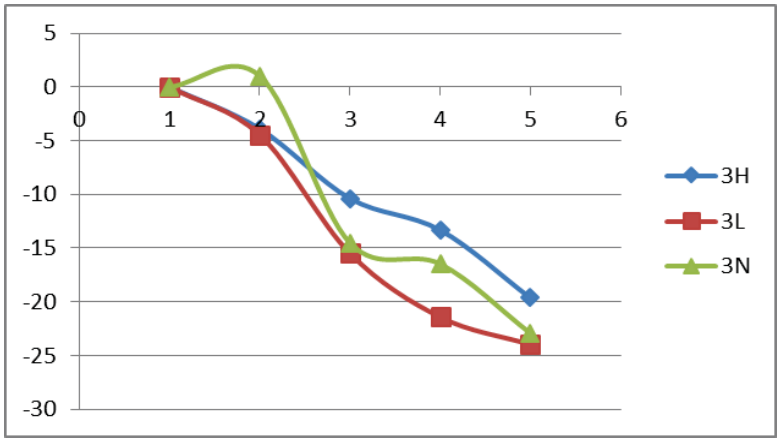
42 GRAPHS OF INDIVIDUAL HORSE DATA FROM MIMETIC PEPTIDE SCRAPE WOUND STUDY



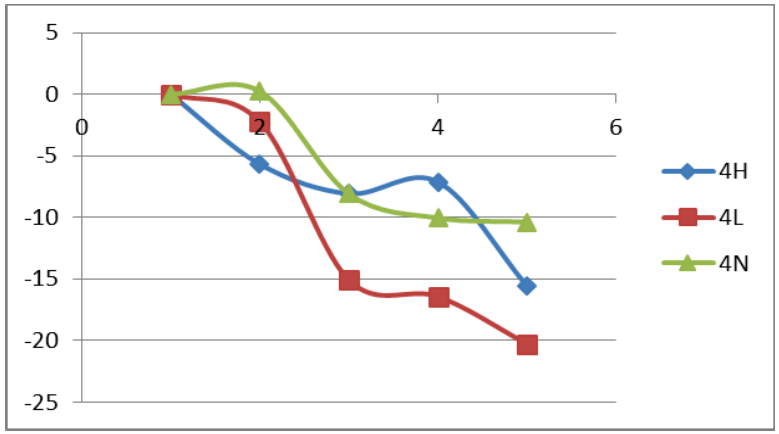
Horse 1



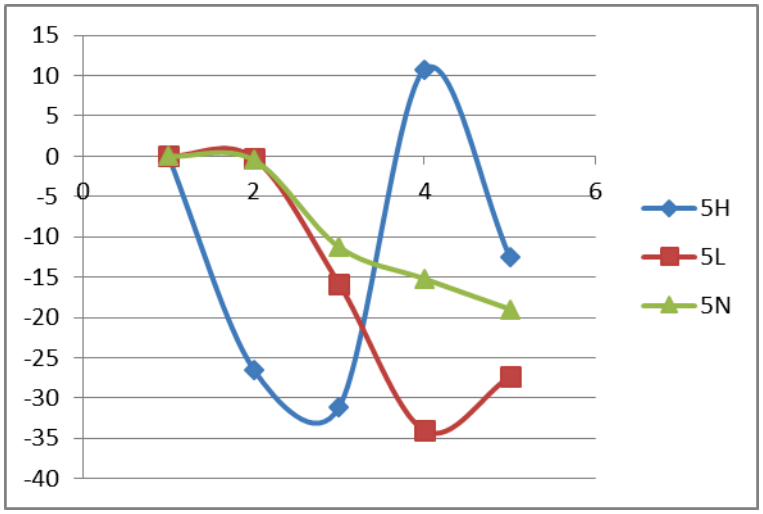
Horse 2



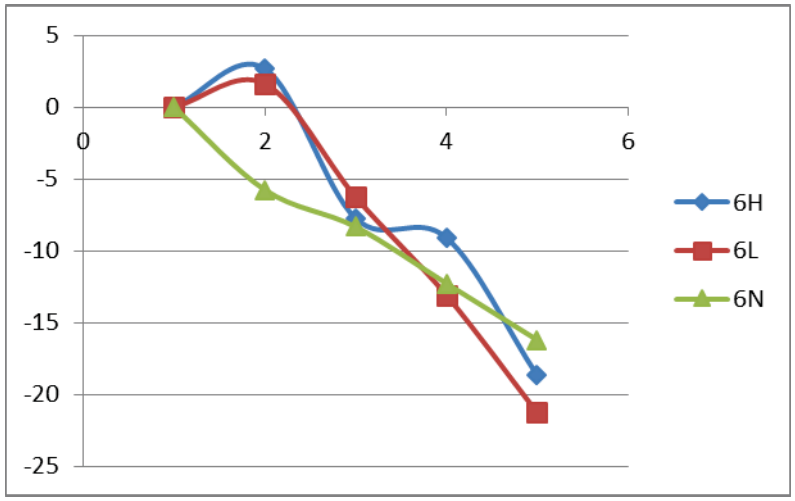
Horse 3



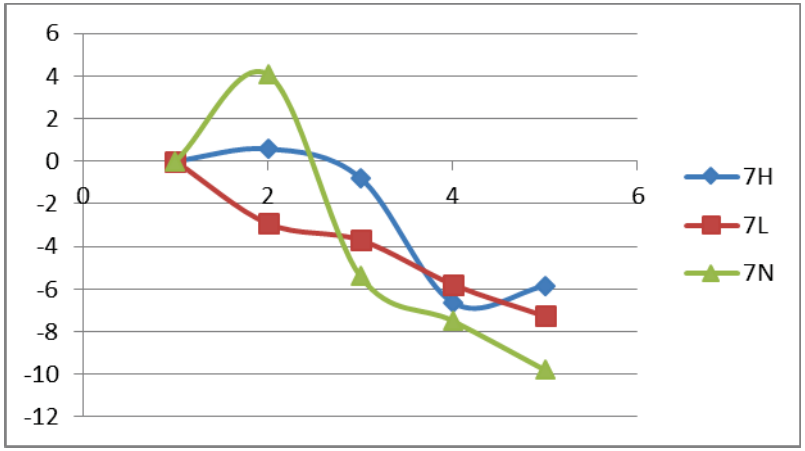
Horse 4



Horse 5 nb 1 well for H group



Horse 6



Horse 7

43 TABLE OF HORSE COLLECTION INFORMATION

ID		DATE	AGE	BREED	EUTH METHOD	EUTH TO COLLECT	GROWTH
Hunt 1	1	26/10/07	15	Tbx	Shot	2	Y
Hunt 2	2	02/11/07	10	Tbx	Shot	1	N
Massey	3	13/11/07	20	Tb	Pentobarb		N
Massey	4	20/11/07	4wks	Tb	Pentobarb		N
Massey	5	22/01/08	4	Tb	Shot		N
Hunt 6	6	05/02/08	20	Tb	Shot		N
Massey	7	08/02/08	4	Tb	Pentobarb		
Abbatoir	8	Sheep			Captive bolt		No
Massey	9	Friesian cow	2	COW ECR	Shot		Yes
Massey	10	29/04/08	20	Pony	Pentobarb	14	Y v few
Massey	11	08/05/08	11	WB	Pentobarb	3	Y
Massey	12	21/05/08	5	Tb	Pentobarb	1.5	N
Massey	13	06/06/08	2	Tb	Pentobarb	Collected under GA	Y
Massey	14	11/06/08	6	TB	Pentobarb	20	Y
Massey	15	27/06/08	2	StB	Pentobarb	26	No

Massey	16	19/09/08	1	Tb	Pentobarb	24	No
Massey	17	20/10/08	5	Tb	Pentobarb	20	Y few
Massey	18	08/04/09	5	Tb	Pentobarb	24	Y
Massey	19	15/07/09	4	Tb	Captive bolt	1	
Gore 1	C1		4	Tb	Captive bolt & exsang		Y
	C2		6	Tb	Captive bolt & exsang		Y
	C3		5	Tb	Captive bolt & exsang		Y
	C4		6	Tb	Captive bolt & exsang		Y
	C5		3	Tb	Captive bolt & exsang		Y
	C6		4	Tb	Captive bolt & exsang		
	C7		2		Captive bolt & exsang		
	C8		4		Captive bolt & exsang		
Gore 2	G1		2	Tb	Captive bolt & exsang		y
	G2		2	Tb	Captive bolt & exsang		Y
	G3		2	Tb	Captive bolt & exsang		Y
	G4		2	Tb	Captive bolt & exsang		Y
	G5		2	Tb	Captive bolt & exsang		Y

44 Collection and Extraction Times for Source Tendons

Horse id				Collection	Start trypsin collagenase digest	Seeded culture flasks
G1	2	Tb	Captive bolt & exsanguination	L 06:20 R 06:30	18:40 18:55	05:00 05:00
G2	2	Tb	Captive bolt & exsanguination	L 06:35 R 06:45	19:20 19:05	05:20 05:20
G3	2	Tb	Captive bolt & exsanguination	L 06:50 R 07:00	20:00 19:45	05:45 05:45
G4	2	Tb	Captive bolt & exsanguination	L 07:15 R 07:25	20:45 20:30	06:10 06:10
G5	2	Tb	Captive bolt & exsanguination	L 07:30 R 07:40	21:35 21:05	06:30 06:30

45 Horse Identification Form

See file 'APPENDIX 45 IDENTIFICATION OF THOROUGHBRED.pdf' on disk

46 RTV Dish Statistics and Video

See folder 'APPENDIX 46 STATS ON RTV DISH' on disk.

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