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Introduction and the Great CT:MR Dilemma
Although both modalities have been in use for a number of years, computed tomography and magnetic resonance imaging are relative newcomers to the armamentarium of diagnostic imaging studies available for evaluation of equine distal limb disorders. Still unanswered are questions regarding the ‘best test’ for a given clinical case or condition and there are relatively few objective comparative studies to address this.1-5 Despite the fact that some individuals or institutions may champion a specific imaging modality, clinical experience tells us that often no “best test” exists and that some combination of clinical and imaging tests may be required to reach a specific diagnosis. The worldly approach to the common conundrum of which test to use (i.e. which imaging modality) would consider the patient and its clinical presentation first and blend this information with the strengths and weaknesses, sensitivity and specificity of the available diagnostic imaging tests.6

From a historical perspective, computed tomography (CT) pre-dates magnetic resonance imaging (MRI) and therefore many orthopedic applications for CT were described initially.1, 7-16 As MRI has become more common in the clinical setting, its use has largely usurped CT particularly in the diagnosis of soft tissue musculoskeletal injuries. It is generally accepted that CT can yield more information regarding the morphology of bone but soft tissue detail of MRI is superior. In equine veterinary medicine, many reports tout the clinical utility of MRI particularly for the diagnosis of tendon and ligament injuries causing foot lameness. CT has been reported for the same purpose but to lesser extent. As stated above, there are few objective reports comparing the relative sensitivity and specificity of the two modalities for diagnosis of specific orthopedic disorders.

Basis for CT and MR Image Formation
The basis for CT and MR image formation are fundamentally different. As such, imaging features of the two modalities often do not correlate when comparing normal anatomy and pathology. Conceptually, CT image formation is the same as for conventional radiography and tissue appearance on an image is directly and predictably determined by tissue density. An x-ray generating tube is located across from a row of x-ray detectors in a circular gantry containing the patient. All of the image display features of digital radiography including window and level (contrast and brightness) adjustments are also applicable to CT images. MR image generation is more complicated and image features vary depending on the chemical composition of, and microenvironment around, the tissues of interest. MR images have much higher contrast resolution whereby tissues of different chemical composition that are immediately juxtaposed can be easily delineated. Another important difference between CT and MR that relates to the physics of image production is that modern helical scanners can produce one image per second or less per
detector row facilitating a complete distal extremity examination over the time period of 45 seconds or less. This excellent temporal resolution is an important consideration in dynamic studies such as selective CT angiography. As multi-row CT technology continues to advance, spatial, contrast and temporal resolution will all continue to improve. Contemporaneous advances in CT image processing have also improved the clinical utility of three dimensional and surface rendering reconstructions.

**Contrast Computed Tomography**

CT produces images that represent the regional anatomy and significant changes in tissue morphology are necessary to identify injury. In order for a tendon tear to be identified on CT it must be sufficiently large and cause enough of a change in density to be detected by the scanner. Likewise, a tendon injury must cause enough disruption of the highly organized tendon fibres to allow protons to move according to the external magnetic field and radiofrequency pulses applied giving off an MR signal. The identification of lesions in both modalities is aided by the recognition of additional basic imaging (roentgen) findings such as changes in size, shape and margination.

In both CT and MR musculoskeletal lesion recognition is further increased by the addition of contrast material (iodine or gadolinium based) through intravascular and intra-articular routes. Intra-articular contrast enhancement helps to delineate intra-articular ligaments such as the intercarpal ligaments or peri-articular soft tissue structures that can be difficult to detect without joint distension or increased intrinsic contrast provided by the contrast media. Intra-articular contrast also improves delineation of articular margins and can improve detection of articular cartilage defects. Intravenous contrast material is usually administered systemically through a peripheral vein, which, in the horse is a very large total body dose (approximately 1L). Under normal circumstances, intravascularly administered contrast medium initially results in vascular enhancement (vascular phase) with eventual redistribution to the extravascular fluid space (tissue or delayed phase) over the first few minutes. Alterations in tissue physiology such as neovascularization, increased blood flow or increased tissue permeability result in qualitative changes in the images, i.e. increased contrast material accumulation in the area. This provides two benefits, firstly, lesions become more obvious and secondly, contrast material provides some understanding of the characteristics of underlying tissue physiology that lead to the alterations seen with disease.

**Computed Tomography Angiography**

CT yields excellent anatomic detail of bone and has been widely used for the evaluation of traumatic bone injuries in people. Veterinary reports also show the superiority of CT over conventional radiographs for fracture depiction and characterization. In people, the configuration of extremity fractures is considered secondarily to the degree of soft tissue and vascular damage and fractures with vascular compromise are associated with higher rates of infection and limb amputation. These characteristics make CT angiography an important component of the pre-operative assessment of distal extremity trauma in horses when it is available.

Our group has developed a technique for the selective regional delivery of contrast material to the distal limb of the horse that has greatly aided the identification of tendon and ligament lesions within the hoof capsule using CT. Different patterns of contrast enhancement are identified in equine tendon lesions and although research is pending at this stage, may represent differences in stages of injury or healing. Soft tissue injuries identified using contrast enhanced computed tomography have had a similar distribution to the reports of the same injuries identified with MRI. Injuries are identified involving the deep digital flexor tendon, the collateral ligaments of the distal interphalangeal joint, the collateral sesamoidean ligament, the navicular impar ligament and the navicular bursa. Also similar to other reports abnormalities were commonly identified in the bone structures in addition to the soft tissues of the foot. Current sports medicine literature has also recognized alterations in blood flow dynamics in tendinopathy such as hyperemia and new vessel formation and these characteristics of tendon healing probably contribute to the contrast enhancing pattern of tendon injuries diagnosed on CT in the horse.
**Interventional Computed Tomography**

The jury is still out regarding the efficacy of many of the systemic and intralesional treatment options in common use for tendon injury. Research continues investigating many different options for tendon lesion injection such as autologous stem cells harvested from bone marrow or fat, platelet rich and platelet free plasma and shock wave therapy. Regardless of the choice of treatment, CT can be used to guide needle placement into the lesion allowing accurate delivery of the medication to lesions above and below the hoof capsule, a previously unexplored application of CT. CT can also be used to guide minimally invasive surgical procedures.

**Summary**

Computed tomography is a versatile, rapid and relatively inexpensive cross sectional imaging modality. Bone detail is excellent and the addition of contrast material improves its utility for the evaluation of the soft tissues. Furthermore, the ability to scan rapidly allows an assessment of underlying vascular physiology that is difficult to obtain with many other imaging modalities. Improvements in software and hardware continue and will likely improve the clinical utility of the modality over time.

**References**

FETLOCK FATIGUE INJURIES IN THOROUGHBRED RACEHORSES

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Fatigue related injuries of the metacarpo/metatarsophalangeal joints such as metacarpal/metatarsal condylar fractures and palmar/plantar osteochondral disease (POD) are common in Thoroughbred racehorses: up to 80% of racehorses suffer from POD in at least one condyle and lateral metacarpal condylar fractures are the most common fatal distal limb fractures in the UK. These injuries are unique to racehorses and are rarely observed in horses that don’t habitually train and perform at high speed.

Metacarpal/metatarsal condylar fractures are rarely associated with a fall or direct trauma to the limb and are typically located in the parasagittal groove or the axial aspect of the condyles. Complete and incomplete fractures occur in the same anatomical location. Chronic cartilage and subchondral bone changes are consistently associated with complete fractures (Radtke CL, Danova NA, Scollay MC, et al.). They are a consequence of repeated high magnitude loading during fast work resulting in damage accumulation.

The highest joint surface loads during locomotion have been measured in the metacarpophalangeal joint (Harrison SM, Whitton RC, Kawcak CE, et al.). Microcracks are found in subchondral bone at sites where condylar fractures and POD occur (Stepnik MW, Radtke CL, Scollay MC, et al.). These microcracks are similar to the fatigue damage detected in experimental animals after repeated loading of bone and corroborate that these injuries are a result of material fatigue of bone.

Fatigue is a process that has undergone much study in material science. Understanding the fatigue properties of a material or structure allows engineers to avoid its catastrophic failure. Fatigue life is an important material property and refers to the numbers of cycles of loading that can be sustained before failure occurs. Many factors affect the fatigue life of a structure including (1) the material properties, (2) the magnitude of the applied load, and (3) the geometry and architecture of the loaded structure. Fatigue life decreases exponentially with increasing load. This is important in horses as loads within the limb increase with increasing speed of gait. Fatigue properties are also inherently variable making predictions in an individual difficult.

Unlike many engineering materials bone is dynamic. It adapts to increased loading by modelling to maintain the strains within the bone at a safe level (Riggs CM and Boyde A). Fatigue damage accumulates quicker and failure is more likely in unadapted bone. Horses are at higher risk of suffering from a fatal lateral condylar fracture in their first year of racing, possibly reflecting insufficient bone adaptation (Parkin TDH, Clegg PD, French NP, et al.). Bone can also repair fatigued matrix through remodelling. Fatigue injuries develop when microdamage accumulates faster than remodelling can repair. The first phase of remodelling is bone resorption which weakens the bone through increased porosity. Remodelling of the equine metacarpus is reduced during race training and accelerated during rest periods (Whitton RC, Trope GD, Ghasem-Zadeh A, et al.). A bone that is porous following a rest period may fail earlier than a fully adapted bone if subjected to the same repetitive high intensity load.

As fatigue injuries are ‘material failure’ they should be preventable. Epidemiologic findings suggest the existence of a fine balance between a minimum and a maximum amount of fast speed work required in order to reduce the incidence of fatigue related bone injuries. Maximising adaptation has been identified as an important part of training young racehorses. However, even well adapted bones accumulate microdamage and therefore working with and encouraging the remodelling process is also important. If remodelling inhibition at the extremes of training is unavoidable then the duration of exposure to high speed work needs to be limited and appropriate rest periods instituted. Equally important is a gradual return to fast speed work after a rest.
It is unknown how different training regimens affect bone remodelling rates. Further research is warranted to elucidate the effect of fast speed work and rest on subchondral bone damage accumulation and repair.

**References**


Clinical significance of subchondral bone lucencies (POD lesions) in the distal McIII in TB racehorses diagnosed with radiography.

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Palmar osteochondral disease (POD) is an increasingly recognized syndrome causing fetlock related lameness and poor performance in racing Thoroughbreds. The pathogenesis of the condition has been well described by Riggs EVJ 2006, and is due to repetitive trauma of the McIII’s distal palmar condyle, during high speed exercise. This phenomenon creates subchondral bone POD lesions which have a spectrum of severity from faint discolouration to more obvious bruising, both visible through the overlying cartilage, to severe osteochondral ulcers.

Faint Discolouration   Obvious Bruising     Ulcerated POD lesion

Clinically, horses with ulcerated POD lesions are usually older (5yrs+), unilaterally lame with moderate to severe effusion of the fetlock joint but are not always sore to flexion. Intra-articular centesis reveals orange coloured synovial fluid, which has an increased protein count on cytology. Intra-articular medication with short acting corticosteroids improves the soundness grade, temporarily, but the joint effusion usually remains. In majority of cases, fetlock intra-articular nerve block only improves the lameness grade by 50% with four point n.b. rendering the horse sound. Differentials for this type of clinical condition are: incomplete lateral or medial condylar fracture, joint sepsis, sesamoid fracture, P1 fracture, or an intra-synovial suspensory branch lesion.

Despite challenges to the clinician to detect early lesions, ulcerated POD can be easily diagnosed with radiography. An ulcerated POD lesion presents as a concave radiolucency within the subchondral bone of the medial or lateral palmar condyle, adjacent to the articular margin. There are four views which can highlight these lucencies in the condyle (Pilsworth et al 1988, Butler et al Clinical Radiology of the Horse 3rd Ed, pages 152-5, Richard et al 2007, Hornof et al 1980) namely: flexed DP view (flexed dorso15°disto-palmaroproximal oblique), flexed lateromedial view, steep DP view (dorso60°proximo-palmarodistal oblique) and the medial or lateral oblique views (dorso30°proximo45°latero-palmarodistalmedial oblique).
The **flexed DP view** (*flexed dorso15° disto-palmaroproximal oblique*) is the easiest view to detect ulcerated POD lesions in the fetlock joint. In my experience, the fetlock is best positioned by raising the foot dorsally and proximally onto a 20cm x 25cm block, locking the shoe so it’s perpendicular to the ground and bringing the cannon bone to almost vertical. The fetlock is then held in a flexed position by the assistant who supports the forearm to prevent excessive fetlock flexion. The x-ray cassette is rested vertically on the horse’s heels in a plate holder. The x-ray generator is positioned on a stand, and angled 15° upwards allowing the beam to pass along a tangential plane through the fetlock articular surface. It is important that the limb is positioned carefully, paying attention to the degree of fetlock flexion and the plate is perpendicular to the beam. The flexed DP view highlights density changes within the subchondral bone of the palmar condyle by elevating the sesamoids away from the joint space. Radiographically, POD lesions appear as a smooth, well defined, concave lucency along the mid-condyle articular margin of distal McIII. Areas of relative radiolucency seen either side of the sagittal ridge are a normal finding.

Like the flexed DP view, the **flexed lateromedial view** also separates the sesamoids away from the palmar aspect of distal McIII and widens the articular space of the metacarpal-phalangeal joint. A POD lesion usually presents as incongruity of the articular surface along the palmar articular 1/3 of the distal McIII. Commonly, a large osteophyte off the distal tip of the proximal sesamoids is also seen in horses with a concurrent POD lesion. This view also allows evaluation of the thickness of the subchondral bone plate and density of the adjacent trabeculae bone. With DR, it is possible to window and level the image as well as use the inverse contrast function – useful to assess the palmar condyle.
The steep DP view (*dorso60°proximo-palmarodistal oblique view*) of the fetlock joint allows for less superimposition of the palmar proximal P1 as well as elevating the sesamoid bones away from the joint surface. A POD lesion in this view appears as a discrete, focal lucency within the distal McIII, midway between the articular surface and proximal sesamoid bone.

Finally, the raised **medial or lateral oblique views** (*dorso30°proximo45°latero-palmarodistalmedial oblique view*) highlight the palmar condyle with no superimposition. In this view, a POD lesion is seen as a focal, circumscribed lucency within the condyle, immediately distal to the highlighted sesamoid bone. A suspected lesion in a condyle in this view should be compared to the contralateral condyle and also seen in one of the 3 other views.
Differential diagnosis to a concave or circumscribed radiolucencies in the distal McIII using these views are: 1) subchondral bone cysts 2) OCD lesions especially those originating from the saggital ridges 3) lateral or medial condylar fractures and 4) POD lesions. Subchondral bone cysts are found in younger horses and consist of a circumscribed lucency, seen on most standard fetlock views and usually don’t involve the palmar articular aspect of the condyle. OCD lesions usually present within the dorsal aspect of the saggital ridge, which are highlighted on a standing or flexed lateromedial view. Finally, medial or lateral condylar fractures usually originate from the articular surface of the condylar groove, not mid condyle. However, surgeons should be cautious prior to McIII lag screw fixation when a suspected condylar fracture radiates from axial part of the condyle. In these cases, the condylar fracture is most likely originating from a POD lesion, which will have a poorer prognosis following surgical repair (Rick 1983).

Clinical Research Study on POD – Does size matter?

Aim: to determine the clinical significance and racing career of Thoroughbred racehorses diagnosed with subchondral bone lucencies (POD lesions) via radiology.

Hypothesis: TB racehorse’s diagnosed with a POD lucency in the distal McIII were less likely to race and were retired soon after diagnosis, no matter the size of the lesion.

Method: The study group consisted of TB racehorse’s, which had race and clinical records from the time of radiographic diagnosis till retirement. POD lesions were confirmed on both flexed DP and flexed...
lateromedial views. The size of the POD lesions, in terms of width and height, were calculated via a CR digital caliper, from the flexed DP view, and measured as a percentage of the retrospective condyle width. Post mortem examinations were conducted after retirement to confirm radiographic diagnosis.

**Results:** 77 racehorses were diagnosed with a subchondral bone lucency (POD lesion) in the distal McIII via radiography, during a fetlock lameness investigation. Horses, which subsequently underwent post mortem (55/70), had their diagnosed radiolucencies confirmed as POD lesions (recorded limb, size and condyle). POD lucencies, which were calculated to be equal or greater than 50% of the width of the condyle, would not race again, with 100% of this group retired due to non-responsive fetlock lameness despite several episodes of intra-articular medication and months of rehabilitation. POD lucencies less than 50% the width of the condyle raced again (median 8 times), earned stake money but required intra-articular medication to remain sound. Horse’s which had a condylar fracture originating from a POD lesion never raced again despite undergoing surgical lag screw fixation and retired due to fetlock complications post surgery.

<table>
<thead>
<tr>
<th>&lt; 50% condyle</th>
<th>&gt; 50% condyle</th>
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<td>Racing prospect with medication</td>
<td>Retirement from racing</td>
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**Conclusion** - This study highlighted the accuracy of using radiography to not only diagnose ulcerated POD lesions, but also determining the prognosis of future racing careers of horses affected with this condition.
Prepurchase Radiographs – Can they provide a black and white answer?

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Radiography is the most common diagnostic tool used to evaluate the musculoskeletal system in the prepurchase examination, in one review radiography was used in 49% of all pre purchase exams. This number appears to be increasing with the introduction of mobile digital radiography. However the correlation between radiographic appearances and clinical findings is poor unless severe changes are seen. Normal radiographs don’t always exclude pathological processes and apparently abnormal radiographic findings may not have current or future relevance.

Three important concepts to consider….  
A prepurchase exam is not a lameness exam. Diagnostic nerve blocks are not performed thus any radiographic abnormalities can not be attributed to a specific lameness.  
Radiographs give information about bone but minimal regarding soft tissue. Radiographic soft tissue swelling has usually already been noted on the clinical exam however boney abnormalities may highlight the presence of a significant soft tissue lesion and further ultrasonographic evaluation is warranted i.e. Roughening of the abaxial margin of the proximal sesamoid and lesions within the corresponding suspensory branches.  
Prepurchase radiographs give current information only. When abnormalities are found especially on radiographs veterinarians are frequently asked to speculate how they will affect future performance. Extrapolations to future soundness can be difficult based on a one time examination, especially when the changes are subtle or do not correlate with the physical exam.

When to perform?
- Abnormal clinical examination findings such as positive flexions tests, joint effusion, lameness on lunge
- Client request due to previous experiences, concerns or desire to re-sell the horse in the future
- In the young horse yet to be broken or older horse that is not in work
- Standard radiographic series required, for example Thoroughbred yearling repository
- Price of horse

What images to take?
Radiographs taken should include all orthogonal views and be of high diagnostic quality. For example when radiographing the front foot, a lateromedial and dorsopalmar are not sufficient and views should also include the dorsoproximal palmarodistal oblique of the third phalanx and the palmaroproximal palmarodistal (flexor) oblique of the navicular bone. Areas radiographed will depend on the clinical exam and the contralateral limb should be radiographed for comparison. Table 1 below describes the suggested anatomical areas to perform survey radiographs depending on the intended use of the horse.
Table 1. Suggested areas to perform prepurchase radiographs depending on horse’s intended use.

<table>
<thead>
<tr>
<th></th>
<th>Front feet</th>
<th>Front pastern &amp; fetlocks</th>
<th>Knee pastern &amp; fetlocks</th>
<th>Hind pastern &amp; fetlocks</th>
<th>Hocks</th>
<th>Stifles</th>
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<tbody>
<tr>
<td>TB yearling</td>
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<td>■</td>
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<td>■</td>
<td>■</td>
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<tr>
<td>Racehorse</td>
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<tr>
<td>SJ &amp; Dressage</td>
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<tr>
<td>Quarter horse</td>
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How to interpret these images?

All radiographic abnormalities need to be described and recorded regardless of the lesion’s clinical significance and risk rating. Lesions that will reduce the athletic performance of the horse include developmental disease, osteoarthritis, evidence of ‘wear and tear’, fractures and bone modelling. It is unlikely that a horse will not have some radiographic changes hence a buyer will expect the veterinarian to predict what impact these changes will have in the future. A risk assessment of low, medium or high should be made in conjunction with clinical findings, age and breed of the horse, intended use and whether the horse is meant for future sale. Radiographs may also be sent for a second opinion or to the client’s own veterinarian as their risk assessment may vary.

The following describe common conditions with variable correlation between radiographic changes and lameness in the horse.

**Navicular syndrome**
Radiographic changes are controversial as can be the same in normal and lame horses. Generally if the following radiographic changes are seen (increased number of enlarged invaginations, medullary sclerosis/loss of corticomedullary definition, medullary lucency and flexor cortex erosions/new bone formation) in combination with clinical signs then there is a high risk for impairment for future athletic performance. Horses with mild navicular changes may be able to be managed appropriately for pleasure or low level riding but are unlikely to be compatible as a high level dressage or showjumper. It must be noted that the radiographic appearance of the distal phalanx and navicular bone of Warmbloods can appear more irregular than Thoroughbreds.

**Tarsal degenerative joint disease**
Degenerative joint disease in the hock, particularly the tarsometarsal (TMT) and distal intertarsal (DIT) joint, is a common cause of hind limb lameness. Depending on the severity of the disease the risk is high for impairment for future athletic performance. Radiographic changes include: 1. Joint margins (shape & opacity), 2. Narrowing of joint space, 3. Subchondral lysis, 4. Trabecular bone sclerosis, 5. Subchondral bone irregularities and 6. Ill-defined subchondral bone/cartilage interface. However correlation between radiographic and clinical signs is low. Remodelling or bony spurs with smooth contours of the dorsal aspect of the TMT and DIT without changes in the articular margin are viewed as low risk. Subchondral bone plate irregularities appear to have greater significance for the DIT than the TMT joint.
Medial femoral condyle cystic lucencies

These appear to be an unpredictable risk for future lameness within young unraced Thoroughbreds. In 2009 a study by Jackson et al reviewing 2401 sets of yearling films found that medial femoral condyle lucencies equal to or less than 6mm in depth did not affect performance but those greater than 6mm in depth were less likely to start a race at 2 and 3 years of age. However due to the small number of horses (n=20) with greater than 6mm lesions other values were lower (race starts, wins, prize money) but not significant. Howard et al (1995) study correlating lesion size and racing performance after arthroscopic debridement portrayed that the amount of cartilage disruption was a better indicator of performance that depth of lesion radiographically. With personal experience lesions less than or equal 6mm depth have a low risk for racing and medium risk for sales whilst lesions greater than 6mm given their unpredictable nature have a medium risk for racing and medium to high risk for sales.

In summary; radiographic lesions or changes can only be interpreted when combined with a thorough clinical exam. All radiographic abnormalities should be recorded regardless of their risk assessment. The aim of prepurchase radiographs is to identify radiographic conditions which carry a degree of risk for future athletic performance. There are numerous factors affecting risk rating such as clinical findings, age and breed of the horse, intended use and whether the horse is being purchased for future sale. By giving the buyer an assessment of risk rather than a ‘pass or fail’ opinion, the veterinarian can help facilitate a successful sale.

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IMAGING OF THE EQUINE SKULL

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Introduction
Although conventional radiographic examination has historically played an important role in initial diagnosis and subsequent monitoring of equine skull disorders, it is an inherently limited diagnostic test in that complex three-dimensional structures of varying density are viewed as a two dimensional image. Image distortion and anatomic superimposition can obscure or make ambiguous important radiographic findings. When supplementary information is necessary to accurately assess the extent of disease or when questions arise regarding the anatomic localization of the lesion, additional diagnostic imaging such as computed tomography (CT) is indicated.1,3

Computed Tomography
Computed tomography (CT) uses x-rays and x-ray attenuation to create the image. The CT gantry houses a row of x-ray detectors across from an x-ray generator. The gantry rotates around the region of interest on the patient creating a ‘cross-sectional x-ray image’. The basic physics of CT are dependent on tissue density, similar to that of planar radiology, but CT’s cross-sectional nature eliminates superimposition of structures and dramatically improves contrast resolution while maintaining excellent spatial resolution. CT is particularly useful for imaging the equine skull where the anatomy is complex but inherent tissue contrast (i.e., large density changes between gas, soft tissue, bone and enamel) is high.

CT image interpretation is based on the fundamental principles of radiology. Normal anatomy is characterized and lesions are detected through the identification of roentgen findings such as alteration in size, shape, number, opacity (density) and margination. Furthermore when well-positioned, cross sectional images of the skull are symmetrical and therefore one side can provide an internal control for the other. Asymmetry can thereby be used to confirm or refute a suspected abnormality. The approach to conventional radiographic image interpretation can be applied to the evaluation of CT images. For example, widening or increased lucency of the periodontal/periapical space occurs on CT images appears much the same as it does on radiographic images, and its presence can be interpreted in a similar manner (i.e. that it most likely represents osseous resorption secondary to infection or periodontal disease.)

There are other advantages to CT that improve the diagnostic use of the modality. It is an inherently digital modality and therefore all of the advantages of digital imaging apply to it. Image-viewing software can be used to alter the window and level (digital imaging terms analogous to contrast and brightness), in addition to features such as zoom, rotate and measure using electronic calipers. CT images are produced using tissue density differences as the basis for image formation and electronic density measurements of various normal and abnormal tissue are both consistent and predictable. Use of tissue density measurements adds another parameter useful for refining a diagnosis. Another useful feature of CT is that the images can be reformatted into different imaging planes, even though they are initially acquired the transverse plane.

Until recently, a major disadvantage of CT was that general anesthesia was required during acquisition of the images. Historically, CT scanners in use in veterinary medicine were older generation axial scanners, which required a considerable amount of time to acquire a diagnostic study. The technology has advanced recently, and many veterinary hospitals offer helical scanning, in which the patient advances through the
center of the gantry as the gantry continues to rotate. This is in contrast to older generation scanners where the gantry would rotate through 360° and then reset with the patient table advancing incrementally through the gantry. Helical scanners are generally capable generating 1-64 images per revolution (revolution time approximately 1 second), and the image collimation thickness can vary from about 0.5-15 mm. The disadvantage of general anesthesia is largely offset by the additional information gained. Often, the CT scan and a subsequent therapeutic procedure can be performed under the same general anesthesia.

More recently, CT technology has been modified to allow for imaging the standing sedated horse. This advancement has the potential to change standard of care for equine patients with disorders of the head since general anesthesia, with all of its added morbidity and mortality risks, is avoided.

There are many different reports in the literature of a variety of conditions of the equine skull that document the value of CT. Multiple reports describe the CT findings associated with dental infections and infections of dental origin affecting the surrounding supporting structures. In general, CT is indicated when secondary infections of the sinuses or paranasal bones compromise radiographic evaluation of the underlying primary problem, and when a draining tract, a supernumerary tooth, or any other dental abnormality cannot be explained by conventional radiography. CT should also be considered as a means to increase the diagnostic certainty in clinical situations where the incorrect treatment is undesirable or potentially harmful to the patient (i.e. extraction of a non-diseased tooth).

CT is also useful for diseases of the paranasal sinuses and ethmoid turbinates. It is used commonly pre-operatively to gain an accurate assessment of the extent of the disease prior surgery. This is especially useful for extensive paranasal sinus cyst or ethmoid hematoma evaluation.

The temporomandibular joint, like most other joints, can be affected by osteoarthrosis. Radiographic changes typical of joint disease, such as joint surface irregularities, sclerosis, narrowing, malalignment, and periarticular osteophytosis, occur in the temporomandibular joint but may not be evident on radiographs until clinical signs of joint disease have been apparent for some time. This articulation is easily imaged using computed tomography, and a few reports demonstrate its usefulness for diagnosing disease of the TMJ. Ultrasound examination of this joint can give an accurate assessment of the joint’s soft tissues and periarticular bone.

An often-overlooked cause of oral dysfunction that can manifest as dysphagia or resistance to bitting is abnormality of the hyoid apparatus. The use of CT for diagnosis and characterization of temporohyoid osteoarthropathy has been reported. Radiography or CT of this structure is often used in conjunction with endoscopic examination of the guttural pouches. CT allows the anatomy to be completely evaluated by eliminating superimposition of the skull.

Summary
The equine skull and the dental structures have a complex structure and can be afflicted by numerous conditions. To make accurate diagnoses and to perform appropriate treatments, many steps, including a thorough clinical examination in addition to diagnostic imaging, should be taken. In some cases, when the clinical situation is complicated, routine radiography is inadequate to accurately define a problem area and CT should be considered.
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UPDATE ON THE PATHOGENESIS OF RECURRENT LARYNGEAL NEUROPATHY

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Overview
Recurrent laryngeal neuropathy (RLN) is a distal axonopathy of unknown aetiology affecting the left and right recurrent laryngeal nerves of large breed horses (Reviewed by Hahn 2003). The resultant denervation atrophy of the target laryngeal muscles causes clinically significant disease resulting from loss of function of the cricoarytenoideus dorsalis muscle (CAD), the single laryngeal abductor. Clinical signs of poor performance and upper respiratory noise are particularly apparent during strenuous exercise as near maximal laryngeal abduction is required for optimal air flow (Rakesh et al 2008). Despite nerve and muscle pathology being described bilaterally, the manifestation of natural disease is generally confined to the left side, consistent with the site of most severe pathological change.

Anatomical considerations
The anatomical pathway of the left and right recurrent laryngeal nerves differ. Axons of the left nerve loop around the aorta whereas the right branch turns at the right subclavian artery (Hackett 2000). The left recurrent laryngeal nerve is 30-40cm longer than the right, and twice as long as other nerves in the horse (Cole 1946). The complexity and length of its pathway is proposed to underlie the pathology of recurrent laryngeal neuropathy. Evaluation of other long peripheral nerves in horses with RLN demonstrated disease was confined to this specific nerve only (Hahn et al 2008). Thus, RLN is a disease process unique to the equine species.

Equine CAD comprises two distinct neuromuscular compartments. The lateral compartment contains fibres running at an angle oblique to the sagittal ridge of the cricoid cartilage and inserting via a tendinous attachment on the dorsolateral arytenoid muscular process. The medial compartment is composed of fibres running acutely to the sagittal ridge. Cheetham et al (2008) demonstrated glottal opening was predominantly influenced by contraction of the lateral compartment suggesting that its function specifically, may be important in expression of disease.

Histopathology
In the CAD muscle from horses with RLN, ATPase staining identified fibre type grouping, evidence of neurogenic muscle disease (Gunn 1972). Further investigations identified concurrent neuropathy along with similar, milder muscle pathology in nerve and CAD of horses without clinical disease (Gunn 1972, Cahill and Goulden 1986b, Duncan et al 1977) suggesting clinically relevant disease represents the ‘end stage’ of a chronic pathological process.

Laryngoscopically, subclinical RLN is assumed to be present when asynchronous or asymmetric laryngeal movement is observed yet full laryngeal abduction is possible (Duncan et al 1977). The majority of these horses are not at substantial risk for developing laryngeal paralysis (Anderson et al 1997, Dixon et al 2002) despite a high incidence (64%) of non-symmetrical movement reported in yearlings (Stick et al 2001).

The incidence of histopathological changes consistent with RLN, in laryngoscopically normal horses ranges from 30-100% (Duncan et al 1977, Piercy et al 2009). Fibre type grouping is considered
pathognomonic for denervation/reinnervation and the hallmark of disease. The wide variation in prevalence may be influenced by the method of analysis. Statistical assessment for the presence of a non-random fibre arrangement has been described (Venema 1992), but rarely used.

Immunohistochemical analyses suggest that in subclinical disease there is near elimination of the fastest (IIX) muscle fibre types with relative increase in type IIA fibres. Using the same methodology, similar IIX fibre loss was seen in four horses with clinical RLN compared with four histologically normal horses (Steel et al 2010). Although these studies suggest loss of IIX muscle fibres is important in disease, phenotypic differences between subclinical and clinical cases are not mirrored by significant differences in muscle fibre types and the reasons for progression to clinical disease are currently unknown.

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EVIDENCE BASED TREATMENT OF DDSP

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Introduction
Over the years, numerous treatment options have been described for treatment of dorsal displacement of the soft palate (DDSP). This in itself usually sends a “warning” that no one treatment is optimal. When making decisions regarding which treatment is most appropriate it is important to understand the rationale and scientific evidence that led to the development of these procedures, as well as being aware of the results of clinical efficacy studies. Whenever possible, clinical decision-making should be based upon the best available evidence. Recently a systematic review of the efficacy of interventions for DDSP was undertaken (Allen et al., 2012). The results of this systematic review revealed that, in evidence-based medicine terms, the quality of the literature was considered to be low. It is therefore difficult from the current evidence to draw firm conclusions regarding the true efficacy of these procedures or to determine which procedures might be the most successful and least harmful for treatment of DDSP. Systematic reviews only include efficacy studies in clinical cases. As a result, several well-conducted research studies performed in clinically normal horses that might provide further evidence to support or refute a treatment were not included. This presentation will attempt to summarise the findings of the systematic review and highlight some of the findings from other research studies in an attempt to draw all the evidence together.

Conservative and medical treatments
A number of conservative treatment methods have been described, including tack alterations such as nosebands, bit attachments, tongue-ties, the use of a bitless bridle or laryngohyoid support device (“Cornell collar”). Other conservative measures include the use of oral glycerine, increasing fitness or resting the horse. Very little clinical research has yet been undertaken to investigate these factors. Most work has focussed on the use of the tongue tie.

The tongue-tie aims to prevent caudal retraction of the tongue and was also thought to pull the hyoid apparatus and larynx forward. A recent study showed that a tongue tie did alter the position of the hyoid apparatus when assessed ultrasonographically (Chalmers et al., 2013). However, previous studies found that application of a tongue tie did not improve airway dynamics in clinically normal horses during exercise (Beard et al. 2001; Cornelisse et al., 2001a) nor did it increase nasopharyngeal diameter or alter hyoid position in anesthetised normal horses (Cornelisse et al., 2001b).

The results of a variety of forms of conservative management (including tongue-ties) in clinical studies are quite variable but in some studies appear similar to those obtained by surgical interventions (Allen et al., 2012). One study did suggest however that the improvement may only be temporary, and it is also noteworthy that many horses undergo surgical interventions when conservative management has been unsuccessful.

Medical treatments include primarily the use of corticosteroids to minimise inflammation that may be causing primary palate dysfunction or dysfunction secondary to neuropraxia of the pharyngeal branches of the vagus. However present there is insufficient evidence to confirm the role of upper airway
inflammation in clinical cases of palatal dysfunction. Only one small study, involving 6 horses, has examined the efficacy of treatment with oral corticosteroids. Treatment was reported to be effective in 100% of cases and this warrants further investigation (Allen et al., 2012).

**Soft palate procedures**

Several surgical treatments (thermal cautery, laser palatoplasty, palatal sclerotherapy, excision palatoplasty, staphylectomy) have been described that aim to increase the tension or stiffness of the soft palate. These methods do not address muscular strength of palatal musculature but aim to reduce the compliance of the soft palate through the induction of fibrosis. The stiffening that results is thought to increase the intrinsic strength of the caudal soft palate so that it is able to resist the large pressure changes that occur within the nasopharynx during strenuous exercise. However, there is presently insufficient evidence to confirm whether or not increasing the stiffness improves palatal function.

No research studies have assessed oral tension palatoplasty techniques (either by thermal cautery or excision) with respect to the histopathological response or the effect on soft palate stiffness. A histological study assessing the effect of diode laser treatment of the nasal surface of the soft palate confirmed that it did induce a fibrous tissue reaction (Delfs et al., 2008). However, loss of soft palate skeletal muscle was also observed secondary to laser-induced thermal injury. Despite the development of fibrosis, the stiffness actually decreased and the soft palates from treated horses were in fact more compliant than those of control horses. It has subsequently been proposed that loss of muscle mass may well be detrimental to soft palate stability and hence this procedure may be detrimental rather than beneficial in the long term. Palatal sclerotherapy has been described using sodium tetradecl sulphate and poly-L-lactic acid as sclerosing agents, which are injected into the submucosa from the nasal aspect. A fibrous tissue reaction has been observed in response to injections of poly-L-lactic acid (Cehak et al., 2006), but no histological response and no increase in stiffness was reported with sodium tetradecl sulphate injections (Munoz et al., 2010).

Staphylectomy has been performed by excision and more recently by laser. The procedure is thought to result in fibrosis and stiffening of the caudal border of the soft palate. Following laser staphylectomy, the free border of the soft palate becomes thickened due to excessive formation of connective tissue. The procedure reduces the length of the soft palate thereby increasing the size of the intrapharyngeum ostium. Rather than preventing displacement, the procedure may appear successful by reducing the degree of obstruction that occurs once displacement has taken place. The staphylectomy procedure impairs upper airway mechanics in clinically normal horses, resulting in significantly increased tracheal and translaryngeal inspiratory impedance during exercise compared to pre-surgery (O’Reilly et al., 1997). The effect on respiratory parameters in clinical cases with palatal dysfunction has not been studied.

Variable success rates have been published for soft palate procedures and are highly dependent on how a diagnosis is achieved and how outcome of surgery is assessed (Allen et al., 2012).

**Procedures affecting laryngohyoid positioning**

Interventions may alter laryngeal position by preventing caudal retraction of the larynx (for example by tenectomy or myectomy of sternothyroideus, sternohyoideus and/or omohyoideus muscles) or by advancement of the larynx by means of the laryngeal tie-forward procedure or use of the laryngohyoid support device. However, to date, the assumption that caudal retraction of the larynx is the cause of DDSP in naturally occurring cases has not been confirmed.

A myectomy/tenectomy of sternothyrohyoideus/omohyoideus may be performed alone or in combination with other surgical procedures. Although the effect of sternothyroid tenectomy or myectomy on the position of the hyoid or larynx at rest has not been studied, resection of the sternothyroides and
sternohyoideus muscles in normal horses increases the translaryngeal and tracheal inspiratory pressures during exercise (Holcombe et al. 1994). These results imply that the sternothyroid and sternohyoideus are important in maintaining the stability of the upper airway and that the myectomy procedure has an adverse effect on upper airway mechanics in normal horses. Results of efficacy studies are again variable with most suggesting only moderate success rates (Allen et al., 2012).

The laryngeal tie-forward (LTF) procedure involves placement of a suture between the thyroid cartilage and the basihyoid bone, in an attempt to recreate the action of the thyrohyoideus muscle. This procedure was developed after placement of the suture was found to prevent DDSP in 5 of 6 thyrohyoideus-resected horses (Ducharme et al., 2003). Radiographs have confirmed that the LTF acts to move the basihyoid dorsally and caudally and to move the larynx dorsally and rostrally at rest. The LTF technique has been modified and most surgeons include a concurrent sternothyroid tenectomy.

Several clinical studies have been undertaken to evaluate the laryngeal tie forward procedure. Although the initial studies suggest good efficacy of the procedure, other studies have reported lower success rates that were not substantially different from those reported for other procedures (Allen et al, 2012).

**Epiglottic surgeries**

It is proposed that epiglottic augmentation increases epiglottic size, thickness and rigidity thus helping to prevent DDSP during exercise. At present there is limited evidence to confirm the role of the epiglottis in dynamic palatal dysfunction. Studies in the resting horse and in horses with epiglottic retroversion suggest the epiglottis does not play a role in soft palate positioning (Holcombe et al., 2002; Terron-Canedo and Franklin., 2012). However a clinical observation study has confirmed an association between epiglottic conformation changes during exercise and the development of DDSP during exercise (Allen & Franklin, 2013).

**Conclusions**

Current evidence places the practicing veterinarian in a difficult position with regard to selection of a treatment that is most likely to be effective in a particular horse. In many cases conservative interventions are trialled before resorting to surgical interventions. Owners and trainers should be made aware of the limitations of surgical procedures. However, because performance is so substantially affected by DDSP, they may be willing to try surgical interventions even though the success rates are only moderate. Ultimately it is hoped that an improved understanding of the aetiopathogenesis of DDSP will allow the development of more effective procedures.

**References:**


Mechanical Evaluation of the Equine Laryngoplasty

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The biomechanical principles behind a prosthetic laryngoplasty are relatively basic. Simply put, a prosthetic material is utilized to replace the abductor muscle function of the larynx to achieve arytenoid abduction thus allowing for effective airflow. However, the surgical techniques employed to achieve this function are extremely varied and have been associated with unreliable results. As a result a consideration of the biomechanics involved with this commonly performed procedure is warranted. In general, biomechanics is concerned with the behavior of a biological body when subjected to forces or displacement and the subsequent effects on the system in question. In recent years new research pertaining to the equine laryngoplasty and its mechanical evaluation has been performed. An outline of these developments and how this information is being used to improve the results of this commonly performed procedure will be discussed.

The basic principles of airflow mechanics will be reviewed and subsequently progress into discussion of the biomechanics of the component parts of a laryngoplasty. Specifically, the component roles of the arytenoid, the cricoid, the cricoarytenoid articulation and the choice of prosthetic material will be reviewed.

This is a technically demanding surgery where attention to detail, knowledge of biomechanical principles and small changes are commonly associated with large alterations in surgical outcome. An outline of the current understanding of laryngeal biomechanics and its relevance to clinical practice will be presented.
Despite much research over past decades, the cause and detailed pathogenesis of recurrent laryngeal neuropathy are unclear. Some horses are mildly affected and never show clinical signs (subclinical cases), whereas other are severely affected resulting in poor performance and upper respiratory noise. One of the major limitations for investigators of this disease is that horses with clinical RLN represent the ‘end stage’ of disease. There is currently no method for serial evaluation of unaffected or subclinical horses, antemortem. Subclinical horses are definitively identified by microscopic evaluation of laryngeal muscle sections which is currently performed post mortem. We know that subclinical disease is prevalent in certain horse populations yet a relatively small proportion of horses progress to clinical disease. Therefore the importance of ‘mild’ RLN is uncertain and the reason for progression unknown. The inability to identify and monitor subclinical cases antemortem clearly limits research progress and must be addressed if we are to make significant grounds in understanding the pathogenesis of this important disease. The aim of this study was to improve our understanding of changes occurring within the diseased CAD muscle. Specifically, we examined the effect of disease on myosin heavy chain gene expression, and pathways of muscle breakdown. We focussed on identifying critical changes that might cause onset of clinical signs and on laying the foundation for future research in the field.

The study was divided into three parts:

Part 1: Gene expression was investigated in age matched cases and controls for a range of genes encoding myosin heavy chain proteins. This is the first step in understanding what differentiates horses with normal laryngeal movement (with or without subclinical disease), from those that are paralysed.

Part 2: Myosin heavy chain gene expression was examined for normal horses of ages ranging from immature to mature adults. Amongst other factors age and training alter muscle fibre type. Our ultimate aim is to assess individual horses repeatedly over time, and to identify horses at risk. It was therefore essential that we investigate if age or onset of training alter laryngeal muscle fibre type.

Part 3: Gene expression encoding key proteins in muscle atrophy (‘atrogenes’) were evaluated in normal horses and those affected by RLN. MyHC and the atrogenes were also examined in a small number of horses that had surgical re-innervation of the CAD, which had failed to restore sufficient function for racing. As muscle atrophies, pathways involved in muscle degeneration are upregulated. Atrogin-1 and MuRF have been recently identified as key regulators of this process (Sacheck et al 2007). Increased expression of these genes in normal horses may suggest subclinical disease and could be a useful marker of disease progression.

Downregulation of gene expression for IIX and slow muscle fibres occurred in RLN. Fast (IIX) fibres are lost early in disease, and appears to continue as clinical signs occur. Downregulation of genes encoding slow fibres occurs less dramatically and we hypothesise that in combination with low IIX expression, the loss of slow fibres is responsible for onset of clinical signs.

The effect of age and training on MyHC gene regulation was examined in normal horses. There was large variability in the magnitude of gene expression in some individuals. Combined with the absence of untrained adult controls, this made identification of an effect based solely on age difficult. Within each age group of trained horses (greater than 2 years old) there was often prominent upregulation of genes encoding Slow and Embryonic MyHC, relative to fast MyHC isoforms. In general, expression for type IIX and IIA were of similar low magnitude suggesting a shift from fast to Slow MyHC occurred. This may explain the ability of many horses to sustain laryngeal function despite significant loss of type IIX MyHC, that was reported in subclinical cases (Rhee et al 2009). If (following muscle adaptation) IIX
fibres contribute only a small part of the CAD muscle belly, it may be unsurprising that their loss can be tolerated during subclinical disease. Atrogin-1 and MuRF1 encode important pathways for muscle atrophy. Horses with RLN did not have significantly greater expression of the ‘atrophy genes’ than controls, though again, highly variable expression was seen. Within the group of controls, there were individuals with dramatic increased expression of both genes and often, MuRF1 expression was of greater magnitude than Atrogin-1. We hypothesise that normal individuals with strong expression of Atrogin-1 and MuRF1 have subclinical RLN and that MuRF1 may be the more sensitive indicator of disease.

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PRELIMINARY FINDINGS REGARDING OVERGROUND ENDOSCOPY PRE- AND POST- UPPER AIRWAY SURGERY

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Summary:
Dynamic respiratory endoscopy (DRE) is used to visualise the equine upper airway during high speed exercise, allowing accurate diagnosis and subsequent treatment of dynamic upper airway conditions. DRE can also be performed following upper airway surgery to evaluate surgical success. This presentation will discuss our findings at a Thoroughbred racetrack and referral practice, focusing on pre- and post-upper airway surgery, including laryngoplasty; tie-forward procedures and resection of the aryepiglottic folds.
OVERGROUND ENDOSCOPY – WHAT’S IMPORTANT?

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Introduction: 
The development of overground endoscopy over the past 5 years has revolutionised the way in which we diagnose dynamic obstructions of the upper respiratory tract (URT). This technology enables horses to be examined in their normal environment and it’s availability enables increased numbers of horses to receive a definitive diagnosis of URT collapse prior to embarking upon surgery. This presentation will cover the top 10 tips for successfully performing overground endoscopy and making a definitive diagnosis.

1) Getting started - which equipment should I buy? 
With a number of systems now on the market there are a number of factors to consider. These include the cost, image quality, ease of use, real-time viewing of the image and ability to flush the endoscope during the exercise test, as well as logistics for on-going support and servicing (especially if importing equipment from overseas).

2) Preparation, preparation, preparation 
Before setting off it is important to check that your equipment is clean and in good working order, battery packs are fully charged and that all of the components are packed. There is nothing more frustrating than to travel 2 hours to examine a horse only to find a crucial component is missing or not working!
Another important consideration is where the exercise test will take place. For examinations at the track, it is important to check with the Stewards in advance and plan your examination towards the end of routine training when the track is quiet.

3) Horse preparation and set up 
Ideally horses should be suitably fit for the assessment being undertaken. If unfit, horses may not be able to exercise at a sufficiently high intensity that would be necessary to illicit abnormalities. Furthermore, it may not be possible to achieve exercise intensities that would be reached during competition.
Application of the equipment is best performed in a quiet environment (e.g., in a stable / stall) rather than in the arena or start of the gallops, where horses are likely to be more excitable. As for resting endoscopy, passage of the endoscope is via the ventral meatus and is usually straightforward in the majority of horses with or without the use of a nose-twitch. The ability to move the endoscope tip is important for accurate positioning. However, in the majority of cases when the position is set, there is little need to alter it during exercise.
Positioning of the tip of the endoscope is critical. If the endoscope is positioned too far rostrally, it may be difficult to observe all of the structures associated with the larynx (e.g., the vocal cords) and beyond (e.g., the crico-tracheal membrane). This is particularly evident if lighting is suboptimal or if there is pharyngeal collapse that obscures the view of the larynx. However, if the endoscope is positioned too close to the larynx, it may not be possible to identify the presence of pharyngeal wall collapse or palatal instability. Ideally, the endoscope should be positioned so that the tip of the epiglottis is clearly visible. However, in some cases it may be necessary to readjust the endoscope and repeat the exercise test to visualise different parts of the upper airway.
4) **Interpretation of resting findings**

Once the endoscope is in position and before the exercise test is performed, it is useful to make some notes on any abnormalities that are obvious at rest. In some cases a definitive diagnosis may be possible from the resting examination. However, in most cases it is worthwhile completing the exercising examination even when abnormalities are observed at rest since many horses have collapse of multiple structures during exercise, which may not be evident at rest.

5) **Planning the exercise test**

The type of exercise test performed is critical in enabling an accurate diagnosis of dynamic airway obstruction to be made. In cases where horses make obvious respiratory noise during training conditions, a diagnosis should be straightforward. However, for investigation of those cases that make abnormal respiratory noise only during competition or racing it is essential to replicate the conditions encountered during competition. This also applies to those horses that present with poor performance that are not reported to make abnormal respiratory noise. If the clinical signs reported during competition are not replicated, false negative findings may occur.

For investigation of poor performance in racehorses, it is recommended that exercise testing be performed at an appropriate track where the distance and speeds encountered during racing can be replicated. In pleasure and sport horses, it is now acknowledged that rider interventions and changes head and neck position may markedly influence the upper airway dynamics. Again, it is important to replicate the conditions under which the problem arises. For dressage horses, this may require the horse to perform certain manoeuvres, whilst for showjumpers it may be necessary to include jumping exercises. For eventers, it is often necessary to recreate the speeds and distances encountered during the cross-country phase.

Where you are located during the exercise test for optimal viewing of the examination will depend on the facilities available. Where horses are being exercised in an arena it is possible to visualise the horse and the upper airway recording simultaneously, throughout the exercise test. It is also possible to listen to any abnormal sounds produced and relate these to the type of exercise being performed and the endoscopic findings. This is not always straightforward on the track, since the telemetric range of the equipment may not enable visualisation of the endoscopic image throughout the entire test. Furthermore, the distance from the horse means that it is not always possible to hear any abnormal sounds produced by the horse. In some cases, it may be possible to drive alongside the horse. However, if this is not possible, it is best to position yourself near the end of the track so that you can hear and watch the image as the horse passes by whilst at peak speed or as it starts to fatigue towards the end of exercise. Noting the timings of the start and end of exercise is useful when replaying the video recording post-exercise. Some equipment incorporates a microphone, which can also be beneficial.

6) **Additional measurements**

A number of additional measurements may help you to achieve a definitive diagnosis, especially in the case of those horses being investigated for poor performance rather than abnormal respiratory noise. The use of a GPS monitor enables you to determine the speed and distance covered by the horse. A heart rate monitor provides useful information relating to the cardiac response to exercise and recovery. Lactate measurements may also be useful in some cases. For horses with a history of poor performance the inclusion of an exercising ECG is important to help rule out possible cardiac arrhythmias. Other examinations that might be carried out post-exercise include examination of the lower respiratory tract, tracheal wash and bronchoalveolar lavage, gastroscopy and blood sampling to check for evidence of subclinical myopathy.

Even in those cases where abnormal respiratory noise is the primary complaint it is important to remember that poor performance is frequently multifactorial and where possible a thorough examination of all body systems is warranted.
7) Interpretation of results
Once the examination has been performed, it is necessary to inform the owner / trainer of your findings and advise the most appropriate course of action. When interpreting the upper airway findings it is important to first consider whether the presenting complaint was replicated during the exercise test. If not, it may be necessary to repeat the examination. In other cases, abnormal sounds may have been heard but no obvious findings are evident on the video recording. Again, a repeat examination is warranted here, with the endoscope position altered in order to visualise different parts of the URT more clearly.
When viewing the recording it is important to determine whether the image is of diagnostic quality. Is the lighting sufficient to visualise all of the structures and is there excessive mucus on the camera that might impair the image? It is then important to review the recording both in real-time and in slow motion in order that you do not miss more subtle abnormalities.
When considering the functional significance of any URT collapse it is important to consider the extent of airway obstruction and the intended use of the horse. For horses undertaking submaximal exercise alone, some forms of dynamic airway collapse may not be clinically significant. It is also important to consider external factors that might influence URT collapse. These include rider interventions and head and neck position. In some cases it may be possible to avoid dynamic URT collapse by making alterations to the way in which the horse is ridden, without the need to resort to surgery. Finally it is important to consider whether or not any other factors may also be contributing to the horse’s poor performance and whether other investigations are also warranted.

8) Post-processing of your images
In this age of digital technology, owners and trainers are frequently keen to have a copy of the videoendoscopic images. Readily available software enables you to now edit the video to include only the salient points and also to superimpose patient details.

9) Cleaning and care of your kit
This is a crucial step that is sadly frequently overlooked in veterinary medicine. However, it is important to consider biosecurity and the risk of transmitting infectious agents between horses. If multiple horses are to be examined during one visit it is important to clean and flush the endoscope between horses. A complete sterilisation should then be performed once you return to the clinic. Saddlery equipment should also be cleaned appropriately.

10) Not sure – ask for help?
Arboviral disease in Australian horses
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Introduction
Extreme weather events in 2010-11 with widespread flooding over south eastern Australia resulted in an unprecedented epidemic of arboviral disease in Australian horses. Several endemic mosquito-borne arboviruses are not only clinically relevant to horses but of serious public health importance. Arboviral disease is generally more prevalent in Northern (or more tropical) regions; however, occasional spread of disease to the southern extent of the continent occurs sporadically. Such spread has been particularly noted following extreme weather events producing excessive summer rainfall and widespread autumnal flooding, as occurred in 1974 and recently in 2011 (Gard, Marshall et al. 1977; Marshall 1988; Knox, Cowan et al. 2012; Roche, Wicks et al. 2013).

Three Arboviruses that have been well associated with neurological and or musculoskeletal disease in horses (and humans) belong to the Flavivirus and Togavirus families. West Nile virus - Kunjin subgroup (WNV(kun)) and Murray Valley Encephalitis virus (MVEV) are Flaviviruses that may cause fatal illness. Ross River virus (RRV) in the Alphavirus genus of the Togaviridae family may cause musculoskeletal disease in horses and humans.

All of these viruses are zoonotic and signs or symptoms of disease tend to occur in the vast minority of infected individuals (Gard, Marshall et al. 1977; Kay, Pollitt et al. 1987; Marshall 1988; Azuolas, Wishart et al. 2003; El-Hage, McCluskey et al. 2008; Knox, Cowan et al. 2012). Heavy rainfall and widespread flooding during the summer of 2010-11 over south eastern Australia created favourable conditions for the arthropod vectors and amplifying hosts of these arboviruses leading to an outbreak of disease in over 1000 horses with a case fatality rate in the order of 10% (Animal Health Australia 2012; Roche, Wicks et al. 2013).

Australian Flaviviruses of Clinical significance in horses

The Flavivirus family includes over 70 species of RNA viruses that tend to be grouped serologically, the Japanese Encephalitis (JE) serogroup includes WNV(kun) and MVEV. Both of these viruses require mosquito – bird cycles of transmission and amplification respectively. Horses (and humans) are termed dead end hosts where infection may be termed a spill- over event (Marshall 1988; Mackenzie, Broom et al. 1998; Hall, Broom et al. 2002).

West Nile virus (Kunjin)
Although closely related within the Flavivirus genus the Kunjin virus and WNV are antigenically distinct. However since phylogenetic evaluation of the virus has established the close relationship between the two strains, Kunjin virus is now classified within the WNV clade and now termed WNV(kun) (Scherret, Poidinger et al. 2001; Scherret, Mackenzie et al. 2002).
Furthermore, the emergence of a more neuroinvasive Kunjin strain (termed WNV(NSW2011)) has led to Australia’s OIE status being re categorised as endemic for West Nile Fever (Frost, Zhang et al. 2012). It is important to note that although WNV(KUN) is considered zoonotic and pathogenic to horses, it has been rarely reported as a cause of disease in humans (or birds). This contrasts with strains circulating in the USA such as WNVNY99 which has a fatality rate several times greater in horses, humans and birds (Frost, Zhang et al. 2012; Roche, Wicks et al. 2013).

**Murray Valley Encephalitis Virus**

Murray Valley Encephalitis virus is endemic to northern Australia causing sporadic neurological disease in both humans and horses. Occasional spikes in prevalence occur often, but not always following extensive flooding as reported in 1974 and 2011 (Gard, Marshall et al. 1977; Roche, Wicks et al. 2013). Serological evidence of infection in horses far exceeds that of clinical disease, however human and horse fatalities are sporadically reported (Gard, Marshall et al. 1977; Knox, Cowan et al. 2012; Anonymous. 2013; Roche, Wicks et al. 2013). MVEV is considered a more virulent pathogen for humans than other endemic arboviruses (Knox, Cowan et al. 2012). Seroconversion in sentinel chickens strategically located, provide some guide as to the potential activity of arboviruses; in particular MVEV (Animal Health Australia 2012; Knox, Cowan et al. 2012; Anonymous. 2013).

**Alphavirus - Ross River virus**

Ross Rover Virus is a Togavirus within the alphavirus genus that includes another endemic zoonosis - Barmah Forest Virus (BFV) which are widespread throughout Australia and tropical regions to the North. Reports of human disease number over one to five thousand on average annually for BFV and RRV respectively (Russell 1998; Russell and Dwyer 2000; Anonymous. 2013). Several mosquito species act as vectors – in particular the aggressive day and night biting saltmarsh mosquito *Aedes camptorhynchus*. Natural reservoir hosts for RRV are considered to be native macropods (wallabies and kangaroos). Humans and horses are again considered spill-over hosts of little relevance to the lifecycle (Russell 1998). Although this stance is oft repeated (and most likely extrapolated from Flavivirus findings), the author considers that further research is required before such statements can be made with any veracity (Kay, Pollitt et al. 1987; Azuolas, Wishart et al. 2003; El-Hage, McCluskey et al. 2008). This is particularly important given the numbers of horses in close proximity to urban fringes.

**Equine Arboviral Disease Epidemic 2011**

Over one thousand horses succumbed to equine neurological and or musculoskeletal disease in the 2011 arboviral outbreak. Most recovered with supportive treatment however approximately 10% of affected horses nationwide died or were euthenased (Roche, Wicks et al. 2013).
Neurological syndrome
The predominant clinical abnormality of horses with disease due to arbovirus was neurological, particularly in NSW and SA.
Clinical signs of affected horses included some or all of the following; ataxia, muscle fasciculations, altered mental state, hypermetria, pyrexia, facial nerve paralysis dysphagia, and blindness. Some horses presented initially with clinical signs referable to colic, lameness, or facial paralysis due to pain and or altered gait respectively (author’s observations). Hendra virus exclusion was performed in the majority of cases (Roche, Wicks et al. 2013).
One feature noted in two horses with fatal flaviviral infections was their peculiar stance described as a “circus elephant on a box” with hindlimbs placed more cranially than normal (Holmes, Gilkerson et al. 2012; Tee, Horadagoda et al. 2012).
Although the majority of horses recovered from illness, recumbency is considered a poor prognostic indicator and the fatality rate was approximately 10%. This was lower than the rate of approximately 30% of WNV disease in US horses, however was considered higher than previous reports of WNV\textsubscript{(kun)} disease prior to 2011 (Long, Ostlund EN et al. 2002; Frost, Zhang et al. 2012). Several strains of WNV\textsubscript{(kun)} were isolated during 2011, and it has been postulated that the emergence of a more neuroinvasive virus, termed WNV\textsubscript{(Nsw2011)} may have been responsible for an increase in diseased horses and higher fatality rate (Frost, Zhang et al. 2012; Roche, Wicks et al. 2013). This increase in virulence was however not noted in humans nor birds.

Pathogenesis
Many of the clinical neurological abnormalities seen in Australian horses with flaviviral disease can be attributed to their predilection for specific regions within the CNS. Inflammation typically occurs in the mid-brain, hind-brain and throughout the spinal cord (Gard, Marshall et al. 1977; Long, Ostlund EN et al. 2002; Carr and Dickason 2011).

Muscloskeletal syndrome
In Victoria and SA a large number of horses with signs including reluctance to move, swelling of distal limb joints and pyrexia were associated with serological evidence of recent RRV infection. Although the virulence of this virus has been questioned, previous reports of such disease in horses were consistent with these signs (Gard, Marshall et al. 1977; Azuolas, Wishart et al. 2003; El-Hage, McCluskey et al. 2008). Several horses were noted to have concurrent infections of RRV and at least one Flavivirus (Department of Environment and Primary Industries 2011; Animal Health Australia 2012).

Diagnosis
Disease due to arbovirus(es) was confirmed using one or several methods that included an array of necessarily complex serology, PCR and Virus isolation. Whilst clinical signs may have been highly suggestive, haematology and biochemical changes were not considered specific for arboviral disease. Specific temporal correlation of infection using various methods were generally required for confirmation.
Assessment of serological responses of horses infected with Arboviruses poses several challenges due to several factors including the presence of background antibodies in horses with and without disease and high level of cross reactivity within the Flavivirus subgroup
Both WNV(KUN) and MVEV are part of the JEV serogroup and their close antigenic relationship results commonly in cross reactivity. This phenomenon resulted in some difficulty establishing the primary infecting virus. A panel of Flavivirus ELISA assays are generally used including a Pan Flavivirus ELISA and individual MVEV, and WNV(KUN) ELISA. Acute and convalescent sera provided valuable information regarding seroconversion and the maturity of the antibody response. Other tests to identify IgM antibody are indicative of recent infection in horses. IgG antibodies on the other hand are slower to detect and may last for years. A distinction between the two may assist in determining whether antibodies may be merely background or significant. Virus Neutralisation type tests are generally performed on samples positive to ELISA assays to provide greater specificity however, these were still not without cross reactivity issues either. Serum for RRV testing involved similar type assays and protocol as the Flaviviruses. Dual or mixed serological responses both within the Flaviviruses and between Flavi and alphaviruses were not uncommon (Department of Environment and Primary Industries 2011). PCR assays were also performed on blood and tissues in addition to virus isolation in cell culture on blood and post mortem derived tissue samples. Immunohistochemistry was also performed in some cases (Dr. Jemma Bergfeld, AAHL).

Pathology
Histopathological lesions were limited to the CNS and typical of viral encephalomyelitis in horses. Such findings often included mild to moderate, multifocal lymphocytic polioencephalomyelitis, with lymphocytic and mononuclear inflammation in the medulla oblongata and pons, basal nuclei, thalamus, and mesencephalon. Microscopic changes were typically multifocal mild to severe perivascular cuffs composed of lymphocytes and macrophages (Gard, Marshall et al. 1977; Carr and Dickason 2011; Department of Environment and Primary Industries 2011; Animal Health Australia 2012; Tee, Horadagoda et al. 2012).

Therapy
No antiviral therapies for arboviral infections in horses have demonstrated efficacy hence treatment is essentially supportive. Non-steroidal anti-inflammatories, anti-oxidant therapy such as DMSO and Vitamin E were often administered with or without corticosteroid therapy. Dexamethasone sodium has been used at doses of 0.05-0.1mg/kg SID IV particularly for recumbent horses, however this remains controversial for viral infections. The use of Interferon alpha has been mooted for Flaviviral diseased horses but there are limited data to support this (Lin, Liao et al. 2004). Recumbent horses or those with cranial nerve deficits need to be monitored and supported to reduce secondary complications.

Discussion
The large number of diseased horses, with approximately 100 fatalities reported, represented an unprecedented outbreak of equine disease. The epidemic served as a sobering reminder of the potential for disease in horses due to Arboviruses. No treatment methods for diseased horses have had demonstrated efficacy and such supportive therapy is the mainstay. Current
emphasis remains on control of vectors and vigilance. Vaccination of horses using commercially available products such as the recombinant canary-pox vectored WNV vaccine was canvassed following the outbreak and it would be likely that such vaccination would protect against local strains of WNV(KUN) (Dr J. Minke, personal communication).

However several sobering aspects remain following this outbreak regarding the increased virulence of the WNV(KUN) strain(s), and the likelihood of recurrence. Although there is no commercial vaccine against MVEV it has been suggested that a chimeric JEV vaccine may afford cross protection in people and offers some prospect for horses. This may be a double edged sword as the cross immunity may interfere with detection of JEV surveillance.

Finally newer therapies such a monoclonal antibody blockade against cytokines known to assist the virulence of WNV and RRV such as macrophage migration inhibiting factor, offer some hope for effective treatment whilst anti-viral therapies are not available (Arjona, Foellmer et al. 2007).

The 2011 outbreak provided much information and insight into arboviral diseases of Australian horses including the range of clinical presentations and promoted advances in diagnostic and prophylactic capabilities. These viruses remain a challenge in terms of prediction and control of disease outbreaks. However the recent outbreak has promoted greater awareness of equine clinicians and access to more rapid and comprehensive diagnosis.

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References


MALE

Seminal plasma has traditionally been removed from semen by centrifugation as a vital step in the processing equine semen for cryopreservation (Amann and Pickett 1984). The removal of seminal plasma in chilled transported semen did stabilise sperm membranes but there was no apparent improvement in motility or fertility (Barrier-Battut, Bonnet et al. 2013). The use of a filter (Sperm Filter, BotuPharma) was used to removed seminal plasma and achieved a higher yield of sperm without affecting motility (Neto, Monteiro et al. 2013; Ramires Neto, Monteiro et al. 2013).

Fractionated centrifugation using a continuous gradient (Equipure™, Nidacon, Sweden) improved sperm number and the morphology of collected sperm (Edmond, Brinsko et al. 2012). Using their new more sensitive test for sperm chromatin damage, (Crespo, Gosalvez et al. 2013) showed that colloidal centrifugation (using Equipure™, Nidacon, Sweden) is better than conventional centrifugation.

The use of a cushion has been used to prevent damage to sperm during centrifugation (Ecot, Decuadro-Hansen et al. 2005; Knop, Hoffmann et al. 2005). The volume of the cushion can be decreased from 3.5ml to 1ml without detriment. The concentration of extended semen can also be as high as 250 million/ml during the centrifugation process without sperm damage (Bliss, Voge et al. 2012).

The use of daily centrifugation, removal of supernatant and resuspension with fresh extender prolonged the life (motility and sperm membrane) of chilled semen. This was useful where a significant portion of seminal plasma (50%) was used in the extender (Love, Blanchard et al. 2012).

Glycerol is widely used as a cryoprotectant for semen preservation. The toxicity of glycerol involves osmotic and nonosmotic effects on sperm actin cytoskeleton, sperm membrane and mitochondria. Recommendations to keep glycerol concentration below 2.5% were suggested (Macias Garcia, Ortega Ferrusola et al. 2012). The amides have recently been used as cryopreservation agents to replace glycerol (Henry, Snoeck et al. 2002; Medeiros, Gomes et al. 2002; Vidament, Daire et al. 2002). Dimethyl formamide (DMFA) at 4% improved the outcome of frozen semen compared to glycerol or a combination of glycerol and DMFA by reducing sublethal damage (improvements in motility, membrane architecture and integrity, mitochondrial membrane potential) (Morillo Rodriguez, Balao da Silva et al. 2012). Dimethyl formamide improved the viability of sex-sorted sperm compared to glycerol (Gibb, Morris et al. 2013).

FEMALE

Physiology

Kisspeptin is a relatively newly discovered hormone that might play the role of gatekeeper in the hypothalamus. Kisspeptin could be responsible for initiating seasonality as it is released from the hypothalamus and has receptors on the GnRH neurons (Magee, Bruemmer et al. 2012). The authors had previously shown that a single dose of kisspeptin could raise LH but only for a short
time, not enough to induce ovulation (Magee, Foradori et al. 2009). The use of repeated kisspeptin injections did not induce early ovulation and interestingly it suppressed oestrus behaviour and oestradiol levels. This interference suggested that the current model of kisspeptin’s linear mechanism (hypothalamic-pituitary signalling) may be an oversimplification (Magee, Bruemmer et al. 2012).

The introduction of colour Doppler to equine reproductive ultrasonography raises questions as to its efficacy. One area is the potential to map the vascularity of the corpus luteum (CL) and associating it with progesterone production. Unfortunately there is no effect of percentage of vascularity of the CL and progesterone production. However when the mares were broken into high and low vascularity, there was higher progesterone in the high vascularity group (Uliani, Sudano et al. 2012). Also there was no correlation between the vascularity of the preovulatory follicle and subsequent CL formation and progesterone level (Romano, Ferreira et al. 2012).

Pathophysiology

A review of ovulations and the formation of corpus haemorrhagica (CH), haemorrhagic anovulatory follicles (HAF) and their ultrasonographic appearances throws more light on detecting aberrations (Cuervo-Arango and Newcombe 2013). Approximately 2/3 of mares develop CH and its diameter is strongly correlated to the diameter of the preovulatory follicle. A CH clots relatively quickly (within 8-16 hours) due to the evacuation of follicular fluid and replacement with pure blood. The fluid contents of HAF do not clot for 32-72 hours due to the presence of follicular fluid which has high heparin-like substance content. The diameter of HAF stops increasing when the contents organise and then starts to decrease. With frequent examinations, the detection of follicular evacuation and collapse indicates ovulation. With increased intervals between examinations, the detection of ovulation relies on observation of an organised blood clot. There is a time in between were there is overlap with both early CH and HAF sharing common characteristics. The luteal border of HAF is thinner than CH and the larger diameter of the HAF can help to differentiate between the two (Cuervo-Arango and Newcombe 2013).

The production of prostaglandins (PG) in the equine endometrium during the oestrous cycle along with alterations due to fibrosis was examined. In the healthy uterus, mRNA transcription increases in mid to late luteal phase and into follicular phase of the cycle. PGE2 is high in mid to late luteal phase with PGF2 later in late luteal phase and follicular phase. PGE2 in other species has an antiluteolytic effect and this might be the case in the mare. This synchrony breaks down with fibrosis. Fibrotic areas become out of step with the oestrous cycle in relation to the timing of PG synthesis. The authors claim this explains cycle aberrations with endometrosis as well as a potential role in early embryo loss with down regulation of PGE2 (a potential antiluteolytic hormone) (Szostek, Siemieniuch et al. 2012).

Phospholipase A2 (PA2) liberates arachidonic acid from phospholipids a first step in the production of prostaglandins. PA2 increases in the endometrium during late dioestrus in the nonpregnant mare but stays low in the pregnant mare. The embryo might be the source of the PA2 inhibitor (Ababneh and Troedsson 2013).

The use of NSAID can be useful in treating endometritis, however repeated use of flunixin meglumine during the periovulatory period can result in ovulation failure and production of haemorrhagic anovulatory follicles (Cuervo-Arango 2012).

The administration of oxytocin during dioestrus was noted to prevent luteolysis (Vanderwall, Rasmussen et al. 2007). A recent study refined the process finding the administration of 60IU daily between days 8 to 14 postovulation was ideal. Mares had lower PGFM (prostaglandin
F2alpha metabolite) levels at day 16, reduced endometrial expression of cyclo-oxygenase 2 suggesting this was the mechanism of action of the oxytocin injections (Keith, Ball et al. 2013). Stem cells (equine adipose derived mesenchymal stem cells) were infused into the uterus to investigate the possibility of a treatment for severe endometrial fibrosis (endometrosis). Stem cells were infused during oestrus and homed in on the periglandular space and uterine glands as the labelled cells were detected in endometrial biopsies up to 3 weeks after infusion (Mambelli, Winter et al. 2013). This experiment shows a proof of principle that the infusion of stem cells into the uterus allowed colonisation of these stem cells into the endometrium of the uterus. The authors did not comment on whether there was any change (improvement) in the health of the endometrium.

Anti-Mullerian Hormone was found to be useful in detecting granulosa cell tumours and potentially more sensitive than inhibin or testosterone (Ball, Almeida et al. 2013).

Pregnancy

It was interestingly to listen to (Hanlon 2010) review of Australasian Thoroughbred Statistics in relation to early postpartum breeding and how the failure to breed on “foal heat” is a wasted opportunity and a reduction in reproductive production. To add fuel to the fire of the ongoing debate (Blanchard, Thompson et al. 2012) report that early breeding is associated with a reduced chance of pregnancy and an increased chance of pregnancy loss. Differences might be explained by management differences between the hemispheres or the difference between looking at the individual or the herd.

The persistence of endometrial cups post abortion has been recognised recently as a possible cause of reproductive failure in following seasons (Steiner, Antczak et al. 2006). Another case of 2 mares (Allen and Wilsher 2012) and the possible use of kerosene as a chemical curettage (Crabtree, Chang et al. 2012) continues to throw more light on this intriguing condition.

Foetal membrane examination of a series of foals born with flexural deformities revealed that the foetal membranes showed one or more of the following characteristics: a reduction in their linear dimensions; folding of the allantochorion over the course of major blood vessels; oedema (Wilsher, Ousey et al. 2013). The authors proposed that relaxin, which has the role of myometrial quiescence but also promoting vascular endothelial factor might play a role in the pathogenesis of this condition.

A case series of 5 hydrallantois demonstrated a strong association with placentitis but it is not know if it is cause or effect (Govaere, De Schauwer et al. 2013). In a series of experimental infectious placentitis cases, serum amyloid A was useful in monitoring severity and also response to therapy (Coutinho da Silva, Canisso et al. 2012).

The use of 3D ultrasonography was described between days 35 and 180. After several methods were investigated, the transrectal use of a bulb-shaped abdominal 3D transducer enabled visualisation without sedation. The entire examination could be performed in less than 10 minutes. For early examinations (days 60-70) the 3D imaging was not as good as conventional 2D ultrasonography (Kotoyori, Yokoo et al. 2012).

EMBRYO TRANSFER

The use of statistics derived over large numbers is useful when looking at benchmarking. A recent survey of 809 uterine flushes and 454 embryo transfers produced data related to recovery rates; degree of synchrony; recipient day post ovulation and pregnancy rates; embryo age at recovery and pregnancy at 60 days. Embryos recovered on day 6 (42%) were lower than days 7-10 (56-66%). Pregnancy rate for +1 to -5 synchrony was similar (61-77%) but lower on -6 (27%). The pregnancy rate from transfers done on recipient day 2 post ovulation was lower
(33%) than days 3-8 (55-66%). Pregnancy rate was higher for day 7 embryos (76%) than day 6 (50%), day 8 (64%) and day 9 (44%) (Jacob, Haag et al. 2012).

The use of a protocol had a high rate of double ovulation in an ET program and increased the embryos recovered. Luteolysis was induced on day 8 post ovulation. Mares were monitored and when the largest follicle was no larger than 25mm and there was a second follicle at least 20mm in diameter, mares were treated with 100ug 12 hourly until 2 follicles were between 33 and 38mm in diameter. Ovulation was induced with hCG (Nagao, Neto et al. 2012).

**CLONING**

One of the barriers to cloning is the perception that donor oocyte mitochondrial DNA might influence the potential of the produced individual. The use of selected donors is limited by the number of suitable oocytes available in comparison with indiscriminate abattoir donors. The Texas A&M Team have shown their cutting edge by maturation of immature oocytes from transvaginal aspiration, in vitro maturation and use as donor oocytes for somatic nuclear transfer (Choi, Norris et al. 2013).

Although only a small study size, 2 mares that were cloned from the same original copy were used for embryo transfer over 3 seasons. Although both were managed similarly and bred to the same stallions, one had a poorer reproductive performance than the other. This poses questions as to why the difference, is it due to the influence of the recipient oocyte and/or other epigenetic factors (Terris, Normandin et al. 2012).

**REFERENCES**


Gastric Outflow Obstruction in Foals

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Equine gastric outflow obstruction is a relatively uncommon condition that can occur as a sequel to gastroduodenal ulceration in suckling and weanling foals. Inflammation, swelling, granulation tissue, and fibrosis associated with severe ulceration of the pylorus and or duodenum cause mechanical and functional obstruction of gastric outflow. Delayed or absent gastric emptying leads to gastric distension, reflux, gastric and esophageal ulceration, aspiration pneumonia, and ascending cholangiohepatis and pancreatitis. Affected foals often have a history of previous illness or musculoskeletal injury and the patient may develop obstruction after fully recovering from conditions such as sepsis, diarrhoea, or musculoskeletal infection or injury. Development of the condition despite prophylactic treatment with anti-ulcer medication is common. Most foals will exhibit signs of persistent bruxism, ptalism and reflux but occassionally will present with non-specific signs of colic. Additional clinical abnormalities often seen include fever, depression, anorexia, diarrhea, and abnormal lung sounds. Differential diagnoses include, ileus, enteritis, oesophageal or jejunal obstruction, and ulceration of the mouth, oesophagus or stomach.

A presumptive diagnosis is made based on persistent clinical signs of reflux, bruxism, and ptalism. Delayed or absent gastric outflow can be confirmed by contrast radiography. Barium suspension (10ml/kg) is administered via nasogastric tube following decompression of the stomach and lateral abdominal radiographs are obtained immediately after administration and at regular intervals up to 90 minutes post administration. A diagnosis of gastric outflow obstruction is confirmed if minimal or no barium is seen aboral to the stomach 30-90 minutes post administration. Gastric lavage should be performed to remove barium from the stomach following the study if surgery is anticipated. Abdominal ultrasound can be used to confirm the presence of gastric distension and to evaluate the duodenum. Identification of thickening or stricture of the duodenum confirms that it is affected but the absence of a lesion does not rule out duodenal involvement as imaging of the entire duodenum is challenging. Gastroscopy commonly reveals linear esophageal erosion and ulceration due to reflux esophagitis and diffuse gastric ulceration. Gastroscopic identification of a duodenal or pyloric ulcer often proves to be difficult as the obstruction blocks passage of the endoscope and visualization.

Initial medical treatment includes frequent gastric decompression, intravenous fluid and electrolyte therapy, broad spectrum antimicrobials, and gastroprotectants, and pro-kinetics such as metoclopramide. Young foals may require parenteral nutrition. The value of orally administered medication is questionable as delayed gastric emptying inhibits absorption. Surgery may be postponed for several days while medical treatment is attempted and a diagnosis is established provided the stomach is decompressed and the patient receives appropriate supportive care.
Surgery is indicated in patients with delayed gastric emptying that are unresponsive to medical treatment. A ventral midline laparotomy extending from the umbilicus to xiphoid and placement of Balfour retractors provides adequate exposure. Routine exploration of the abdomen should be performed along with thorough palpation of the pylorus and duodenum. The location of the obstruction is confirmed by visual inspection and palpation of thickening, stricture, adhesions, proximal distension, or perforation of the duodenum and or pylorus. Obstruction may occur solely at the pylorus. However, multiple sites of obstruction and obstruction of the duodenum are more common. Obstruction of the pylorus and proximal 1-2cm of duodenum may be bypassed by gastroduodenostomy. Duodenal obstructions are bypassed by performing a gastrojejunostomy. When a gastrojejunostomy is performed, a jejunojejunostomy may be performed as well to prevent the formation of a blind loop and reduce backflow into the stomach. Post-surgical complications that may necessitate repeat celiotomy include entrapment of the jejunum in the loop created by the anastomosis, small intestinal volvulus, obstruction of the common bile duct, and perforation.

Overall long term survival 2 years after surgery is favourable (69%) and survivors appear to have normal athletic careers. Factors significantly associated with survival include location of the lesion, presence of adhesions, and development of post-operative reflux. Pyloric obstruction bypassed by gastroduodenostomy carries an excellent prognosis. Whereas, only 59% of foals with duodenal obstruction survive long term. Long term survival is reduced to 33% when adhesions to the duodenum are identified at surgery. The most common cause of fatality is perforation of an ulcer along the anastomosis at 4-7 months post operatively.

References:
SCINTIGRAPHIC UPTAKE WITHIN THE FETLOCK JOINT AND THE EFFECT ON PERFORMANCE FOLLOWING DIAGNOSIS

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Reasons for performing study:
To describe patterns of radiopharmaceutical uptake (IRU) in fetlock joints of Thoroughbred (TB) racehorses and to evaluate post-scintigraphy performance.

Hypotheses:
IRU in the metacarpal (MC) and metatarsal (MT) condyles is more common than IRU in the parasagittal grooves and is associated with poorer post-diagnosis performance than controls.

Methods:
Location of IRU within the fetlock region was identified and graded subjectively in TB racehorses presenting for scintigraphic examination. Performance variables were determined from race records for horses with moderate/marked MC/MT condylar IRU (cases), all other horses undergoing scintigraphy (scintigraphy controls), and age/sex matched controls from the last race in which a case participated (controls). Statistical analysis included quantile regression, Fisher's exact test, Kaplan-Meier survival curves, log-rank test and Cox regression.

Results:
Metacarpal/MT condylar IRU was identified in 103/220 horses with only 3/220 having parasagittal IRU. Moderate/marked IRU was identified in the forelimb MC and hindlimb MT condylar regions in 62 and 39 horses respectively with palmaromedial and plantarolateral IRU most common. Forelimb cases had fewer starts (P=0.003), reduced earnings (P<0.001) and reduced earnings per start (P<0.001) than controls. Hindlimb cases had fewer starts (P=0.017), reduced earnings (P<0.001) and reduced earnings per start (P<0.001) than controls. Cases were more likely to return to racing later than controls following a rest period (P=0.003). Cases were older than scintigraphy controls (P<0.001).

Conclusions:
In TB racehorses presenting for lameness or poor performance MC/MT condylar IRU is the most common abnormality identified within the fetlock joint and condylar fractures are rarely identified using scintigraphy. Racehorses with moderate/marked condylar IRU have a shortened racing career, are less successful than age/sex matched controls and are older than other racehorses presented for scintigraphy.

Potential Relevance:
Overload of the MC/MT condyles is a common and significant problem in TB racehorses that is readily identified with scintigraphy. Scintigraphy of horses that are lame or performing poorly is less useful for screening for potential condylar fractures.
Supraventricular digital flexor tendon (SDFT) injury is a frequent age- and exercise-related occurrence in equine athletes with many lesions involving the core of the midmetacarpal region of one or both fore limb tendons. Reported incidences of SDFT injury in racehorses have ranged between 8 and 43% (Dowling et al., 2000; Patterson-Kane and Firth, 2008), with increased risks of injury in older animals being measured in a number of studies in a number of countries (Jeffcott et al., 1982; Rossdale et al., 1985; Cohen et al., 1997; Williams et al., 2001; Kasashima et al., 2004; Perkins et al., 2005; Lam et al., 2007; Singer et al., 2008; Ely et al., 2009; Avella et al., 2009; O’Meara et al., 2010). The high prevalence of SDFT injury with long recovery periods and high recurrence rates causes significant wastage of equine athletes and raises public concerns regarding animal welfare (Jeffcott et al., 1982; Rossdale et al., 1985; Cohen et al., 1997; Cohen et al., 2000). There is currently no consistently successful or scientifically based method of treatment, and the prognosis for an SDFT lesion is largely dependent on the initial severity of the injury (Marr et al., 1993).

One of the basic problems is failure of the tendon healing process to reconstitute normal structure and mechanical properties. There tends to be long-term persistence of scar tissue with inappropriate matrix composition and increased levels of poorly organised type III collagen (Williams et al., 1980; Williams, McCullagh and Silver, 1984; Dahlgren, Brower-Toland and Nixon, 2005). Type III collagen forms smaller fibrils and does not have the tensile strength of type I collagen that predominates in the matrix of normal adult tendon (Williams et al., 1980; Williams, McCullagh and Silver, 1984; Birk and Mayne, 1997). As a result injury recurrence rates of 40-80% have been reported (Rooney and Genovese, 1981; Marr et al., 1993; Dowling et al., 2000).

1.1.1 Why is the equine SDFT prone to injury?
Due to the conflicting requirements of strength and elasticity, the SDFT has narrow mechanical safety margins. In vitro studies have shown that SDFT failure occurs at strains of 12-20% (Riemersma and Schamhardt, 1985; Wilson and Goodship, 1994). Strains of 11.5% and 16.6% respectively were measured in two galloping horses using surgically implanted force transducers in the SDFT (Stephens et al., 1989; Platt et al., 1994). This implies that the mechanical safety margins are very narrow, even though experimental error precludes direct comparison of these figures. Additionally, the SDFT demonstrates an inefficiency of energy storage of approximately 5-10% (Riemersma and Schamhardt, 1985). Heat generated during exercise can be substantial; with temperatures of 45°C being recorded in the core of the mid-metacarpus segment of the SDFT during protracted galloping as opposed to 38°C in the periphery of the tendon cross-section (Wilson and Goodship, 1994). It has been postulated that tendon hyperthermia may play a role in tendon injury. In one study equine SDFT cells were more resistant than other fibroblasts to temperatures of that magnitude; however cells in suspension were used, which are known to be more heat resistant (Smith et al., 1993; Birch, Wilson and Goodship, 1997). Rates of diffusion of fluorescent molecules through gap junctions of confluent monoculture SDFT tenocytes were markedly reduced within 30 minutes of heating to 45°C for 10 minutes. Although communication was re-established after 2 hours, by 24 hours 80% of cells had died (Burrows et al., 2008). Hyperthermia was therefore responsible for initial disruption of intercellular
communication. Subsequent cell death may have been due to death signals being passed from injured to healthy cells through new or reopened gap junction channels (Burrows et al., 2008). It is not known how such temperatures influence cellular metabolism in vivo. Regional and/or local hypoxia, acidosis, variations in extracellular matrix (ECM), or poor vascularisation have also been proposed but remain unproven (Riemersma and Schamhardt, 1985). Study results in terms of vascularity have been inconsistent, perhaps due to the techniques employed, but indicate that tendon blood flow is better than initially thought (Stromberg, 1971; Kraus-Hansen et al., 1992). Histological and micro-radiographic evaluation has shown that the mid and distal portion of the SDFT have less vascular tissue but functional blood flow exists in these areas; exercise did not dramatically increase blood flow within these regions (Stromberg, 1971). The relevance of blood flow and therefore hypoxia and nutrition in the aetiology of tendon injury has yet to be substantiated (Smith and Webbon, 1996).

1.2 Anatomy and physiology of the equine superficial digital flexor tendon
1.2.1 Digital tendon function in the horse
1.2.1.1 Mechanical support and energy storage
Horses have evolved to run fast over short distances to evade being eaten by predators. This is reflected in their unique limb anatomy and function. Tendons are structures that link muscle to bone and transmit force between them to enable joint movement. Tendons are fibro-elastic in nature and are resistant to mechanical loads. The size, shape and attachment to bone vary dependent on the function of the muscle to which they are attached. Powerful muscles tend to have short, broad tendons whilst those engaged in subtle movements have long, thin tendons (Kannus, 2000).

Strain-bearing tendons such as the equine SDFT have special properties, including viscoelasticity, that enable them to withstand high strains and to store energy that can be subsequently released to increase the efficiency of locomotion (Hooley et al., 1980; Woo et al., 1982; Shadwick, 1990; Alexander, 2002; Birch, 2007). Horses have developed an elongated digit with long tendons attached to short muscles to enhance elastic energy savings and force-generation (Biewener, 1998; Minetti et al., 1999). Tendon and muscle stresses increase with changes of gait and are highest during galloping (Biewener, 1998). The apparent efficiency of mechanical work in horses increases from 10% during walking to close to 100% at a gallop. This is due to a combination of storage and release of elastic energy in the tendons of the limb, high anaerobic output and the ability to exchange potential and kinetic energy at high speeds. The muscular work of galloping in horses is halved by storing and returning elastic strain energy in spring-like muscle-tendon units. This mechanism is optimised by unique musculoskeletal adaptations: the digital flexor muscles have extremely short fibres and significant passive properties, whereas the tendons are very long and span several joints. The long fibres of tendons store and return elastic energy during the stance phase and the short fibres of muscle reduce the energetic cost of force-generation (Minetti et al., 1999; Alexander, 2002).

The SDFT flexes the digit during the swing phase of locomotion and contributes to prevention of over-extension of the metacarpophalangeal (fetlock) joint during the stance phase along with the deep digital flexor tendon (DDFT) and suspensory ligament (SL) (Batson et al., 2003; Butcher et al., 2007). Experimentally, the SDFT is often compared with the anatomically apposing common digital extensor tendon (CDET) that is loaded only by muscular contraction during the swing
phase of the gait for correct positioning of the digit prior to weight-bearing. Because the SDFT largely acts passively via a check ligament attached to the radius, the superficial digital flexor muscle is thought to act as a dampener of high-frequency limb oscillations that occur at high-speed gaits (Wilson et al., 2001).

Elastic recoil contributes up to 36% of the work required for galloping, and increased elastic storage at faster speeds contributes to a reduction in the metabolic demand as horses move faster (Biewener, 1998). Approximately 93% of energy stored is released in elastic recoil while the remainder is lost as heat (Alexander, 2002). The SDFT therefore needs to have low stiffness and be very elastic to store the maximum amount of energy (Batson et al., 2003). It has a conflicting function however, as in assisting prevention of over-extension of the fetlock it must have high tensile strength. Such tendons typically have low mechanical safety margins. Tendons that merely position the limb, including the CDET, are rarely injured. Viscoelasticity of tendons is responsible for time-dependent mechanical behaviour; the relationship between stress and strain is not constant but depends upon the time of displacement or load. The viscous component shows linear resistance to shear flow and strain whereas the elastic component strains instantaneously when stretched and instantaneously returns to original length when the stress is removed. Viscoelasticity is characterised by creep and stress relaxation. Viscoelastic materials also show hysteresis, which is the energy lost or dissipated during loading and is identifiable by the unloading curve not following the loading curve (Fung, 1993).

Creep, a critical viscoelastic property of equine SDFT (Kasashima et al., 2008), is increasing deformation under constant load; when stress is held constant, strain increases with time, which contrasts with an elastic material, which does not exhibit increased deformation no matter how long the load is applied. Creep is time-dependent and long term physiological loading results in some lengthening of tendon but with return to normal length when loading ceases. Loading that exceeds physiological strains may lead to non-recoverable tendon lengthening and pathology (Riemersma and Schamhardt, 1985; Wilson and Goodship, 1994). Stress is defined as load exerted per unit area, and strain as elongation as a percentage of initial length. This change in shape is also called deformation. Stress relaxation means that the stress will be reduced or will relax in response to constant deformation i.e. when strain is held constant, stress decreases with time. The ratio of these two parameters can be used to describe the elastic modulus of tendon, which is a useful index of the material stiffness since stress:strain and elastic modulus are all independent of tendon size. The elastic modulus should not be confused with the more general term stiffness, which is the force required to cause a unit change in length and so dependent on tendon size. Comparing the SDFT as a whole with the CDET shows that although the SDFT is more compliant as a material due to differences in the matrix molecular composition including water and total sulphated glycosaminoglycan (GAG) contents, the whole tendon has a higher cross-sectional area than the CDET and is therefore stronger (Batson et al., 2003).

1.2.1.2 Tendon structure

Tendon has a hierarchical structure with the largest subunits being longitudinally orientated fascicules measuring 80-500μm in diameter that are approximately hexagonal in cross-section (Evans and Barbenal, 1975; Kastelic et al., 1978; Benjamin and Ralphs, 1997). Tendons are surrounded by loose areolar connective tissue called the paratenon, which consists of type I and III collagen fibrils and elastic fibrils and it is lined on the inside by synovial cells to allow free
movement in some zones (Kannus, 2000). The epitenon lies beneath the paratenon as a fine connective tissue sheath that envelops the whole tendon; it is contiguous with the paratenon and endotenon. The endotenon is a thin reticular network of connective tissue inside the tendon that carries blood vessels, nerves and lymphatics and wraps around each fascicle (Kannus, 2000). Intrinsic vessels enter the tendon at the musculotendinous and osseotendinous junctions and the extrinsic supply comes from the paratenon and associated structures. The vascular supply is located only within the connective tissue sheaths and does not enter the fascicles (Stromberg, 1973; Kraus-Hansen et al., 1992; Crevier-Denoix et al., 1998).

1.2.1.3 The extracellular matrix

The extracellular matrix comprises most of the tendon structure. The major component of the ECM is collagen with type I predominating in the adult SDFT and only a small amount of type III being present (Parry, Craig and Barnes, 1978; Birch et al., 1998; Birch et al., 1999; Birch et al., 2007). Substantial variations in composition and quantity of ECM components exist along the length of the SDFT, with these variations being significantly influenced by the predominant force, tensional or compressional, that is applied to the tendon at a particular site (Riemersma and Schamhardt, 1985; Riemersma and De Bruyn, 1986; Vogel and Koob, 1989; Goodship et al., 1994).

Collagen accounts for 65-80% dry weight of the tendon matrix (Goodship et al., 1994). Soluble tropocollagen molecules form cross-links to create insoluble collagen molecules, which then aggregate into microfibrils, subfibrils and then fibrils that measure 35-550nm diameter in the adult equine SDFT (Parry, Craig and Barnes, 1978; Parry, Barnes and Craig, 1978). Collagen fibrils are the sub-microscopic units of tensile strength, and are aligned longitudinally within fascicles in a matrix comprised of water, proteoglycans and glycoproteins (Kastelic et al., 1978). In the adult SDFT the collagen fibrils have a bimodal distribution, with large diameter fibrils providing tensile strength and small diameter fibrils elasticity (Parry, Craig and Barnes, 1978). Large diameter fibrils are stronger because they have a higher density of intrafibrillar covalent cross-linkages; small diameter fibrils have a greater surface area allowing greater interaction with the interfibrillar matrix to prevent permanent slippage of fibrils past one another (Parry, Craig and Barnes, 1978). In a rested state, fibrils can be seen to follow a planar waveform or “crimp” along the longitudinal axis of the fibril (Diamant et al., 1972; Wilmink et al., 1992). Opening out of the crimp when the tendon is stretched acts as a safety mechanism, allowing the structure to be subjected to higher levels of strain. Elastic fibres are found between collagen fibres and constitute 1-2% of the dry weight of tendons; they may contribute to crimp recovery (Wilmink et al., 1992; Butler et al., 1978). The larger elastic fibres are found near the cells or at the periphery of tendon and smaller ones deeper within tendon (Parry, Barnes and Craig, 1978). Proteoglycans are made up of a protein core to which one or more glycosaminoglycans (GAGs) are attached. They are usually large (106Da in humans), negatively charged and hydrophilic (Kannus, 2000). They can contain 50 times their weight in water and are mainly situated within and around the collagen fibrils. Their high charge and repulsion allows them to contribute to resistance of the ECM to compressive and tensile forces (Kannus, 2000).

1.2.1.4 Tendon fibroblasts (tenocytes)
Tenocytes comprise 90-95% of the cellular population in tendons and are organised in longitudinal rows within fascicles between collagen fibrils (McNeilly et al., 1996). Long cytoplasmic projections maintain contact between cells in those in adjacent rows. Tenocytes are responsible for synthesis, degradation and turnover of the ECM. There appears to be three categories of tenocytes, relative proportions of which relate to their anatomical location within the tendon and therefore whether they are subjected to tensional or compressional forces. Type 1 and 2 tenocytes have spindle-shaped and cigar-shaped nuclei respectively and can be identified in tensional and compressed areas of tendons; type 3 tenocytes are characterised by plump, rounded nuclei and are found only in areas where compressional forces are present (Smith and Webbon, 1996). Although type 1 cells have been referred to as tenocytes and type 2 as tenoblasts, both cells may be a continuum (different stages of maturation) of the same cell line, rather than two distinct populations. It has been suggested that type 1 cells may be sensing cells, similar in function to osteocytes in bone, whereas type 2 cells may act similar to osteoblasts or osteoclasts, which are effector cells (Holmes, 1971; Smith and Webbon, 1996; Bernard-Beaubois et al., 1997; Ko and McCulloch, 2001; Cheun et al., 2004; Stanley 2005; Stanley et al., 2007; Stanley et al., 2008). Type 3 cells demonstrate a chondrocyte-type phenotype and produce type II collagen and aggregan, whereas types 1 and 2 produce type I collagen. In recent studies cells were identified that appeared to be of an intermediate type (morphologically), which suggests a progression from one type to another (Screen et al., 2003; Stanley, 2005). Numbers of tenocytes per unit area are reduced significantly with maturation and ageing, and there is a concurrent increase in the ratio of type 1 to type 2 cells in addition to reductions in cellular activity (Stanley, 2005; Stanley et al., 2008). In addition to tenocytes within fascicles, there are fibroblastic cells in epitenon, endotenon and paratenon, the morphology and functions of which have not been defined.

1.3 Pathophysiology of tendonitis
1.3.1 Superficial digital flexor tendon injury
It is generally agreed that SDFT injury in many cases follows a period of accumulation of microdamage that weakens the matrix and predisposes the tendon to rupture during normal athletic activity (Riemersma and Schamhardt, 1985; Stephens et al., 1989; Wilson and Goodship, 1994). Various authors have argued that tendonosis or tendonopathy are more useful terms than tendonitis because inflammation is not thought to be a feature of initial tendon injury. Pathology, in the great majority of circumstances is consistent with degeneration. However, early disease has been poorly investigated because it is frequently asymptomatic and therefore there is a paucity of histopathology in such cases. It may be that inflammation is present at the initiation of the degenerative process but there is a lack of good quality histological data from symptomatic tendon disorders of short duration. Three main theories have been advanced in attempting to explain the aetiology of tendonopathy with respect to mechanical, vascular and neural influences; however these are not necessarily relevant to equine disease (Rees et al., 2006).

It is generally agreed that there is likely to be an imbalance between fatigue damage and routine repair with the damage being excessive and/or the ability of cells to repair it compromised. Tenocytes must constantly repair the tendon matrix otherwise all tendons would eventually fail, however a threshold of loading may exist above which a tendon cannot fabricate enough ECM to keep pace with the rate of damage, leading to a decline in the tendon’s mechanical properties (Ker, 2002). There is growing evidence for the key role of the tenocyte in both the normal
physiological homeostasis and regulation of the tendon matrix and the pathological derangements that occur in disease. In particular, the tenocyte is considered to have a major role in effecting the sub-clinical matrix degeneration that is thought to occur prior to clinical disease, as well as in the severe degradative events that occur in the tendon at the onset of clinical disease. Furthermore, the tenocyte is likely to have a central role in the production of the biologically inadequate fibrocartilaginous repair tissue that develops subsequent to tenonopathy (Clegg et al., 2007).

Cultured fibroblasts from injured human patellar tendons are hyper-proliferative compared to tendon fibroblasts from healthy control patients and this appears to be due to sustained over-expression of platelet-derived growth factor-β (PDGFR-β), which in turn is upregulated by transforming growth factor-β1 (TGF-β1) (Rolf et al., 2001).

1.3.2 The role of age and exercise
A number of controlled exercise studies and surveys of post-mortem tissue have demonstrated the role of both age and galloping exercise in accumulation of microdamage in the injury-prone mid-metacarpal SDFT core. Post-mortem studies identified discoloured zones within tendons of asymptomatic horses, and these areas were histologically abnormal with increased collagen type III levels consistent with fibrous scar formation (Webbon, 1973; Peloso et al., 1994; Webbon, 1977; Birch et al., 1998). These findings support the suspicion that pre-existing injury occurs and may precede overt injury. The bilateral presentation of most tendonopathies is also inconsistent with an acute traumatic injury without prior pathology (Smith et al., 2002). Irregularities in racing and timed work have been noted prior to injury in horses involved with race/training fatal musculoskeletal injury or ligamentous/tendinous rupture (Estberg et al., 1996). At a microscopic level, the angle of the crimp waveform of collagen fibrils in the tendon core was found to reduce after 10 years of age in wild horses; this change occurred at a significantly earlier age in racehorses and horses subjected to treadmill exercise (Patterson-Kane, Firth et al., 1997; Patterson-Kane, Parry, Birch et al., 1997; Patterson-Kane et al., 1998). In theory, fibrils with a lower crimp angle will straighten and subsequently fail at lower levels of strain, so it was hypothesised that the reduction in crimp angle constituted micro-damage secondary to repetitive loading over a protracted period of time (Wilmink et al., 1992; Patterson-Kane, Firth et al., 1997; Patterson-Kane, Parry, Birch et al., 1997; Patterson-Kane, Parry, Goodship et al., 1997). Similarly, in horses exercised on a treadmill for an 18 month period there was a reduction in collagen fibril mass-average diameter in the SDFT core, which is also consistent with mechanical weakening (Patterson-Kane et al., 1998). In the same horses, training did not reduce CDET fibril mass-average diameters, supporting the concept that the functionally distinct nature of the CDET and SDFT results in fundamentally different responses to high-speed exercise regimens (Edwards et al., 2005). The age-related increases in SDFT injury rates can be explained by this concept of the accumulation of micro-damage, although this relationship is complicated by the fact that older racehorses are likely to have undergone greater amounts of training and racing exercise.

1.3.3 Epidemiological studies in Japan, USA, Hong Kong (HK), New Zealand (NZ), Australia and the United Kingdom (UK)
Most epidemiological studies probably still underestimate the true incidence of SDFT injuries in racehorses as many cases occur during training as well as in racing (Kasashima et al., 2004;
Perkins et al., 2005; Lam et al., 2007). Tendon injuries have a very significant financial impact on the equine industry. Career earnings in racing Thoroughbreds in Hong Kong that were retired due to tendon injury were less than half those of horses retired for other reasons. Almost one third of the tendon injury group earned no money during their career and had fewer race starts (Lam et al., 2007). There is not only the loss of race and competition earnings to consider; veterinary fees and the cost of the long convalescence periods (generally 9-12 months) may also be considerable.

Career racing and training injuries involving the SDFT occurred in 11.1% of racing Thoroughbreds in Japan in a one year study of 10,262 horses (Kasashima et al., 2004). The prevalence of tendon injuries in the USA has been estimated at approximately 2/1000 race starts (Peloso et al., 1994). In Hong Kong SDFT injury was the most common reason for retirement of racehorses and accounted for a total of 14.2% of all retirements in a study spanning 12 years (Lam et al., 2007). There was also an increase in risk of tendon injury from 23-42% over the 12 year period, which may have been an actual increase or due to better clinical recognition of the injury (Lam et al., 2007).

The incidence of all injuries was 6.9/1000 race starts in national hunt racing in the United Kingdom and the SDFT was the most frequently injured structure (Pinchbeck et al., 2004). Most SDFT injuries are non-catastrophic but race-related conditions may not be clinically apparent for several hours after racing. Deterioration and euthanasia that occur away from the racetrack will not have been recorded in most studies (Peloso et al., 1994; Williams et al., 2001). Injury involving the SDFT is also common in elite eventing and show-jumping horses (Dyson, 2004; Murray et al., 2006; Singer et al., 2008).

1.3.4 Risk factors
Epidemiological studies performed in many countries have identified similar age, exercise and sex-related factors. An increase in injury rate with increasing age over all race types has been reported in studies performed in the USA, Australia, UK, NZ and Japan (Mohammed et al., 1992; Bailey et al., 1998; Cohen et al., 2000; Williams et al., 2001; Kasashima et al., 2004). Horses 4 years of age and older than 5 years of age were 6.76 and 15.26 times more likely, respectively, to incur injury to the SDFT in a longitudinal study performed in NZ involving 1,571 horses and 554,745 study days (Perkins et al., 2005). Epidemiological data have shown a strong association between age and exercise and strain-induced tendonopathy in humans (Gibbon et al., 1999). There was a higher proportion of horses retiring due to tendon injury at 3 (19.7%) and 4 (17.4%) years of age when compared with 2 (5.6%) year-olds or those older than 5 (11.1%) years of age in a retrospective study of Hong Kong racetracks from 1992-2004 (Lam et al., 2007). The median time in training for horses retired with tendon injuries was 29 months, which was 25% less than the median time in training for the population retired for other reasons (Lam et al., 2007). The premature retirement of 2 and 3 year-old horses because of SDFT injury in the HK study compared to other studies, possibly reflects management and financial considerations specific to HK because long-term convalescence required for tendon repair is problematic.

The association of injury with cumulative high speed exercise appears to vary between zones in the USA (Cohen et al., 2000). SDFT injuries resulting in retirement were more likely to have
occurred in the fore limb than hind limb (Williams et al., 2001; Kasashima et al., 2004; Perkins et al., 2005; Lam et al., 2007). Racing in a clockwise direction was associated with a risk of retirement due to SDFT injury 1.75 times greater in the right forelimb (Lam et al., 2007). This is the leading limb but the non-leading limb is exposed to greater ground reaction forces at the canter (McGuigan and Wilson, 2003; Witte et al., 2004). However, the difference between the two limbs decreased with increases in speed (Witte et al., 2004). The combination of exercise at racing speed and the distance travelled during a racing career predispose Thoroughbred racehorses to fatal musculoskeletal injury (Estberg et al., 1996). This suggests an impact-related cause. One study performed in the 1980s showed that SDFT injury is more commonly associated with softer surfaces, which is not supportive of impact-related injury, however very few studies have addressed that factor (Rooney and Genovese, 1981). An Australian study showed an increase in fatal musculoskeletal injuries as track surface became firmer (Bailey et al., 1998).

Injury of the SDFT may be more common on softer surfaces because these conditions result in greater energy expenditure per stride resulting in more rapid fatigue of the DDFT and increased strain on the SDFT (Butcher et al., 2007). SDFT injury often occurs towards the end of a race and it has been suggested that this may be associated with fatigue of the fast-twitch fibres of the DDFT, which reduces fetlock support and increases SDFT loading (Butcher et al., 2007). Numerous studies have demonstrated an increase in likelihood of SDFT injury in colts and stallions (Cohen et al., 2000; Kasashima et al., 2004; Perkins et al., 2005; Lam et al., 2007). However, others have not shown this to be the case (Rooney and Genovese, 1981; Mohammed et al., 1992; Bailey et al., 1998).

1.3.5 Why does micro-damage occur?

It would appear that while an energy-storing tendon can respond to the mechanical forces applied to it during growth, there is no evidence that it can do so after skeletal maturity (Birch, Rutter and Goodship, 1997; Patterson-Kane et al., 1998; Kasashima et al., 2002; Birch, Wilson et al., 2008). The ability of tenocytes to synthesise and turn over matrix components in the adult SDFT appears to be low, particularly when compared with the CDET, a positional tendon, whose tenocyte density is lower than the SDFT (Batson et al., 2003; Goodman et al., 2004; Birch, 2007; Birch, Worboys et al., 2008; Young et al., 2009; Thorpe et al., 2010). As well, tenocyte density in the SDFT decreases with age (Stanley et al., 2007). This may explain the frequency with which an imbalance between fatigue damage and routine repair appears to develop. If either the damage resulting from the loading regimen is excessive or the ability of the cells to repair damage is compromised, the damage may accumulate and result in tendonopathy. A threshold of loading may exist above which a tendon cannot fabricate enough extra-cellular matrix to keep pace with the rate of damage, leading to a decline in the tendon's mechanical properties (Ker, 2002). Other possible negative influences on tenocyte reparative ability could include hypoxia, ischaemia with reperfusion injury, hyperthermia (as stated above), oxidative stress, abnormal regulation of apoptosis, inflammatory mediators, metalloproteinases (MMPs) and some drugs (particularly fluoroquinolone antibiotics) (Goodship and Wilson, 1994; Birch, Rutter and Goodship, 1997; Stone et al., 1999; Yuan et al., 2002; Yuan et al., 2003; Yoon et al., 2004; Bestwick and Maffulli, 2004; Sode et al., 2007). Impaired or dysfunctional protein synthesis by tenocytes may lead to weaker tendon tissue and in turn increase the risk for tendon rupture (Yuan et al., 2002; Yuan et al., 2003); this has been referred to as the ‘tendonosis cycle’. Some significant recent work has demonstrated the possible role of MMPs, the enzymes that degrade matrix proteins. Extremes of physiological loading can result in isolated collagen fibril damage,
which alters normal cell-matrix interactions in the damaged areas. If these fibrils do not impart normal loads to tendon cells, cellular understimulation may produce a catabolic state with increased release of prostaglandins and MMPs, and increased apoptosis (Yuan et al., 2002; Yuan et al., 2003; Arnoczky, Lavagnino and Egerbacher, 2007).

1.3.6 Pathology
Gross appearance, histology and ultrasonography have been used to define the various stages of repair in spontaneous SDFT injury. Tendon core colour changes (pink and purple) are often evident on the cut surface and this is frequently observed in horses that have recently been racing. More chronic lesions are paler. Some lesions have a well demarcated pale area with a darker inter-fascicular pattern giving a marbled appearance and these tendons are palpably soft. The degree of fibrosis is proportional to the chronicity and severity of the lesion (Webbon, 1977). The specific ultrasonographic appearance representative of the various stages of tendon healing has been described in 28 cases of spontaneous SDFT injury (Marr et al., 1993). Acute lesions of less than 2 weeks duration were characterised histologically by haemorrhage, oedema, fibrolysis, fibrin deposition and inflammation. Ultrasonographically, acute lesions were generally identified as anechoic or hypoechoic areas (both could be present and very complex), which were often well defined and lacking normal linear echoes. Lesions of 4-20 months duration histologically showed fibroplasia, granulation tissue and immature fibrous tissue. Often, there were foci of recent haemorrhage and haemosiderin deposition, indicative of on-going damage, most likely due to the low strain tolerance of immature (reparative) tendon tissue. In mild injuries of 6 months duration, tenocytes tended to be larger and rounder. Ultrasonographically, these lesions were uniformly or heterogeneously hypoechoic and moderately well defined, with poorly organized short linear echoes. Chronic lesions were characterised by fibrosis, increased number of cells with rounded nuclei, irregular arrangement of collagen, widespread scar formation, and prominent endotenon tissue with haemosiderin deposition.

Repair was not always complete and occasionally granulation tissue was detected as long as 14 months post-injury (Marr et al., 1993). Chronic fibrosis was represented ultrasonographically by heterogeneously echogenic areas transversely, and disorganised linear echoes longitudinally. Large acellular scars were seen as hyperechoic foci (Marr et al., 1993). Most injuries involved central fibres and the mid-metacarpal region of the tendon, and the region with the smallest crosssectional area was the site most commonly injured (Webbon, 1977). The fibroblasts in scar tissue were found to be larger and more basophilic than normal tenocytes, to have large vesicular nuclei and to resemble the 'myofibroblasts' described in scar tissue elsewhere. The cell to matrix ratio in scarred zones of tendon was found to be increased and the concentration of collagen in these areas was less than in normal tendon. However, the scar tissue collagen was more readily extractable and contained a different pattern of collagen types with 20-30% type III collagen. As a result of the presence of type III collagen, the scarred tendon was likely to have less tensile strength than normal tendon (Williams et al., 1980).

1.4 Gap junction structure and function
1.4.1.1 Gap junction intercellular communication (GJIC)
Gap junctions (GJs) facilitate cell-to-cell communication by mediating the direct passage of chemical or electrical signals from the cytoplasm of one cell to another (Saez et al., 2003). Gap junctions are involved in development and patterning, in maintaining tissue homeostasis,
synchronisation of responses and in disease processes (Risek et al., 1992). Gap junctions are hydrophilic channels composed of protein monomers termed connexins (Cx). Connexins by themselves appear to have a significant role in cell growth, differentiation, tumorigenicity, injury, and apoptosis (Sohl and Willecke, 2004; Jiang and Gu, 2005). Connexins may assemble into hexamers called connexons or hemichannels (Evans and Martin, 2002). Hemichannels can function independently and communicate with the extracellular space (Jiang and Gu, 2005; Evans et al., 2006; Spray et al., 2006). A connexon can also dock with a connexon of an adjacent cell to form an aqueous transmembrane channel spanning the two plasma membranes.

Assembly of these GJ channels is a complicated, highly regulated process that includes biosynthesis of the connexin subunit proteins on endoplasmic reticulum membranes, oligomerisation of compatible subunits into hexameric hemichannels (connexons), delivery of the connexons to the plasma membrane, head-on docking of compatible connexons in the extracellular space at distinct locations, arrangement of 10 to several hundred channels into dynamic, spatially and temporally organized GJ channel aggregates (so-called plaques), and coordinated removal of channels into the cytoplasm followed by their degradation (Musil and Goodenough, 1993; Lauf et al., 2002; Segretain and Falk, 2004). Connexin expression can be altered by various mechanisms: transcription, translation, protein turnover and degradation. Phosphorylation and ubiquitation can affect the expression of Cx43 protein; modification of serine or tyrosine residues in the intra-cytoplasmic carboxy terminal region alters plasma membrane assembly, protein degradation and GJIC (Kim et al., 1999; Lampe and Lau, 2000; Solan and Lampe, 2009; Willis et al., 2010; Su and Lau., 2012; Thevenin et al., 2013). Connexons can be made up of one type of connexin protein or different types of connexin proteins. Connexons of uniform connexin composition are called homomeric. When different connexins join together to form one connexon, it is called a heteromeric connexon. When two identical connexons come together to form a gap junction, it is called a homotypic gap junction. The channel is heterotypic if the connexons differ. Junctions formed from different connexins have different transmission characteristics. Particular tissue types or cell types express a limited range of connexins, but the opportunity of forming homomeric or heteromeric connexons and homotypic or heterotypic GJs allows for a wide range of permeability characteristics (Evans and Martin, 2002; Goodenough and Paul, 2003). The Cx subunit is a four-transmembrane spanning unit protein harbouring two extracellular, one cytoplasmic loop and one cytoplasmic N- as well as one C-terminal region. Within both extracellular loops, the order of three cysteine residues is highly conserved. Opposing cysteines in both loops form disulphidebridges stabilising the loops during the docking of two connexons (Kovacs et al., 2007).

In 2007, a single nomenclature system was adopted for the identification of human and mouse connexin genes and therefore by extension, those of domestic livestock (www.genenames.org/genefamily/gj.php). The single nomenclature is proposed to replace previous nomenclatures based on 1) the molecular mass of the connexin, for example Cx43, and 2) sub-classes identified by the Greek alphabet (Kumar and Gilula, 1996). Human Cx43 and Cx32 are now designated as GJA1 and GJB1 respectively. To date the family of Cx genes comprises 20 members in the mouse genome and 21 members in the human genome. The structure of connexin genes is relatively simple. In most Cx genes, the 5’ -untranslated region (UTR) designated as exon 1 is separated from the complete connexin coding region and the subsequent 3’ -UTR located on exon 2. Exceptions to this format have been identified in some
Cardiomyocytes express Cx40, Cx43, and Cx45 (Beyer, 1993; Darrow et al., 1995; Darrow et al., 1996; Martinez et al., 2002; Lin et al., 2010), and smooth muscle cells express Cx37, Cx40, Cx43, and Cx45 (Beyer et al., 1992; Rennick et al., 1993; Little et al., 1995; Kilarski et al., 1998a; Kilarski et al., 1998b; Nakamura et al., 1999; Nakamura and Shibata, 1999; Gemel et al., 2004), suggesting that contractile cells co-express Cx40, Cx43, and Cx45. Connexin43 is the most widely expressed Cx and many cells, including contractile cells such as keratinocytes, fibroblasts, osteoblastic cells, and pancreatic islet cells, express Cx43 (Beyer, 1993; Vozzi et al., 1995; Donahue et al., 1996; Wiszniewski et al., 2000; Pirraco et al., 2012). In somatic cells, Cx26 and Cx32 have a close relationship with secretory tissues. Hepatocytes and acinar cells of the pancreas express Cx26 and Cx32 (Perez-Armendariz et al., 1991; Beyer, 1993; Zhang and Thorgeirsson, 1994; Kojima et al., 1995), as do acinar cells in salivary glands (Muramatsu et al., 1996; Shimono et al., 1996). Periodontal ligament fibroblasts also express Cx32 and are known to secrete collagen type I, collagen type III and fibronectin, and other bone-related proteins (Basdra and Komposch, 1997; Carnes et al., 1997; Morishita et al., 1998; Chien et al., 1999; Sawa et al., 2000; Yamaoka et al., 2000; Yamaoka et al., 2002; Mamalis et al., 2011). Connexin32 is only expressed at low levels in the skin (Di et al., 2001; Kretz et al., 2003; Kretz et al., 2004).

Most cells express two or more connexins (McNeilly et al., 1996; Bruzzone et al., 1996; Su et al., 1997; Nagy et al., 1997; Nagy et al., 1999; Yellowley et al., 2000; Kretz et al., 2004; Martin and Evans, 2004; Waggett et al., 2006; Hervé et al., 2007; Lin et al., 2010; Hervé and Derangeon, 2012). Connexin genes show inter-species variation and orthologous connexins are not necessarily expressed in the same tissue or cell types between species (Eiberger et al., 2001; Sohl et al., 2003). In certain situations the absence of one Cx isoform can be compensated for by another (Reaume et al., 1995; Minkoff et al., 1999; Sohl and Willecke, 2004). Compensation, not always total, has been demonstrated in knock-out and knock-in experiments but the complexity of connexin/connexon interactions complicates interpretation. Redundant function in one tissue may be associated with specific and irreplaceable function in another tissue (Sullivan et al., 1998; Plum et al., 2000; Winterhager et al., 2007; Frank et al., 2010; Laird, 2010; Degen et al., 2011; Bedner et al., 2012).

1.4.1.2 The functional role of gap junctions

The multiple communication channels of a GJ plaque enable ions and small molecules including amino acids, sugar, nucleotides, and steroids to pass directly from one cell to another without the need for such constituents to enter the extracellular space. Most gap junction channels are calcium sensitive, and increasing [Ca2+] after cell injury results in channel closure (Maurer and Weingart, 1987; Noma and Tsuboi, 1987; Harris, 2001; Nicholson, 2003). Conversely, it would appear that elevated intracellular [Ca2+] triggers (uncoupled) Cx32 and Cx43 hemichannels to open (De Vuyst et al., 2006; De Vuyst et al., 2009; Scemes, 2012; Bennett et al., 2012).
1.4.2 Gap junctions in tendon tissue
Tenocytes are in intimate contact longitudinally within rows, and laterally lateral cell processes extend between rows that are proposed to be sheet-like (Merilees and Flint, 1980; Strocchi et al., 1991; McNeilly et al., 1996). Tenocytes in rats, birds, horses and humans have been shown to express Cx32 and Cx43 (McNeilly et al., 1996; Banes et al., 1996; Chuen et al., 2004; Stanley, 2005; Wall, Otey et al., 2007; Maeda et al., 2010). Cx32 is found in GJs that form between tenocyte cell bodies in the same row, whereas Cx43 is found in GJs forming both within and between rows. Such organisation in theory provides a tendon-wide communicating cellular network (McNeilly et al., 1996). Tenocyte gap junctions are protected from mechanical damage by intracellular longitudinally-orientated actin contractile stress fibres linked by adherens junctions (Ralphs et al., 2002).

Phenotypical variation may occur within the one tendon. In many tendons, including the equine SDFT, there are compressional, tensional and transitional zones, which reflect the forces applied to the tendon at those sites (Benjamin and Ralphs, 1997; Ralphs et al., 1998; Perez-Castro and Vogel, 1999; Batson et al., 2003). In those zones subjected only to tensional force (such as the mid-metacarpal region of the horse SDFT) there is higher expression of GJ connexin proteins (Ralphs et al., 1998). Gap junction expression also varies with age and tendon function. In the SDFT, Cx43 and Cx32 expression, measured as plaque area (mm2) per cell, decreases conjointly with the age-related decrease in tenocyte numbers but although cellularity also decreases in the CDET, there is an increase in Cx43 expression per cell (Young et al., 2009).

Gap junctions and their proteins are necessary for collagen synthesis by tenocytes. In one study Cx43 was up-regulated by mechanical loading of cultured tendon cells. Gap junction blockade reduced collagen expression in response to mechanical loading in cyclically loaded tendons in vitro (Banes, Weinhold et al., 1999). Results from one study of avian tenocytes subjected to cyclical loading indicated that Cx32 has a stimulatory effect on collagen synthesis, while Cx43 is inhibitory (Waggett and Ralphs, 2001). A reduction in expression of Cx43 following the application of antisense oligodeoxynucleotides (asODNs) led to a 500% increase in collagen synthesis (Waggett and Ralphs, 1999). Potentially, tenocytes could have a basal level of ECM synthesis, maintained by systems involving Cx32 signalling, that is enhanced by load. Connexin43 signalling then becomes active, dampening down the load response and maintaining control. Individual tenocytes have an independent capability to respond to load but GJ function greatly enhances the cell response to mechanotransduction and growth factor signalling (Banes, Weinhold et al., 1999; Waggett and Ralphs, 2001; Waggett et al., 2006). It should be noted however, that findings in one avian in vitro system do not necessarily translate to equine tendon tissue and that blocking Cx43 and Cx32 respectively in that system was not complete.

In the equine SDFT significant reductions in Cx43 and Cx32 protein expression per tenocyte were measured in one study between foetuses and young foals (1-6 months), implying that the synthetic capacity of cells in the adult tendon is significantly reduced (Stanley et al., 2007). It should be noted that GJ plaque numbers and size do not necessarily correspond with communication efficiency (Rufer et al., 1996).
1.5 Antisense oligodeoxynucleotides
1.5.1 Gap junctions in wound healing

Wound healing is a complex process requiring the coordinated efforts of multiple cell types. Gap junction intercellular communication (GJIC) has been shown to have a pre-eminent role in this process, influencing inflammation, differentiation, proliferation, migration and tissue contraction (Goliger and Paul, 1995; Kumar and Gilula, 1996; Saitoh et al., 1997; Lucke et al., 1999; Ehrlich et al., 2000; Oviedo-Orta et al., 2000; Oviedo-Orta et al., 2001; Oviedo-Orta et al., 2002; Oviedo-Orta and Evans, 2002; Govindarajan et al., 2010).

Dynamic changes in Cx isoform expression occur in the epidermis and dermis of the skin and accompany fibroblastic proliferation, migration and differentiation during healing of excisional wounds in rodents. Connexin26 and Cx30 appear to be associated with cell migration, Cx43 in proliferation and migration and Cx31.1 in terminal differentiation (Mori et al., 2006; Becker et al., 2012). Connexin26 and Cx31.1 were shown to have a negative correlation with proliferation, while Cx43 had a positive correlation (Coutinho et al., 2003).

1.5.2 Downregulation of gap junction activity using oligodeoxynucleotides

Antisense oligodeoxynucleotides represent a potentially powerful molecular biological technique to reduce (knock-down) protein expression during the inflammatory process (Paterson et al., 1977; Stephenson and Zamecnik, 1978; Ma et al., 2000; Juliano et al., 2009; Becker et al., 2012; Juliano et al., 2012).

Two Cx43 antisense oligomer sequences have been published in the scientific literature. One early sequence used was an antisense sequence covering the start codon (Yasui et al., 2000; Villars et al., 2002; Frantseva et al., 2002). A separate connexin43 specific antisense oligomer, DB1, has been shown to have significant biological effects on the developing chicken embryo in ovo (Becker et al., 1999) and in wound healing (Moyer, Davis et al., 2002; Moyer, Saggers et al., 2002; Qiu et al., 2003; Coutinho et al., 2005; Mori et al., 2006). DB1 is an unmodified antisense oligodeoxynucleotide (asODN) 30 bases long. It is effectively a short, singlestranded fragment of DNA which is delivered using pluronic gel [Pluronic F-127 (Poloxamer) gel] (Green et al., 2001). Pluronic gel, at 30 % v/v, solidifies as it warms to physiological temperatures and provides for sustained delivery of the asODN at low concentrations. The gel is a mild surfactant, which aids delivery, and is typically used with asODN at 1–15 μM concentrations. Antisense oligodeoxynucleotides in their unmodified form have a short intracellular half-life of approximately 20 minutes but the gel-asODN combination permits sustained delivery (Green et al., 2001). Connexin43 protein knock-down is seen within 2 hours and in most tissues is maximal at 6–8 hours. Connexin expression returns to normal by 24 hours (Green et al., 2001).

In a skin wounding model in mice, application of Cx43 asODN caused rapid downregulation of Cx43 protein expression in the epidermis and dermis, which persisted for at least 24 hours (Mori et al., 2006). This model showed enhanced proliferation and subsequent migration of fibroblasts into the wound, accelerated angiogenesis, reduced inflammation, and increased mRNA for collagen α1 and general collagen content at the wound site. Antisense-treated wounds healed more rapidly due to enhanced proliferation and maturation of granulation tissue, faster wound contraction and accelerated re-epithelialisation. Promoting GJIC between wound fibroblasts in granulation tissue increases the rate of collagen maturation (Moyer, Davis et al., 2002; Moyer, Saggers et al., 2002).
Connexin43 needs to be down-regulated at the leading wound edge for reepithelialisation to occur. Chronic skin wounds that are very slow to heal do not down-regulate Cx43 at the wound edges (Becker et al., 2012). Application of Cx43 antisense ODN enhanced the rate of down-regulation of Cx43 in the leading edges of the wounded epidermis (prior to re-epithelialisation) and reduced its up-regulation in the dermis and its vasculature (Qiu et al., 2003). In burns, there was a reduction in blister size and reduced spread of the injury. In both burns and lesions, the initial inflammatory response was reduced in deep dermal wound zones as shown by decreased numbers of neutrophils (Coutinho et al., 2005; Mori et al., 2006). Subsequently, epidermal proliferation was enhanced and wound re-epithelialisation accelerated (Qiu et al., 2003; Coutinho et al., 2005; Mori et al., 2006). Connexin43 asODN has been shown to reduce inflammation and the spread of injury in models of optic nerve and spinal cord trauma (Danesh-Meyer et al., 2008; Cronin et al., 2008; Zhang, O’Carroll et al., 2010).

Gap junction intercellular communication appears critical for fibroblast progression from migratory cell through to apoptosis as granulation tissue matures into scar tissue, but importantly GJIC also influences the cells of the connective tissue and subsequently the ECM. This close relationship suggests that while GJIC plays a key role in tissue maintenance processes in normal tendon, it may also be an extremely important factor in control of tendon injury repair. Modulating of connexin expression could therefore be used to improve tendon healing.

GAP JUNCTION EXPRESSION IN NORMAL, INJURED AND CONTRALATERAL EQUINE SUPERFICIAL DIGITAL FLEXOR TENDONS

In an unpublished pilot study, PCR was used to look for eleven connexin isoform genes in the SDFT and/or common digital extensor tendon (CDET) of twelve horses, who included one foetus, three foals (aged 1-6 days) and eight adults (aged 2-8 years). Connexin43, Cx46, Cx37, Cx40, Cx50, Cx32, Cx26, Cx31 and Cx30 were identified in all or some SDFTs; Cx30.3 and Cx31.1 were not identified in any SDFTs examined. Two of the eleven Cx isoform gene sequences (equine Cx43 and equine Cx32) were sourced from gene banks but the genetic sequences of the other nine Cx isoforms were deduced using human Cx gene family RT-PCR profiling and the design of their PCR primers was based on human sequences (Dr. N. Young, pers. comm.). The current study, utilising similar methodology, set out to determine whether Cx gene expression profiles identified in the SDFTs of the normal horses of the pilot study differed following SDFT injury. These connexin isoforms were analysed as they were considered most ubiquitous (Cx43) or likely to be involved in tendon tissue (Cx32, Cx26) and vascular bed (Cx43, Cx40, Cx37) based upon published studies. Cx46 and Cx50 are primarily eye lens proteins and, although reported in PCR screens of ischaemic tissues and in other tissues such as testis, were included as negative controls. It was unexpected that these showed a positive expression in the tendon and some care is required with interpretation of those results. Other connexin isoforms were not included as they tend to be tissue specific (Cx36, Cx47 and Cx29 in the nervous system for example) or rare and unlikely to play major roles in tendon response to injury.

Equine SDFT tendon fibroblasts (tenocytes) have been shown to express Cx43 (Stanley, 2005; Stanley et al., 2006; Stanley et al., 2007; Young et al., 2009). Similarly, Cx43 expression was observed in all equine SDFTs sampled in the current study. Reverse transcription polymerase chain reaction products of appropriate size were obtained from five equine SDFTs for Cx40 but
were not sequenced to confirm their identity due to time constraints. Although expression of Cx40, Cx43 and Cx45 often occurs in the same tissues and colocalisation of Cx43 and Cx45 has been observed in adult human ventricular myocardium (Coppen et al., 1998; Grikscheit et al., 2008), Cx45 has not been identified in tendon from any species. Connexin32 expression has previously been identified in equine SDFTs (Stanley, 2005; Stanley et al., 2006; Stanley et al., 2007; Young et al., 2009). Forty five of the 49 equine SDFTs evaluated in the present work showed Cx32 expression.

Polymerase chain reaction product compatible with the expected size for Cx26 was observed definitely in one and possibly in two of the equine SDFT samples but sequencing was not undertaken. Connexin26 has been identified in rat tail tendon (Maeda et al., 2010). Sequencing of PCR products confirmed that both Cx31 and Cx31.1 were expressed in equine SDFTs. Connexin31 is a non-cardiac connexin with prominent expression in the skin, cochlea, uterus and placenta (Evans and Martin, 2002). It can be regulated by phosphorylation and mouse Cx31 hemichannels cannot form functional heterotypic channels with any other connexin (Elfgang et al., 1995a). It is postulated that Cx31.1 has a role in coordinating apoptosis as it has been identified in the granular layer of dying skin and with atretic ovarian follicular cells (Goliger and Paul, 1994; Coutinho et al., 2003; Wright et al., 2009).

It was surprising that Cx37 was not detected in SDFTs as it has previously been identified in equine SDFTs (Dr N. Young, pers. comm.). Connexin37 is expressed in vascular endothelial cells, particularly those subjected to shear stress; frequently Cx37 is coexpressed with Cx43 (Johnstone et al., 2009; Pfenniger et al., 2012). Although the ECM of uninjured SDFT is of low vascularity, neovascularisation is characteristic of injured SDFT and expression of Cx37 would be expected. Lack of detectable Cx37 may have been due to inadequate PCR optimisation or perhaps shear stress is not a significant factor in injured SDFT neovascular blood flow. Connexin46 has also previously been identified in SDFT (Dr N. Young, pers. comm.). Connexin46 would appear to be important in tissues associated with low oxygen tension as it is expressed by cells in the lens and by tumour cells (Tenbroek et al., 1992; Banerjee et al., 2010). It has been postulated that low oxygen tension exists in normal tendon tissue, particularly during exercise when ischaemia may be present, and hypoxia has been suggested as a possible contributing factor in the development of tendonopathy (Riemersma and Schamhardt, 1985). Failure to optimise the PCR may again account for lack of Cx46 expression.

In uninjured equine SDFT, Cx43 measured by qPCR was the predominant connexin gene expressed and was expressed in all the samples tested; no other Cx isoform gene was expressed in every sample. Connexin31 and Cx31.1 genes were expressed more commonly and for Cx31.1, measured by qPCR, at higher levels than any of the other Cx isoforms except for Cx43. Connexin32 gene expression was not identified in all uninjured SDFT samples tested and when expressed was often at low levels. Failure to identify Cx32 gene expression in some of the samples was most likely due to technical issues associated with qPCR because all samples showed Cx32 protein expression, albeit some at low intensity. Primer design and PCR protocols can influence detection and/or quantification of gene expression. It may well be that optimisation of the PCR was not achieved for all the Cx isoform genes, either at all or in some reactions, and therefore detection of gene expression may have failed.
Except for Cx43, there was no consistent pattern to the detected gene expression of those Cx isoforms that were identified as being expressed; expression was not always detected in the injured SDFTs. Conversely, expression was not detected in some uninjured SDFTs but was identifiable in others. Inconsistency of detection may also be a reflection of the abundance of the target connexin gene rather than its absence. Low expression of the target gene may not allow PCR amplification and the complexity of the tissue further compounds the issue as subpopulations of cells will influence mRNA expression (Karrer et al., 1995). The SDFT contains subpopulations of tenocytes, some of which are postulated to be more metabolically active than others. Tenocyte subpopulation numbers and their metabolic activity vary with anatomical location within the tendon and with age (Stanley et al., 2006; Stanley et al., 2007; Stanley et al., 2008).

Increased connexin gene expression usually, but not always, leads to increased expression of the protein that is encoded by the gene. However, the expression ratio is often not 1:1 (Hervé, 2007; Herve et al., 2007). Connexin43 and Cx32 protein expression was assessed quantitatively using immunohistochemistry; uninjured equine SDFTs were compared with injured equine SDFTs and the contralateral limb SDFTs to both uninjured and injured SDFTs, the sampling population was the same as used for Cx isoform PCR product identification and sequencing, and Cx qPCR gene expression. Connexin monomers usually assemble into functional GJ plaques. Gap junctions, particularly those of Cx43, frequently clump together to form plaques. In uninjured SDFT, these plaques were usually 1-3 per cell in number and were predominantly located at either end of the cell; occasionally they were identified at other locations. Polar distribution indicates longitudinal contact with cells in the same row whereas non-polar distribution suggests contact with cells in adjacent rows although this could not be conclusively established. A number of SDFT cryosections were labelled for Cx43 and stained for intracytoplasmic actin with phalloidin and nucleic DNA with Hoechst 33342, the rationale being that actin anchored to the cell membrane would enable the outline of cells to be determined and permit identification of the spatial orientation of Cx43 GJs. Unfortunately, the degree of actin staining was not adequate to identify cellular boundaries or cytoplasmic processes and consideration will be given to using Texas-red-X-conjugated phalloidin to visualise cytoplasmic processes and determine cell volume and area in future studies (Sugawara et al., 2005).

Based on visual appraisal of CLSM images of labelled cryosections, Cx43 protein expression was significantly upregulated in injured and injured contralateral SDFTs when compared with uninjured SDFTs. In injured SDFT, number and size of Cx43 GJ plaques appeared substantially increased and the location of Cx43 GJs appeared more widespread. Gap junctions are involved in intercellular communication and therefore the observed upregulation in Cx43 protein expression suggests a dramatic increase in the level of intercellular communication but GJs may not have been functional. However, there was an obvious increase in tendon cellularity in injured SDFT and this could bias the observer’s perception of there being both an absolute and relative (per tenocyte) increase in Cx43 protein expression. Increased tenocyte numbers are characteristic of tendonopathy in energy storing tendons such as human Achilles and patellar tendons (Kannus and Jozsa, 1991; Cook et al., 2004; Maffulli et al., 2011) and equine SDFT (Webbon, 1973; Marr et al., 1993) although chronically injured tendons with large amounts of scar tissue may show reduced cellularity (Webbon, 1973; Marr et al., 1993).
It has been postulated that Cx43 GJs play an inhibitory role in collagen synthesis and may be particularly important in the synthesis of collagen type III in injured tendon (Waggett and Ralphs, 2001; Ralphs et al., 2002; Waggett et al., 2006). The increased Cx43 protein expression paralleled the increased Cx43 gene expression and was present at the same time that collagen type III gene expression was upregulated. This will be discussed in Chapter 4. Once again, occasional inconsistencies were observed in the level of Cx43 protein expression; areas immediately adjacent (peripheral) to the site of injury, identified ultrasonographically, sometimes showed greater expression but usually the most intense expression was at the site of injury (central region). Frequently, Cx43 protein was diffusely expressed within the injured tendon although there was a decreasing gradient from the central area of injury to more distant sites. This may be a reflection of the sampled population where chronic and recurrent injuries were more common than first-time recently injured SDFT but may also be an indicator of more generalized disturbance of homeostasis. Connexin43 protein expression was also increased in the contralateral SDFT. The increase in Cx43 protein expression in contralateral SDFT did not appear to be associated with an increase in cellularity compared to uninjured SDFT although cell density counts were not undertaken. Upregulation of Cx43 GJ activity in injured SDFT was often associated with tenocytes that were of the type 2 phenotype. Tenocyte phenotypic change, where nuclei become rounder and the cell less elongated has been described in both asymptomatic (Cook et al., 2004) and symptomatic tendonopathies in human and horses (Webbon, 1973; Marr et al., 1993; Cook et al., 2004; Maffulli et al., 2011). It has been suggested that type 2 tenocytes migrate from the endotenon and/or epitenon into the tendon parenchyma as part of the reparative process (Webbon, 1973). Extrinsic healing of injured tendon is characterised by migration of peripherally-located fibroblasts, originating from the epitenon and endotenon, into perifascicular and intrafascicular locations; these cells are subjected to different loading patterns and show increased synthetic activity (Jones et al., 2003).

Confocal microscopy images of injured SDFT showed upregulation of Cx43 and Cx32 protein expression involving both type 1 and type 2 tenocytes but differences, if any, in expression volume and distribution between tenocyte types were not explored. Loading of rat tail and bovine extensor tendons at 5% gross strain (2-3% strain at the cellular level) resulted in tenocyte deformation manifest as slightly longer and thinner cells and nuclei (Screen et al., 2003) and therefore older horses who have exercised over a number of years could be expected to show differences in tenocyte phenotype when compared to younger horses. There appears to be a regional effect because tenocytes in the mid-metacarpal region of both the equine SDFT and CDET have been shown to be longer and thinner than is seen in other zones of either tendon (Stanley, 2005) despite there being substantial differences in applied strain between the two tendons (Stephens et al., 1989). Tenocyte length in horses, irrespective of type, increases with age (Stanley, 2005) but tenocyte number per unit area in horses decreases with age and there is a concurrent increase in the ratio of type 1 to type 2 cells (Stanley, 2005; Stanley et al., 2007; Young et al., 2009). Age-related changes in tenocyte morphology have been identified in humans and rabbits; tenocytes became longer and thinner (Strocchi et al., 1991; Nakagawa et al., 1994). Functionality of type 1 and 2 tenocytes may differ with regards metabolic activity and ECM synthesis. Type 2 tenocytes were shown in one immunohistochemical study to express more PINP (Chuen et al., 2004). The expression pattern of Cx32 GJs in normal SDFT was distinct from that of Cx43; polar distribution predominated but Cx32 GJs tended not to aggregate into clearly defined plaques. Connexin32 protein expression visually was considerably
upregulated in injured SDFT when compared with normal SDFT but the increase in cellularity may have resulted in an error of estimation. In injured SDFT, the distribution of Cx32 GJs was substantially increased. Others have suggested that Cx32 GJs are only involved in communicating with cells in the same row and not with cells in adjacent rows (McNeilly et al., 1996). Immunofluorescence was detected more extensively than expected in injured SDFT with widespread but low-intensity distribution along what was assumed to be the plasma membrane of tenocytes. However, cytoplasmic projections were not visualised and the observed diffuse immunofluorescence may have been intracytoplasmic Cx32 protein expression. Alternatively, the observed diffuse immunofluorescence may have been Cx32 protein localised within hemichannels. The distribution of Cx32 protein expression in injured SDFT was similar to that seen with Cx43 with sites adjacent and distant to the identified site of injury often showing upregulation but usually at a lower level than seen at the central zone of injury. As with Cx43, increased Cx32 protein expression was frequently observed in the contralateral SDFT. This increase in Cx32 protein expression did not appear to be associated with an increase in cellularity compared to uninjured SDFT but counts to establish cell density were not done. Although not quantified, there was an apparent increase in tenocytes with more rounded nuclei (cigar-shaped) in the central zones of contralateral SDFT. Persisting but diminished Cx32 protein upregulation was observed in more chronically injured SDFT. Interestingly, Cx32 protein expression in some of the uninjured tendons examined was at a very low level but this was not surprising considering the low level of Cx32 gene expression measured with qPCR in some samples. Connexin32 gap junctions have also been postulated to play a role in ECM collagen synthesis (Waggett et al., 2006) and therefore significant upregulation could be expected to markedly influence collagen synthesis in injured SDFT.

In equine SDFT significant reductions in Cx43 and Cx32 protein expression per tenocyte were measured in one study between foetuses and young foals (1-6 months), implying that the synthetic capacity of cells in the adult tendon is significantly decreased (Stanley et al., 2007). Horses in the present investigation were at least two years old and the mean and median ages were 4.6 and 5 years respectively and therefore the hypothesis that tenocytes become less metabolically active with age should have been satisfied. However, the increased Cx protein and gene expression in injured SDFT was suggestive of considerable metabolic activity although it may not have been appropriately directed or of sufficient magnitude for adequate repair. The patency and functionality of either Cx43 GJs or Cx32 GJs remains to be established. If Cx43 GJs exert an inhibitory effect on collagen synthesis and Cx32 GJs are stimulatory, then the balance between Cx43 and Cx32 expression would be highly significant but this remains to be determined. It should also be noted that in the lung, changes within the ECM influence GJIC. Connexin expression in alveolar epithelial cells is regulated by the ECM (Guo et al., 2001) and cellular interaction of integrins α3β1 with laminin 5 promotes GJIC (Lampe et al., 1998). It is probable that similar feedback mechanisms exist in tendon.

3.6 Conclusion
Upregulation of Cx43 and Cx32 gene and protein expression not restricted to ultrasonographically-detected SDFT pathology indicated diffuse metabolic alterations in injured SDFT. The finding that Cx protein expression is upregulated in the contralateral SDFT suggests that the contralateral tendon is being subjected to increased load and therefore at greater risk of developing pathology and supports the clinical finding that many horses with an SDFT injury of
one fore-limb will simultaneously or subsequently show an SDFT injury of the opposite fore-limb.

**COLLAGEN EXPRESSION IN NORMAL, INJURED AND CONTRALATERAL EQUINE SUPERFICIAL DIGITAL FLEXOR TENDONS**

Procollagen amino-propeptide types I and III are the translational products of collagen type I and III gene expression respectively and the outcomes observed in this study show that gene and protein upregulation for both collagen types I and III was present in the central zone of injured SDFTs and correlated with the upregulation of GJIC previously described in Chapter Three. Increased collagen type I gene expression observed at other zones in injured SDFTs was not associated with significant increases in PINP expression. However, the sampled population was biased towards chronic or recurrent SDFT injury due to the difficulty of accessing acute SDFT injuries. Because of the mixed nature of the SDFT injuries, often recurrent injuries were superimposed on chronic injuries and therefore inconsistencies of gene and protein expression were apparent within an injured tendon.

Gap junction intercellular communication appears to be essential in the response of tendon fibroblasts to mechanical stimulation and subsequent maintenance of the ECM (Elfsgang *et al*., 1995a; Elfsgang *et al*., 1995b; McNeilly *et al*., 1996; Banes, Weinhold *et al*., 1999; Waggett *et al*., 2006; Richardson *et al*., 2007). It has been postulated that Cx43 gap junctions alter the ratio of collagen type I: collagen type III synthesis (in favour of type III) during the repair process in injured tendons. Antisense-mediated downregulation of Cx43 gene expression in avian tenocytes led the authors to conclude that Cx43 inhibited collagen synthesis whereas Cx32 was stimulatory; complete downregulation of Cx43 gene expression however was not achieved (Waggett *et al*., 2006). Chemically-induced, total GJ blockade of tendon and dermal fibroblasts results in reduced collagen type I and total collagen synthesis (Banes, Weinhold *et al*., 1999; Ehrlich *et al*., 2006).

Genetically-manipulated Cx43-deficient mice show reduced gene expression of collagen type I along with several other genes involved in bone formation, which suggests that upregulation of Cx43 expression by itself is not likely to be inhibitory for collagen synthesis (Lecanda *et al*., 2000). The application of connexin43 antisense oligodeoxynucleotide (Cx43 asODN) to skin wounds led to increased collagen 1α1 gene and total collagen protein expression by dermal fibroblasts and reduced inflammation (Mori *et al*., 2006). The findings of these two studies are in contradiction to the conclusions drawn from the previously discussed avian tenocyte study (Waggett *et al*., 2006) but tissue-specific differences may be responsible. Wounds of the skin are a complex environment and significant elevation of TGF-β1 was noted in the Cx43 asODN study of skin wound repair (Mori *et al*., 2006). The findings of these two studies are in contradiction to the conclusions drawn from the previously discussed avian tenocyte study (Waggett *et al*., 2006) but tissue-specific differences may be responsible. Wounds of the skin are a complex environment and significant elevation of TGF-β1 was noted in the Cx43 asODN study of skin wound repair (Mori *et al*., 2006). Transforming growth factor is a profibrotic cytokine and therefore increased production would be expected to increase collagen synthesis. Downregulation of Cx43 was shown to decrease TGF-β in a mouse model of myocardial infarction with subsequent reduction in fibrosis (Zhang *et al*., 2010). Connexin43 expression and GJIC was reduced following TGF-β1 stimulation in rat hepatic stellate cells and in human bladder smooth muscle cells but the converse was observed in human aortic cells (Rama *et al*., 2006; Neuhaus *et al*., 2009; Lim *et al*., 2009). It has also been shown in bone that the promoter region of the collagen 1α1 gene responds to Cx43 resulting in increased collagen synthesis (Stains *et al*., 2003). In osteoblasts, the α1 chain of collagen type I contains a Cx-responsive
promoter region (Stains et al., 2003); perhaps similar zones exist in the Col1α1 gene in tenocytes. Therefore, the degree of Cx43 regulation, the relationship between Cx43 and profibrotic comediators such as TGF-β and pSMAD, and the tissue/cell type all influence the potential fibrotic response following injury.

Transforming growth factor-β appears to play a significant role in collagen synthesis in tendons by influencing scleraxis and tenomodulin expression (Shukunami et al., 2006; Pryce et al., 2009; Maeda et al., 2010; Mendias et al., 2012); TGF-β is in turn influenced by GJ activity (Dai et al., 2007; Asazuma-Nakamura et al., 2009). Gap junction protein Cx43 has been shown to mediate TGF-β activity by competing with Smad 2/3 for binding sites on microtubules. This results in accumulation of Smad 2/3 and Smad 4 in the nucleus of the cell and leads to the transcription of TGF-β genes (Dai et al., 2007).

In the current study, the same injured equine SDFT were used to investigate connexin and collagen gene and protein expression. In Chapter Three, it was noted that Cx43 gene and protein expression were significantly upregulated and Cx43 gene expression, measured with qPCR, was ten-fold greater than Cx32 gene expression. Therefore, if Cx43 negatively influences collagen synthesis, increased Cx43 expression would be expected to inhibit collagen synthesis; however results did not support this outcome as both PINP and PIIINP expression was increased in SDFTs. Connexin32 gene and protein expression were also shown to be upregulated in the injured tendons and therefore it may be the balance of Cx43 and Cx32 expression, along with other factors, which influence collagen synthesis. The status of the ECM has been shown to influence GJIC in other tissues (Guo et al., 2001; Imbeault et al., 2009; Bowers et al., 2010) but this has not been investigated in tendons. Interestingly, the increased Cx43 and Cx32 protein but not Cx gene expression quantified in contralateral SDFTs described in Chapter Three was not accompanied by significant changes in collagen gene and procollagen protein expression in these same tendons. It was posited in the previous chapter that the elevated Cx protein expression was the consequence of increased loading of the contralateral SDFT following ipsilateral SDFT injury. The observed disparity between Cx and collagen protein expression indicates the relationship between GJIC and ECM maintenance is complex and is impacted by numerous factors influencing gene transcription, and protein translation and degradation.

Indicators of collagen synthesis in the normal equine SDFT have been shown to decrease with age, neutral salt-soluble collagen, collagen type I mRNA, PINP per mm2 of tendon tissue, PIIINP per mm2 of tendon tissue and PINP per tenocyte are at lower levels in adult SDFT compared with juvenile SDFT (Young et al., 2009). Decreased collagen synthesis in adult SDFT supports the hypothesis that this tendon becomes metabolically less active with aging, which may result in reduced capacity for repair. Additionally, the ratio of PINP to PIIINP synthesis is altered in adult SDFT so that PIIINP synthesis predominates, which is likely to lead to increased synthesis of collagen type III and an ECM containing greater numbers of smaller diameter fibrils thereby increasing the risk of injury (Young et al., 2009). Normal SDFTs were harvested from horses 2-7 years of age with substantial variability in exercise status. Therefore, some age- and exercise-related differences in ECM gene and protein expression would be expected and as will be discussed further in Chapter 6, the SDFTs of Horse 19 responded to the application of repetitive cyclic strain unlike the others. The SDFTs of this horse were “stiffer” indicative of considerable maturity of cross-linkages and therefore high content of insoluble collagen.
Typically, tendonopathies demonstrate elevated expression of mRNA for collagen type I and III genes, which is accompanied by increased synthesis of PIIINP and subsequent ECM deposition of collagen type III but not increased ECM content of collagen type I (Riley et al., 1994; De Mos et al., 2007). Despite evidence of increased collagen synthesis, total collagen did not change in degenerative disease involving the equine SDFT (Birch et al., 1998) and human Achilles tendon (Ireland et al., 2001; De Mos et al., 2007) but was decreased in human supraspinatus tendonopathy, possibly in response to the intra-tendonous corticosteroid administration used in treatment (Riley et al., 1994; Bank et al., 1999). Tendonopathy has been shown to be associated with higher collagen turnover and higher amounts of damaged and degraded collagen (De Mos et al., 2007). Increased collagen solubility due to reduced cross-linkages is characteristic of tendonopathy, solubility increases in acetic acid, pepsin and cyanogen bromide (Williams, McCullagh and Silver, 1984; Riley et al., 1994). These changes are consistent with new collagen synthesis and indicate that a cohort of tendon cells is capable of an altered pattern of collagen and matrix synthesis. It may be that collagen type III can be more rapidly synthesised and therefore the rate of repair is accelerated at the cost of some initial loss of strength at the repair site. In tendon evolution, injury due to repetitive cyclic activity may have been of less significance than direct trauma.

Various techniques are available to determine the age, and rates of synthesis and degradation, of collagen in connective tissue including tendons (Becker et al., 1976; Bada, 1984; Birch, Worboys et al., 2008; Sivan et al., 2008; Young et al., 2009; Thorpe et al., 2010). Quantification of collagen type can be undertaken in a number of ways ranging from colorimetric assays to polyacrylamide gel electrophoresis (Ramshaw and Werkmeister, 1988; Walsh et al., 1992; Houghton et al., 1996; Chen et al., 2009). The sirius red dye technique, a colorimetric assay, was employed because of its simplicity as a screening regimen; it was also much less labour intensive than gel electrophoresis. Sirius red dye selectively binds to collagen; the dye-bound soluble collagen is precipitated and treated with an alkali, which releases the dye. Biochemical analysis of the collagen content of injured, contralateral and uninjured SDFTs was undertaken using a commercially available kit. The Sircol™ Soluble Collagen Assay utilises neutral salts and dilute acids to selectively precipitate collagen from tendon tissue. Precipitation enables the determination of the molecular species of collagen present in complex mixtures. Essentially, biochemical digestion was used to separate the three major constituents of soluble collagen, these being neutral salt-soluble (nssc), acid-soluble (asc) and pepsin-insoluble collagen (psc). The remaining collagen in this study was called insoluble (isc).

Extracellular matrix is heterogeneous with regards type and age of collagen but recent synthesis and degradation will influence the fractions present. Accretion occurs in the ECM and therefore with age, inter-fibrillar linking increasingly occurs resulting in greater insolubility (Davidson and Cooper, 1968). The various solvents used here sequentially cause increasing disaggregation of cross-linkages (nssc < asc < psc). Newly synthesised collagen molecules have immature cross-links initially, which progressively develop into mature cross-links. It should be noted that nssc may also represent the degradation product of insoluble collagen and not just newly synthesised collagen with immature cross-links; degradation products will also have less or weaker cross-linkages (Jackson and Bentley, 1960). The typical picture with new collagen synthesis should show increased total collagen and increased nssc (measured as μg of collagen/mg of tendon tissue) accompanied by a decrease in psc, but with minimal or no change in asc. However
increases in nssc, asc and psc, accompanied by a decrease in total collagen content, suggests increased degradation of insoluble collagen. Essentially, nssc represents the earliest form of extracellular collagen in the synthetic pathway. Solubility of collagen in acetic acid is determined not only by the degree of non-covalent bonds but also by the stability of covalent aldime cross-linkages. Therefore, increases in asc suggest formed but premature cross-linkages or increased degradation products. Pepsin is responsible for more aggressive proteolytic degradation than acetic acid; pepsin cleaves the non-helical zones of the collagen molecule, which are known to contain inter- and intra-molecular cross-linkages. The level of mature cross-links, such as those formed by hydroxypyridinium, is the determinant of collagen insolubility (Jackson and Bentley, 1960; Davidson and Cooper, 1968). Accumulation of crosslinkages increases resistance to enzymatic digestion (Laurent, 1987; De Mos et al., 2007). Tendons with high levels of cross-linkages contain more insoluble collagen and show increased “stiffness” (decreased compliance) (Reddy et al., 2002; Reddy, 2004).

Additional characterisation of collagen may have been achieved by employing more aggressive tissue digestion. Only one pepsin application was used; a second pepsin digestion is likely to have yielded more solubilised collagen. Further solubilisation of collagen could have been achieved by employing selective salt precipitation, which involves the use of gradient molar concentrations (salt solutions of increasing ionic concentrations) of sodium chloride (NaCl) to identify specific collagen molecules, including procollagens. Neutral salts disrupt intermolecular forces; the greater the ionic strength of the salt solution, the greater the disruption and resultant collagen extraction. Salt solutions of low molarity will only disrupt the weakest of bonds, which are in the youngest collagen. Cyanogen bromide digestion has also been used to solubilise collagen constituents that are insoluble after acetic acid and pepsin digestion; much of the insoluble collagen in acetic acid and pepsin digest is extractable (solubilised) following cyanogen bromide digestion (Bornstein et al., 1966).

Higher mean values for nssc in all zones of injured SDFTs compared with central zones of uninjured SDFTs and equivalent zones of contralateral SDFTs are indicative of increased recent collagen synthesis and/or increased collagen degradation. The increased collagen type I and III gene expression and PINP and PIIINP protein expression measured in central zones of injured SDFTs supports the conclusion that increased nssc is due to recent collagen synthesis but associated accelerated collagen degradation cannot be discounted. Although mean values for isc were not remarkably or significantly different between central zones of injured SDFTs and uninjured SDFTs, mean values for isc in central zones of paired injured and contralateral SDFTs were different with values being lower in injured SDFTs. Increased collagen solubility is characteristic of new collagen when insufficient time has elapsed for development of mature crosslinkages and/or accelerated collagen degradation with cross-linkage disruption, both of which could be expected to occur in injured SDFTs.

Insoluble collagen tended to represent a greater portion of the total collagen in normal SDFTs when compared with that in injured SDFTs but between-horse variability negated significance. Calculated concentrations for both soluble and insoluble collagens were, in almost all cases, much lower than published values for normal equine SDFTs (Young et al., 2009). The Sirius red dye-binding assay has been found by various authors to be reliable, accurate and repeatable (Walsh et al., 1992) but others have described its limitations (Kliment et al., 2011). Its
application in this investigation was difficult and there were a number of steps in the assay where error could be introduced, perpetuated and amplified, including errors related to volume control, calculation and capture of released collagen.

Horses used in the present study appeared to represent a spectrum of SDFT injury and hence the significant variability observed in the Time 0 (at harvest) data. It has been postulated that tendonopathy has three stages, which form a continuum (Cook and Purdam, 2009). Firstly, there is reactive tendonopathy, which is characterized by tenocyte proliferation and phenotypic alteration (Cook et al., 2004). Increases in non-collagenous elements of the ECM lead to tendon thickening and generally there is minimal collagen disruption. The second stage is referred to as tendon disrepair and involves similar elements to the first stage but is associated with greater disruption of the ECM and collagen separation. Degenerative tendonopathy is the final stage and is recognised by matrix heterogeneity (Cook and Purdam, 2009). Examples of the three stages were represented in the injured SDFTs examined but stages 2 and 3 predominated. This was not surprising considering the difficulty of obtaining horses with acutely injured SDFTs. Typically, donated and purchased horses with injured SDFTs had a history of initial injury, being rested for variable periods of time and then returned to work with subsequent SDFT re-injury.

4.6 Conclusion
Both PINP and PIIINP were identified within the cytoplasm of tenocytes and SDFT injury was significantly correlated with upregulation of both PINP and PIIINP expression. Increased expression of procollagen amino-propeptides I and III proteins as de facto representatives of collagen type I and III synthesis in injured SDFTs paralleled the increased collagen type I and III gene expression measured with qPCR. The expression of procollagen proteins and collagen genes in injured SDFTs was diffuse and extended beyond the limits of injury as defined ultrasonographically.

THE EFFECT OF CONNEXIN43 ANTISENSE OLIGODEOXYNUCLEOTIDE ON GAP JUNCTION AND COLLAGEN EXPRESSION IN EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON IN VITRO EXPLANTS
In the present investigation, in vitro SDFT explants were harvested in a manner that created a six-sided wound with four long sides and two (short) ends; epitenon was removed but endotenon was not. The reparative process could therefore be expected to involve all sides and the two ends but a previous study showed that fibroblastic proliferation and collagen synthesis was mainly associated with the two ends of the explants and areas adjacent to the endotenon (Murphy and Nixon, 1997). This is perhaps not surprising though as transverse cuts will sever a large number of longitudinally oriented cells across the whole explant. Incisions in the longitudinal direction of the tendon will only damage those cells immediately at the cut surface.

The culture medium was serum-deprived for twenty four hours in both untreated and Cx43 asODN-treated explants as serum reduces asODN activity by accelerating nucleotide degradation (Juliano et al., 2009; Juliano et al., 2012). Serum supplementation exceeded physiological levels, particularly for tenocytes, and may have had mitogenic effects. Addition of tissue factors has been shown to positively influence cell metabolism and hasten the reparative process in an in vitro SDFT explant model (Dahlgren et al., 2001) but supplementation was greater than physiological levels and various authors have postulated that such levels may influence cell
population dynamics and ECM-mediated collagen type I inhibitory effects on cell cycle (Schocklmann et al., 2000; Costa et al., 2006). One study investigating the survivability and longevity of in vitro SDFT explants discussed the pro et contra of tissue culture media as a means of improving their model (Riley et al., 1996). Extensive manipulation of tissue culture media was not attempted in the current investigation and tissue culture conditions used were based on those shown to successfully maintain SDFT tendon fibroblast monolayer cultures (Burrows, 2009). Equine SDFT in vitro explants have been shown to remain viable and metabolically active for up to one month (Riley et al., 1996; Samiric et al., 2004). However, in the period immediately after harvest, cellular metabolic function decreases and some cellular death occurs due to hypoxia, ischaemia, Ca2+ flux and disturbances of pH. Cells in tendon explants show phenotypic drift, particularly if the explants are not being mechanically stimulated, and this may influence gene and protein expression. Several studies have shown GJIC decreases in explants of spinal cord and cornea for a period after harvesting and then recovers (Zhang, O'Carroll et al., 2010; Deva et al., 2012). Gap junction activity was shown to initially decrease following partial hepatectomy in rats but increased again after two days (Traub et al., 1989). A similar decline was observed in the current study with an inevitable decrease in Cx43 gene and protein expression immediately after harvest. By 24-48 hours after harvest, Cx43 protein expression had increased in untreated SDFT explants. Cell density and growth phase influences the amount of collagen synthesized (Kirchhofer et al., 1986; Ben-Ze’ev et al., 1988). Cell density and GJ expression decrease with increasing age in the equine SDFT (Stanley et al., 2007; Young et al., 2009). Although explants were harvested from mature horses, there was an age range from 3-6 years and this may have influenced cell density, cellular activity and GJ expression, and subsequent collagen synthesis.

Procollagens and their subsequent collagen end-products would not only have been incorporated into the ECM of the explants but would also have leached into and accumulated in the tissue culture medium (Lareu et al., 2007). This was not measured in the present study.

Uninjured freshly harvested equine SDFTs were used to produce in vitro explants and Cx43 asODN was applied to these explants. Unmodified Cx43 asODN was used because it is less likely to bind to proteins other than its specific target, which reduces the likelihood of non-specific inhibition; it also shows better cellular penetration but is more susceptible to serum/plasma and tissue enzyme (nuclease) degradation and is quite unstable (Milligan et al., 1993; Wagner, 1994; Juliano et al., 2009). Simple (unmodified) phosphodiester asODN supports RNase H activity, which enables degradation of complementary mRNA and subsequent gene expression effects (Juliano et al., 2009). Antisense oligonucleotides are large polar molecules, which are taken into the cell by endocytosis. Once inside the cell they are trafficked within vesicles and endosomal release occurs within the cytoplasm. Any of these steps can be rate limiting and asODN efficacy is not solely a product of total cell uptake but may be substantially influenced by intracytoplasmic processes. Antisense oligonucleotides show both productive and non-productive pathways within the cytoplasm, which influences the magnitude of their transcriptional and translational effects; asODNs may exert both nuclear and cytoplasmic effects (Juliano et al., 2009; Juliano et al., 2012).

Technically, Cx43 asODN application was challenging as timing was critical. Connexin43 asODN was applied in pluronic gel, which is liquid at temperatures below 4°C but rapidly
solidifies at higher temperatures. Pluronic gel is used as a vehicle and ensures some persistence of the asODN at the site of application; it has been shown to be a very stable platform for the delivery of Cx43 asODN (Green et al., 2001; Ratkay-Traub et al., 2001; Qiu et al., 2003; Coutinho et al., 2005; Law et al., 2006). Premature application of the Cx43 asODN/PG solution ran the risk of the solution flowing into the tissue culture medium and being diluted; being tardy resulted in the solution solidifying in the tube, which precluded application. Prechilling the syringe used to apply the Cx43 asODN reduced the frequency of mishap. In the current situation, downregulation of Cx43 protein expression was expected to occur within several hours of Cx43 asODN application and duration of effect was considered to be 24-72 hours (Qiu et al., 2003; Coutinho et al., 2005; Cronin et al., 2008; Danesh-Meyer et al., 2008). The benefits of Cx43 asODN have been shown to extend well beyond persistence of the Cx43 asODN (Qiu et al., 2003; Deva et al., 2012). Reduced corneal scarring secondary to decreased myofibroblasts activity was observed at 5 and 21 days following single application of Cx43 asODN (Deva et al., 2012). Connexin43 asODN in PG has been shown to penetrate to a depth of at least 10 μm into corneal epithelium one hour after application (Law et al., 2006) but it is not known how far Cx43 asODN is able to penetrate into dense connective tissue such as equine SDFT.

There is no evidence from previous studies that Cx43 asODN directly influences Cx32 gene and/or protein expression. In the current study, Cx32 protein expression declined with time in untreated (control) SDFT explants but also appeared to be variably influenced by concentration-dependent Cx43 asODN application. Increased production of pro-inflammatory cytokines such as TNF-α by cultured endothelial cells decreased Cx32 expression but did not appear to influence Cx43 expression; conversely, upregulation of Cx32 expression decreased production of TNF-α (Okamoto et al., 2011). Downregulation of Cx43 expression following Cx43 asODN application in skin, cornea and spinal cord results in anti-inflammatory effects including diminished inflammatory cell invasion and activation (Qi et al., 2003; Cronin et al., 2008; Danesh-Meyer et al., 2008; Grupcheva et al., 2012). Mimetic peptides that decrease Cx43 expression show similar anti-inflammatory properties (O’Carroll et al., 2008; Danesh-Meyer et al., 2012). Therefore, in the current investigation, the balance between increased pro-inflammatory cytokine production and Cx43 asODN-induced anti-inflammatory effects may have influenced Cx32 expression. Interestingly, decreased Cx43 gene and protein expression in cultured tenocytes subjected to hyperthermia from normal equine SDFTs was associated with increased Cx32 gene and protein expression suggestive of a compensatory response by Cx32 (Burrows, 2009).

Inter-relationships between connexins are complex and are characterised by species and tissue differences. Specific downregulation of Cx43 in cultured rat epidermal keratinocytes resulted in the concurrent downregulation of Cx26 (Langlois et al., 2007) although heterotypic Cx43/Cx26 GJs are not predicted to form in such circumstances (Gemel et al., 2004). In another study, Cx31 deficient mice showed reduced levels of Cx43 (Kretz et al., 2003). Mice deficient in Cx31 do not show dysfunctional epidermal development (Plum et al., 2001), whereas the absence of Cx31 in humans is associated with skin disease (Richard et al., 1998), highlighting species differences in connexin interactions. The response of connexins may differ significantly between tissues; Cx36 has been shown to be compensatory for Cx45 in the retina but not in the heart where absence of Cx45 is lethal (Frank et al., 2010). Perhaps, the altered Cx32 expression in explants following
the application of Cx43 asODN is an indicator of the specific and complex relationship that exists between Cx43 and Cx32 in the equine SDFT.

Why was there not significant attenuation of Cx43 protein expression in SDFT explants following Cx43 asODN application? Loss of Cx43 asODN into the tissue culture medium was certainly a possibility, particularly early in the study when expertise in handling the pluronic gel was low and premature application resulted in the solution being more liquid than gel. This too would have influenced persistence of Cx43 asODN. The intracellular half-life of Cx43 asODN has been shown to be about 20-30 minutes but a reservoir of Cx43 asODN in the pluronic gel permits intracellular top-ups (Wagner, 1994; Qiu et al., 2003). Although delivery of Cx43 in pluronic gel has been well established (Green et al., 2001; Ratkay-Traub et al., 2001; Qiu et al., 2003), Cx43 asODN has not been used in the horse and not in tissue as dense as equine SDFT. Mice, rats and rabbits have been the predominant investigational species, often neonates, and involving soft tissues such as cornea (Danish-Meyer et al., 2008; Deva et al., 2012), spinal cord (Cronin et al., 2008; Zhang, O'Carroll et al., 2010) and skin (Qiu et al., 2003; Coutinho et al., 2005). In wounds involving the skin of neonatal mice, Cx43 asODN was associated with various cell types up to 100 μm from the wound edges (Qiu et al., 2003). There may have been a failure of Cx43 asODN to penetrate the dense equine SDFT explants, particularly those of the older horses, but a pilot study using fluorophore-tagged Cx43 asODN indicated that penetration was achieved up to 20 μm in depth at two hours post administration. It is also important to note that Immunohistochemistry may not reveal connexins in the hemichannel format where they are dispersed in themembrane and do not form plaques which can be visualised. The fact that [30 μM] treatments resulted in significant reduction in mRNA levels suggests this could be the case.

Application of Cx43 asODN directly on to cultured juvenile avian tendon fibroblasts did not appear to achieve maximal Cx43 downregulation based on absence of significant alteration in GJ dye transfer (Waggett et al., 2006) and therefore it would not be surprising if asODN exerted less effect on tissue explants, particularly those of the older horses. Application of Cx43 asODN to equine SDFT in vitro explants demonstrated inconsistent and unpredictable effects that could be attributed to alterations in Cx43 and Cx32 GJ activity. The observed changes in Cx43 gene, Cx32 protein and PINP and PIIINP protein expression should not readily be dismissed as being solely an explant-related effect i.e. the decline in cell function following explant harvest.

Gap junction signalling has been shown to influence both neighbouring cell death and survival (Rawanduzy et al., 1997; Frantseva, Kokarvtseva, Naus et al., 2002, Frantseva et al., 2002; Naus et al., 2001; Nakase et al., 2004; Cronin et al., 2008; Danesh-Meyer et al., 2008; Danesh-Meyer et al., 2012). Initial diminution, recovery and subsequently increased Cx43 GJIC following explant harvest is likely to have influenced cell survival and homeostasis and complicates interpretation of the effects of Cx43 asODN application. Cell death, injury and insult following explant harvest are likely to have resulted in the production of pro-inflammatory cytokines, which would also influence GJIC. Fibroblasts in injured tendons are capable of synthesising pro-inflammatory cytokines (Hosaka et al., 2005a; Hosaka et al., 2005b; John et al., 2010) and SDFT explants contain macrophages, neutrophils and endothelial cells, which also produce pro-inflammatory cytokines (Stanley and Lacy, 2010). Connexin43 gap junctions in endothelial cells mediate the spread of proinflammatory signals in rat lung (Parthasarathi et al.,
and although explants capillary circulation would have ceased post mortem, capillaries would be perfused with culture medium, which would ensure their endothelial cells remained metabolically active.

Inadequate or no attenuation of Cx43 protein expression may occur in higher mammals because the tertiary structure of Cx43 mRNA appears to be more folded than is the case in lower mammals. Despite very substantial Cx43 mRNA sequence homology between mammalian species, the complexity of the structure in higher mammals may limit access to the applicable region of the molecule thereby limiting effectiveness of asODN (pers. comm. Prof. Colin Green). Differences in structure have been identified between mouse and rat Cx43 mRNA (Law et al., 2006) and higher concentrations of Cx43 asODN have been required to achieve comparable downregulation of Cx43 expression in rabbits compared to mice ([10 μM] and [1 μM] respectively) (Qiu et al., 2003; Coutinho et al., 2005; Deva et al., 2012). Connexin43 asODN has not previously been used in the horse. It may be that the mechanisms involved in cellular penetration and/or intracellular trafficking do not result in the efficient release of monomeric oligonucleotides within horse tenocytes. Redesigning the Cx43 asODN to be more specific for horse mRNA may markedly improve downregulation.

The two concentrations of Cx43 asODN used in the main study were based on results obtained from a concentration-response pilot study in which [2 μM], [10 μM] and [30 μM] concentrations of Cx43 asODN were applied to in vitro explants obtained from uninjured left and right forelimb SDFTs of one two-year-old TB filly. Quantitative CLSM showed that [2 μM] Cx43 asODN was not effective but [10 μM] and [30 μM] caused downregulation of Cx43 protein expression. When applied to mice spinal cord in vitro explants, Cx43 asODN demonstrated a hyperbolic doserresponse curve whereby doses less than [2 μM] and greater than [2 μM] but less than [25 μM] did not exert any significant effect on spinal cord swelling and Cx43 protein expression; [2 μM] was more effective than [25 μM] (Zhang et al., 2010). The optimal micromolar concentration of Cx43 asODN to cause marked downregulation of Cx43 protein expression in equine SDFT in vitro explants may not have been established in the present study. Additional research is necessary to determine the efficiency of Cx43 asODN uptake into equine SDFT and half-life within SDFT. A non-significant gel effect was noted when Cx43 asODN in pluronic gel was applied to mice spinal cord in vitro explants; PG alone reduced spinal cord swelling, possibly due to an osmotic effect, which prevented water (culture medium) entering the explant (Zhang, O’Carroll et al., 2010). No consistent pluronic gel effect was observed when PG was applied as a vehicle without Cx43 asODN to in vitro SDFT explants in the current investigation. The same volume of PG was used for both treated and untreated explants.

Modest increases in cell surface Cx protein can lead to rapid increases in gap junctional plaque size due to the cooperative insertion of functional intercellular channels (Castro et al., 1999). Although considerable evidence suggests that connexin protein half-life is short there is contrary evidence that, in certain circumstances, gap junctional plaques can be quite long-lived with half-lives of 12 hours or longer (Epstein et al., 1977; Flagg-Newton and Loewenstein, 1981; Flagg-Newton and Loewenstein, 1981; Wang et al., 2000). Some of these long-lived plaques were characterised by their exceptionally large size (Flagg-Newton et al., 1981). These findings suggest there is capability for upregulation of connexin stability and assembly thereby maintaining GJIC, particularly in those circumstances where protein synthesis is inhibited either
directly or indirectly, although transcriptional amplification (increased levels of transcripts within the cell’s gene expression program) cannot be discounted. Perhaps the observed increase in Cx43 GJ plaque volume following application of [30 μM] Cx43 asODN is a response to the reduced Cx43 gene expression and is a compensatory mechanism to maintain GJIC. In a knock-down experiment where siRNA was used to investigate Cx43 GJIC between mast cells and dermal fibroblasts, intracellular Cx43 protein persisted in the mast cells considerably beyond the expected half-life suggesting that Cx turnover varies between cell types (Pistorio and Ehrlich, 2011). This variation may possibly influence the interpretation of the effect of knock-down by Cx43 asODN on Cx43 protein. The rate of protein turnover also appears to vary between connexins (Law et al., 2006). Stretched cardiomyocytes show protracted persistence of Cx43 GJ assembly hours after the cessation of stretch and despite decreasing expression of Cx43 mRNA (Wang et al., 2000).

Heat stress causes disassembly of GJs and other stressors, such as hypoxia/anoxia and ischaemia, also result in increased degradation of GJs (Laing and Beyer, 1995; Beardslee et al., 2000; Zeevi-Levin et al., 2005; Burrows et al., 2008). Murine ventricular myocyte Cx43 GJ conduction was unaffected by fifteen minutes of hypoxia despite increased protein expression of both the phosphorylated and nonphosphorylated Cx43 isoforms. However, after 5 hours of hypoxia, total Cx43 protein was decreased by 50% with the Cx43 non-phosphorylated isoform protein expression being unchanged. Although there was no significant alteration in Cx43 gene expression, CLSM quantified large decreases in Cx43 fluorescence, GJ plaque number and GJ plaque size (Zeevi-Levin et al., 2005). It should be noted that that GJIC is controlled not only by the number of GJs but also by their permeability. Channel opening is influenced by a number of factors including the constituency of the channels; heteromeric and homomeric GJs have different permeabilities (Gemel et al., 2004; Ayad et al., 2006; Gemel et al., 2008; Ek-Vitorin and Burt, 2013). Cell swelling, actin dynamics and the production of nitrous oxide also influence channel opening (Waschke et al., 2005; Kameritsch et al., 2005; Derangeon et al., 2008; De Mello, 2010). Gap junctional plaque size is not necessarily representative of GJIC (Rufer et al., 1996) but GJIC is certainly influenced by the rate of Cx protein turnover and reduced Cx degradation has been shown to increase GJ assembly and function (Musil et al., 2000); control of Cx degradation provides an additional posttranslational mechanism by which GJIC can be increased. Downregulation of Cx43 gene expression in the in vitro SDFT explants would potentially lead to decreased Cx43 protein expression and subsequent reduction in GJIC. All observations in this project emphasise the significance of Cx43 in GJIC in the equine SDFT; no other Cx is similarly expressed in normal and injured SDFTs. Therefore, it would not be surprising that conservation of Cx43 protein (and therefore GJIC) secondarily to decreased degradation would lead to increased Cx43 GJ volume following downregulation of Cx43 gene expression after Cx43 asODN application. Gap junction activity has been shown to modulate gene expression (Becker and Mobbs, 1999; Becker et al., 1999; Lecanda et al., 2000). Production of the ubiquitous transcription factors Sp1 and Sp3 is influenced by gap junction intercellular communication; they are downstream targets of signalling events when GJ activity is disrupted. Downregulation of Cx43 GJs in bone has been shown to support the recruitment of Sp3, which is recognised as a repressor of gene transcription. Alterations in the ratio that favour the recruitment of Sp3 can lead to repression of the collagen 1α1 gene promoter with subsequent inhibition of collagen 1α1 gene transcription leading to reduced collagen type I protein expression (Stains et al., 2003). Therefore, Cx43 gap junctional activity is important in collagen
type I expression but Cx43 asODN downregulation is transient and therefore unlikely to decrease collagen type I gene and protein expression in the long-term. It is interesting to speculate on the influence of Cx43 asODN in SDFT repair. The increased collagen type III production observed in acute injury and the hyperfibrotic state of chronic injury are deleterious to the resumption of an athletic career. In the current investigation, Cx43 asODN influenced both PINP and PIIINP synthesis and although it may be advantageous to limit collagen type III expression in the early stages of repair, it may not be beneficial to alter collagen type I expression concurrently. However, reduced collagen type I expression may also indicate a reduced inflammatory response (owing to connexin hemichannel blockade) and it is the ratio of type I to type III which is the key factor. The fact that type III collagen expression was reduced to a greater extent than that of type I collagen is encouraging. Cumulative microdamage leading to clinical tendonopathy is a very different reparative environment to that of acute skin wounds though where Cx43 GJ downregulation has been shown to accelerate repair and decrease scarring. Would Cx43 asODN be delivered systemically or locally? Direct intratendonous administration would reduce the risk of systemic toxicity but create additional tendon pathology. Systemic administration may not result in appropriate biodistribution. Downregulation of Cx43 using an asODN approach results in more rapid repair with less scarring in skin wounds and burns (Coutinho et al., 2005; Mori et al., 2006). However, variability in experimental protocols, species and tissues used, and the complexity of the wounded skin environment make comparisons with the present investigation difficult and the findings that Cx43 asODN and Cx32 asODN differentially influenced collagen synthesis in an avian in vitro tendon fibroblast model do not necessarily translate to an equine in vitro SDFT explant model. It should be noted that in a previous investigation, collagen synthesis differed between SDFT tenocyte monocultures and SDFT tissue explants; the significant age-related differences in PINP and PIIINP protein synthesis in explants was not observed in monocultures (Young et al., 2009). However cells derived from immature SDFT, when subjected to cyclic strain and supplemented with TGF-β, showed increased collagen type I and III synthesis (Goodman et al., 2004). Therefore, size of explant may and culture type and environment certainly does influence tenocyte metabolism in vitro.

Typically, new collagen synthesis biochemically is characterised by increased nscc, decreased psc and little or no change in asc content (Jackson and Bentley, 1960; Davidson and Cooper, 1968; Vogel, 1974; Reddy et al., 1999). It was not surprising that collagen synthesis decreased in recently harvested explants because of cell death due to necrosis and apoptosis. Hypoxia, ischaemia, Ca2+ fluctuations and alterations in pH would disrupt metabolism in surviving cells; metabolic perturbation was demonstrated by the observed immediate post harvest reduction in Cx43 GJ protein expression. The absence of mechanical stimulation would have been an additional inhibitor of collagen synthesis (Amiel et al., 1982).

Tenocytes within the explants were supported by the natural three dimensional scaffold of the extra cellular matrix of the tendon tissue but culture conditions may have been less than optimal to support collagen synthesis (Riley et al., 1996). Serum was withheld for 24 hours post harvest in both treated and untreated (control) explants and deprivation would negatively impact on collagen synthesis but this effect should have been short term as various studies have shown that collagen synthesis in cell culture is stimulated within 24 hours of the addition of tissue growth factors (Goodman et al., 2004; Cui et al., 2011). Viable cells were present in the SDFT explants because enzymatic tissue digestion and subsequent cell culture demonstrated that tenocytes
survived at Day 10 post harvest. Other studies have shown that collagen synthesis decreases over time in animal tendon explants (Abrahamsson et al., 1991; Abrahamsson, 1997), including explants obtained from equine SDFT (Bosch et al., 2007). After 6 weeks of culture, collagen synthesis decreased to 10% of the initial level in rabbit tendon explants supplemented with serum, but to 3% in non-serum supplemented conditions (Abrahamsson et al., 1991). Neutral salt-soluble collagen is more soluble than acid- and pepsin-soluble collagens and therefore some nssc may have leached from the explants into the culture medium, but this was not assayed.

The lack of change in explant asc content in the current study suggests that either minimal collagen degradation was occurring or that measurement of asc is not a particularly robust indicator of collagen degradation. Matrix turn-over in the equine SDFT in vivo has been shown to be very slow, particularly with increasing horse age, although accumulating degradation products have been postulated to play a role in the age-related reduced responsiveness of the equine SDFT to exercise-induced microdamage (Thorpe et al., 2010). Measuring the racemisation of aspartic acid, which determines collagen age, and defining the content of collagen degradation markers such as collagenase-generated neoepitope and cross-linked telopeptide of collagen type I provide comprehensive information on collagen degradation (Thorpe et al., 2010). The rate of collagen degradation has not been measured in equine SDFT explants. Decreasing collagen synthesis in the absence of collagen degradation would manifest as decreased nssc content with no alteration in asc, which was observed in this experiment. The absence of detectable alteration in psc content of explants also indicated that mature explant collagen was not being degraded; well-established cross-linkages were not being broken-down. Insoluble collagen content represents aged, mature collagen with well-established cross-linkages. It is well documented that isc content of tendons increases with age, indicative of mature cross-linkages. The SDFTs used in the current model were from mature horses and therefore high isc content would be expected. The lack of reduction in isc indicated that the well-developed cross-linkages of mature collagen were not being disrupted.

Assayed concentrations for nssc, asc, psc and isc were all substantially lower than the values published by others for equine SDFT (Young et al., 2009), most likely due to technical difficulties associated with the assay.

5.6 Conclusion

Harvesting, creating and maintaining the in vitro SDFT explants were technically demanding but with practice explant reproducibility was achieved. The model also negates many of the problems that are inherent in using cell monolayer cultures: Tenocytes are in a natural scaffold, which maintains the normal relationship between cells and ECM; aggressive tissue digestion, which potentially damages cells and influences cell phenotype, is not required to release cells, and, based on CLSM observations, less phenotypic drift occurs.

There was considerable variability within the study because of dependence on donation of tissue samples and thus unavoidably coming from a population of animals of varying age, sex and exercise history. Variability was controlled to some degree by including the Time 0 (at harvest) data but is still likely to have influenced detection of real differences between treated and untreated explants. The results do however suggest that the Cx43 asODN used in this study may have some effectiveness with a reduction in transcript level noted although expression of Cx43
protein was not evident at the immunohistochemistry level. This is not inconsistent with the application of Cx43 asODN where mRNA is reduced, and so hemichannel expression is less but Cx43 GJ protein is relatively conserved within GJ plaques. Most importantly, the reduced Cx43 mRNA levels correlated with reduced type I and type III collagen expression, which may indicate a reduced inflammatory response, but more importantly with a greater reduction in type III collagen which may reflect a better quality of repair, Whilst not definitive, the results do suggest that further work with an equine optimised asODN, and/or connexin mimetic peptide hemichannel blockers (which are less species specific) may be worth pursuing.
**Update on Regenerative Medicine**
Dr Benjamin Ahern,
Randwick Equine Centre, 3 Jane Street, Randwick, NSW 2031, Australia

Regenerative medicine pertains to the stimulation and support of damaged tissues ability to self-repair whilst concurrently inhibiting any ongoing detrimental processes. The overall goal of regenerative medicine is to achieve a healing response that is superior to conservative management options. Clinical therapies currently available consist of a range of biomedical products including but not limited to autologous conditioned plasma, interleukin-1 receptor antagonist protein (IRAP) to the broad (and expanding) array of stem cell products. This presentation will give brief overviews of the different biologic agents currently available and summarize some of the pertinent literature with regards to treatment methodology and clinical expectations. Special consideration will be given to stem cells, what different types are there, what sources are available, and compare and contrast ‘off the shelf’ and autologous options. This will be done with an eye towards what ‘works’ and what does not based on the literature currently available.
An update on laminitis pathophysiology and prevention
Dr. Andrew van Eps BVSc, PhD, MACVSc, DACVIM, School of Veterinary Science, The University of Queensland, Australia

Laminitis Pathophysiology
Laminitis is a common and frustrating complication of a variety of primary diseases in the horse. Regardless of the inciting cause, the resultant lesion is the same: laminitis is ultimately a failure of the attachment between epidermis and dermis, and therefore a failure of attachment between hoof wall and distal phalanx.
At the interface between the dermis and the lamellar epidermal basal cells (EBCs) lies the basement membrane (BM) (Pollitt 1994). Lysis of the BM, as well as its detachment from the underlying EBCs is a key event in the development of laminitis (Pollitt 1996). Recently, lamellar BM changes have been detected very early in the developmental period of experimentally induced (alimentary oligofructose overload) laminitis: approximately 12 hours or more before even the earliest clinical signs of laminitis would be expected (Visser 2009). There appears to be not only degradation and lysis of the BM, but also separation of intact BM from the underlying EBCs indicating failure/dissolution of hemidesmosomes (HD), which are responsible for anchoring the BM to the cytoskeleton of the EBCs. This is consistent with previous work documenting HD dissolution later in the course of the disease (French and Pollitt 2004; Nourian et al. 2007).
The exact mechanisms that cause the BM and HD failure (and ultimately the acute laminitis lesion) remain unclear. In recent years, researchers have come to realise that there are important differences in the pathophysiology of laminitis depending on the inciting cause. This has divided laminitis into essentially three forms, based upon the purported basic mechanisms of disease (sepsis, hyperinsulinaemia/endocrinopathy and supporting limb laminitis).

Laminitis secondary to sepsis
Laminitis secondary to diseases characterised by the systemic inflammatory response syndrome (SIRS), and in particular sepsis, has been the most extensively studied form of laminitis, and perhaps has the most complex pathophysiology. Recent evidence suggests that this form of laminitis has much in common with the end organ dysfunction that occurs in human sepsis (Belknap et al. 2009). A similar, complex interaction of bioenergetic failure, microvascular dysfunction, inflammation and enzymatic degradation appears to contribute to lamellar failure. In septic horses, the severity (and clinical importance) of damage to the lamellae over other “end organs” is likely due to the addition of mechanical distraction (caused by weight bearing) in this tissue. Researchers studying sepsis-related laminitis have largely departed from the traditional hypothesis of ischemia and reperfusion injury in favour of pursuing inflammation and enzymatic activation/degradation as key early pathophysiological events.
Enzymatic degradation of components of the dermo-epidermal junction was first suggested by Pollitt as a potential pathogenesis for laminitis (Pollitt 1999). Matrix metalloproteinase enzymes (MMPs) were originally implicated. Although MMPs undoubtedly play a role in perpetuating lamellar damage, their role as initiators of laminitis has been recently challenged: new evidence places MMP-2 and MMP-9 production and activation many hours later than other molecular events in the oligofructose CHO model (Visser 2009). Recently, ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) has been...
identified as an enzyme capable of processing and destabilising the proteoglycans and glycoproteins of the lamellar BM. A marked increase in the expression of ADAMTS-4 mRNA has been identified early in experimental laminitis development, as well as in natural cases of the disease (Budak et al. 2009; Coyne et al. 2009; Visser 2009). This enzyme may play a central role in initiating the laminitis lesion.

The central role of inflammation in the pathogenesis of acute laminitis has been highlighted experimentally. (Belknap et al. 2007; Black 2009; Black et al. 2006; Blikslager et al. 2006; Faleiros et al. 2011; Faleiros et al. 2009a; Faleiros et al. 2009b; Fontaine et al. 2001; Waguespack et al. 2004a; Waguespack et al. 2004b) Endothelial activation, cytokine and chemokine upregulation, and leukocyte emigration into the lamellar tissue occur early during the development of experimentally induced laminitis. Leukocyte accumulation occurs during experimental CHO laminitis induction close to the onset of lameness (Belknap et al. 2007; Leise et al. 2010). Once they have emigrated into the lamellar tissue, leukocytes (particularly neutrophils) produce reactive oxygen species, enzymes and proinflammatory cytokines that contribute to inflammation and tissue damage (Belknap 2008). The initiators of systemic and local inflammation have not been identified, however a range of bacterial products including endotoxin have been suggested. Clinical signs consistent with endotoxemia were the major risk factor and the common clinical finding in hospitalized horses that developed laminitis in an epidemiological study (Parsons et al. 2007). In a recent study utilising the oligofructose CHO model, Bailey and colleagues demonstrated the presence of endotoxemia, platelet activation and TNF alpha within the blood early in the developmental period (Bailey et al. 2009). Endotoxin and other bacterial products undoubtedly contribute to initiation of the early inflammatory changes observed in experimental models of acute laminitis, and also the pathogenesis of naturally occurring laminitis due to sepsis.

The traditional notion that ischemia is an initiator and a key event in the development of sepsis-related laminitis is no longer accepted by most research groups. There is no doubt, however, that changes in blood flow to the lamellae occur in association with the development and progression of laminitis. The fundamental question of whether lamellar ischemia is actually occurring as an initial event in the pathogenesis has been addressed directly by Loftus et al. (2007) in the black walnut extract (BWE) model: the authors were unable to demonstrate molecular biochemical or metabolic evidence of ischemia during the developmental phase. This work, however, has not been repeated in the CHO model or in naturally occurring laminitis. In addition to reduced blood flow, disturbances of mitochondrial function and cellular energy metabolism may occur in the presence of adequate blood flow/nutrient delivery, as is the case in human sepsis related organ dysfunction (Belknap et al. 2009). The contribution of disturbed glucose metabolism and indeed the contribution of insulin to sepsis-related laminitis has not been investigated; however increases in serum insulin levels during the development of OF-induced laminitis were minor and not considered biologically significant (van Eps and Pollitt 2006). Endotoxin has been shown to reduce insulin sensitivity after experimental infusion (Toth et al. 2008).

**Laminitis associated with hyperinsulinemia**

Laminitis that occurs in horses with pars pituitary intermedia dysfunction and in horses with equine metabolic syndrome (EMS) has been linked to insulin resistance/ hyperinsulinemia and derangements of glucose regulation (Asplin et al. 2007; Johnson et al. 2010; McGowan 2010; Treiber et al. 2006a). Although the exact mechanisms leading to pasture-associated laminitis are still unclear, EMS (and hyperinsulinemia) is implicated as a risk factor for the development of
pasture-associated laminitis (Geor and Frank 2009; Treiber et al. 2006b). Glucocorticoid-associated laminitis can be iatrogenic or related to pituitary pars intermedia dysfunction (PPID). In both cases, laminitis can be linked to the ability of corticosteroids to induce insulin resistance and therefore hyperinsulinemia (Johnson et al. 2004).

Hyperinsulinaemia itself appears to be necessary for the development of laminitis in this form of the disease as evidenced by the studies of Asplin et al. (2007), and de Laat and Pollitt (2010a). In these studies the authors report the induction of laminitis in healthy ponies and healthy adult Standardbred horses, respectively, with the administration of a constant rate infusion of insulin, whilst also sustaining euglycaemia through glucose administration (prolonged euglycemic-hyperinsulinaemic clamp). Laminitis was also able to be induced by constant infusion of exogenous glucose alone in Standardbred horses, which increased endogenous serum insulin concentrations (de Laat et al. 2012a)

The exact mechanisms by which hyperinsulinaemia leads to laminitis are still unclear. There is little evidence for inflammatory or enzymatic mediation (de Laat et al. 2012b; de Laat et al. 2010a). Asplin et al. (2011) showed that lamellar tissue relies on non-insulin dependent glucose uptake. An association between vascular dysfunction and/or an altered blood supply and the development of disease as a result of hyperinsulinaemia has been suggested (de Laat et al. 2010b). Based on the nitric-oxide-mediated vasodilatory effect exerted by insulin (Muniyappa et al. 2007), a potential increased delivery of laminogenic substances to the hoof is hypothesized (de Laat et al. 2010b). Although glucose toxicity has been suggested as a possible mechanism, there was little evidence for this (or oxidative stress) in a recent study (de Laat et al. 2012b). Dysregulation of epidermal cell growth and division through activation of insulin like growth receptor (IGF-1) has been suggested (De Laat 2012) as a potential mechanism for the laminitis lesion associated with hyperinsulinemia.

Supporting Limb Laminitis

Although the severity and duration of lameness are considered risk factors (Peloso et al. 1996; Redden 2004), the development of supporting limb laminitis (SLL) is still unpredictable, both in terms of timing and also with respect to which cases will succumb to it (and what degree of pain exhibited in the limb with the primary condition is necessary for SLL development) (Richardson 2008). This type of laminitis tends to be commonly associated with rapid and severe failure of the lamellae, with subsequent distal displacement (“sinking”) of the distal phalanx (DP) within the hoof capsule.

It is well accepted that in the standing horse, the body mass is divided between the fore and hind limbs in a 60:40 ratio. Peak ground reaction forces increase to be equivalent to 0.25 x bwt at the walk, 0.5 x bwt at the trot, and up to 3 x bwt at the gallop. It therefore seems unlikely, from a mechanical perspective, that compensatory load redistribution in the standing horse bearing weight on a single limb could exceed the mechanical “strength” of the lamellar tissue.

Almost 30 years ago it was first demonstrated that there was a valve-like mechanism in the digital arteries of the loaded limb leading to a cut-off of arterial blood supply during the loading phase (van Kraayenburg 1982). This work has been repeated by Pollitt using 2D angiography, and more recently using 3D computed tomography (CT) studies (van Eps et al. 2010). To avoid digital ischemia, it appears that cyclic loading of the feet plays an essential role in digital homeostasis for the horse at rest. Reduced frequency of this cyclic loading is thought to be a major risk factor for the development of SLL (Redden 2004), and may represent the major
pathophysiological mechanism. Whilst standing, the normal horse will shift weight in the forelimbs approximately 1 to 5 times per minute (Pietra et al. 2004), presumably to maintain adequate blood flow to the foot. Reduced limb load cycling has an effect on local energy metabolites in the lamellar tissue, as measured by tissue microdialysis (van Eps - unpublished data), and the exact requirements for limb load cycling in order to maintain a viable energy state in the lamellar tissue are under investigation in the author’s laboratory – preliminary results will be presented.

Lamellar tissue has a high requirement for glucose, yet there is no means for local glycogen storage, therefore reduced supply of blood glucose may rapidly lead to energy failure. The formation and maintenance of the bonds between epidermis and dermis (hemidesmosomes) is an energy dependant process, and therefore prolonged negative energy balance may lead to a loss of these bonds and ultimately separation of epidermis from dermis (laminitis) as has been documented in vitro (Pass et al. 1998). It should be recognised that infections are a common component in cases at high risk of SLL, and that stress-related hyperinsulinemia is also a consequence of hospitalisation and surgery, therefore there may be contributing factors to SLL that are common elements of other forms of laminitis.

**Laminitis prevention and treatment**

Efforts should be preferentially aimed at prevention, as once the laminitis lesion is present it cannot be reversed. In the absence of total destabilisation, healing processes begin within the lamellar tissue over the ensuing days after the development of acute laminitis. Proliferation of EBCs and repopulation and repair of BM occurs; however the original architecture of the lamellar interface is lost and the resultant disorganised structure lacks the surface area and strength of the original attachment (Van Eps and Pollitt 2009b).

Several studies have demonstrated that digital hypothermia reduces the severity of laminitis lesions when initiated early in the developmental stage of oligofructose (OF)-induced laminitis (van Eps and Pollitt 2004; Van Eps and Pollitt 2009a; van Eps et al. 2012). Digital hypothermia is now commonly used clinically in the horse at risk of laminitis; however, it remains unclear whether there is still a beneficial effect when cryotherapy application is delayed until the detection of lameness. A recent study in the author’s laboratory has demonstrated that digital hypothermia effectively prevents the progression of lamellar injury (and even structural failure) when initiated at the detection of lameness in an acute laminitis model (van Eps and Belknap – unpublished data). This study provides the first evidence to support the use of digital hypothermia in clinical acute laminitis cases.

The key to prevention of laminitis associated with insulin resistance/hyperinsulinaemia is likely to be early identification of horses at risk, with subsequent restriction of diet (including selective pasture access) as well as controlled exercise and weight control. Drugs aimed at increasing insulin sensitivity (metformin and pioglitazone) have so far failed to demonstrate a consistent useful effect in the horse (Durham 2012; Durham et al. 2008; Tinworth et al. 2012; Wearn et al. 2012a; Wearn et al. 2012b). Control of PPID is best achieved through early diagnosis and management with oral pergolide.

The key to supporting limb laminitis prevention may lie in strategies to monitor and increase load cycling in the supporting limb. Monitoring should include some form of serial assessment of limb cycling – this can easily be achieved using commercially available inexpensive human pedometers. These devices can be taped to the limb just above the carpus/hock, with readings recorded at set intervals. The pedometer readings provide a
measurement of limb load cycling frequency that can be followed over time. Providing some regular walking exercise is likely to be of some benefit, however requirements for type and frequency are yet to be determined, and for each case are likely to be dependent on the nature and severity of the primary condition. It is well recognised anecdotally that horses that spend more time recumbent appear to have a reduced incidence of SLL. Encouraging recumbency with deep bedding and a quiet, isolated stall environment may have some effect. Sedation may also encourage recumbency, however it also reduces voluntary exercise and limb load cycling in the stall and therefore may be contraindicated. Forced recumbency has been investigated experimentally by Wattle and colleagues, and appears to be well tolerated for up to 3 days (Wattle et al. 1995). Depending on the nature of the primary condition, forced recumbency may, however, be detrimental to healing.

References


RESIDENT/RESEARCH PRESENTATIONS

THE EFFECT OF CIPROFLOXACIN ON THE MARE’S ENDOMETRIUM AFTER INTRAUTERINE INFUSION FOR THREE DAYS

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Introduction
Bacterial endometritis continues to be a major cause of equine infertility and contributes to major reproductive wastage within the equine breeding industry. Intrauterine antimicrobial treatment in addition to uterine lavage and the use of ecbolic agents forms the current mainstay of endometritis therapy (Le Blanc, 2003). Ciprofloxacin (CIP) is a synthetic, broad spectrum antimicrobial agent which may have advantageous properties for the treatment of bacterial endometritis due to its extended lack of acquired resistance and its high efficacy against gram negative organisms including Pseudomonas aeruginosa. While ciprofloxacin may be effective against bacteria commonly associated with endometritis in mares, it has a low pH of 3 and its influence on the endometrium is undocumented. The present study was designed as a preliminary investigation into the effects of Ciprofloxacin on the endometrium of the mare after intrauterine administration.

Materials and Methods
2.1 Treatment groups
Seven non-pregnant, Standardbred mares of unknown reproductive history were recruited for the study and a commercially available cyprofloxicin preparation, Ciproxin®IV 100 (Bayer Australia, Pymble, Australia) was utilised for the treatments. The mares were separated into three groups; Group 1 (n=2) received an infusion dose of 50mg (0.1mg/kg) diluted in saline and buffered with sodium bicarbonate to provide a pH range of 5.9 -6.2, Group 2 (n=4) received 25mg (0.05mg/kg) diluted and buffered as for Group 1. The last mare (Group 3, n=1) was infused with pure ciprofloxacin solution at a dose of 50mg (0.1mg/kg) to determine if the drug could be administered intrauterine without the need for buffering. Infusions were started on day one of oestrus as determined by behaviour, manual and ultrasonographical evaluation of the reproductive tract.

2.2 pH analysis of the uterus
Uterine fluid samples were collected by passing an insemination pipette through the cervix and into the uterus, using minimal contamination techniques. Suction was achieved with the use of a 30mL syringe attached to the end of the pipette. Uterine fluid was immediately centrifuged (Qik Spin Micro Centrifuge, Edwards Instrument Co. Australia) and the pH of the supernatant measured (Oakton millivolt pH meter, Oakton Instruments, USA).

2.3 Endometrial biopsies
Endometrial biopsies were collected before treatment (T0), which acted as individual controls for each horse. Post treatment biopsies included 3 hours (T3) 6 hours (T6), 12 hours (T12) 24 hours (T24) and 48 hours (T48) after the last infusion to evaluate acute effects of treatment. The last 21 day (T21) biopsy was collected to assess chronic changes associated with ciprofloxacin infusion.
Biopsies were then prepared for grading histologically using a “modified Kenny and Doig” category scale or Ciprofloxacin quantification by High Performance Liquid Chromatography (HPLC). Endometrial samples were graded based on inflammation and fibrosis.

2.4 Plasma collection
Blood was collected via a jugular catheters (BD Angiocath™ IV cannula, 14G 5 ½ inch, Franklin Lakes, USA). Samples were collected at 0 (baseline), 1, 2, 3, 4, 6, 12 and 24 hours into pre-labelled 9mL lithium heparin vacutainer tubes (BD vacutainer®, Franklin Lakes, USA). The collection tubes were stored on ice and centrifuged (Eppendorf Centrifuge 5810R, Eppendorf South Pacific Pty Ltd., Humbug, Germany) at 4000 rpm for 10 min within two hours of collection. Plasma was then decanted and stored at -21 °C until analysis.

2.5 Quantification of ciprofloxacin in endometrium and plasma using HPLC
The concentration of ciprofloxacin in plasma and endometrial biopsies was measured using HPLC. Standard curves were created by spiking blank plasma and endometrial samples with known amounts of ciprofloxacin. Precision was expressed as the percentage residual standard deviation, and was determined by analysing single extracts of spiked plasma and endometrial tissue on each day of analysis. Accuracy was defined as the closeness between the experimentally measured and true concentration values. Ciprofloxacin was then extracted from the tissue or plasma using a solid phase extraction technique. Mean efficacy of extraction from endometrium and plasma was calculated as 81% and 86% respectively, when compared to CIP standards prior to commencement of sample analysis. The eluate was then evaporated, reconstituted, and filtered before analysis. The concentration of antibiotic in the biological tissue was then established by comparison against the standard curve. Extraction efficiency was determined by comparison of the peak curve areas obtained from spiked plasma and endometrial tissue blanks with those given by equivalent authentic standard solutions. The limit of quantification was defined as the lowest drug concentration on the standard curve that could be measured with precision and accuracy.

2.6 Statistical analysis
2.6.1 pH analysis
Data were analysed using ANOVA to determine the effects of day and time of collection on mean uterine pH values. A confidence interval of 95% was used and significance was determined when p<0.05.

2.6.2 Endometrial biopsy
A linear mixed regression analysis (GenStat©2000-2012 VSN International Ltd, Hemel Hempstead, UK) was performed on the data to investigate the association of fibrosis and inflammation with time after intrauterine infusion of ciprofloxacin. Individual horse was incorporated as a random effect; to investigate potential differences in individual responses of the outcome variables evaluated, given each horse had five time measures. Statistical significance was considered at P-value < 0.05.

2.6.3 Quantification of ciprofloxacin
Due to the small sample size, statistical analyses were not performed on the endometrial ciprofloxacin concentrations, with raw data presented.

Results
3.1 pH analysis
The pre-treatment intrauterine pH of mares in oestrus during this study ranged from 6.9 to 7.9. A significant effect of individual mare on uterine pH was identified and due to the low numbers of
mares included in the study, mares were assessed individually. There was no effect of time or day on the pH of uterine fluid in normal healthy mares. It was noted that mares developing endometritis throughout the treatment period demonstrated fluctuations in pH in comparison to normal oestrus mares and demonstrated consistently lower pH in comparison to previous samples taken prior to inflammation. However, no statistical trend or significance could be identified with the sample size used in this study. To identify a significant (p<0.05) decline in pH following ciproxin infusion, approximately 23 mares would need to be included in each treatment group (endometritis vs healthy endometrium) for required experimental power. Following ciprofloxacin infusion, no significant differences in uterine pH was observed in any of the subjects compared to their pre-infusion levels.

**Endometrial biopsy**

Histological examination of the endometrial tissue demonstrated an increase in inflammation between six and 48 hours post infusion with Ciprofloxacin (Figure 1). The inflammatory response was significantly higher in mares <24 hours post infusion in comparison to 48 and 504 hours post infusion (p < 0.041).

![Inflammatory Score](image)

**Figure 1:** Mean endometrial inflammatory response to ciprofloxacin infusion

A significant difference (p>0.035) in fibrosis grade was identified at 504 hours post ciprofloxacin infusion in comparison to previous time points (0 - 48 hours, Figure 2). The fibrotic score was highest in four (1, 3, 4, 6) horses at 504 hours, whilst one (5) peaked at 24 hours. Horse 2 demonstrated two peaks in fibrosis both in the pre-treatment measurement (baseline) and at 48 hours. Horse (7) did not have fibrotic lesions at any time.
**Figure 2:** Endometrial fibrosis response to ciprofloxacin in mares

A summary of fibrosis grades obtained from individual biopsies during the trial is shown in Table 1. There was an initial increase in fibrotic grade directly post infusion between 6 and 48 hours but no effect of time after treatment on the level of fibrosis was identified ($P > 0.05$). However, the peak in fibrosis at 504 hours post ciprofloxacin infusion was statistically significant compared with the fibrosis response at 0 to 48 hours ($P = 0.035$). The fibrotic score was highest in four (1, 3, 4, 6) horses at 504 hours, whilst one (5) peaked at 24 hours. Horse 2 was an outlier, in that the highest score was evident at both the pre-biopsy (baseline) and 48 hours. Horse (7) did not have fibrotic lesions at any time. There was no evidence of individual variation among inflammation or fibrosis responses.

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Table 1: Fibrosis grade given to each mare for each biopsy time point

Treatment with 50mg buffered ciprofloxacin produced the most severe fibrosis scores (3 and 3.5), followed by treatment with 25mg buffered ciprofloxacin (score 2 for all mares), whilst treatment with 50 mg un-buffered ciprofloxacin did not induce endometrial fibrosis (score 0, Figure 3). The small sample size precluded statistical analysis of treatment group comparisons.
**Figure 3:** Comparison of final fibrosis grade between treatment groups

3.3 **Quantification of ciprofloxacin**

Due to unforeseen laboratory error during the extraction process, only endometrial samples from two of the seven horses produced valid results. Ciprofloxacin was detected in endometrial biopsies from mare 2 and mare 6 at all sample time points. The maximum concentration ($C_{\text{max}}$) of Ciprofloxacin in endometrial biopsies was achieved one hour post infusion at both 50mg and 25mg doses (Figure 4).

![Figure 4: Endometrial ciprofloxacin (CIP) concentration over time following intruterine infusion of CIP in mares 2 and 6.](image)
The mean endometrial ciprofloxacin concentration was greater than MIC for the four most common bacterial causes of endometritis in both mares for at least one hour. Endometrial CIP concentration from mare 6 was greater than MIC for *S. zooepidemicus* (1.0µg/g) at 3 hours post treatment and again at 24 hours post treatment. The mean endometrial concentration from mare 2 was less than MIC for *S. zooepidemicus* after one hour. However, the mean endometrial concentration remained above MIC for *E. coli*, *K. pneumoniae* and *P. aeruginosa* (0.1µg/g) at all sample times.

Laboratory error also occurred during the extraction process for plasma and samples from only three of the seven mares (1, 2 and 6) produced valid results. Ciprofloxacin was undetectable in all samples, at all time points. At no time were the plasma CIP concentrations close to required MIC for treatment of the four uterine pathogens of interest.

3.4 Additional findings

Foreign material within endometrial tissue was noted occasionally during histological examination of post-treatment biopsy samples. In these samples there were multiple small circular, non-staining refractile foreign bodies containing variable amounts of radiating striated basophilic material. This basophilic granular material was highly suggestive of mineral. Surrounding this material was a large number of inflammatory cells, predominantly neutrophils, which were variably degenerate (Figure 5 A and B).

![Figure 5: Ciprofloxacin crystals within the endometrium are washed away during fixation of histopathology blocks, leaving tissue with occasional crystal remnants (A). Circular foreign bodies found within the endometrium (arrows) surrounded by a high number of degenerative inflammatory cells and cellular debris 24 hrs post intrauterine infusion of buffered Ciproxin (B).](image)

On further examination using light microscopy the material was determined to be tiny crystals. Immediately after buffering the Ciproxin solution but before intrauterine infusion, crystals were approximately 80 µg in length. The tiny pieces aggregate quickly into large 3-dimensional crystals (Figure 6 A and B) 15-20 minutes after buffering. It was this large 3-dimensional form that was infused into the uterus.
Discussion
At the commencement of this study, there was concern that the pH of the ciprofloxacin infusion may have an adverse effect on the endometrium. Thus, varying concentrations and buffered or un-buffered formulations were investigated. Paradoxically, the least irritation to the endometrium was noted with the un-buffered formulation.

pH analysis of the uterine fluid retrieved from the mares demonstrated that the intrauterine buffering mechanisms are very effective at maintaining pH homeostasis. Furthermore, no diurnal variation of samples taken during the normal oestrus period were identified (P<0.05). These results are in accordance with Penrod, Dever and Prendergast et al. (2007), demonstrating that the uterine pH of mares did not differ throughout the oestrous cycle. However, it was noted that variations in the uterine environment, as diagnosed by biopsy and cytological analysis of uterine fluid, may compromise the uterine buffering capacity, resulting in deviations from the normal uterine pH in these mares.

Results pertaining from the pH analysis were not statistically significant due to limited data availability and further work is required to compare intrauterine pH between healthy mares and those diagnosed with endometritis. Previous studies have shown that intrauterine pH of the mare in oestrus with endometritis ranges from 6.7 – 7.0 while a pH of 6.4 was describe during dioestrus. A pH of 6.4 is also the optimal pH for bacterial proliferation (Pycock and Allen, 1990). The normal pH of the uterus in oestrus mares is higher than that described for endometritis mares, suggesting that normal uterine pH is unfavourable for bacterial colonisation. The results of the present study and those in the literature support the importance of equine uterine pH in detecting uterine health during oestrus.

Intrauterine infusion of buffered Ciproxcin caused a statistically significant inflammatory response (p = 0.041) between 0-24; 48; 504 hours. An initial inflammatory response was observed (Figure 1) within 6 hours of the final infusion. This inflammatory response involved a neutrophilic infiltration consistent with acute inflammation (R. M. Kenney, 1978). Interestingly, by 48 hours post infusion the mean inflammatory response was not different to that of the initial biopsies (an average score of 5.5) and by 21 days the mean inflammation was less than that of the initial grade (4.5). The decrease in final inflammatory scores below that of pre-treatment levels may be due to influences on the endometrium associated with the additional sampling for the concurrent studies of pH and quantification of ciproxin within the uterine endometrium. The entry of foreign objects such as pipettes and gloved hands may have contributed to an initial inflammatory reaction of pre-treatment biopsies higher than expected. Alternatively the difference observed may have been due to the different stage in the oestrous cycle that the 21 day

Figure 6 A and B: Micrograph of solution zero (A) and 20 minutes (B) after Ciproxcin was buffered with sodium bicarbonate.
A significant increase in fibrosis score between initial and final biopsies was consistent in 3 of the 6 horses (Figure 2, Table 1) infused with buffered ciprofloxacin (p = 0.035). All of these mares began the program with a fibrosis score of zero. The fibrosis score of Horse 5 did not differ between pre and final biopsy samples (score 2). Fluctuation in fibrosis scores between biopsy intervals within the same mare was a common trend in this study. The initial biopsy of Horse 3 identified frequent periglandular fibrosis that was not identified again in subsequent biopsies. Likewise, Horse 1 had a consistent fibrosis score of 3.5 in post treatment biopsies, with the exception of the 48 hour biopsy (2) and Horse 5 had a single rise in fibrosis score at 24 hours from 2 to 3.5. These observations, combined with a general initial trend demonstrating a preliminary peak in fibrosis score (mean 1.9) at 6 hours makes interpretation difficult. Unlike inflammation, endometrial fibrosis is a progressive, irreversible change directly associated with reduced conception rates and ability to maintain a pregnancy to term (Ball, 1988; R. M. Kenney, 1978;).

When examining raw data, the degree of fibrosis as an outcome of intrauterine Ciproxcin infusion was different between treatment groups (Figure 4). However, this difference was not statistically significant. Treatment Group 1, involving intrauterine infusion of 25mLs of 50mg of buffered ciprofloxacin, initiated severe endometrial damage evident in Horse 1 who began the trial with a fibrosis grade of zero and concluded with a score of 3.5. Infusion of 12.5mL of 25mg of buffered Ciproxcin in Group 2 resulted in fibrotic change, but to a lesser extent as demonstrated by the Group 2 mean final fibrosis score of 2. A statistical comparison between treatment groups was not performed due to the invalidity of statistics associated with the small sample size of each group. Treatment Group 3 (un-buffered 50mg Ciproxcin infusion) did not develop fibrosis; however the group only involved one mare. These trends need to be interpreted in the light of the small sample size of each group. However the results support further investigation with a larger group of mares is required to assess the effects of intra-uterine infusion of ciprofloxacin on the fertility of the mare.

Ciprofloxacin was detected in endometrial tissue at levels above the required mean inhibitory concentrations for common bacterial causes of equine endometritis for up to three hours post infusion in two mares. The maximum ciprofloxacin concentration was observed in endometrial biopsies one hour post infusion. Ciprofloxacin was detected above MIC at 1, 3 and 24 hours post treatment, in biopsies obtained from mare 6. However, concentrations declined to below MIC in the 6 and 12 hour biopsies. This result may be explained by the precipitation of Ciproxcin in the buffered infusion, which meant that only the surface in contact with crystalline Ciproxcin would have detectable concentrations on analysis. Where Ciproxcin is in an
unbuffered solution it is distributed evenly in its soluble molecular form. The crystalline precipitant is not evenly distributed, which will affect the ability to evenly contact surfaces. Samples at 6 and 12 hours may have been taken from an endometrial surface that had not contacted crystalline Ciproxcin. Gonzalez, Moreno and Small et al. (2010), were able to detect both Enrofloxicin and Ciproxcin in endometrium at levels above MIC for the same pathogens, following i.v. administration at doses 25 and 5-fold to those used in this trial for up to 48 hours. No detectable uptake of ciprofloxacin was identified in the plasma samples which is consistent with results of other studies (Gonzalez, Moreno and Small et al., 2010; Dowling, Wilson and Tyler et al., 1995). In both of these studies, tissue concentrations of Ciproxcin were found to be significantly higher at all times in comparison to plasma concentrations. Weese, Kaese and Baird et al. (2002) concluded that systemic administration of ciprofloxacin is suspected of causing increased risk of antibiotic-associated gastrointestinal disease. Consequently, the negligible systemic uptake of Ciproxcin after intrauterine administration in this study indicates that there would be minimal risk of these side-effects.

In this study a number of adverse effects after buffered ciprofloxacin intrauterine infusion were observed. These include straining to urinate (4/7), uncomfortable behaviour (2/7) sloughing of endometrial tissue (3/7), and vaginal bleeding (6/7). Hysteroscopy was performed to investigate the extent of uterine damage and to localise the source of bleeding and uterine sloughing. Grossly, endometrial necrosis was not evident in these mares during hysteroscopic examination. Instead, endometrial necrosis was present in Horse 3, independent of any clinical adverse effects. Horse 3 suffered from concurrent endometritis, which may be a cause of the necrosis. Interestingly however, endometrial necrosis was not seen during hysteroscopy of Horse 5 who also suffered from concurrent endometritis. Hysteroscopic examination revealed macroscopic evidence of endometrial damage after Ciproxin infusion. The most common pathological lesions of the endometrium present in ciprofloxacin treated mares (treatment Groups 1 and 2) were hyperaemia, ecchymosis, ulceration and blood vessel congestion. Investigation into these observations indicated that the dilution and buffering of Ciproxin®IV caused the solution to precipitate and act as an endometrial irritant. It was hypothesised that the buffering process may be the cause of the significant endometrial inflammation, as a reduction in the initial dose of buffered Ciproxin continued to produce clinical adverse effects. Hence, the addition of a third treatment group (Group 3), where intrauterine infusion of 50mg of un-buffered Ciproxcin in isotonic saline was administered. Due to time restraints and mare availability, only one mare was infused with unbuffered Ciproxcin to investigate this theory. The observational results from this trial infusing unbuffered Ciproxin concur with Fumuso, Breda and Redolatti et al. (2010) and support the safety of its use as an intrauterine infusion. The dosage used in this trial was much higher than that used by Fumuso, Breda and Redolatti et al. (2010), yet there were still no observed adverse effects.

Although a chemical burn injury or the presence of foreign material are postulated as causes of endometrial irritation subsequent to buffered ciprofloxacin administration in this trial, the exact mechanism is unknown. Under light microscopy, crystals were identified within the endometrium (Figure 5 B) and were seen to embed themselves into the endometrium. All acute samples from buffered ciprofloxacin treated mares contained a degree of necrotic debris, degenerative or apoptotic cells. Furthermore, the crystals appeared to attract a significant cellular infiltrate encircling the foreign material. Although the crystals also appeared to be washed out by the histological processing, large cavities were left by the crystals within the endometrium (Figure 5 A). This observation suggests the number of crystals (particularly smaller
ones) was considerably higher than was visualised on microscopic examination. Consistently the foreign material and tissue destruction was evident in the acute phase between the 6 and 48 hour biopsy times. Upon inspection of the buffered Ciproxin but before intrauterine infusion, crystals were measured to be approximately 80 µg in length (Figure 6A). Over the following 20 minutes, these smaller crystals aggregated quickly forming large 3-dimentional crystals (Figure 6B). It is hypothesised that these large 3-dimentional crystals were the cause of significant irritation and formation of fibrosis when the buffered Ciproxin was infused into the uterus.

**Conclusion**

The results of this study conclude that the administration of either buffered or unbuffered ciprofloxacin did not alter the intrinsic intrauterine pH of the mare, suggesting a robust buffering capacity within the uterus. This finding also suggests that the endometrial damage associated with the administration of buffered ciprofloxacin is due to factors other than alterations to uterine pH, supporting an hypothesis that crystal formation may be resulting in endometrial irritation. Although Ciprofloxacin is detectable within endometrial tissue following intrauterine administration, we conclude that buffered Ciproxin is not a suitable preparation for the intrauterine treatment of endometritis in the mare due to fibrotic changes that are likely to compromise future fertility. However, once daily intrauterine treatment of 50mg un-buffered Ciproxin for three consecutive days may be a suitable clinical option for the treatment of equine endometritis. In the one mare in which intrauterine infusion of un-buffered Ciproxin was trialled, there was clear improvement in histological, hysteroscopic and clinical pathology. These results suggest the safe use of this un-buffered formulation, warranting further investigation into dose, safety and efficacy. If successful, the results of the present study suggest that the MIC of the common bacterial causes of endometritis will be reached, and maintained, for up to three hours after the final infusion, with the maximum concentration occurring by one hour in all mares. Due to the negligible systemic uptake of the drug, the risk of morbidity and mortality of antibiotic associated gastrointestinal disease is minimised. Further research is required to investigate the potential of Ciproxin as a suitable intra-uterine antimicrobial and the use of pH analysis to identify endometritis in the mare.

**References**


LONG TERM FOLLOW UP OF MEDICAL TREATMENT AND RACE PERFORMANCE FOLLOWING LARYNGOPLASTY AND VENTRICULECTOMY IN 176 THOROUGHBRED RACEHORSES IN AUSTRALIA

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2 School of Veterinary Science, University of Queensland
3 School of Animal & Veterinary Sciences, Charles Sturt University

Introduction: Laryngoplasty is a common surgical treatment for recurrent laryngeal neuropathy. The procedure carries a risk of numerous short and long term complications aside from prosthesis failure. Complications include incisional seroma and infection, nasal discharge and potentially chronic serious coughing, dysphagia and pneumonia (Hawkins, Tulleners, Ross et al. 1997). While increased incidence of excessive tracheal mucous and epistaxis has been reported in racing Thoroughbreds post-operatively (Mason, Riggs, Cogger 2013), effect of post-operative complications on race performance has not been evaluated.

Aim: To investigate short and long term complications, management, and long term race results of Thoroughbred racehorses following combined left laryngoplasty and left ventriculectomy under Australian conditions.

Methods: A retrospective case series was conducted between August 1999 and December 2009. Case records and race results were evaluated for 176 Thoroughbred racehorses that underwent left sided laryngoplasty and left ventriculectomy performed by the same surgeon. Short and long term medical records were reviewed for Warwick Farm Equine Centre (WFEC) cases. Performance indices were compared between groups using the Wilcoxon rank sum test, or the Wilcoxon sign rank test for paired data. Significance was set at P=0.05.

Results: Complications occurred in 14% (8/58) of WFEC cases during hospitalisation. Following hospital discharge, 37 cases were available for medical follow-up. Twenty-four percent required further endoscopy with subsequent administration of medication due to the presence of lower respiratory tract (LRT) disease. Presence of managed LRT disease did not significantly affect race results (see Table 1). Two horses had persistent nasal discharge of feed post-surgery, requiring regular treatment for LRT disease; they had racing careers of 45 and 276 days, median 6 starts, and 1 had earnings at 1 start being a win in 1 race.

Table 1: Post-operative race results for WFEC cases with and without managed LRT disease

<table>
<thead>
<tr>
<th></th>
<th>LRT Disease</th>
<th>No LRT Disease</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median starts (IQR)</td>
<td>21 (5-22)</td>
<td>7 (1.5-15)</td>
<td>P=0.09</td>
</tr>
<tr>
<td>Median wins (IQR)</td>
<td>1 (0-1)</td>
<td>0.5 (0-2)</td>
<td>P=0.9</td>
</tr>
<tr>
<td>Median places (IQR)</td>
<td>4 (0-5)</td>
<td>1 (0-5)</td>
<td>P=0.5</td>
</tr>
<tr>
<td>Median earnings $ (IQR)</td>
<td>6,800</td>
<td>3,625</td>
<td>P=0.6</td>
</tr>
<tr>
<td></td>
<td>(0-17,450)</td>
<td>(0-17,325)</td>
<td></td>
</tr>
</tbody>
</table>
Following surgery, 79% (139/176) of horses raced, 59% (104/176) started more than five races and 44% (77/176) won a race. Raced horses performed better following surgery than unraced in terms of return to racing, number of starts and post-surgery earnings (see Table 2).

Table 2: Post-operative race results for raced and unraced cases

<table>
<thead>
<tr>
<th></th>
<th>Raced</th>
<th>Unraced</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion return to race %</td>
<td>86</td>
<td>65</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Median starts (IQR)</td>
<td>7 (4-19)</td>
<td>4.5 (0-14)</td>
<td>P=0.04</td>
</tr>
<tr>
<td>Median wins (IQR)</td>
<td>0 (0-2)</td>
<td>0 (0-1)</td>
<td>P=0.2</td>
</tr>
<tr>
<td>Median places (IQR)</td>
<td>2 (0-5)</td>
<td>0 (0-4)</td>
<td>P=0.08</td>
</tr>
<tr>
<td>Median earnings $ (Range)</td>
<td>4,727 (0-733,617)</td>
<td>100 (0-2,650,755)</td>
<td>P=0.03</td>
</tr>
<tr>
<td>Median starts earning prize money (IQR)</td>
<td>0 (0-3)</td>
<td>0.5 (0-6)</td>
<td>P=0.07</td>
</tr>
</tbody>
</table>

Conclusions: Horses with medically managed LRT disease raced as effectively as those requiring no further treatment. Persistent nasal discharge of feed post-surgery did not preclude a racing career, although performance appeared reduced in this small group. A good prognosis for return to racing can be expected following laryngoplasty and unilateral ventriculectomy, with significantly better results for previously raced horses.

References:


COMPARISON OF MELOXICAM AND PHENYLBUTAZONE ON LIPOPOLYSACCHARIDE INDUCED SYNOVITIS IN HORSES

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Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for the management of lameness, which is the most common problem faced by performance and racing horses. The beneficial effects of NSAIDs are achieved by inhibition of the cyclooxygenase (COX) enzyme, which catalyst the formation of pro-inflammatory prostanoids. Meloxicam (MEL), a COX-2 selective agent, has been advocated for this purpose and previous studies by our group (D’Arcy-Moskwa, Noble, Weston, et al. 2012; Noble, Edwards, Lievaart et al. 2012) have demonstrated that the drug may be safer than less selective COX inhibitors. MEL has been demonstrated to be an effective anti-inflammatory in vitro and in vivo, however comparisons with drugs in more common usage are lacking. Our aim, over the course of two studies, was to objectively assess the efficacy of meloxicam in horses, by comparison to a positive control (phenylbutazone, PBZ).

An initial pilot study was conducted to compare two in vivo methods for the induction of reversible lameness and synovial inflammation in horses. Four horses were subjected to injection of 10 mL autologous blood (Judy & Galuppo 2005) and injection of lipopolysaccharide (LPS) in contralateral limbs. Data from this study demonstrated that LPS was the more suitable method, yielding an obvious but transient lameness, as well as increases in synovial fluid nucleated cell count and synovial fluid protein concentration.

A further 8 horses were assigned a treatment based on drug (PBZ or MEL) and limb (left or right) in a crossover study using a randomised block design. Response to treatment was assessed by use of a modified composite orthopaedic pain scale (Bussieres, Jacques, Lainay 2008) as well as measures of stride length, joint flexion, joint circumference and joint effusion. Horses were fitted with an in-shoe pressure mapping system (Tekscan®, Hooft™ System, Boston, USA) and exercised on a treadmill where vertical force at the walk and trot were measured. Lameness was subjectively graded according to AAEP descriptors by one investigator (SLR) in real time, as well as by independent review of randomised de-identified video footage of each treadmill run by two investigators (SLR and GDT). Synovial fluid (SF) was collected immediately prior to injection of LPS, and at 4, 8, 24, 48 and 72 hours following induction of synovitis, for assessment of gross characteristics and routine cytology.

A marked lameness and inflammatory response was elicited by the intra-articular injection of LPS. Whilst the observed lameness grade and force plate measurements were substantively ameliorated by both drugs, the associated synovial inflammatory response was of similar magnitude to that observed in the pilot study (no NSAID treatment). MEL was associated with lower lameness scores than PBZ treatment. Peak reduction in vertical force was evident at 8-hours post induction of synovitis for both drugs, and no treatment effect was observed at a walk or trot. Similarly no significant differences attributable to NSAID treatment were evident for stride length, joint flexion, joint effusion or joint circumference. Using the adapted orthopaedic pain scale, discomfort was most notable at 4-hours post LPS injection with both drugs. A greater peak in pain score was observed with MEL, however pain resolved more quickly than with PBZ treatment. Gross characteristics of SF, SF protein concentration (P=0.837) and total nucleated...
cell count (P=0.424) were not different between treatments. All horses recovered well from all procedures.

In this study, both meloxicam and phenylbutazone treatments attenuated lameness induced by the intra-articular injection of LPS, but did not have an appreciable effect on SF gross or cytological characteristics. The results of both subjective and objective observations in this study suggest the overall clinical efficacy of meloxicam in horses with LPS-induced synovitis was comparable to that of phenylbutazone, supporting the clinical use of this agent for management of lameness in horses.

References
TRIPLEX DOPPLER ASSESSMENT FOR UMBILICAL CORD BLOOD FLOW IN THE POST-PARTUM FOAL: A PRELIMINARY STUDY

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Introduction: Considerable controversy surrounds the issue of early versus delayed umbilical cord clamping (DCC) in the human neonate, but the actual presence of umbilical cord blood flow is not disputed (Philip, 2006; McDonald & Middleton, 2009). Within the literature, placental transfusion via umbilical cord circulation during the immediate post partum period in the equine neonate is either very important (Rossdale & Mahaffey 1958) or does not occur (Doarn, Threlfall & Kline 1987). Publications from the UK support the early work by Rossdale & Mahaffey (Noakes, 2001) whilst most US based publications corroborate the study conducted by Doarn et al. (Bartholomew et al. 2010; Youngquist & Threlfall, 2006).

Aim: To attempt to establish the presence or absence of true blood flow within the umbilical cord of the equine neonate during it’s adaptive phase.

Methods: Thoroughbred foals (n=24) born from spontaneous parturitions on one commercial stud farm in the Hunter Valley were assessed from the end of stage 2 parturition until natural umbilical cord separation using high quality spectral (pulse wave) and colour doppler ultrasound (Zonare One, CA USA) in triplex mode. Measurements of flow rate (ml/min) in the umbilical vein and arteries between the foals’ abdomen and point of amniotic attachment on the cord were fully digitally recorded and retrospectively analysed using on-board processing software as well as OsiriX (v.5.6 32-bit ©Pixmeo SARL) viewing software. Scores (0-3) for pulsation, tension, blood flow and quality of the examination were assigned. Some foal and mare parameters were also recorded. Variables were compared with t-tests calculated on Wizard (Version 1.3.5 - E Miller).

Results: Post-partum umbilical blood flow (PUF) as well as palpable pulsations were clearly detected in the 23 of 24 foals. Cord separation occurred at a mean of 7.5 ±1.0 minutes after birth. The mean time of cessation of flow was 6.5 ±0.8(15) minutes after birth. Heart rate, quality scores, and tension scores were not associated with time over the first 5 minutes. Venous PUF rate (ml/min) and PUF score were correlated (P<0.001 Fig 1). Tension and PUF scores were correlated when grouped into high tension and low tension groups (P<0.05 Fig. 1). There was a negative linear association between tension score and the quality score for the umbilical vein. The quality score for the artery was positively affected at tension score 1 and negatively affected at tension score 3 (P<0.05). PUF scores and Pulse scores were associated (P<0.05). Other foal and mare parameters were within reported normal ranges (Rossdale & Ricketts, 1980).

Conclusion: Colour and pulse wave doppler readings indicate that there is blood flow within the umbilical cord of the post partum equine neonate. Analysis of the challenges associated with each examination and the observations made may help to partly explain the lack of flow or the lack of clinical significance of placental transfusion obtained by groups such as Doarn et al. and Bartholomew et al. and help clarify why there is such discrepancy within the scientific literature.
REFERENCES:


Supporting limb laminitis (SLL) occurs in the contralateral limb of horses that have sustained painful limb injuries [1]. It is a success-limiting complication during treatment of otherwise manageable equine limb pathologies [1; 2]. The proposed triggers of SLL are altered load distribution and uninterrupted weight bearing (decreased load cycling) which are thought to reduce perfusion of the digit [3]. Inadequate perfusion could result in metabolic derangements that lead to lamellar failure and consequently SLL [4-6]. With a high lamellar glucose consumption [7] and loss of mechanical strength demonstrated after glucose deprivation in vitro [8], a decrease in glucose delivery due to hypoperfusion may be an important mechanism of lamellar failure in SLL.

Tissue microdialysis samples the interstitium and can assess local energy metabolism and document tissue ischemia and bioenergetic failure [9]. Using the urea clearance method [10], microdialysis can also directly assess local perfusion. Lamellar microdialysis has been previously described [11-13]. The aim of this study was to assess the effects of unilateral weight bearing, altered limb load cycling, and modified vasomotor tone on lamellar perfusion and bioenergetics using tissue microdialysis.

Coaxial microdialysis probes (0.5 mm diameter, 10 mm long, 100 kDa cut-off) were inserted into the dorsal lamellae of one forelimb and the dermis of the skin over the tail base (as a control) in 9 clinically normal Standardbred horses. Using a precision pump, probes were continuously perfused at a rate of 1 µl/min with an isotonic, polyionic perfusion fluid containing 40 g/L Dextran-70 to prevent fluid loss [14], and 20 mmol/L of urea to assess local blood flow using the urea clearance method [10]. After probe placement, a 2-hour stabilization period was allowed. Thereafter, a sample (microdialysate) was collected every 15 minutes for a 24-hour study period.

The experiment consisted of a randomized latin square design, in which horses were subjected to nine consecutive interventions with a washout period between them (table). Concentrations of glucose, lactate, pyruvate and urea in the microdialysate were immediately measured using a clinical microdialysis analyser[^7], and standard indices of energy metabolism (lactate:glucose [L:G] and lactate:pyruvate [L:P] ratios) were calculated. Data were converted to a percentage of the baseline value immediately preceding each intervention and analysed non-parametrically. Significance was set at p < 0.05.

In the lamellar dialysate, glucose concentration decreased, and lactate, L:G, L:P and urea increased significantly (compared with baseline) during application of the tourniquet (p<0.05). During walking, lamellar glucose and pyruvate increased, and urea decreased significantly (p<0.05). Glucose increased and L:G decreased significantly during static limb load cycling (p<0.05). Glucose decreased and L:G increased significantly during perfusion with norepinephrine (NE) (p<0.05) [16]. During the other interventions (including unilateral weight bearing) there were no significant variations in the lamellar analytes compared with baseline. In the skin dermis, there was a significant decrease in glucose and significant increases in lactate, L:G, L:P and urea during perfusion with NE (p<0.05). The decrease in L:G was significant
(p<0.05) and the increase in glucose approached significance (p = 0.10) during local vasodilation with KCl. Urea concentration during the baseline period was significantly higher (p<0.05) in the lamellae compared to the skin, consistent with comparatively reduced urea clearance in the lamellar tissue.

The results show that the lamellar microdialysis technique is capable of detecting ischaemia: the decrease in glucose, increase in lactate and increase in L:G and L:P noted in lamellar samples during tourniquet application is a metabolic pattern that has been attributed to ischaemia in other tissues [15]. The decrease in urea clearance during tourniquet application demonstrates that this method can detect profound lamellar hypoperfusion consistent with ischaemia [10]. The decrease in glucose and increase in L:G during NE delivery to the lamellae are compatible with a reduction in perfusion; however, although a trend towards a decrease in urea clearance was apparent, it did not change significantly, suggesting that the urea clearance method might be less sensitive than energy metabolite changes for the assessment of less profound reductions in lamellar perfusion. Unilateral weight bearing did not induce significant changes in metabolite concentrations or urea clearance compared with baseline. Interestingly, walking was the only intervention that caused a statistically significant increase in lamellar urea clearance (increase in lamellar perfusion). Furthermore, glucose and pyruvate concentrations also increased profoundly during walking, corroborating the presence of increased perfusion compared with standing in the stocks. There was a similar, less profound metabolic effect with static limb load cycling. In contrast, there was a complete lack of lamellar response to pharmacological vasodilation (KCl, acepromazine and the perineural block) suggesting that there are mechanical impediments to lamellar perfusion in the static, standing horse that cannot be overcome with pharmacological vasodilation alone. In addition, the significantly lower lamellar urea clearance (compared with the skin dermis) suggests that lamellar perfusion is comparatively poor in the static, standing horse.

These results suggest that lamellar perfusion and energy balance are closely linked to limb load cycling and are particularly affected by walking. Short term unilateral forelimb weight bearing (increased load on the digit) did not have a detectable effect on lamellar perfusion beyond that of simply standing confined in stocks. If lamellar hypoperfusion and energy failure are key contributors to SLL, these results indicate that developing methods or strategies to increase limb load cycling may be important for preventing the disease.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Perineural block (to produce vasodilation)</td>
<td>-</td>
</tr>
<tr>
<td>2 Tourniquet (hypoperfusion)</td>
<td>30</td>
</tr>
<tr>
<td>3 Opposite limb held off the ground (unilateral weight bearing)</td>
<td>60</td>
</tr>
<tr>
<td>4 Enhanced static limb load cycling (instrumented limb lifted q10s)</td>
<td>30</td>
</tr>
<tr>
<td>5 Detomidine sedation (reduced limb load cycling)</td>
<td>-</td>
</tr>
<tr>
<td>6 Walking (hand lead, uninterrupted )</td>
<td>30</td>
</tr>
<tr>
<td>7 KCl perfusion (15 mmol/L in perfusate; vasodilation)</td>
<td>45</td>
</tr>
<tr>
<td>8 Norepinephrine perfusion (0.5 mg/ml in perfusate [16]; vasoconstriction)</td>
<td>45</td>
</tr>
<tr>
<td>9 Acepromazine (0.1 mg/Kg IV; vasodilation)</td>
<td>-</td>
</tr>
</tbody>
</table>
Footnotes:

a ISCUSflex Microdialysis Analyzer, CMA Microdialysis AB, Solna, Sweden.

References:


Nanoparticles for targeted drug delivery in equine laminitis

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Laminitis is a crippling disease of the equine foot. At present there is no pharmaceutical means of laminitis prophylaxis. Several candidate anti-laminitis drugs have been identified (Pollitt CC, Pass MA and Pollitt S 1998, Wang L, Pawlak EA, Johnson PJ et al. 2013). Their use is precluded by expense, systemic side effects and rapid clearance after intravenous dosing. Liposomes are phospholipid nanoparticles used for targeted drug delivery. They extravasate at sites of increased vascular permeability, including inflamed tissues (Laverman P, Dams ETM, Storm G et al. 2001). The aim of this study was to determine whether intravenous (IV) liposomes accumulate in lamellar tissue during laminitis development, and hence assess whether they have potential for targeted lamellar drug delivery.

Ten horses were enrolled in the study. Six horses received 10g/kg oligofructose (OF) via nasogastric tube to induce laminitis. PEG-liposomes were prepared via the film hydration method and labelled with 8.5 GBq ⁹⁹mTc-hexamethyl-propylene-amine-oxime. Horses received 300 µmol ⁹⁹mTc-PEG-liposomes plus 5.5 µmol/kg PEG liposomes by slow IV infusion at the time of OF administration (n=2), 12 hours after OF administration (n=2) and 18 hours after OF administration (n=2). Four control horses received sham treatment of water via nasogastric tube followed by IV liposome infusion. Scintigraphic imaging was performed at 0, 6 and 12 hours post-infusion. Regions of interest were drawn over the hoof, fetlock and metacarpus and the count density per second (CD/s) was recorded at each site. The horses were euthanized at 12 hours post infusion (n=8) apart from the 2 horses that received liposomes at the time of OF administration (n=2). These horses were euthanized at 24 hours post infusion. The percentage of the injected dose of liposomes per kilogram (%ID/kg) of tissue was calculated for multiple tissues. Lamellar samples were collected for histology and graded based on the severity of laminitis (Pollitt 1996). Data were analysed non-parametrically, with significance set at p<0.05.

All OF horses developed clinical and histologic signs of laminitis (Obel grade 2-3, median histologic score 1.5). The half-life of liposomes in the blood was 24 h. Radiopharmaceutical uptake in the hoof (measured as CD/s) increased with time in the OF horses (p=0.04), but decreased with time in control horses (p=0.01). The %ID/kg of lamellar tissue was significantly higher in the OF group compared to control horses (median [interquartile range], 0.21 [0.14-0.3]% vs. 0.07 [0.06-0.11]%, p=0.02). The %ID/kg was also significantly higher in OF vs. control skin (0.17 [0.15-0.28]% vs 0.08 [0.07-0.10]%, p=0.01), muscle (0.04 [0.03-0.06]% vs 0.02 [0.01-0.02]%, p=0.03), jejunum (0.2 [0.17-0.25]% vs. 0.11 [0.09-0.13]% , p=0.048), colon (0.27 [0.25-0.41]% vs 0.14 [0.13-0.17]% , p=0.03), and kidney (1.37 [1.06-1.47]% vs 0.82 [0.74-1.03]% , p=0.04).

This study demonstrates that PEG-liposomes accumulate in lamellar tissue during OF induced laminitis development and therefore have potential for lamellar drug delivery. Liposomes accumulate in inflamed tissues due to increased vascular permeability. Therefore, these findings indicate lamellar inflammation and increased lamellar vascular permeability occur early in the
developmental phase of laminitis. Liposome accumulation also occurred in the skin, muscle, jejunum, colon and kidneys, demonstrating the presence of systemic inflammation.