AUSTRALIAN COLLEGE OF VETERINARY SCIENTISTS

COLLEGE SCIENCE WEEK SCIENTIFIC MEETING

Thursday 5 July to Saturday 7 July 2007

EQUINE CHAPTER PROCEEDINGS

GOLD COAST INTERNATIONAL HOTEL, SURFERS PARADISE
Cnr Gold Coast Highway & Staghorn Avenue, Surfers Paradise

Scientific Meeting Equine Chapter Program Coordinator – John Chopin

Proceedings Editor – John Chopin
## PROGRAM FOR EQUINE CHAPTER – Margot’s, First Floor

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MANAGEMENT OF EQUINE SKULL FRACTURES USING FIXATION WITH POLYDIOXANONE SUTURES

Kylie Schaaf BVSc(Hons) BSc(Vet) MACVSc (Equine Surgery), Nicholas Kannegieter BVSc, DipVetClinStud, PhD, FACVSc (Equine Surgery), David Lovell BVSc(Hons) QDAH, MACVSc (Equine Surgery)

Fractures of the facial bone are commonly encountered in equine practice. The inevitable cause is direct trauma, usually due to a kick from another horse or collision with a stationary object. This can result in severely comminuted fractures, often with a large number of small, unstable pieces of bone. There is usually an obvious depression in the facial bones. If there is serious instability of bones over the nasal passages or paranasal sinuses, there may be obvious movement of the skin and bone as a result of negative pressure created during inspiration. If the fractured fragments are wedged in depression, severe dyspnoea may be encountered. There may also be moderate to severe nasal haemorrhage. However, the most serious and urgent presenting complication is trauma to the eye with depression or prolapse of the globe following fractures of the bony orbit. While these fractures appear severe, provided there is no ocular involvement or dyspnoea, they are not usually life threatening and can be repaired several days post injury if required. If there is any ocular involvement or dyspnoea, immediate attention is required.

While these fractures appear severe, provided there is no dyspnoea, they are not usually life threatening and can be repaired several days post injury if required.

Examples of skull fractures into the paranasal sinuses and nasal cavity

Further examples of depression fractures distorting the nasal passages (left) and the bony orbit (right)
While facial bone fractures appear dramatic they usually have a very good prognosis. Nearly all will eventually heal. The advantages of surgical fixation include a superior cosmetic result, improved airway dynamics and a faster healing time.

We describe a technique used for surgical fixation of unstable skull fractures. The affected bones included the frontal bone, the supraorbital process of the frontal bone, the nasal, maxilla, lacrimal and zygomatic bones. Complicating factors included paranasal sinus, nasal cavity and ocular involvement.

An incision is made over the fractures that provides the best exposure of all the fracture sites. The incision may be straight, curved, or sometimes multiple incisions are required. Care should be taken to ensure all skin flaps have a good blood supply. The periosteum should also be preserved, as this is important for bony healing. The fracture fragments are elevated back to their normal position. Any pieces devoid of periosteum should be removed. The sinus and nasal cavity should be flushed to remove multiple small fracture fragments. In many situations, simple elevation will provide suitable stability to allow fracture healing. However, if the fracture fragments are unstable, our preferred technique is fixation with polydioxanone sutures.

Elevation of the fracture fragments and fixation with PDS sutures

A small diameter (2.7-3.2mm) hole is drilled in the opposing edges of the fracture fragments. Depending on the level of stability required, 1-3 strands of polydioxanone (2-PDS) is pre-placed in the holes in both sides of the fracture. Pre-placing the sutures also allows for easy placement of multiple sutures. The fragments are then elevated and secured into position. The wound is extensively lavaged with balanced electrolyte solution. The periosteum and subcutaneous tissue is closed with 0-vicryl in a simple continuous pattern. The skin is closed with stainless steel staples. If seroma formation is a concern, small stab incisions rostrally provide the necessary drainage. With severe fractures we will apply cushioning with padding and adhesive bandages to protect the repair during the recovery period. A tracheotomy may be necessary in severe cases of bilateral fractures.
Complications that may be encountered include bone sequestration and damage to the orbit as a result of the initial trauma. Occasionally with severe trauma and sequestration over the paranasal sinuses a fistula may form. This can be repaired with a periosteal advancement flap. Discharge over a PDS suture was also encountered in one horse. The offending suture was removed with the horse standing under local anaesthetic and sedation.

This case series demonstrates that unstable skull fractures can be successfully repaired with polydioxanone suture fixation. The cosmetic result in all cases was considered acceptable and all horses were returned to their intended use.
LACK OF VETERINARY HEALTH CARE OF PONY CLUB HORSES IN REGIONAL AUSTRALIA

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Abstract

Results from a qualitative survey of Pony Club horse owners’ perceptions of horse health and performance raised concerns about the role of veterinarians in preventive health care of these animals (Buckley 2004). This study further investigated the type of routine health care received by a cohort of Australian Pony Club horses, and the persons providing such care.

A prospective longitudinal study of 84 Pony Club horses belonging to 41 families in the Wagga Wagga district of NSW was conducted from June 00 to July 01 to investigate health and performance of Pony Club horses. Participating families kept daily records of routine horse health care administered to study horses, disease occurrence, person administering routine health care and person attending a sick horse. These records were collected and clarified during monthly visits by the chief investigator to horse and property. To be classified as healthcare, a preventive event had to be administered to the horse, and owners had to consider it a routine event rather than a treatment.

The health care data reflect the experiences of the 47 horses that spent ≥10 months in the study. The most commonly administered healthcare procedures were foot care (with 75% of horses having their feet trimmed and or shoed on ≤7 occasions over 10 months), anthelmintic administration (90% of horses received ≤4 treatments over 10 months), “other care” (28% of horses received other care, which involved principally chiropractic manipulation), dental care (26% received some form of dental care over the 10 months) and vaccination (only 8% of horses were vaccinated during the study).

Veterinarians rarely administered routine health care to Pony Club horses, and when they did, it was limited to the administration of vaccines against tetanus and strangles. Chiropractors provided more health care than either veterinarians or equine dentists, but equine dentists provided all the dental care. Of all animal health care providers, farriers had the most regular contact with these horses and their owners; they administered either shoeing or hoof trimming at approximately 8-weekly intervals.

Veterinarians were only peripherally involved in routine health care of these Pony Club horses, supporting the view that equine veterinarians are seen by horse owners as disease experts rather than health experts (Buckley 2004). Equine veterinarians are missing
opportunities for the provision of basic health care advice and education to horse owners through lack of regular contact with horse and owner. Increased involvement of equine veterinarians in preventive horse health care programs offers prospects for improving the health and welfare of these animals. For example, veterinarians are uniquely placed to help horse owners adopt more strategic approaches to parasite control in the face of widespread anthelmintic resistance.

Veterinarians could develop strategic health care programs targeting Pony Clubs and market such services as extending beyond the treatment of sick animals. A pre-purchase examination, rarely requested for Pony Club horses in this region (Buckley 2004), might provide a starting point from which to launch regular comprehensive health checks. This might help establish a regular presence as an advisor in a preventive context. Targeting horse health at this “grass roots” level may even lead to equine health benefits in other sectors of the Australian horse industry, as young riders progress beyond Pony Club.

Acknowledgements
The authors would like to thank participating Pony Clubs, David Buckley, Garry Anderson, Charles Sturt University and the Rural Industries Research and Development Corporation.

REPEATED OBSERVATIONS OF NATURALLY OCCURRING LAMINITIS IN PONY CLUB HORSES IN REGIONAL AUSTRALIA

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Abstract
This is the first report of repeated observations of naturally occurring laminitis in a cohort of horses.

A prospective longitudinal study involving 84 Pony Club horses in regional Australia was conducted from June 00 to July 01 to investigate health and performance of Pony club horses. Participating families kept daily records of horse nutrition, exercise, housing, routine health care and disease occurrence. Horses were subjected to a monthly veterinary examination, during which bodyweight and condition score were evaluated and recorded. Monthly observations of pasture biomass and pasture composition were also recorded.

To enable accurate descriptions of naturally occurring cases of laminitis, additional categories were created, chronic inactive laminitis, chronic active laminitis, and chronic inactive laminitis with non-laminitic lameness.

A case of acute laminitis was defined as a horse showing characteristic lameness, heat and bounding digital pulses in at least two feet.

A case of chronic inactive laminitis was defined as a horse showing several of the following characteristics: abnormal angle of hoof wall, loss of concavity of sole, depression above coronet, abnormal hoof wall growth lines, and absence of lameness.

A case of chronic active laminitis was defined as a horse showing several of the following characteristics: abnormal angle of hoof wall, loss of concavity of sole, depression above coronet, abnormal hoof wall growth lines as well as characteristic lameness, heat and bounding digital pulses in at least two feet.

A case of chronic inactive laminitis with non-laminitic lameness was defined as a horse with chronic inactive laminitis as well as lameness without signs supporting acute laminitis.

Of 84 study horses, 23.8% (20/84 animals) experienced 32 laminitis events from June 2000 to July 2001. Acute and chronic active cases of laminitis occurred in winter or spring. The duration of a laminitic episode varied from 14 days to 99 days. Two of the horses were lost to
follow up. Of the remaining horses, in only one animal was a single instance of chronic inactive laminitis not followed by further recurrence of either laminitis or lameness during the study period. The majority of laminitis cases (89% or 16/18) progressed to one and up to four more bouts of laminitis and/or recurring lameness during the study period.

Laminitic Pony Club horses represent a considerable burden and source of frustration for their carers/owners due to the loss of considerable numbers of riding days. Horses that have experienced an episode of laminitis are very likely to experience further episodes. The presence of chronic inactive laminitis is an important finding during a pre-purchase examination. Prospective owners should be cautioned of the high likelihood of recurring bouts of laminitis and lameness, and the difficulty of prevention, especially where pasture based-laminitis is suspected.

Acknowledgements
The authors would like to thank participating Pony Clubs, David Buckley, Garry Anderson, Charles Sturt University and the Rural Industries Research and Development Corporation.
ENDOCRINOPATHIC LAMINITIS IN HORSES

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Introduction

Laminitis is a devastating disease, and one that has not yet revealed all its secrets. Most work to date has focussed on models of laminitis that are associated with perturbations of the gastrointestinal tract (carbohydrate overload or oligofructose models) or inflammatory (Black Walnut model). Yet less is known about potentially the most common form of laminitis, endocrinopathic laminitis. Endocrinopathic laminitis has been differentiated from laminitis occurring in association with pro-inflammatory and intestinal conditions and is defined as laminitis developing from putative hormonal influences (Johnson et al. 2004). Conditions associated with endocrinopathic laminitis include Equine Cushing’s Syndrome (or PPID), Insulin Resistance (or Equine Metabolic Syndrome or Pasture-Associated Laminitis) and iatrogenic corticosteroid administration. In common to all these conditions are disturbed glucose and insulin regulation, and, most importantly, the development of insulin resistance.

Equine Cushing’s syndrome

Laminitis is one of the most common signs of equine Cushing’s syndrome (ECS) and occurs in approximately 80% of affected horses (McGowan and Neiger 2003). The disease is characterised by loss of tonic inhibition of pituitary pars intermedia activity by dopamine leading to excessive hormone production and subsequent hyperplasia of that part of the pituitary gland. These include β-endorphin, α-melanocyte stimulating hormone and corticotrophin-like intermediate peptide. While the proportion of ACTH produced from the pars intermedia is small, the marked over activity of the ECS pars intermedia results in excess ACTH production. Additionally, despite low or normal resting cortisol concentrations in affected horses, there is a loss of diurnal rhythm of cortisol concentration, particularly failure of cortisol concentrations to lower diurnally.

Many of the clinical signs of ECS, including laminitis are attributed to excessive action of cortisol, and, in fact, use of a cortisol inhibitor has been shown to be effective in reducing the clinical signs of laminitis in affected horses (McGowan and Neiger 2003).

Iatrogenic corticosteroid induced laminitis

Similarly to Equine Cushing’s syndrome, iatrogenic corticosteroid administration has been postulated to cause laminitis, although in reality, the prevalence of this is likely to be low in normal horses and probably occurs indirectly. Despite the low prevalence, iatrogenic corticosteroid induced laminitis is receiving increasing attention and when it does occur, can be devastating to both clinician and client (Bathe 2007).
Glucocorticoids and Laminitis

The common link between both ECS and iatrogenic corticosteroid administration is the ability of cortisol to induce insulin resistance. There is a well-established link between insulin and cortisol, which have opposing actions on glucose metabolism, such that hyperinsulinaemia and insulin resistance are potential consequences of ECS or corticosteroid treatment (Bailey and Elliott, 2007). Emphasising the importance of insulin resistance is the importance of insulin as a prognostic indicator for survival in horses with ECS. Horses with abnormally high levels of insulin (>180 nmol/l) are much more likely to develop laminitis and survive less than two years after diagnosis, than those with only moderate elevations or normal insulin levels (<60 nmol/l) (McGowan et al 2004).

Insulin resistance syndrome

Insulin resistance syndrome in man is something that has recently received a lot of attention due to its relationship with cardiovascular disease. Approximately 30% of the USA human population are insulin resistant (Reaven 2004). Insulin resistance is important not only because it can lead to the development of Type 2 diabetes, but even people who are able to maintain blood glucose concentration may suffer from a syndrome of compensatory hyperinsulinaemia that results in increased triglyceride production from liver, abnormal lipoprotein profile, endothelial dysfunction and a procoagulant state. People with this syndrome are predisposed cardiovascular disease, hypertension, fatty liver, sleep apnoea and cystic ovarian disease.

The term Metabolic Syndrome is really a series of some of the signs of insulin resistance syndrome that predispose humans to cardiovascular disease (Reaven 2004, 2005). In other words, it provides a set of criteria for diagnosis of people who require lifestyle change to reduce incidence of cardiovascular disease.

Therefore insulin resistance syndrome is probably a better term in horses.

Insulin Resistance Syndrome in Horses

Insulin resistance in horses manifests differently from that in man and was first reported as “Laminitis, Hypothyroidism and Obesity: a peripheral cushingoid syndrome in horses” (Johnson 1999) which described a syndrome of obese, cresty horses with laminitis. The pathogenesis was related back to omental Cushing’s in man which is a syndrome of obesity and abnormal cortisol activity due to activation by the adipose tissue, especially omental adipose tissue as the name suggests. Since then the more popular term has been “Equine Metabolic syndrome” (Johnson 2002, 2003), based on the then current trends in human research. However, irrespective of the name of the condition, the condition seen clinically is defined by insulin resistance, recurrent painful laminitis despite good management and veterinary care and often obesity (historically or currently). Despite sometimes appearing similar to horses with ECS, horses with insulin resistance are:

- negative to equine Cushing’s syndrome on specific endocrine tests (i.e. low dose dex suppression, basal [ACTH], NOT basal insulin)
- not hirsute
- younger than horses with ECS

Of potentially greater interest is that these clinical cases probably represent the tip of the iceberg and there are a number of horses with subclinical disease waiting for the right pasture conditions to develop overt pasture-associated laminitis. These horses have been described by
Treiber et al. (2005, 2006) as having ‘Prelaminitic Metabolic Syndrome’ and among other signs, ponies with this Syndrome had twice the normal blood concentration of insulin when grazing on winter pasture. In spring, when lush pasture was available, these ponies developed laminitis and had even greater insulin concentration on basal testing (more than five times the normal values) (Treiber et al. 2006).

**Mechanism of Endocrinopathic laminitis**

There are 2 main theories of the mechanism of endocrinopathic laminitis, glucose uptake impairment vs. glucotoxicity, and both theories are associated with insulin resistance.

**Glucose uptake impairment**

Glucose uptake impairment is the classical sign of insulin resistance, due to the principal effects of insulin in stimulating glucose dispersal into the tissues via GLUT4 transport proteins, particularly in muscle and adipose tissue. The underlying problem of insulin resistance is then potential glucose deprivation of tissues, starving them and causing cell death or damage. In support of this, is the research that has shown that healthy hoof tissue has an absolute requirement for glucose, such that when hoof explants are incubated in the absence of glucose, or in the presence of a glucose uptake inhibitor, the layers of tissue separate rapidly, as they do when laminitis occurs (Pass et al. 1998). Additionally, the hoof utilises glucose at an exceptionally fast rate compared with most other tissues (Wattle and Pollitt 2004), so in theory, even a small decrease in the rate of glucose uptake could be extremely damaging.

However, recent research at our lab has shown that the hoof lamellae are insulin-independent (Asplin et al. 2007) based on a number of experiments that indicated that glucose uptake in the hoof is neither dependant on insulin, nor is it influenced by the presence of insulin. Further, when the glucose transport proteins were examined, there was a predominance of GLUT1 in lamellae, which are insulin independent, consistent with the hoof having such a high metabolic demand for glucose (Wattle and Pollitt 2004). However, these results indicate that laminitis is cannot be caused by glucose deprivation, resulting from insulin resistance.

**Glucotoxicity**

Glucotoxicity is a major problem in human diabetics and sufferers of insulin resistance syndrome. As mentioned above the key problems associated with glucotoxicity are vascular due to both pro-coagulant activity and oxidative endothelial damage. The same may occur in horses and Johnson (2004) has speculated that obese horses or horses undergoing stress have a series of metabolic events that precipitate abnormal adipose tissue endocrine function - setting up vicious cycle of insulin resistance and secondary effects of insulin resistance syndrome as occur in man – except that it is manifest as laminitis and not cardiovascular disease. The key events to precipitate laminitis in such a model could include either vascular damage and ischaemic damage, or pro-inflammatory effects and subsequent matrix metalloproteinase (MMP 2 and 9) activation and basement membrane separation.

**Conclusion**

Endocrinopathic laminitis is a common and insidious problem in horses. While the exact mechanism has not yet been elucidated, it is clear that insulin resistance is a key and common underlying problem. Effort to detect and monitor insulin resistance in predisposed horses is an essential part of diagnosis and treatment of endocrinopathic laminitis should include methods of decreasing factors contributing to insulin resistance including attention to diet and exercise.
REFERENCES
RECENT ADVANCES IN UNDERSTANDING LAMINITIS IN HORSES

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Introduction

Laminitis is the most serious disease of the equine foot and causes pathological changes in anatomy that lead to devastating loss of function. The simplest definition of laminitis is: failure of the attachment between the distal phalanx and the inner hoof wall. A horse has laminitis when the lamellar architecture of the inner hoof wall, which normally suspends the distal phalanx from the inner surface of the hoof capsule, fails. Without the distal phalanx properly attached to the inside of the hoof, the weight of the horse and the forces of locomotion drive the bone down into the hoof capsule, shearing and damaging arteries and veins, crushing the corium of the sole and coronet, causing unrelenting pain and a characteristic lameness.

The phases of laminitis

A developmental phase, during which lamellar separation is triggered, precedes the appearance of the foot pain of laminitis. This is around 24 - 40 h in the case of excessive ingestion of high starch grain (Garner et al. 1975; Obel 1948; Pollitt 1996) or fructan (van Eps and Pollitt 2006) and within 6 h after ingestion or dosing with an extract of the heart wood of black walnut timber (Galey et al. 1991). During the developmental phase and prior to the clinical appearance of foot pain the horse or pony usually experiences a problem with one or more of the following organ systems: gastrointestinal, respiratory, reproductive, renal, endocrine, musculoskeletal, and immune. Multi-systemic aberrations in organs anatomically remote from the foot result in the lamellar tissues of the feet being exposed to factors which lead to separation and disorganisation of lamellar anatomy. The exact nature of the laminitis trigger factors, apparently reaching the lamellar tissues via the circulation, has yet to be elucidated. Sometimes no developmental phase can be recognized: the horse or pony is discovered in the acute phase with no apparent ill-health or inciting problem occurring beforehand. Obesity and related endocrinopathic problems have recently been incriminated in the pathogenesis of this insidious form of laminitis (Asplin et al. 2007; Johnson 2002; Johnson et al. 2004). Grass founder can also appear without warning and this has now been linked to seasonal variations in the concentration of the soluble sugar fructan by temperate pasture species (Longland and Byrdy 2006). Fructan can suddenly reach very high concentrations in the stems of grass and trigger a laminitis inducing gastrointestinal disturbance when consumed by horses and ponies. That laminitis can be induced by such sugars has been verified experimentally using oligofructose, a closely related compound (Pollitt et al. 2003; van Eps and Pollitt 2006). The parenteral injection of potent long acting corticosteroid preparations for the treatment of skin disease may precipitate iatrogenic acute laminitis (Eustace and Redden 1990).
The developmental phase merges into the acute phase of laminitis which lasts from the onset of clinical foot pain and lameness at the trot, to the time when there is clinical (usually radiological) evidence of displacement of the distal phalanx within the hoof capsule. After the acute phase, if the horse does not die from the disease process inciting the development of laminitis, it can make an apparent complete recovery or develop palmar/plantar displacement of the distal phalanx, the hallmark of chronic laminitis. The chronic phase can last indefinitely with clinical signs ranging from persistent, mild lameness, continued severe foot pain, further degeneration of lamellar attachments, recumbency, hoof wall deformation and even sloughing of the hooves (Hunt 1993). It is important to realize that the process initiating the destruction of the lamellar attachment apparatus begins to operate during the developmental phase before the first clinical sign of laminitis, foot pain, is apparent. During the developmental phase the specific problems of the horse, often have to be attended to urgently (e.g. acute abdomen, grain overload acidosis, electrolyte imbalance, rhabdomyolysis, retained placenta) and unfortunately the feet are often left out of therapeutic equation until the first signs of foot pain (shifting weight from one foot to the other, lameness when trotted out, especially when turned) appear. By the time foot pain is apparent lamellar pathology is underway. In other words foot pain is the clinical sign that lamellar disintegration is occurring. To wait and see if foot pain is the sequel to a metabolic crisis is to miss the opportunity to prevent or at least ameliorate lamellar pathology. There is a good correlation between the severity of laminitis histopathology, as seen with the microscope, and the degree of lameness (Obel 1948) shown by the horse (Pollitt 1996). When a horse first starts to show laminitic pain, the anatomy of the hoof wall lamellae is being destroyed. The higher the lameness grade, the more severe the microscopic damage. Any activity that places stress on an already weakened lamellar attachment apparatus (such as forced exercise) causes further damage and is contraindicated. The use of nerve blocks to eliminate pain will also encourage locomotion and precipitate more damage.

The Pathophysiology of laminitis

The spectacular disintegration of the lamellar attachment apparatus, initiated during the development phase of laminitis, compromises a normally robust and trouble free hoof, distal phalanx attachment apparatus in a surprisingly short period of time. Logic suggests that it is a normally tightly controlled metabolic process that is thrown into disarray to cause the lamellar specific lesion of laminitis during its developmental phase (Pollitt et al. 2003). The enzymatic (MMP) remodelling of the epidermal lamellae, assumed to be mandatory as the continually proliferating hoof wall (Daradka and Pollitt 2004) moves past the stationary distal phalanx, appears to be accidentally recruited in the pathogenesis of the laminitis disease process. MMPs are found in increased quantities from lamellar tissues affected by laminitis (Pollitt et al. 1998). The epidermal cells of other species have been shown to readily increase their production of MMP when exposed to inflammatory signalling proteins cytokines. Cultures of human oral mucosal keratinocytes respond to the addition of the cytokines tumour necrosis factor (TNF), interleukin -1 (IL-1 ) and transforming growth factor - 1 (TGF-1) by increasing production of MMP-9 (Pirilä 2003). Lamellar tissues affected by laminitis also increase transcription of MMP (Kyaw-Tanner and Pollitt 2004) and produce MMPs in their active forms (Pollitt et al. 1998) but whether in response to circulating cytokines or some other trigger factor is yet to be established. Evidence from our in vitro studies, using equine lamellar explants, suggests that lamellar MMPs are not activated by exposure to either human cytokines (Mungall et al. 2001) or selected recombinant cytokines eg. IL-6 (Visser and Pollitt, unpublished data). Recently however we have shown very large increases in the
lamellar transcription of proinflammatory cytokines at the time of lameness; notably interleukin-6 and 8 (IL-6 and IL-8) (Belknap et al. 2006). Increased cytokine expression may lead to downstream events resulting in lamellar failure, similar to organ failure in human sepsis.

The enzymatic theory of laminitis aetiology based on lamellar MMP activation challenges the alternative view that laminitis develops because of vascular pathology affecting the circulation of the foot. A current theory is that vasoconstriction and high hydrostatic interstitial fluid pressure (compartment syndrome) impede the flow of blood in the lamellar microcirculation to cause ischemic necrosis of epidermal lamellae (Allen et al. 1990). Current data do not support a role for global ischemia of the laminae in the development of laminitis induced with BWE. Lamellar genes expected to be upregulated if hypoxia/ischaemia were occurring are not (Cochran et al. 2006). The authors rightly question treatments aimed at ameliorating ischemia (vasodilators) in the early stages of laminitis.

Epidermal cell necrosis, intravascular coagulation and oedema were not identified in sections made from tissue in the early stages of laminitis (Pollitt 1996). The vessels in the primary dermal lamella, even the smallest, were generally dilated without evidence of microvascular thrombi (Weiss et al. 1994). Further, no abnormalities in the systemic coagulation and fibrinolytic cascades are found in horses with carbohydrate induced acute laminitis (Prasse et al. 1990). The gross anatomical appearance of freshly dissected laminitis tissue is one of dryness. Sometimes the lamellae peel apart. Tissues affected by a compartment syndrome exude fluid.

**Laminitis trigger factors**

What are the laminitis trigger factors? Since the carbohydrate overload model of laminitis is characterized by endotoxin production it would seem a safe presumption that macrophages in the peritoneal cavity and elsewhere in the body would be subject to endotoxin stimulation just as they are during other acute gastrointestinal diseases (Barton et al. 1996). Macrophages express tumor necrosis factor along with other cytokines such as interleukin within minutes of exposure to endotoxin. The cytokine cascade originating from an acute abdomen is responsible for most of the pathological effects of endotoxemia. However laminitis has never been triggered by the experimental administration of endotoxin into the bloodstream (Hunt et al. 1990) or the peritoneal cavity and the actual trigger factors of laminitis remain unidentified. What appears certain in the light of recent research is that the lamellar disintegration of laminitis is mediated by the uncontrolled release of excess MMP (Kyaw-Tanner and Pollitt 2004).

**Laminitis in vitro**

We have successfully developed a test tube or in vitro model (Mungall et al. 2001; Pollitt et al. 1998) for equine laminitis using small explants of tissue taken from the inner hoof wall of normal, freshly killed, abattoir horses. Each explant consists of hoof and its lamellar layer and the sub-lamellar connective tissue. After incubation for 48 h in tissue culture medium, plus the laminitis trigger factor under investigation, each explant is subjected to tension. The force required to separate epidermal from dermal lamellae is recorded. When dermal-epidermal lamellar separation occurs readily (as occurs in field cases of laminitis) we consider the tissue to have developed in vitro laminitis. Lamellar explants can be cultured for up to 7 days in normal medium and no lamellar separation occurs. It is virtually impossible to
separate normal lamellar explants. One event that readily causes separation of lamellar explants is MMP activation. The addition to the culture medium of an MMP activator, readily induces explant lamellar separation. The presence or absence of MMP activation in the tissue culture fluid is detected visually using zymograms. Histological sections show a clear zone of complete separation between the basement membrane and the basal cells of the epidermal lamellae. This is a characteristic of \textit{in vitro} laminitis and resembles the basement membrane lesion of natural \textit{in vivo} laminitis.

We have used the \textit{in vitro} laminitis explant model to investigate most of the proposed causes of equine laminitis. The equine lamellae have tested resistant to virtually all known cytokines, tissue factors and prostaglandins. Gram negative bacterial endotoxin and even anaerobic culture conditions fail to induce lamellar separation or significant MMP activation. There is one notable exception however. A factor present in the supernatant of cultures of \textit{Streptococcus bovis} isolated from the equine caecum activates equine hoof MMP-2 and causes lamellar separation (Mungall \textit{et al.} 2001). During grain overload \textit{S. bovis} is the principal microorganism responsible for the rapid fermentation of carbohydrate to lactic acid in the equine hindgut. In the presence of virtually unlimited substrate its population explodes exponentially. We are currently investigating the role of the \textit{S. bovis} MMP activator in natural cases of equine laminitis. If it crosses the mucosal barrier of the hindgut and enters the circulation it may be a “cause” of laminitis (at least in the carbohydrate overload model). In other words it may be an exogenous laminitis trigger factor (LTF). On the other hand fermentation by \textit{Strep bovis} of large quantities of carbohydrate substrate severely damages the epithelium of the hindgut and creates a leaky mucosal barrier. Rapid epithelial remodelling of a repairing hindgut may be a source of factors that ‘overflow’, via the circulation, and trigger ‘accidental’ remodelling of hoof lamellae. The low pH of the hindgut during carbohydrate overload results in ‘die-back’ of the \textit{Strep bovis} population (Milinovich \textit{et al.} 2007; Milinovich \textit{et al.} 2006) and peptidoglycan exotoxin and toxic microbial nuclear degradation products released from dying microbes are also candidate LTFs or at least factors that may stimulate a cytokine cascade.

\textbf{MMPs and laminitis}

We have recently cloned the genes responsible for MMP expression in lamellar hoof (Kyaw-Tanner and Pollitt 2004). Horses with acute laminitis show increased expression of the MMPs, 48 hours after alimentary carbohydrate overload. For MMP gene expression to have doubled by the time lameness is manifest implies that the factors signalling the increased expression have been present for some time. This places perturbation of MMP equilibrium early in the cascade of events leading to the foot pain of acute, clinical laminitis. Indeed biopsies of lamellar tissue taken as laminitis develops all show some of the histopathology of the laminitis grading systems (French and Pollitt 2004a; Pollitt 1996). At 24h, lamellae had intact basement membranes but SELs were attenuated with round basal cell nuclei. At 36h, SEL attenuation had progressed and SEL basal cells with rounded nuclei were disorganized; SEL tips were pointed instead of rounded. Only at 48h was the BM not attached to SEL basal cells suggesting that the dysadhesion process commenced somewhere between 36 and 48h (Croser and Pollitt 2006). However the molecular and biochemical events contributing to BM disattachment, as evidenced by nuclear rounding and SEL attenuation, were in place by 24h. The basement membrane lesion of laminitis is insidious in nature and well under way by the time clinicians are aware of laminitis foot pain. Any preventive (van Eps and Pollitt 2004; van Eps \textit{et al.} 2004) or treatment strategies must be in place before overt foot pain develops if horses are to survive the development phase of laminitis without significant lamellar damage.
MMP inhibitors

There is a wide range of chemical agents capable of inhibiting MMP activity both in vitro and in vivo (Roach et al. 2002). We have shown that one of these (Batimastat or BB-94, British Biotech, Oxford) blocks the activity of the laminitis MMPs in vitro and has the potential to be a useful tool in the prevention and management of acute laminitis (Pollitt et al. 1998). Trials to test whether MMP inhibitors can prevent or ameliorate field cases of laminitis are currently underway in the Australian Equine Laminitis Research Unit at The University of Queensland.

The ultrastructure of laminitis

Laminitis studied by transmission electron microscopy (TEM) and immunofluorescence microscopy (IFM) has provided new insight into the mechanism of the disease. The hemidesmosome (HD) is the attachment plaque responsible for maintaining contact between the SEL basal cell and its underlying basement membrane. In lamellar SEL samples taken at the onset of acute laminitis many HDs are absent or disrupted. Loss and disruption of HDs is accompanied by BM separation, cytoskeleton damage and rounding of the basal cell nucleus (Nourian et al. 2007).

Hoof lamellae cultured in vitro without sufficient glucose separate under tension and the intracytoplasmic components of their HDs fade and collapse (French and Pollitt 2004b). Glucose starvation may be operating in vivo when toxaemia and the various endocrinopathies associated with laminitis limit the supply of lamellar glucose. Activation of constituent lamellar MMPs also causes lamellar separation under tension but without affecting HD ultrastructure. Activated MMPs appear to cleave laminin5 anchoring filaments and set the BM adrift; also a process now shown to occur in vivo (French and Pollitt 2004a).

Laminitis therapy

From the outset it must be stated that a therapeutic regime, using biological or chemotherapeutic agents, able to arrest or block the triggering of laminitis, does not exist. On the other hand, there is a plethora of remedies, used empirically, that symptomatically help the horse after it has acquired laminitis. It is more the extent and severity of the lamellar pathology that influences the outcome for the horse, not the treatment regimen itself. An effective laminitis preventive may emerge when the mechanism behind the disintegration of the anatomy of the hoof wall lamellae is fully understood. Our discovery that a class of enzymes appears to be involved in the lamellar failure of laminitis has led us to commence trials of proteinase inhibitor therapy, specifically targeted at hoof wall matrix metalloproteinases.

Since laminitis usually develops as a sequela to a disease process in a body compartment other than the foot, it is of paramount importance that the primary disease is treated urgently and effectively. If the duration and severity of the primary disease can be reduced by intensive therapy, there is a strong chance that the severity of lamellar pathology may also be reduced, thus improving the prognosis for the horse. Nevertheless, severe laminitis is sometimes the outcome despite the best of current therapy.

When the laminitis process is triggered, there is virtually nothing, by way of drug therapy, that will stop its relentless progress. The administration of a nonsteroidal anti-inflammatory drug (NSAID) like phenylbutazone, during the developmental/acute stages, will abolish foot
pain and create a more comfortable-looking horse, but the disease continues unabated. This creates an ethical dilemma; balancing the need to alleviate pain and suffering against the realisation that most of what is administered is only palliative. When NSAIDs are in use, the patient should be confined to a stall with deep bedding. Exercise, while under the influence of painkillers, such as phenylbutazone, is contraindicated.

Cryotherapy

The results of experiments at the AELRU, continuously evaluating foot temperature (and by implication foot circulation), as horses developed laminitis, showed that vasoconstriction during the developmental stage of laminitis may have had a protective effect (Pollitt and Davies 1998). The induction of digital vasoconstriction may be a useful preventive strategy in the developmental phase of laminitis. Limited anecdotal evidence from practicing veterinarians suggests that cryotherapy may halt the development of the disease. The profound hypometabolic effect of cryotherapy is considered to be the most important mechanism by which cold limits the severity of an injury. Tissue metabolic rate and oxygen consumption are inversely related to temperature. A reduced requirement of cooled tissue for oxygen, glucose and other metabolites enhances the survival of cells during periods of ischemia. This mechanism is thought to protect tissue and is the basis for the use of cryotherapy in organ transplant surgery. A reduction in metabolic enzymatic activity of approximately 50% has been observed with a reduction in tissue temperature of 10°C. The activity of collagenases and pro-inflammatory cytokines is significantly reduced at lower temperatures.

Cryotherapy causes potent local vasoconstriction. This is largely mediated by sympathetic nervous control; however, a direct constrictive effect on blood vessel walls may occur, particularly at lower temperatures. Clinical recommendations for the duration and temperature of cryotherapy in horses are extrapolated from human medicine. Our recent studies have challenged these recommendations.

Cryotherapy: Potential Mechanisms for Preventing Lamellar Damage

The precise, molecular pathogenesis of acute laminitis is unknown. The diverse effects of cryotherapy, however, have the potential to interrupt many of the pathophysiological mechanisms that have been hypothesized to occur during the developmental and acute phases of the disease. A summary is presented in Table 1. Enzymatic degradation of lamellar attachments by matrix metalloproteinases (MMPs) forms the basis of our pathophysiological theory for developmental laminitis. It is hypothesized that the inappropriate release of excess, activated MMPs is mediated by “laminitis trigger factors” delivered to the foot via the digital circulation during developmental laminitis. The delivery of these triggers, which may include cytokines, protein fragments or bacterial products of hindgut origin, appear to be limited by cold-induced digital vasoconstriction during the developmental phase of laminitis. This was the basis for evaluating the use of cryotherapy for the prevention of laminitis. The potent local hypometabolic effect of cryotherapy could augment the vasoconstrictive effect on the digital vasculature. A cold-induced reduction in the local production and activity of MMPs would limit degradation of the lamellar attachments. A digital hypometabolic state would also limit the local production and activity of pro-inflammatory cytokines, such as interleukin and tumor necrosis factor, during the developmental stage of laminitis. Cryotherapy could also limit secondary inflammatory damage caused by white blood cell infiltration. Similar mechanisms are believed to be the basis for the efficacy of scalp cryotherapy in preventing alopecia in cancer patients undergoing chemotherapy. Vasoconstriction apparently reduces...
delivery of the chemotherapeutic agent to the scalp, and cellular uptake and metabolism are reduced when residual drug reaches the hair follicles.

The alternate pathophysiological theory for laminitis proposes that digital hypoperfusion during the developmental stage leads to lamellar ischemia and necrosis. Profound, cold-induced vasoconstriction would seem contraindicated if digital hypoperfusion was the primary mechanism behind the development of laminitis. However, despite a reduction in digital perfusion, the hypometabolic effect of cryotherapy could protect the lamellar tissue from ischaemic damage. Similarly, a profound cold-induced reduction in metabolism could protect the lamellar tissue from a lack of glucose (proposed as an initiator of lamellar separation in one study). Until the true pathophysiology of laminitis is discovered, the apparent resilience of the equine distal limb to prolonged, extreme cold may hold the key to successfully preventing the disease. Continuous distal limb cryotherapy during the developmental stage of laminitis has the potential to preserve the lamellar tissue until the systemic insult, occurring elsewhere in the body, has abated.

The Efficacy of Continuous Distal Limb Cryotherapy for the Prevention of Acute Laminitis.

Experimental Data. We have completed two controlled studies on the efficacy of cryotherapy for the prevention of laminitis. In the first study laminitis was induced in six horses using the oligofructose overload model. Each horse had one forelimb immersed in ice and water (mean temperature 0.5-1.7°C) for a 48 hour experimental period, achieving a mean internal hoof temperature of 3.5-0.9°C. All horses developed clinical and histological laminitis in one or more of the untreated limbs. The cooled limbs did not develop clinical laminitis and had significantly reduced lamellar histological damage. The study also showed significantly reduced up-regulation of lamellar MMP mRNA in the cooled limbs when compared with the untreated limbs. Although cryotherapy markedly reduced the severity of laminitis it did not completely prevent minor histological changes in 4 of the 6 horses.

In a second study cryotherapy was applied to all 4 limbs of 6 horses for 72 h. Laminitis was induced as before and the observation period was extended until 7 days post oligofructose dosing. The horses showed either no or very mild clinical signs of laminitis and histology of lamellar tissues taken 7 days post induction showed no laminitis. Control horses were lame at 7 days and had moderate to severe laminitis histopathology (van Eps et al. 2004).

Cryotherapy was instigated immediately following administration of the carbohydrate induction bolus in these studies. In a clinical case of grain overload or acute colitis such prompt initiation of cryotherapy may not be possible. It is unclear whether such a potent prophylactic effect would occur if cryotherapy was initiated later in the course of the disease when lameness was already present. Thus the potential of cryotherapy to prevent laminitis has been demonstrated and further clinical evaluation of the technique is justified.

Clinical Data
Anecdotal evidence of the successful use of cryotherapy to prevent acute laminitis has surfaced following the initial evidence-based recommendations for its use. The authors have trialed continuous distal limb cryotherapy for the prevention of laminitis in 7 cases of acute colitis (5 Thoroughbred geldings, 1 Thoroughbred colt and 1 Arab mare). All cases presented
with fever (>39.5°C), profuse watery diarrhea and signs of endotoxaemia and circulatory shock (injected mucous membranes with poor capillary refill time, rapid heart rate and depression). Only one horse had signs of laminitis before the initiation of cryotherapy. This horse had increased intensity of digital pulses in all four limbs, though lameness was not obvious. All cases were placed into a plastic tub with a rubber floor. Shoes, if present, were not removed. Water, then cubed ice, was added to the tub to submerge the fore and hind limbs. The level of ice and water was maintained at the upper third of the cannon bones. Approximately 100 kg of cubed ice was required to cool the water initially. Subsequently, 50 kg of ice was added at 4- to 8-hour intervals to maintain the temperature within the bath at less than 5°C.

All horses were treated (while in the cold bath) with intravenous polyionic fluids and plasma, antibiotics, NSAIDs and activated charcoal and paraffin oil by nasogastric tube. Lucerne hay and water were provided ad libitum. The cases were monitored constantly and remained in the cold bath for a minimum of 72 hours. All horses tolerated the cold bath well, without attempting to escape. The decision to remove the horses from the cold bath after the 72-hour period was based on resolution of clinical signs. Each horse was removed when the rectal temperature stabilized below 38.5°C, the manure was formed, and the mucous membranes returned to normal color. Five of the horses were removed at, or shortly after, 72 hours. The remaining 2 horses were removed from the bath at approximately 96 hours. None of the horses were lame on removal from the cold bath; however, all had increased intensity of digital pulses in all four limbs for the ensuing 24 hours. Variable distal limb oedema was also present. One horse that had signs of incipient laminitis before commencement of cryotherapy was mildly lame between 12 and 24 hours after removal from the cold bath. The lameness disappeared over the subsequent 10 days of hospitalization and radiographs of this horse revealed no displacement of the distal phalanx within the hoof capsule. It is unclear whether cryotherapy reduced the severity or had no effect on the development of laminitis in this case.

The remaining 6 horses were sound throughout the hospitalization period, and no lameness was detected on subsequent re-examinations 4 to 6 weeks later. All horses have returned to athletic activity, reportedly at previous levels. At the time of publication, three of the Thoroughbred horses have won metropolitan races since discharge. After examination of hospital records, the authors estimate the incidence of acute laminitis in previous similar cases of acute colitis (that were not treated with cryotherapy) to be 40 to 50%. Although these are very limited numbers, the authors believe the prophylactic use of continuous distal limb cryotherapy in similar cases at risk of developing laminitis is worthy of further clinical evaluation.

Application Methods
Any means by which the distal limbs can be continually exposed to temperatures of 0 to 5°C is acceptable. The cooling method should include the hoof and its solar surface. We suggest cooling the limb up to the top of the cannon, as this appears to result in more effective cooling of the lamellar region. Cooling just the feet is not enough. Ice and water immersion is effective, practical and inexpensive. Commercial cryotherapy cuff devices could be modified to include the hoof, though this is practically difficult. These devices are usually designed for compression as well as cooling. The effects of prolonged compression on the equine distal limb are currently unknown. The authors have had experience with a range of boots and tubs for ice and water immersion.
We have found that the use of a tub, 200 cm long, 80 cm wide and 50 cm high, most practical for prolonged, continuous application of cryotherapy to all four limbs. A water-tight door at one end for ease of access, and a rubber floor are suggested. Temporary or permanent stocks, together with cross-tying the head may assist in keeping the horse stationary. A refrigerated pump, re-circulating water at around 2°C, can reduce or replace the requirement for ice. Overall, vigilance should be exercised to maintain immersion temperatures below 5°C to maximize the protective effect.

Continuous distal limb cryotherapy shows considerable promise as a technique for preventing acute laminitis. The authors continue to evaluate cryotherapy in clinical cases at risk of developing laminitis, and welcome correspondence from others engaged in similar pursuits. Currently the most challenging aspect of cryotherapy in the clinical situation is the identification of cases that will develop laminitis, and subsequently deciding when to initiate and cease cryotherapy in these cases. A biological marker to identify horses at imminent risk of developing laminitis is needed. Such a marker would define the clinically silent developmental phase of laminitis in individual cases, and greatly improve the potential for prevention of the acute disease. Undoubtedly genetic markers exist for the early identification of horses developing laminitis. Up-regulation of MMP-2 mRNA early in the acute phase of laminitis has been demonstrated in lamellar tissue. If this process begins during the developmental phase of laminitis, particularly within the blood, skin, ergot or chestnut tissue, a diagnostic potential exists. The eventual discovery of the exact pathophysiology of laminitis will surely lead to effective and direct methods of prevention and therapy. In the meantime, the apparent resilience of the equine distal limb to prolonged, extreme cold can be harnessed and may hold the key to successfully preventing the disease.

Management practices to avoid pasture associated laminitis.
There is now strong circumstantial evidence that fructan in the hindgut of horses may trigger laminitis. Horses can ingest fructan rich pasture rapidly in amounts exceeding (Longland and Byrdy 2006) that used to induce experimental laminitis (van Eps and Pollitt 2006).

Owners of horses predisposed to laminitis should develop strategies to reduce risk. Most horse owners in New Zealand and Australia are committed to pasture feeding regimens throughout the year so a combination of both pasture and horse management practices need to be considered. The aim is to reduce the concentrations of water soluble carbohydrate (WSC) in pasture and to prevent its consumption by the grazing horse.

Pasture factors
Some pasture species are notorious fructan accumulators (they are selected and bred for this) and if possible should not be fed to horses. The WSC content of grass can reach 56% of its total dry matter (DM) of which fructan can be 44%. Grass that is actively growing tends to store less WSC. Maintaining soil moisture and fertility and keeping grass short by mowing or grazing encourages leaf growth and WSC consumption (Watts and Chatterton 2004). WSC accumulation in grass is driven by photosynthesis and takes time to occur. It peaks in the afternoon and early evening and high WSC intake can be avoided by allowing grazing only in the early morning. Likewise pasture shaded by tree-lines and windbreaks accumulates less WSC and susceptible horses can be strip grazed behind electric fences in these areas. Some horse managers will poison selected paddocks to eliminate pasture altogether and at time of high risk keep their horses on these “dry lots”. Times of risk are conditions of high light intensity and low ground temperatures such as in spring and autumn and particular care is indicated at these times. Under these conditions photosynthesis and WSC production is
relentless but growth and metabolism is slow; hence WSC accumulation. Using a cash flow analogy; the bank balance is greatly in credit – cash income exceeds expenditure. Drought or periods of low soil moisture may also drive WSC accumulation and even dry looking pasture can have a high WSC concentration. Drought breaking rain can also be a trap. WSC accumulated in subsoil roots during dry times is rapidly mobilized to new shoots and many a pony has foundered on insignificant looking pasture after rain. Another trap is slashed or heavily grazed pasture or stubble after harvesting. Most of the WSC of grass is stored, not in the green leaves, but in the lower, pale green stems that as a WSC reservoir. Grass that has gone to seed in summer is usually low in overall WSC content in its leafy tissues but could still pose a risk from the starch in the seeds. A yield of starch from the seed of perennial ryegrass has been estimated at 360kg/ha per growing season (Longland and Byrdy 2006). Horses will selectively strip seed from standing pasture and could conceivably consume sufficient starch to trigger laminitis from hindgut fermentation.

**Horse factors**
Grazing muzzles have been successfully used to limit grass and thus WSC intake by horses at pasture. The hole in the muzzle limits intake and confines it to leafy tops that are lower in WSC content. When horses and ponies have no access to pasture and are yarded or confined to dry lots what are they to be fed? The usual solution is grass or forage hay. However the haymaking process may not always reduce WSC and sometimes the most innocent looking hay may have dangerous WSC levels. If possible choose hay made from mature seeded, pasture made in summer. Hay could still be dangerous if harvested during periods of plant stress such as autumn and spring. Analysis of the WSC content of such hay is warranted but not always practical. Fortunately soaking hay in fresh water leaches out WSC (but not starch) and reduces the WSC content significantly. Sixty minutes of soaking and draining removed an average of 31% of the soluble sugars from 15 hay samples (Watts and Chatterton 2004).

Pony breeds in particular are prone to obesity and insulin resistance and obese individuals are at high risk of developing laminitis. The diet of obese individuals can be modified so that energy is derived from fat and fibre rather than from high glycaemic sources. Owners should monitor the body weight and learn to condition score their horses aiming for more optimum weights. Insulin resistance can be reversed by weight reduction and regular exercise.

“Founderguard” is an antibiotic formulation that can be fed to horses and ponies at pasture and when present in the hindgut limits the proliferation of *Streptococcus bovis*. When ‘predosed’ it may control hindgut carbohydrate fermentation to levels that prevent serious laminitis.

**Conclusions**
Ideas on laminitis pathophysiology abound (Hood 2004) and this review has focused on the MMP, enzymatic theory of laminitis pathogenesis, an hypothesis that depends on the generation of circulating toxins, or proinflammatory mediators (laminitis trigger factors) in the gastrointestinal (carbohydrate overload) or reproductive (septic metritis) tracts. A weakness of this hypothesis is how can laminitis trigger factors pass through the lung, kidney and liver without inducing significant pathology (Hood 1999)? Perhaps it all comes down to the unique anatomy of digitigrade equids. MMP activation and basement membrane dysadhesion may be ubiquitous to the epithelia of many organs but without the influence of weight bearing any resultant pathology is transient. However weight bearing BMs such as those within the lamellar dermal epidermal interface of horse’s feet, separate under tension, a process that may escalate into a cascade of ever increasing severity. The validity of this
proposition will be tested when veterinary researchers learn how to unload the feet of horses during the developmental phase of laminitis.

Half of laminitis science is incorrect – but which half? Out of the nebulous laminitis data of the last 60 years patterns and trends are appearing from which principles are crystallizing into veterinary consciousness. Although we have made some progress towards achieving the goal of the AELRU there are still gaps in our knowledge. The biological basis of laminitis has become molecular and the discipline of molecular biology has laminitis in its cross-hairs. These are exciting times to be involved in equine research – we now have tools our forefathers would not have thought possible. A coherent body of knowledge will soon emerge that will demystify laminitis.

Ethics statement
All experiments on horses, conducted by AELRU, are approved by The University of Queensland Animal Ethics Committee (constituted as per the National Health and Medical Research Councils "Australian code of practice for the care and use of animals for scientific purposes" which is embedded in the "Queensland animal care and protection Act 2001") and all horses under experimentation are inspected by an Animal Welfare Officer.

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References


EQUINE MESENCHYMAL STEM CELLS: WHAT ARE THEY? WHERE ARE THEY? HOW DO YOU FIND THEM?

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Introduction
In recent years, stem cell technologies have been developed for a range of pathological conditions, including the repair and regeneration of neuronal and musculoskeletal tissues, prevention of myocardial fibrosis following coronary infarction, stimulation of neo-angiogenesis after ischemic insults, and immune-mediated diseases such as Crohn’s disease and Host vs Graft Disease. This therapeutic versatility follows stem cells’ capacities for substantial proliferation, differentiation along several cell/tissue lineages, their stimulation of host reparative processes and their suppressive influence on dendritic cells and T lymphocytes.

This presentation will focus on adult equine mesenchymal stem cells, since their capacity to differentiate into bone-, cartilage- and tendon/ligament-forming cells is particularly relevant to musculoskeletal problems experienced in equine practice.

Mesenchymal Stem Cells: What are they?
Stem cell populations vary considerably in the putative properties listed above. Embryonic stem cells (ESC) are derived from the inner cell mass of early blastocysts. ESCs are “totipotent” in that they are capable of generating all cell types in the body. This property is assayed experimentally through the ability of ESCs to form teratomas following subcutaneous injection in SCID mice. ESCs also retain high telomerase activity and are capable of limitless replication; in effect, ESCs are immortal. “Pluripotent” stem cells are defined as being capable of generating tissues representative of all three germ lineages, ectoderm, endoderm and mesoderm. Finally, “multipotent” stem cells are capable of restricted differentiation along several cell lineages within a developmental group. As examples, hematopoietic stem cells (HSC) are conventionally considered to be able to generate all blood cell types, such as neutrophils, lymphocytes, erythrocytes and platelets. HSCs can be identified, isolated and sub-typed by fluorescence-activated cell sorting (FACS), utilizing a battery of cell surface proteins, or CD markers, that correlate with phenotype and differentiation potential.

Mesenchymal stem cells (MSCs) are capable of differentiating along several connective tissue lineages, such as osteoblasts (bone), chondrocytes (cartilage), adipocytes (fat), myoblasts (muscle) and teno-fibroblasts (tendon, ligament and meniscus). In contrast to the sophisticated FACS typing developed for HSCs, there is no agreed-upon cell sorting procedure for MSCs. The cell surface markers linked to MSCs are predominantly expressed after these cells have been placed in in vitro culture and so cannot be used to screen biological samples at the time of collection.
Recent work has suggested that stem cells are capable of considerable phenotypic plasticity beyond the confines implied by “pluri-“ and “multi-“potency, but cells in both these categories lack telomerase activity and are, as a consequence, subject to replicative senescence like other somatic cells.

Equine MSCs: Where are they?
Much of the research on MSC isolation and differentiation processes has been carried out with cells isolated from bone marrow aspirates. In contrast to the known locations stem cells that support intestinal mucosa and skin, the specific location(s) of resident MSCs within the bone marrow cavity, the so-called stem cell “niche”, is not known but is a topic of considerable interest at the moment. The marrow HSC niche is provided by osteoblastic stromal cells, but it is not known whether MSCs share this location, occupy a distinct milieu or inhabit a diffuse “niche” within the bone marrow compartment.

The latter option seems likely, in light of the fact that MSCs can also be isolated from a wide range of tissues and body fluids, including adipose tissue, skeletal muscle, periosteum, peripheral blood and synovial membrane. The Stewart lab has successfully isolated MSC-like cells with substantial proliferative capacity and multi-lineage differential potential from tendon tissue and from synovial fluid. It can probably be assumed that MSCs exist at low cellular concentrations in almost all tissues and body fluids. Estimates of stem cell prevalence vary between studies and anatomical locations, but most estimates fall within the range of 0.01-0.001% of total nucleated cell numbers in any given cell preparation.

Equine MSCs: How do you find them?
As noted above, hematopoietic stem cells can be identified and isolated using panels of CD surface markers, with FACS or magnetic beads. Unfortunately, similar recognition panels have not yet been developed for MSCs. The established techniques for isolation of MSCs are based on the high proliferative capacity of MSCs relative to other somatic cell types.

Bone marrow-derived MSCs (BM-MSCs) are usually isolated from horses from sternal or tuber coxae aspirates, collected through Jamshidi biopsy needles. Marrow aspirates contain many cells of the hematopoietic lineage, and so many researchers separate these cells from putative MSCs using Ficoll gradient centrifugation. MSC isolation protocols from other tissues and body fluids usually dispense with gradient selection and depend upon relative proliferation rates over several passages to select for stem cells. Selective adherence to plastic substrates or surfaces coated with matrix proteins such as fibronectin or laminin have been used to add an additional level of selection for stem cells, but these approaches are not consistently applied and are probably not necessary.

Accepting that MSCs are present at concentrations of between 1:10 000 and 1:100, 000, considerable proliferative selection pressure needs to be placed on an initially mixed cell population to generate a predominantly MSC population. Given that MSCs are not immortal and are subject to replicative senescence, it is not surprising that this selection process fails on occasion.

The designation of any given cell as a bona fide “mesenchymal stem cell” is a contentious issue at this point in time, since no unequivocal genetic or expressed markers have been identified to date. Many cell populations are capable of exhibiting multipotentiality, in a qualitative sense, in standard in vitro differentiation assays, but there is little correspondence
between *in vitro* activities and *in vivo* differentiation and regenerative capacity. The necessity for pure populations of MSCs is itself a debatable issue, since there have been a number of claims for clinical success using isolated cell preparations that are self-evidently not pure MSC populations. Non-stem, stromal “contaminant” cells might also play a role in providing signaling cues to co-transplanted MSCs, as is believed to occur in marrow transplantation procedures that follow radiation or chemo therapy-induced hematopoietic depletion.
MESENCHYMAL STEM CELLS: DO THEY WORK?... IF SO, HOW?

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Introduction.
Stem cells have received a great deal of coverage in recent years due to the arguably unlimited potential they hold for reparative and regenerative biomedical applications and, in part, the ethical controversy associated with their use. Stem cells exhibit two properties that are of considerable value to regenerative applications. Firstly, they are capable of substantial proliferation, although only embryonic stem cells can be considered to be “immortal” in this respect. Secondly, stem cells are capable of differentiating along many cell/tissue lineages. Embryonic stem cells are totipotent in that they are capable of generating all tissues of the body. In contrast, adult mesenchymal stem cells (MSC) are considered to be multipotent, since their differentiation potential is restricted to several mesenchymal lineages, such as bone, fat, cartilage and ligament.

In fact, stem cell therapy has been applied for decades, with bone marrow reconstitution following radiation therapy and bone marrow grafting for orthopaedic procedures. More recent applications, however, involve ex vivo isolation of specific stem cell subpopulations, identified by surface protein epitopes and FACS segregation. Further, tissue engineering applications can entail in vitro expansion of stem cell populations, followed by directed differentiation along the desired tissue lineage. More recent discoveries suggest that stem cells more valuable in clinical settings through their ability to modulate host inflammatory, reparative and immune processes, as discussed below.

Strategies for therapeutic MSC use.
Stem cells as tissue builders. The use of MSCs in tissue engineering strategies is largely predicated on the capacity of these cells to differentiate along multiple tissue lineages, in particular, the osteoblast (bone), chondrocyte (cartilage) and tenocyte/desmocyte (tendon and ligament) cell types. There is a great degree of variation between studies in the amount of ex vivo processing of MSCs carried out prior to administration. At one extreme, MSCs are isolated and expanded, then induced to undergo differentiation along the desired tissue lineage before implantation. The in vitro requirements for MSC differentiation along the chondrogenic and osteoblastic lineages and assays for phenotypic assessment have been pretty well established. It should be noted that, although MSCs from a wide range of sources are capable of expressing chondrogenic and osteogenic markers and express these phenotypes in a qualitative sense, differentiated stem cells are biosynthetically far inferior to native chondrocytes and bone cells in terms of tissue synthesis and organization.

MSCs can also be implanted or injected into lesions in as undifferentiated cells, with the expectation that local, tissue-specific cues will provide the necessary differentiation stimuli.
A recent study by Wilke et al, from the Nixon lab at Cornell University (18), is an example of this strategy. In both these approaches, the cell preps can be administered along with recombinant proteins or genetically engineered to express growth factors, to improve subsequent differentiation and biosynthesis. At the other extreme, entire nucleated cell populations from bone marrow aspirates or fat biopsies can be immediately administered, without any significant attempt at stem cell isolation or differentiation. The current Vet-Stem protocol, in all likelihood, follows this approach. Although “purer is better” makes some intuitive sense in this context, it is probable that contaminating, non-stem “stromal” cells

**Stem cell as recruiters and coaches.** It has become increasingly apparent as a result of early tissue engineering attempts that, although MSCs can substantially improve reparative and regenerative responses, they are not themselves directly responsible for synthesizing the newly formed tissue. Rather, MSCs home to sites of damage (15) and, in turn, recruit host-derived cells to stimulate repair. Cell-labelling experiments have demonstrated that exogenous MSCs are able to home to local sites of injury after systemic administration, and it is a safe assumption that endogenous MSCs behave similarly. Based on these observations, it is now widely considered that MSCs exert their beneficial effects by recruiting host cells to sites of injury, modulating the inflammatory and neo-angiogenic responses and accelerating tissue repair by host cells (7), rather than directly synthesizing the regenerate/repair tissue themselves; activities collectively referred to as trophic effects by Caplan and Dennis (2).

Perhaps the best example of this application is MSC therapy for myocardial ischemia. Following coronary artery occlusion, the ventricular myocardium is subject to ischemia, followed by post-ischemic fibrosis. There is now a large body of evidence demonstrating that stem cells delivered intravenously, via intra-coronary catheterization or by direct intramyocardial injection, can significantly reduce ventricular myocardiocyte death and ventricular fibrosis, and improve cardiac function (6,12). Osiris Therapeutic’s “Chondrogen” clinical trial for MSC-mediated meniscal regeneration is also based on this strategy. The experimental data that supports the current clinical trial suggests that intra-articularly injected allogeneic MSCs act on synovial cells adjacent the meniscal lesion to effect repair (8).

Research on spinal cord regeneration from Mark Noble’s lab is also highly instructive regarding the specific conditions that might need to be developed for clinical success in any given regenerative context. The Noble group have determined that neuroepithelial stem cells need to be induced to undergo partial differentiation, with a restricted commitment to the glial cell lineages (oligodendrocytes and astrocytes), to effect optimal repair/regeneration (3). Further, co-culture experiments indicate that mesenchymal stem cells are able to mediate this lineage commitment (1,10), suggesting that effective spinal cord regeneration might require cooperative interactions between MSCs and resident neural precursors.

**Stem cells as immunosuppressive agents.** One of the obvious anticipated limitations of stem cell therapy relates to the induction of a host immune response to non-self, allogeneic donor cells. However, a number of studies have demonstrated that stem cells are both hypo-immunogenic and, more importantly for clinical use, are actively immunosuppressive (5,11,17). MSCs do not express high levels of the MHC class II antigens that induce non-self immune reactions. Further, MSCs suppress dendritic cell (antigen-presenting cell) activities and also inhibit T cell proliferation and activation, through secretion of interferon \( \gamma \) and interleukin 10.
Much of the data on MSC immuno-tolerance is based on in vitro mixed lymphocyte reaction assays. In vivo studies addressing this phenomenon are less convincing, suggesting that MSCs induce recipient responses through alternative immunological pathways (4, 9). MSC immunomodulatory activities are currently being assessed in human clinical trials for the treatment of the immune-mediated diseases Graft vs Host Disease (GVHD) that follows hematopoietic stem cell transplantation and Crohn’s Disease.

**Current stem cell applications in equine medicine and surgery**

To date, MSC therapies in horses have focused on the treatment of musculoskeletal injuries. Roger Smith and colleagues, in association with VetCell Bioscience, has developed a protocol for treating SDFT injuries with autologous, in vitro-expanded, bone marrow-derived MSCs (13,14). In this protocol, MSCs are injected directly into SDFT core lesions, around 1-2 months after the acute injury. The outcomes of over 100 cases suggest that ultrasonic indices of healing are accelerated and the prognosis for successful return to racing is improved; however, no prospective, blinded trials incorporating appropriate control groups have been reported to date.

The Vet-Stem protocol is based on the isolation of progenitor cells from subcutaneous fat biopsies. This therapy has been advocated for the treatment of digital flexor tendon strains, suspensory desmitis and osteoarthritis. An impressive collection of case report/testimonials are available for perusal on the Vet-Stem website, but no published studies are available to date. A case series of suspensory desmitis treated with the Vet-Stem product was presented at the 2007 Veterinary Orthopaedic Society conference, compiled from cases managed by six US equine specialty practices. Clinical success was achieved in approximately 70% of the cases, a figure that compares very favourably with outcomes reported using alternative therapies, but again, no appropriately controlled study is available for assessment.

Alan Nixon’s group recently published the results of a study assessing articular cartilage repair in an equine femoropatellar defect model (18). Fibrin gels seeded with bone marrow-derived MSCs stimulated the early phases of cartilage repair but did not appear to result in long-term improvements in the quality of repair.

**Conclusions**

The remarkable proliferative and multi-lineage capacities of stem cells promise substantial advances in regenerative medicine and surgery in the next decade, ethical issues notwithstanding. It is also obvious that we currently have a very limited grasp of the underlying biological activities of various stem cell populations. The recent exploitation of stem cell immunomodulation suggests that future use of stem cell technologies might have little or nothing to do with their ability to undergo differentiation and that maintaining “stemness” in these cells after transplantation might prove to be the primary objective.

The technical aspects of widespread stem cell therapeutic use are daunting at this point in time. Protocols for collection, storage and administration are still being optimized in human applications, and the costs of establishing these clinical infrastructures are enormous. Our own experiences with MSC collection from several sites in horses demonstrate that there is marked variability in the efficiency of “stem cell” isolation with current procedures. This variability may well be a biological reality rather than a remediable problem with collection techniques.
A clear indication of the relative benefits of stem cell therapies in horses, or any other species for that matter, will require randomized, blinded, prospective studies with carefully selected and matched patient bases and appropriate controls… far easier said than done. The problems with these requirements in an equine practice context are self-evident, particularly in light of the fact that “stem cell therapy” is already commonplace in some areas. Funds for large-scale equine experimental model-based trials are difficult to come by and, from a purely financial perspective, are not likely to be funded by the companies marketing equine stem cell products. Accepting these realities, we should maintain an objective perspective, if not a healthy scepticism, towards the reported outcomes of stem cell therapeutics (16).

References.


ISOLATION OF MULTIPOTENTIAL MSCS FROM EQUINE TENDON

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Introduction.
Mesenchymal stem cells (MSCs) are a promising resource for cell-based repair, particularly with musculoskeletal injuries. These progenitor cells are thought to be the predominant cell type involved in repairing native tissue. Isolation of stem cells and ex vivo expansion may be used to obtain higher numbers of stem cells to augment healing in tissues with low cellularity. Isolation of autogenous MSCs obviates immuno-compatibility and disease transmission problems with minimal morbidity to the donor site.

The majority of research addressing MSC isolation and biology has focuses on stem cells isolated from bone marrow. Bone marrow-derived MSCs are multipotential, and are able to differentiate along osteoblastic, adipogenic, and chondrogenic lineages. In recent years, it has become apparent that MSCs are present in low numbers in most tissues and body fluids, including fat, cartilage, muscle, and synovium. Stem cell isolation from mature tendon has not been reported. The purpose of this study was determine whether MSCs could be isolated from equine digital flexor tendons and to compare their multipotentiality for differentiation with that of bone marrow-derived stem cells collected from the same donors.

Materials and Methods.
Tissues were isolated from 2 normal horses for MSC isolation: tendon was obtained from the superficial digital flexor tendon, and bone marrow aspirates from the tuber coxae. Each horse was treated as a separate experiment. Tendon samples were digested with collagenase and filtered to remove the undigested debris. Both the tendon-derived and bone marrow aspirate cell samples were subjected to a preplating selection for 7 days to enrich the stem cell population. Tendon and bone marrow-derived MSCs were each passaged once to generate at least 25 x 10^6 cells for subsequent differentiation experiments.

To document the capacity of the tendon-derived cells to differentiate along multiple mesenchymal cell lineages, the expanded cells were placed in Adipogenic Induction Medium (AIM), Osteogenic Induction Medium (OIM), and Chondrogenic Induction Medium (CIM) at the following respective cell densities 2.1 x 10^4 cells/ cm², 4.2 x 10^3 cells/ cm², and 2.5 x10^5 cells/ pellet. Tendon-derived MSCs were supplemented with basal media and allowed 2 days to recover from trypsinization as a negative control. This experimental design was replicated with bone marrow-derived MSCs as a known positive control.

Immunofluorescence microscopy was performed for FABP-4 immunolocalization in the AIM-supplemented MSCs. FABP-4 is a marker of the adipogenic lineage. Osteocalcin immunolocalization was used to assess osteogenic differentiation in the OIM-supplemented
cultures, while aggrecan was used as a phenotypic marker of chondrogenesis for the CIM-supplemented MSCs. In addition, samples were stained with Oil red O for lipid, Alizarin red for bone mineral deposit, and Toluidine blue for proteoglycan content. RNA was collected from the CIM supplemented samples and analyzed for aggrecan and collagen type II expression.

**Results**
Both tendon and bone marrow-derived MSCs showed early signs of cytomorphologic differentiation following differentiation media supplementation. After 10 days, MSCs from both bone marrow and tendon were positive for FABP-4 immunofluorescence (Figure 1).

**Figure 1. Adipogenesis of tendon-derived MSCs.** Tendon-derived MSC monolayers cultured in adipogenic medium for 10 days stained positively for the adipocyte marker FABP-4. Bone marrow-derived MSCs were similarly positive for this marker.

Osteogenic differentiation of both tendon- and bone marrow-derived MSCs, as assessed by osteocalcin detection, was evident on days 14 to 21. Cell staining with alizarin red correlated with the immunofluorescence data (Figure 2).

**Figure 2. Alizarin Red staining of mineralized matrix in osteogenic cultures.** Both bone marrow-derived and tendon-derived MSCs stained positively for mineralized matrix after 14 days maintenance in osteogenic medium. These data were consistent with immunodetection of the osteoblast-specific marker, osteocalcin.

Tendon and bone marrow MSCs both increased expression of the chondrocytic markers aggrecan and collagen type II over time, as determined by real-time PCR analyses of mRNA levels (Figure 3).
**Figure 3. Induction of collagen type II and aggrecan mRNAs** in bone marrow-derived (BM-) and tendon-derived (tendon-) MSCs maintained in chondrogenic pellet cultures for 14 days.

**Discussion**

The results of these experiments indicate that multipotential stem cells can be isolated from flexor tendons of mature horses. These cells have the ability to differentiate along adipogenic, osteogenic and chondrocytic pathways with efficiencies comparable to bone marrow-derived MSCs. Preplating and other selection techniques will continue to be developed to maximize the yield of stem cells from tendon. Further work is necessary to determine the capacity of tendon-derived MSCs to aid in repair of tendinous and ligamentous tissues.
CURRENT ADVANCES IN INFLAMMATORY AIRWAY DISEASE

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Lower respiratory tract disease continues to be a major source of wastage in the performance horse industries, where the impact of even small decreases in pulmonary function can have large consequence on performance outcomes. This has led researchers in diverse geographical regions to investigate this syndrome and apply the broad term inflammatory airway disease (IAD) to horses with lower airway inflammation. However, a universally accepted definition of IAD did not exist until a consensus statement was recently published. These authors more narrowly defined IAD as \textit{non-septic inflammation} in horses with poor performance, exercise intolerance or coughing and which may or may not have excess tracheal mucus. Furthermore, the authors determined that this inflammation should be detected by cytologic examination of \textit{bronchoalveolar lavage fluid (BALF)} or via evidence of pulmonary dysfunction based on evidence of lower airway obstruction, airway hyperresponsiveness, or impaired blood gas exchange at rest or during exercise. One result of this consensus statement is that conclusions drawn from many of the studies conducted in Australasia and the United Kingdom on lower airway inflammation in young racehorse cannot be applied to IAD as investigators used tracheal aspirates (TAs) to detect the presence of lower airway inflammation. However, as these studies are most relevant to the situation in Australian performance horses, these results will provide the focus for this brief review and the term airway inflammation in racehorses (AIR) will be used instead of IAD.

\textbf{Definition and Prevalence}

Most studies on the prevalence of airway inflammation in young performance horses in Australian and the UK have defined disease as either the presence of increased proportions of inflammatory cells [neutrophils (>20%), mast cells (>2%) or eosinophils (>1%)] within fluids obtained from the lower airways and/or the presence of increased amounts of mucopus within the trachea. Given these definitions, airway inflammation causing clinical or subclinical disease is common in young, performance horses and has been reported in 11.3 to 50% of Thoroughbred and Standardbred racehorses. Furthermore, respiratory disease was found to be second only to musculoskeletal disease as the most common cause of poor performance, interruption of training, days lost to training, and premature retirement among racehorses in the UK and Australia. The resultant economic losses attributable to AIR are likely to be considerable, and originate from the costs attributable to poor performance, days lost to training, as well as the direct cost of veterinary fees, treatment and control measures.

\textbf{Risk Factors}

A number of management and training practices are reported to predispose racehorses to the development of lower airway inflammation. These include racing, training or intense exercise, long distance transport and co-mingling of horses. In addition, the age of horses appears to influence the development of AIR where studies have found that younger horses (two and three year olds) are more at risk of respiratory tract inflammation. Finally, the stable
environment has been implicated as a risk factor for development of respiratory disease in young horses. Many of these horses spend most of their day in stables and are therefore exposed to aerosolised foreign organisms, particles and gases in a continuous and cumulative manner. The potential consequences of this exposure to noxious stimuli are diverse and the resulting effects on airway health may be immediate or delayed.

**Aetiology**

There has been considerable debate in recent literature regarding the pathogenesis of lower airway inflammation in young performance horses and the exact aetiological agents contributing to its development. This debate has been further muddied due to the confusion regarding a definition of IAD and many different diseases are likely to be included under this term. It should be remembered that, by definition, IAD and AIR are merely ascribing the presence of inflammation. Therefore, any agent that is capable of inducing lower airway inflammation in horses is a potential cause of these syndromes. What is most important is how commonly these different agents are involved, as this information will influence treatment and management. Furthermore, it is important to recognise that this contribution is likely to vary considerably from country to country and even region to region. Finally, although there have been many proposed aetiological causes (see Figure 1), few of these have been substantiated by experimental or epidemiological studies. More research is needed to better define the role of these different agents, and particularly to determine if there are differences in the site of inflammation (large vs small airways) or the cell type involved (neutrophils vs eosinophils vs mast cell vs macrophage/lymphocyte) induced by these agents.

**Viruses:** For many years most respiratory disease and loss of performance in racehorses was attributed by owners, trainers and veterinary surgeons alike to viral infections. However, recent studies have questioned the role of viral infections in AIR and have revealed that most cases of lower airway inflammation occurring in young performance horses in Australia cannot be attributed to known equine respiratory viruses. The viruses investigated in these studies included equine influenza virus (EI), EHV-1, EHV-4, equine rhinitis A and B viruses, and equine adenovirus. In contrast, association between lower airway inflammation and respiratory viruses (EHV-1 and -4, EI) have been found in studies in the UK, but viral infections were significantly less common than bacterial infections.

**Bacteria:** In contrast to respiratory viral infections, a number of recent studies have shown that clinical or sub-clinical bacterial infections may be important contributors to AIR. The bacteria found to have a statistically significant association with lower airway inflammation in racehorses include *Streptococcus zooepidemicus*, *S. pneumoniae*, members of the Pasteurellaceae (including *Actinobacillus spp*), and *Bordetella bronchiseptica*. Furthermore, these same bacteria have been identified in studies in Australia, the UK and the USA. The role of anaerobic bacteria in IAD is less clear. Although it is well documented that these bacteria play an important role in pleuropneumonia, their isolation from horses with AIR is rare as significant parenchymal damage must be present before invasions can occur. Recent studies in the UK have demonstrated a role for Mycoplasma infections in IAD, particularly *M. felis* and *M. equirhinis*. However, these infections were relatively uncommon and one study in Australia failed to isolate Mycoplasma in any cases of AIR, though a second study did isolate these bacteria from a number of horses. This may reflect either regional differences or difficulties in isolation of these organisms. Alternate diagnostic testing modalities e.g. PCR may be required to better define their role in disease.
**Parasites:** Given the routine management procedures carried out in racing establishments, parasites are an unlikely to be a common cause of bronchitis and pneumonitis in young racehorses. However, moderate to marked numbers of eosinophils are occasionally found in TAs of racehorses in Australia and may be due to either pulmonary parasites or a hypersensitivity reaction. The most likely parasite involved in pulmonary disease of young racehorses is *Parascaris equorum*. Often it is difficult to arrive at a definitive diagnosis in these cases, but care must be taken not to rule out involvement of *P. equorum* due to a negative result for faecal floatation as the prepatent period for this parasite is 71 to 110 days, but pulmonary migration occurs 7 to 14 days after ingestion of infective larvae.

**Environmental Agents:** The environment in which racehorses are housed and the management procedures to which they are exposed is frequently at odds with the requirement of optimal lung function during maximal performance. Inhalation of a variety of different non-infectious agents has been proposed to cause AIR and many of these agents are found in the stable and training environment. The organic and inorganic compounds proposed to induce airway inflammation include bacteria, viruses, moulds, mite debris and their faeces, plant material, bacterial endotoxin, β-glucans and inorganic dusts. These airborne compounds are potentially able to induce airway inflammation either by initiating infection, by inducing allergy, by direct toxicity, or indirectly by overwhelming pulmonary defence mechanisms. Of these environmental agents, the most studied has been *endotoxin* and a number of recent investigations have highlighted its ability to induce airway inflammation and hyper-reactivity in either directly or in association with respiratory allergens.

**Exercise:** Exercise alone can result in non-specific airway inflammation. One study found horses undergoing 10 weeks of training had more airway inflammation than non-exercised stable mates. The cause of this inflammation was not documented, but exercise may cause increased deposition of irritant particles in the distal airways associated with the 20-fold or greater increase in minute ventilation that occurs during strenuous exercise. Strenuous exercise also impairs pulmonary macrophage function, alters peripheral lymphocyte function, and increases the concentration of cortisol in serum for up to 24 hours all of which may help predispose to infection or inflammation.

**Allergens:** There is much speculation that IAD is a precursor to the development of heaves and the same may be true of AIR. Therefore, the underlying pathogenesis may be similar and involve exposure to several factors acting in concert including aeroallergens, endotoxin, and small particles and fibers. The presence of elevated numbers of eosinophils or mast cells in TA and/or BALF of horses with poor performance, and the association of BALF mastocytosis with airway hyper-reactivity is supportive of a possible role of an allergic response in the pathogenesis of IAD/AIR.
Figure 1: Proposed aetiologic agents of lower airway inflammation in young racehorses (AIR).

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<tr>
<th>Infectious</th>
<th>Non-Infectious</th>
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<tr>
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<td>Equine influenza virus</td>
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<td>Equine herpesvirus-1</td>
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<tr>
<td>Actinobacillus spp</td>
<td>Equine influenza virus</td>
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<td>B. bronchiseptica</td>
<td>Equine influenza virus</td>
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<tr>
<th>Aerosols</th>
<th>Non-Infectious</th>
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<tr>
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<td>Equine influenza virus</td>
</tr>
<tr>
<td>Pollutants (ozone, SO₂, NO₂, CO)</td>
<td>Equine herpesvirus-4</td>
</tr>
<tr>
<td>Noxious gases (H₂S, NH₃)</td>
<td>Equine herpesvirus-1</td>
</tr>
<tr>
<td>Moulds, mites, pollen</td>
<td>Equine influenza virus</td>
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AIRWAY INFLAMMATION
Clinical Signs
The clinical signs of AIR may differ depending on the site of inflammation, the aetiological agent involved and the degree and type of inflammation present. Clinical signs are often subtle and may be easily missed or overlooked by the trainer, horse owner and veterinarian. Affected horses are typically afebrile, bright and responsive and have a normal appetite. Auscultable pulmonary abnormalities are rarely present, though abnormal breath sounds (e.g. wheezes) and mildly increased respiratory efforts may be present on rare occasions. When a re-breathing bag is used to facilitate auscultation of lung sounds, a cough may be elicited. This hyper-reactivity of the airways may also be detected upon palpation of the trachea. A slight abdominal lift on expiration may be observed intermittently and a mild elevation in respiratory rate may be present. However, the absence of bronchospasm and airway obstruction resulting in an overt and prominent increase in breathing effort distinguishes this syndrome from RAO or heaves. Mild serous to mucoid to mucopurulent nasal discharge may be observed in some horses with AIR. Occasionally horses with AIR may have more severe clinical signs and include fever, signs of depression, and anorexia. In these cases bacteria, and possibly viruses, are more likely to be involved.

Coughing may be acute or chronic and may occur intermittently. It is often observed while eating or early during exercise. **Coughing during exercise is considered the most useful indicator of lower airway disease.** Although coughing is the most common clinical sign of respiratory disease reported by racehorse trainers, it remains a relatively insensitive indicator and is only reported between 38% to 50% of the time. However, although coughing is relatively insensitive, it is specific indicator with up to 80% of horses that cough having AIR.

Finally, many racehorses with AIR do not exhibit overt clinical signs, and reduced exercise tolerance or poor performance may be the only sign reported. Evidence of airway inflammation via endoscopy or within a TA, a BAL or both has been reported in 65 – 75% of racehorses referred for poor performance but with no clinical signs or history of respiratory disease. These findings indicate that sub-clinical disease may be a common manifestation of AIR and the use of endoscopy and cytological evaluation of samples collected from the lower airways are necessary in order to fully investigate respiratory health of horses, particularly those with a history of poor performance.

Diagnosis
A variety of techniques have been used to detect lower airway inflammation in racehorses. However, interpretation of results from the various techniques is currently highly contentious. It must be remembered that different techniques sample different areas of the lung, which influences the results obtained. Therefore, these techniques are not interchangeable, and airway inflammation as determined by one method may not equate to a diagnosis of inflammation with other techniques. In addition, the various diagnostic techniques have different sensitivities and specificities for detection of airway inflammation. Thus, the method of diagnosis will influence the reported prevalence of AIR. Finally, strong debate exists regarding the definition of normality in terms of physical properties and cytological values for the different techniques. Evidently, the relationship
between “abnormal” values and respiratory health and performance requires further clarification.

**History and Clinical Examination:** A detailed history and thorough physical examination must be performed on all suspected cases of AIR. Careful auscultation of the lung fields, including use of a re-breathing bag, is recommended. However, other causes of pulmonary pathology found in young racehorses (e.g. EIPH) may present with similar history and clinical signs. Therefore AIR cannot be diagnosed from the history and clinical signs alone.

**Haematology and Biochemistry:** Horses with AIR rarely have systemic manifestations of disease with the results of haematology and serum biochemical examinations usually being normal.

**Endoscopy:** The use of endoscopy for examination of upper and lower airways and the guided collection of guarded samples from the lower airways should be a routine part of diagnosis of AIR. Mucociliary clearance mechanisms in normal horses are efficient, where mucus elimination keeps pace with production and secretion. When pulmonary irritation occurs, mucus production increases with resultant accumulation of mucus within the airways. Therefore excess mucus in the trachea and mainstem bronchi has been used as evidence for the presence of airway inflammation. Excess tracheal mucus is a common endoscopic finding in racehorses and has been reported in 22-50% of racehorses examined at rest via endoscopy. In addition, horses that cough frequently have excess mucus in their lower airways, with up to 55% of coughing horses having moderate to marked quantities of mucus.

**Cytological Evaluation of Lower Airways Secretions:** Cytological evaluation of secretions collected from the lower airways is commonly used for diagnosis of AIR. However, there is currently considerable debate regarding the technique of choice for detection of lower airway inflammation and subsequent interpretation of cytological findings. The two most frequently used techniques are TA and BAL. However, each of these techniques samples disparate areas of the lung and there is no correlation between the cytological findings of these samples when collected sequentially from an individual animal. Consequently, one should not assume that a “normal” finding obtained by either of these techniques indicates absence of disease in the entire lung. Collection of both samples concurrently is usually indicated in order to assess the overall health of the lower airways, particularly where a specific diagnosis is not readily evident, for example in cases of poor performance or coughing during exercise.

**Tracheal Aspirates:** Tracheal aspirates collect secretions, cells and debris that accumulate in the distal trachea and bronchi but which may also be derived from the more distal airways and alveoli. As such they provide a non-homogenous sample and are not representative of any one segment of the lung. Routine collection of TAs in horses in race training should be performed using guarded catheters via endoscopy. Technical prowess definitely influences the quality of sample obtained using guarded catheters, especially if bacteriological cultivation of these samples is required. Factors that help prevent contamination from occurring include rapid collection of the sample, small volume of infused sterile isotonic saline (10 to 15 ml), and advancement of only the inner catheter into the tracheal ‘puddle’ rather than the catheter as a whole. In
addition, if the horse has coughed frequently during the procedure, an increased risk of contamination with oro-pharyngeal organisms is likely, and these samples are rarely appropriate for bacteriological cultivation.

**Interpretation of Tracheal Aspirates:**

**Mucus:** Care must be taken in the interpretation of mucus present in the airways a few single mucus droplets in the trachea is considered normal and may reflect proximal movement of mucus during exercise. In addition, some racehorses living in stables, without overt signs of airway disease, occasionally have small ventrally confluent accumulations of mucus (Grade 1). Pooling of mucus, especially when this extend a considerable distance in the trachea (Grade 2 and 3), is regarded as abnormal and indicative of airway inflammation. Furthermore, Grade 2-4 tracheal mucus scores are associated with poor racing performance in thoroughbred horses.

**Epithelial Cells:** Tracheal aspirates from normal horses contain low to moderate numbers of epithelial cells, although increased numbers may be obtained when using endoscopic methods of collection. The epithelial cells present in TA’s are predominantly ciliated epithelial cells from the trachea and bronchi, but cuboidal cells from the smaller airways may also be observed. Squamous epithelial cells should not be present in TA’s from normal horses, but may be observed when oropharyngeal contamination occurs at the time of sampling or when there is upper airway dysfunction. As these cells are frequently covered by bacteria, samples in which these cells are observed should not be cultivated for bacteria.

Epithelial cell atypia may occur in normal horses (in low numbers) or in the presence of airway inflammation. However, there are many causes of airway inflammation and the presence of epithelial atypia is not specific for a particular diagnosis or aetiology. Claims that increased numbers of non-ciliated cells and ciliated tufts are observed in TAs from horses with viral infections and/or poor performance, and are therefore indicative of this condition, remain unsubstantiated. Furthermore, isolated ciliated tufts may be seen in any specimen where there is fragmentation of ciliated cells, for example acute bronchitis, and alone are not indicative of viral infections.

**Macrophages:** Pulmonary alveolar macrophages (PAM) are the most abundant type of inflammatory cell present in TAs from normal horses. Their presence, together with ciliated epithelial cells, is a pre-requisite for interpretation of TA cytology, as they originate from the terminal area of the respiratory tract and therefore indicate that all levels of the pulmonary tree have been sampled. Although PAMs are the most common inflammatory cell type in normal horses, increased numbers are rare in young horses with AIR and their significance is unknown.

**Lymphocytes:** Lymphocytes are present in low numbers in normal TAs and they may be difficult to accurately differentiate from other cell types present such as small macrophages, and epithelial cells. The numbers of lymphocytes may increase in cases of respiratory tract disease, but this is variable and no correlation has been made between cytological observations of this cell population in TA and specific disease processes.
Neutrophils: Although a population of moderately preserved neutrophils reside in horses’ airways, the relative percentage of these cells in normal horses is reported to be low. However, these cells respond to a large variety of stimuli, and their numbers may fluctuate rapidly. In addition, in normal horses neutrophils are generally found in higher proportions in TAs than in BALs. This possibly reflects the greater exposure to noxious influences, including bacteria, which may be present in the larger airway. Alternatively, tracheal secretions can derive from many areas of the lung, and increased numbers may therefore reflect this increased area of sampling.

Differential cell counts are frequently performed on TA samples to assist in the diagnosis of AIR. The dilemma with interpretation of these relative counts is to determine what value (if any) represents a significant change. Large variations in the relative percentage of neutrophils in TAs from apparently healthy horses have been observed within and between studies. In addition, there is no association between the relative number of cells in TA (including neutrophils) and poor performance, indicating the presence of these cells may not influence lung function. However, other studies in younger, more homogeneous populations of horses have found smaller variations in neutrophil ratios in normal horses, where the majority of clinically normal horses have fewer than 20% of the neutrophils in TA samples. Furthermore, a strong statistical association between presence of >20% neutrophils in TA specimens and signs of respiratory disease (i.e., coughing) in young racehorses and the likelihood of isolating significant numbers of bacteria exists. Further studies are required to better elucidate the role of this cell type in impairment of performance.

Eosinophils: Studies of clinically normal adult horses indicate that eosinophils are present in very low numbers in TAs. Increased numbers of eosinophils are considered abnormal and may occur in cases of ascarid migration and lung worm infestation. In these cases the relative percentage of eosinophils may be as high as 85% of cells. Due to management procedures practiced for racehorses, infestation with *D. arnfeldii* is unlikely in the majority of cases. However, identification of adult ascarids in the small intestine of racehorses at post mortem is not an uncommon finding. Smaller elevations in the number of eosinophils in TA occur in the absence of parasitic infections and are interpreted as evidence of a type I hypersensitivity response to inhaled allergens.

Mast Cells: Mast cell may be identified by their characteristic staining granules, which are more easily observed where metachromatic stains are used (e.g., Toluidine blue or Leishmann’s stain). Mast cells are rare in TAs from normal horses, and is in contrast to samples obtained by BAL where higher numbers of mast cells (up to 2%) may be present. This difference may be explained by the predominant distribution of equine mast cells within secretions of the smaller airways and alveoli. There is little information on normal or abnormal percentages of mast cells in TA from horses, or the significance of alterations in mast cell numbers.

Bronchoalveolar Lavage: As stated earlier, inflammation of the lower airways is surprisingly compartmentalised, therefore BAL gives new information that cannot be obtained by TA. In addition, different cytological pictures of inflammation have been recognised in studies of BALF from young racehorse, leading to the speculation that different aetiological agents or environmental exposures may be important to inflammatory phenotypes diagnosed with this technique.
Bronchoalveolar lavage yields cellular constituents and extracellular proteins from the epithelial surface of the pulmonary alveoli and terminal airways (segmental bronchi and bronchioles) and as such reflects the changes occurring in small airways in the region of the lung that is lavaged. In studies of horses with diffuse pulmonary disease, cytological examination of BAL fluid accurately reflects histological abnormalities and it is a more reliable technique than TA for investigation of peripheral or chronic pulmonary inflammation. Furthermore, a single BAL sample has been shown to reflect the composition of BAL fluid throughout the lungs in horses with diffuse pulmonary disease such as heaves, but the equivalent study has not been performed in younger horses with AIR.

In young racehorses without signs of lower respiratory tract disease the majority of cells observed in BALF are macrophages and lymphocytes with differential cell counts of macrophages (59-68%) and lymphocytes (28-32%) reported. Macrophages should appear uniform with minimal cytoplasmic vacuolation. The majority of lymphocytes in equine BAL fluid are T lymphocytes and approximately half are helper T cells (CD4+) lymphocytes, and half are suppressor T (CD8+) lymphocytes. Non degenerate neutrophils constitute less than 5% of the total cells, and occasional eosinophils and mast cells are observed, where values of <2% for mast cells and <1% for eosinophils have been advocated as normal in young racehorses. However, higher values for the relative numbers of mast cells have been reported by others in normal horses but may reflect an older population of horse and the environment in which these horses were maintained. Relatively few epithelial cells are observed in BALF from normal racehorses and these are predominantly cuboidal or non-ciliated columnar epithelial cells. Identification of squamous epithelial cells or feed material in cytological preparations may indicate pharyngeal contamination of the BAL sample during catheter passage.

The significance of airway inflammation observed in BALF in young performance horses is not well defined. However there is an increasing body of evidence to suggest that changes in cell populations in samples obtained by BAL may reflect physiologically significant processes. For example, horses presented for poor performance and endoscopic evidence of airway inflammation have elevated neutrophils (>5%), lymphocytes and macrophages in BALF. In addition, airway hyper-reactivity occurs in young performance horses with exercise intolerance and is correlated with elevations in either eosinophils, mast cells or lymphocytes in BALF. Finally, horses with elevated total nucleated cell counts or absolute neutrophil counts in BALF have more severe exercise-induced arterial hypoxaemia as compared to control horses.

**Lung Function Testing:** Lung function tests such as measurement of maximal change in pleural pressure during tidal breathing, pulmonary resistance and dynamic lung compliance have been used to document the severity of airway obstruction in horses with heaves. However, the variability in these functional tests makes them of little value for confirmation of diagnosis unless the animal is so severely affected that affirmation is unnecessary. It is not therefore surprising that these tests have little value for the diagnosis of AIR where the pulmonary pathology and structural changes are significantly less than observed in heaves.
Since the early 1990s several groups around the world have attempted to determine the usefulness of alternate techniques for diagnosis of airway dysfunction in horses with AIR. Airway hyper-reactivity, in response to provocation with histamine or metacholine, has been demonstrated in groups of young performance horses with exercise intolerance. In the differential cell count of BALF obtained from these horses either eosinophils, mast cells or lymphocytes predominated. However, although this technique reveals differences between groups of horses (IAD vs control) it lacks appropriate sensitivity for demonstration of significant differences between individuals. Further refinement of this test, or examination of alternate techniques such as capnography or scintigraphy, may help resolve this issue. Thus, although the volume of information relating to respiratory function testing in horses continues to grow, in particular with reference to IAD, at this time the sensitivity of reported techniques limits their efficacy as routine clinical tools for assessing horses with this syndrome.

Treatment

Treatment strategies for AIR will be predicated to a large degree by the inciting cause of the inflammation, the horse’s clinical signs, type of inflammation demonstrated in the lower respiratory tract, owner/trainer/manager compliance, cost of medication and prior experiences of the attending clinician. As outlined earlier, there is a growing body of evidence to suggest that most cases of AIR in young performance horses are not the result of viral infection. Environmental factors are implicated as being key in the pathogenesis of this disease, with bacteria being variably implicated. As a result the major aims of treatment should revolve, where possible, around identification of aetiological agents, removal of the inciting cause, and management of airway inflammation. In addition, the presence of low-grade airway obstruction due to bronchoconstriction is postulated to occur in some cases as a result of airway inflammation, and may be contributing to the clinical effects observed. Although this effect is by no means proven in all cases of lower airway inflammation, the use of bronchodilators may be indicated as part of the therapeutic plan for this syndrome. Thus, given our current understanding of AIR, therapy for this syndrome should be designed to achieve three main goals; environmental management, treatment of airway inflammation and bronchodilation.

Environmental Management: Ideally medical management should be performed in conjunction with procedures to decrease exposure to pro-inflammatory agents present within the horse’s environment. This may involve a change in the horse’s environment, but will be dictated by facilities available and ambient environmental temperatures. For example in more temperate climates and where space is readily available, it is advisable to house horses with AIR either outdoors or in stables with an open configuration thereby allowing optimal air quality. In addition, bedding known to have lower concentrations of respirable dust or other airway irritants such as endotoxin (e.g., large wood shavings, paper, peat moss) versus those likely to be contaminated with airway irritants (e.g., poor quality or old straw bedding, rice hulls or deep litter systems using shavings) should be used. Appropriate ventilation of the stable environment is essential also, with at least 8 air changes per hour required. Use of low-dust forages is recommended as traditional feedstuffs such as hay are likely to increase the concentration of pollutants and allergens in the horse’s breathing zone by 3–6-fold when compared to low dust alternatives. Alternate methods for reducing dust challenge to horses via alterations in feed include use of complete pelleted feeds or
silage and are preferable to wetting/soaking hay or use of artificially (i.e., kiln dried) cured hay.

**Medical management:** Judicious use of drugs selected for their anti-inflammatory and bronchodilator effects have become central to the management of asthma in humans and heaves in horses. Not surprisingly, a similar approach to AIR in performance horses has been adopted. However, care must be taken with assuming a close association between airway inflammation and bronchoconstriction in these younger horses and this association needs further investigation to improve recommendations for treatment. In addition, horses with a bacterial aetiology of their airway inflammation require judicious antimicrobial therapy.

It should be noted that most of the drugs and dosages recommended for medical management of lower airway inflammation are based on studies performed in horses with heaves. Despite this shortcoming, ‘good’ clinical responses in these younger horses have been reported when these treatment guidelines are adopted (Tables 1 and 2). In addition, sufficient evidence currently exists to suggest that non-steroidal anti-inflammatory and ‘anti-histamine’ drugs are ineffective for the treatment of AIR.

Medications for lower airway inflammation may be administered parenterally or directly into the airways in the form of aerosols. Administration of therapeutic substances via inhalation has the advantage of delivering high concentrations of the drug directly into the airways while minimising the amount absorbed systemically. This latter effect should optimise the therapeutic effect whilst reducing the risk of adverse side effects. Therapeutic aerosols may be produced by nebulising a solution or using pre-packaged solutions delivered via metered-dose inhalers (MDI). An alternative delivery system utilises dry powder inhalers (DPI) where the drug is inhaled as powder form. A variety of devices have been developed to improve delivery of aerosol to the lower airways of the horse and includes face masks with spacers and holding chambers (e.g., Aeromask®) and nose pieces (EquineHaler®, 3M Equine Inhaler®).

**Corticosteroids:** Given the current state of knowledge relating to lower airway inflammation in young performance horses, glucocorticoids should be considered one of the core therapies for this syndrome. Corticosteroids may be administered systemically (usually oral, intravenous or intramuscular routes) or via inhalation, with the risk of systemic effects and longer elimination half-times being greater when glucocorticoids are administered parenterally. In general, use of the shorter acting forms of these agents should be considered as it is anticipated the AIR will be reversible, unlike heaves. Three systemically administered corticosteroids are indicated in the treatment of IAD (see Table 2). These are prednisolone, given orally, dexamethasone and dexamethasone-21-isoniconitate. Dexamethasone may be given orally, via intravenous or intramuscular routes whereas the dexamethasone-21-isoniconitate is given intramuscularly. If dexamethasone is administered per os, an appropriate increase in the dose should be prescribed given the drug is about 50% bio-available when given by this route. Prednisone, an agent popular in the USA for many years for management of a variety of inflammatory disorders, has recently been shown to be ineffective in reducing airway inflammation and as such has no indications for use in horses with lower airway inflammation.
Generally, systemic and aerosol treatment of horses with AIR involves 5-7 days of medication with re-evaluation of airway inflammation at this time if possible. A recent study of inhalation therapy in horses with AIR showed most inflammation will be resolved by 7 days, but some horses required up to 14 days of therapy. This was particularly the case if horses were kept in fast work during therapy. Close monitoring of the efficacy of treatment is recommended. Traditionally horses have been ‘weaned’ off exogenous systemic corticosteroids using a tapering dosage schedule. Debate remains as to the necessity for this practice following short-term systemic administration of corticosteroids.

**Bronchodilators:** Bronchodilators may be indicated in some cases of AIR as they relax airway smooth muscle and relieve airflow obstruction, the latter being a likely consequence of airway inflammation. At present two main classes of bronchodilators have been used in the horse: β₂-agonists and anticholinergics. In general, these products appear to give best results when given via inhalation. However, there are products available for systemic use. It should be stressed that bronchodilators should not be used as the sole therapy for AIR because they do not suppress airway inflammation and do not reduce airway hyper-reactivity. In addition, prolonged use of β₂-agonists without corticosteroids has been found to induce receptor down regulation, which inevitably will render the drug(s) ineffective. In horses with significant airway obstruction, it is logical to administer bronchodilators prior to corticosteroids in order to optimise lung deposition of the latter product.

**Sodium Cromoglycate:** This agent is a mast cell stabiliser and has been recommended for use in horses with AIR involving a relative increase in mast cells (mast cells >2% in samples collected from the lower airway, particularly in BALF). In these cases administration of sodium cromoglycate (200 mg twice daily) has been shown to improve clinical manifestations of respiratory disease and bronchial hyper-reactivity. Sodium cromoglycate can be administered via a nebuliser or metered dose inhaler (MDI) using a face-mask. As sodium cromoglycate is a mast cell stabiliser, clinical effects are dependent on the prevention of mast cell degranulation and as such onset of action may be delayed for several days following onset of treatment. Again, it needs to be stressed that this agent will only be effective when airway inflammation is the result of histamine release from degranulated mast cells.

**Antibiotics:** Bacteria may be isolated from TAs collected aseptically in up to 50% of cases of AIR and therefore antimicrobial agents should be used in these selected cases. Bacteria most commonly involved include *Streptococcus* spp, *Pasteurella* spp, and *Actinobacillus* spp. These organisms probably colonise the lower respiratory tract from their normal site of residence in the oropharynx. *Bordetella bronchiseptica*, members of the Enterobacteriaceae and *Mycoplasma* spp may also be isolated from horses with AIR. Given the bacterial species involved, the antimicrobial agent of choice is usually penicillin G (see Table 2). However, administration of this drug, particularly to competition horses, may be difficult due to prolonged withholding periods. Several alternatives exist with one of the most popular choices being ceftiofur sodium due to a spectrum of activity suitable for bacteria routinely isolated from horses with AIR. Combinations of trimethoprim and sulfonamides are also utilised widely in horses with respiratory infections. Although this combination has a broader spectrum of activity than penicillin G, evidence exists that up to 30% of *Streptococcus spp* may have variable resistance and so their efficacy may be limited in some cases.
Oxytetracycline is commonly used to treat horses with LRT bacterial infections, as like trimethoprim-sulfonamide, it has a short withdrawal time prior to competition. However, the bacteria routinely isolated from cases of AIR have variable sensitivities to this antibiotic. Finally, enrofloxacin should rarely be used for respiratory infections in the horse and only if there is evidence of bacteria not sensitive to more commonly used antimicrobials.

Regardless of the antimicrobial agents selected for use in horses there is always the risk of adverse side effects. One of the most common and potentially devastating side effects is diarrhoea. In the majority of cases diarrhoea is readily reversible when the drug is withdrawn. Other rare yet reported side effects include ‘procaine’ reactions and 1 hypersensitivity reactions.

**Table 1:** Drugs given by inhalation suggested for use in horses for the management of AIR.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose delivered per actuation</th>
<th>No of doses per canister</th>
<th>Dose</th>
<th>Length of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bronchodilators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albuterol + Ipratropium</td>
<td>120 µg (+ 21 µg ipratropium)</td>
<td>200</td>
<td>1-2 µg/kg</td>
<td>1-3 hr</td>
</tr>
<tr>
<td>Ipratropium bromide</td>
<td>18 ug 0.02% solution for nebulisation</td>
<td>200 2.5 ml vial</td>
<td>0.5-1 µg/kg, 2-3 µg/kg</td>
<td>4-6 hrs, 4-6 hrs</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>25ug</td>
<td>120 (13g canister)</td>
<td>0.2-1 µg/kg</td>
<td>6-8 hrs</td>
</tr>
<tr>
<td><strong>Corticosteroids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beclomethasone</td>
<td>42ug</td>
<td>200 (16.8 g canister)</td>
<td>1-3 µg/kg, q12h</td>
<td>8-12hrs</td>
</tr>
<tr>
<td>Fluticasone</td>
<td>220 ug</td>
<td>120 (13 g canister)</td>
<td>2-4 µg/kg q12h</td>
<td>8-12 hrs</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium cromoglycate</td>
<td>0.02% solution for nebulisation</td>
<td>2 ml vials</td>
<td>200mg, q12h, 80 mg, q24h</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Drugs given parenterally suggested for use in horses for the management of AIR.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Route</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Corticosteroids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>1-2 mg/kg, q12-24h</td>
<td>Oral</td>
<td>Cheap; Well tolerated in feed or as paste; Well absorbed from GIT</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.05 – 0.1 mg/kg, q 24h</td>
<td>Oral, IV, IM</td>
<td>Effective; no more than 2-3 doses required</td>
</tr>
<tr>
<td>Dexamethasone-21-isoniconitate</td>
<td>0.02-0.04 mg/kg, q3d</td>
<td>IM</td>
<td>Should not require more than one dose. Effect may not be evident for 2-3 days</td>
</tr>
<tr>
<td><strong>Bronchodilator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>0.8 – 3.2 μg/kg q12h (oral); 0.8 μg/kg (IV) q24h</td>
<td>Oral, IV</td>
<td>Enhances mucociliary escalator function; some side effects – sweating, tremors with IV and high oral doses</td>
</tr>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethoprim-sulfonamide</td>
<td>15-30mg/kg q12h</td>
<td>Oral, IV</td>
<td>Active against many of the bacteria likely to be associated with AIR. Easy to administer per os, therefore popular. Diarrhoea reported.</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>22 mg/kg q12h for procaine salt. 22 mg/kg q6h for aqueous salts</td>
<td>IV aqueous salts IM procaine</td>
<td>Active against many bacteria associated with AIR. Procaine has long elimination time, restricting use close to competition</td>
</tr>
<tr>
<td>Ceftiofur sodium</td>
<td>2.2-4.4 mg/kg q12-24h</td>
<td>IV or IM</td>
<td>Active against many bacteria associated with AIR. Not registered for IV use. IM injections may cause muscle soreness. Diarrhoea reported.</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>6.6 mg/kg q24h</td>
<td>IV</td>
<td>Active against most gram negative organisms associated with AIR. Often combined with penicillin to broaden spectrum.</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>6.6 mg/kg q12-24h</td>
<td>IV</td>
<td>Popular with many racetrack veterinarians for low grade respiratory infections, due to rapid withdrawal times, IV administration and apparent efficacy. Diarrhoea reported.</td>
</tr>
</tbody>
</table>
Recommended Reading:
TREATMENT OF EQUINE SEPTIC ARTHRITIS AND TENOSYNOVITIS BY LOCAL ANTIBIOTIC DELIVERY VIA INTRA-ARTICULAR CATHETERS.

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Introduction
Septic arthritis and tenosynovitis are potentially lethal problems in horses that occur secondary to wounds, surgery, intra-articular injections or a hematogenous infection. In septic arthritis, irreversible damage to the cartilage matrix can occur within 24 hours, resulting in progressive deterioration of the joint. In light of the potentially serious consequences, early aggressive therapy is warranted in all cases of synovial infection in the horse.

Recent advances in the treatment of septic synovitis have focused on improving the delivery of antibiotics to the site of infection. Antibiotic delivery systems that have been utilized to deliver antibiotics to the joint include intra-articular catheters, constant joint infusion systems, intra-articular antibiotic-impregnated beads, and regional limb perfusion. The synovial concentrations of gentamicin have been evaluated for most of these delivery systems. The standard MIC for gentamicin is 2-8 μg/ml and concentrations greater than 8-10 times MIC are desirable to resolve infection and decrease emergence of resistant bacterial strains. Constant joint infusion systems deliver antibiotics continually to the joint. These systems result in extremely high gentamicin synovial concentrations, greater than 400 μg/ml. However, the joint infusion system allows only one antibiotic to be used at a time and the small diameter of the tubing prevents adequate flow of fluid to lavage the joint. When intra-articular antibiotic beads containing 300 mg of gentamicin were placed intra-articularly, synovial concentrations of only 27.9 μg/ml were attained. In addition, intra-articular beads caused a diffuse synovitis and superficial cartilage erosions. Regional limb perfusion with 1 gram of gentamicin infused into a distal limb vein attained synovial concentrations of 221 μg/ml. Levels of synovial gentamicin obtained with distal limb perfusion are significantly lower than levels obtained with an intra-articular injection. Furthermore, phlebitis is a common complication associated with regional limb perfusion and often limits repeated treatment.

The use of an intra-articular catheter for local antibiotic delivery offers several advantages; the system allows multiple antibiotics to be given concurrently. In addition, the diameter of the catheters utilized in the current study allows daily lavage of the joint without the need for general anesthesia. Finally, the concentration of antibiotics delivered to the joint through an intrasynovial catheter is equivalent to levels administered with the joint infusion systems or direct injections.
This clinical study assessed the efficacy of intra-articular catheters for delivery of intra-articular antibiotics in the treatment of septic arthritis in 29 horses. The outcomes were compared with the results obtained in a control group of cases treated with conventional, systemic antibiotic administration and joint lavage at the same institutions.

**Clinical Outcomes**

*Intra-articular catheters (IS Group)*

Twenty-seven out of twenty-nine (93%) horses treated with an IS catheter had clinical resolution of sepsis. One horse improved in lameness but developed colitis and died after 3 days of treatment. This horse was not included for further evaluation in the study. Eighteen (62%) horses were sound after treatment, while eighteen horses remained lame. One horse was lost to follow-up, and two horses were euthanized (1 foal and 1 adult). Both euthanasia cases had periarticular osteomyelitis in addition to septic arthritis. Of note, six horses (20.7%) treated successfully with an IS catheter had previously failed to respond to conventional treatment methods. Three horses had an infected synovial structure with a concurrent articular fracture. All three of these horses developed marked subsequent osteoarthritis despite resolution of the infection. One horse developed laminitis on the opposite support forelimb.

*(Control group)*

Twenty-one of thirty-one (67%) horses treated by conventional methods had clinical resolution of sepsis. Ten of thirty-one (32%) horses with synovial infections did not resolve. Sixteen horses (51%) were sound after treatment, eleven horses remained lame, and four horses were euthanized. At the time of follow-up the horses with persistent infection that were not euthanized had marked lameness (average grade of lameness 3.5/5).

*(Comparison of IS group to Control group)*

The use of an intrasynovial catheter (IS catheter group vs. control group) to administer intra-articular antibiotics 3-4 times a day significantly improved the clinical resolution of infection. In addition, the number of days for clinical resolution of infection was significantly shortened by treatment with the IS catheter. The IS catheter group had a mean of 4.2 days of treatment to resolution of infection with a standard deviation of 2.5 days. In comparison, the control group had a mean of 9.5 days of treatment to resolution of infection with a standard deviation of 9.2 days.

Despite expectations to the contrary, there was no effect of “time to presentation” (immediate vs. delayed) on outcome. The specific synovial structure involved (joint vs. tendon sheath) also had no effect on the likelihood of a successful resolution of infection. This remained true whether the treatment groups were combined or the IS group and the control group were assessed individually.

**Discussion**

The results of this study suggest that intra-articular catheters are a clinically useful means for delivering antibiotics to synovial cavities. Based on these outcomes, antibiotic delivery via IS catheters can be effective in chronic cases where the response to systemic therapy has been inadequate, although early and aggressive therapy is recommended in all cases of septic arthritis and tenosynovitis in horses.
Antibiotic delivery via IS catheters is not recommended in cases where co-existent osteomyelitis is present, since antibiotic penetration into necrotic bone is unlikely. Surgical intervention and debridement of infected bone is necessary for successful resolution of these cases.
INTRODUCTION. Septic arthritis generally presents as an acute onset of severe lameness with overt peri- and intra-articular swelling, heat and local pain. In neonates, septic arthritis occurs secondarily to bacteraemia and seeding of the synovium and/or subchondral vasculature and can affect one or several joints. In foals, this form of septic arthritis often follows or is concurrent with diarrhea, umbilical infection or pneumonia and is predominantly caused by enterobacterial species, such as Salmonellae and E.coli. In adult horses, septic arthritis typically follows direct trauma to a joint or as an extension of periarticular infection. This form of the disease is particularly common in the lower limbs of horses. Septic arthritis also occurs as an infrequent but alarming consequence of arthrocentesis or intra-articular surgery. Staphylococcal and streptococcal species are the most common pathogens in these cases.

Regardless of the etiology, septic arthritis is an expensive disease to treat and carries a guarded prognosis, particularly if athletic ability needs to be restored. Resolution of septic arthritis is often protracted due to compromised antibiotic delivery to the primary site of infection and the rapid, irreversible damage that frequently develops in articular tissues; capsular fibrosis, periarticular osteophyte formation and, most importantly, rapid and severe depletion of articular cartilage proteoglycan levels. Aggrecan-tethered sulfated glycosaminoglycans (sGAGs) bind water and provide resistance to compressive load. In septic arthritis, the aggrecan core protein is degraded, resulting in sGAG loss. This depletion can lead to persistent arthritis despite eradication of the active infection. In recent years, the major proteases responsible for aggrecan degradation in articular cartilage have been identified; members of the “a disintegrin and metalloproteinase with thrombospondin motifs” (ADAMTS) protease family, specifically ADAMTSs 4 and 5 (1). These proteases, rather than cartilage-associated MMPs, appear to be the primary aggrecanases responsible for pathological cartilage degradation.

TLRs are cell membrane-bound receptors that recognize selective pathogen-associated ligands (2). TLR2 is generally considered to recognize epitopes from Gram positive bacteria, such as peptidoglycan G (PepG) and lipoteichoic acid (LTA; 3), whereas lipopolysaccharide (LPS), or endotoxin, is the primary ligand for TLR4 (4). Activation of TLRs results in the expression of a range of inflammatory cytokines, interleukins, chemokines and other effectors that stimulate the host immune response.
Intriguingly, articular chondrocytes express several TLRs, despite residing in an avascular, alymphatic and aneural environment. TLR activation has been implicated in the pathophysiology of rheumatoid arthritis and crystal-associated arthropathies (5,6), but their role in sepsis-driven arthritis is not known. Therefore, this study addressed the role of TLR expression and signaling in equine septic arthritis.

**Methods**

**Joint tissue collection.** Articular cartilage and synovium was collected from clinical cases of joint sepsis immediately after euthanasia. Normal tissues from contralateral joints were also collected for comparisons. The tissues were snap-frozen in liquid nitrogen. Total RNA was isolated from these samples using routine GITC/phenol extraction followed by isopropanol precipitation.

**Chondrocyte collection and culture.** Articular cartilage was collected from the femorotibial joints of skeletally mature horses euthanized for reasons unrelated to musculoskeletal disease. For explant cultures, cartilage was diced into approximately 1mm-thick pieces and immediately placed in culture medium. Articular chondrocytes were isolated by sequential incubation in trypsin/EDTA followed by overnight digestion in collagenase type II in Opti-MEM, supplemented with antibiotics, at 37°C. Isolated chondrocytes were cultured as non-adherent aggregates in a defined, serum-free medium supplemented with ascorbic acid and antibiotics. Cartilage explants were cultured in the same medium. As required, cultures were treated with LPS, LTA, PepG, heat-killed Staph aureus or a neutralizing TLR2 antibody.

**RNA isolation and Northern blot analyses.** Chondrocyte aggregates were pelleted by low-speed centrifugation, followed by aspiration of the medium. Two ml of TriZOL was added to each tube and the cell pellet was immediately homogenized. Further processing of these samples followed the manufacturer’s recommended protocols. Gel electrophoresis and Northern blot analyses of RNA samples were carried out according to standard protocols. Radio labeled probes for equine Coll II, and EF1 alpha were prepared from cDNA templates using 32P-dCTP and random hexanucleotide primers. Prehybridization, hybridization and wash conditions followed protocols recommended by the manufacturer of the nylon membranes. Quantitative PCR was carried out by measuring Sybr green incorporation into newly synthesized cDNA, using a BioRad Real-time iCycler. Primers targeting 150-250 bp cDNA products were generated to assess expression of TLRs 2 and 4, MMPs 2, 3, 9 and 13, ADAMTS 1, 4 and 5, and the reference gene EF1 alpha.

**Sulfated GAG measurements.** Levels of sulfated GAGs present in the pericellular aggregate matrix and released into the culture medium were measured by the colorimetric dimethylmethylene blue (DMMB) assay (7). Serial concentrations of bovine chondroitin sulfate were assayed in parallel to generate a standard curve for these analyses.

**Results**

**TLR2 and TLR4 mRNAs are up regulated in articular cartilage of septic joints.** We compared TLR mRNA expression in cartilage and synovium from septic joints and in uninfected tissues from the contralateral joints, from three clinical cases of equine septic arthritis that were euthanized without treatment. Both TLR 2 and 4 mRNA transcripts were up regulated at least 5-10 fold above expression levels in
contralateral normal tissues from all clinical cases. The Gram category of the bacterial isolate did not appear to correlate with specific TLR up-regulation. Of note, TLR expression appeared to be highest in acutely infected joint tissues, suggesting that TLR up-regulation is an early response to bacterial infection. The up-regulation of both TLRs 2 and 4 in the cartilage indicates that articular chondrocyte and synovial TLR expression is responsive to joint sepsis.

**Bacterial ligands up regulate TLR expression in articular chondrocytes in vitro.** Chondrocyte aggregate cultures were treated with bacterial ligands or heat-killed Staph aureus for 24 or 96 hours. TLR ligand administration induced receptor mRNA up-regulation, suggesting the existence of a positive-feedback loop in this signaling pathway that might serve to amplify the immediate response to bacterial exposure by articular chondrocytes.

**Bacterial ligands stimulate articular cartilage matrix depletion.** Chondrocyte explants were maintained in control medium or treated with bacterial ligands for 48 hours. GAG released into the medium following aggrecanolyis was measured by the DMMB assay. As expected from the clinical consequences of joint sepsis, the TLR ligands directly stimulated GAG release from the explants. Exposure to TLR ligands also suppressed collagen type II mRNA levels in a dose-dependent manner. These results confirm that bacterial TLR ligands induce the critical pathological change evident in septic arthritis; aggrecan degradation and consequent GAG release from the extracellular matrix.

**Bacterial ligands stimulate expression of chondro-degradative enzymes.** The effect of bacterial ligand administration on expression of enzymes known to degrade articular cartilage was assessed by QPCR analyses. LPS, LTA and heated-killed Staph aureus resulted in significant increases in expression of MMPs 2, 3, 9 and 13, and of ADAMTS 1, 4 and 5. These results indicate that bacterial ligands are able to stimulate expression of degradative enzymes that cleave both collagen and aggrecan substrates in cartilage.

**TLR2 blockade inhibits the degradative response to heat-killed Staph aureus.** To test the functional significance of TLR activation in response to bacteria, articular chondrocytes were exposed to heat-killed Staph organisms in the presence or absence of an antibody that neutralizes TLR2 activity. The obstructive antibody significantly reduced sGAG loss in response to exposure to bacteria, indicating that TLR signaling is active in mediating cartilage degradation in joint sepsis.

**Discussion**

The results from these experiments indicate that TLRs are up-regulated in the articular cartilage and synovium of septic joints. Further, exposure of healthy articular chondrocytes to a range of known bacterial TLR ligands results in increased sGAG loss, suppression of collagen type II mRNA expression and up-regulation of several known degradative enzymes. Most importantly, TLR2 blockade reduced the catabolic effects of heat-killed Staph aureus in articular chondrocytes, indicating that TLR signaling is directly required for the degradative responses to bacteria.

TLRs are potential therapeutic targets for neutralizing agents (8) for chondro-protection, in concert with appropriate antibiotic therapy, for managing septic arthritis in horses.
References:
BMP SIGNALING AND MAINTENANCE OF THE ARTICULAR CHONDROCYTIC PHENOTYPE.

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Introduction
Articular chondrocytes (ACs) are highly specialized cells, responsible for synthesizing and maintaining articular cartilage (1). Interest in culturing articular chondrocytes have been fuelled by the need to apply tissue engineering principles to the treatment of articular cartilage injuries, as well as their use in research to better understand articular cartilage diseases. The differentiated AC phenotype is defined by its rounded cell morphology, and its expression of unique extracellular matrix genes such as collagen type II (Coll II) and aggrecan (Agg). Maintenance of the differentiated AC phenotype in vitro is highly sensitive to culture conditions. Retention of the phenotype requires a three-dimensional culture model, whereas culture under conventional monolayer conditions results in loss of the differentiated phenotype, or dedifferentiation.

Bone Morphogenetic Proteins (BMPs) are members of the TGF-β superfamily and have been shown to play a role in chondrocyte differentiation and matrix maturation. Exogenous BMPs exert considerable influence on the AC phenotype in vitro, suggesting that these reagents compensate to some extent for changes in endogenous BMP expression.

In previous studies, we have demonstrated a differential effect of exogenous BMP-2 on expression of AC-specific genes in monolayer cultures, where expression of these genes is routinely lost, and in non-adherent culture conditions that reliably support the differentiated phenotype (2). These data are consistent with the notion that endogenous BMP expression is lost in monolayer cultures, with this loss being mitigated by exogenous BMP administration, whereas nonadherent culture conditions support endogenous, autocrine expression of BMPs that preempt responses to any exogenous ligand. With these data in mind, the objective of this study was to determine whether endogenous BMP activity is required for the support of the AC phenotype in vitro.

Materials and Methods.
Chondrocyte collection and culture. Articular cartilage was collected from the femorotibial joints of skeletally mature horses ranging from 2 years to 10 years, euthanized for reasons unrelated to musculoskeletal disease. Articular chondrocytes
were isolated by sequential incubation in trypsin/EDTA followed by overnight digestion in collagenase type II in Opti-MEM, supplemented with antibiotics, at 37°C.

Isolated chondrocytes were cultured in a defined, serum-free medium supplemented with 50 µg ascorbic acid and antibiotics. Serum (FBS)-supplemented cultures had 10% FBS added to the media. Non-adherent aggregate cultures were maintained in hydrogel-coated low-attachment plates. Monolayer cultures were maintained in conventional 100-mm² culture dishes. As required, cultures were treated with recombinant human BMP-2, recombinant murine Noggin or soluble BMP receptor (R&D Systems, Minneapolis, MN) at the indicated concentrations.

RNA isolation and Northern blot analyses. For total RNA isolation from monolayer cultures, TriZOL was added directly to the cell layer. The lysate was collected using a cell scraper and the transferred to 15 ml Falcon tubes. The TriZol/cell lysate was homogenized and then stored at -80°C until further processing. For total RNA isolation from aggregate cultures, the chondrocyte aggregates were pelleted by low-speed centrifugation, followed by aspiration of the medium. Two ml of TriZOL was added to each tube and the cell pellet was immediately homogenized. Further processing of these samples followed the manufacturer’s recommended protocols.

Gel electrophoresis and Northern blot analyses of RNA samples were carried out according to standard protocols. Radio labeled probes for equine Coll II, Agg, BMP-2 and EF1 alpha were prepared from cDNA templates using 32P-dCTP and random hexanucleotide primers. Prehybridization, hybridization and wash conditions followed protocols recommended by the manufacturer of the nylon membranes. Northern blot results were quantitated by phosphor-imaging of membranes after post-hybridization washing.

Collagen type II ELISA. Coll II protein deposition was measured using the commercially available Native Type II Collagen Detection ELISA kit, according to the manufacturer’s suggested protocol.

Sulfated GAG measurements. Levels of sulfated GAGs present in the pericellular aggregate matrix and released into the culture medium were measured by the colorimetric dimethylmethylene blue (DMMB) assay (3). Serial concentrations of bovine chondroitin sulfate were assayed in parallel to generate a standard curve for these analyses.

Statistical analyses. The significance of differences between the quantitative data from the DMMB and collagen type II ELISA assays was determined by one-way ANOVAs, followed by Dunnett’s post hoc test, where appropriate. A p value less than 0.05 was considered to be significant. Each type of experiment was carried out at least twice, using cells isolated from different donors, to verify consistency in cellular responses.

Results.
Exogenous BMP-2 has differential effects on articular chondrocyte monolayers and aggregates. As expected, FBS supplementation strongly down-regulated collagen type II and aggrecan mRNA expression in articular chondrocyte monolayer cultures after 10 days in culture. Treatment with 100 ng BMP-2/ml increased
expression of these phenotypic markers in both serum-free and serum–supplemented cultures, but did not prevent the down-regulation of collagen type II or aggrecan mRNAs in serum-supplemented cultures. In contrast, levels of collagen type II and aggrecan mRNAs in aggregate cultures were not noticeably affected by FBS supplementation nor by BMP-2 administration.

**Endogenous BMP-2 expression is lost in monolayer cultures.** BMP-2 mRNA expression was undetectable by 7 days in monolayer cultures and paralleled the down-regulation of phenotypic marker genes. BMP-2 expression was consistently higher in articular chondrocyte aggregates and reached relatively consistent levels by day 7, concurrently with stable expression of both collagen type II and aggrecan mRNAs.

**BMP antagonists inhibit expression of the articular chondrocytic phenotype.** The functional importance of endogenous BMP expression was assessed by exposing aggregate cultures to known BMP antagonists, Noggin and a soluble BMP receptor chimeric protein. Both these agents compete with cell membrane-bound BMP receptors for endogenous BMP ligand and consequently inhibit BMP signaling activity. Both these BMP inhibitors dose-dependently suppressed collagen type II and aggrecan mRNA expression at both days 4 and 8, although there was evidence of reduced sensitivity to the inhibitors at the later time point.

**BMP inhibition reduces cartilage matrix synthesis.** The effects of BMP antagonism on cartilage matrix synthesis was assessed by measuring the synthesis and secretion of collagen type II protein and sulfated GAGs by articular chondrocyte aggregates exposed to Noggin. Noggin significantly suppressed collagen type II secretion at day 8. Deposition of sulfated GAGs into the pericellular matrix was also reduced by higher doses of Noggin at both 4 and 8 days in culture. Noggin also significantly suppressed the levels of sulfated GAG in the medium compartment, indicating a marked effect on net synthesis.

**Discussion**

The experiments presented in this study show that exogenous BMP administration has considerable phenotype-sparing activity in monolayer cultures, but has little or no augmentative effect in aggregate cultures, in which the articular chondrocytic phenotype is intrinsically supported. Endogenous BMP-2 expression is lost in monolayer cultures, coincident with down-regulation of phenotypic markers. These findings suggest that the observed effects of exogenous BMP in monolayer cultures represent, to some extent, compensation for the loss of endogenous ligand expression and secretion.

Both the BMP antagonists used in these experiments suppressed expression of collagen type II and aggrecan. These genes are used as cardinal markers of the articular chondrocytic phenotype and the products of these genes constitute the major collagenous and non-collagenous proteins of the cartilage matrix, respectively. Noggin also reduced the secretion and deposition of collagen type II protein into the pericellular matrix of chondrocyte aggregates, consistent with the transcriptional data. Together, these results support the conclusion that BMP activity is critical to support the differentiated articular chondrocytic phenotype *in vitro* and biosynthesis of cartilage-specific matrix components.
The results of this study are of considerable importance to cell-based strategies developed for the repair or regeneration of articular cartilage. Articular chondrocytes must be implanted under conditions that support expression of the autocrine mediators that maintain the specialized characteristics of these cells. Our results indicate that the cellular environment must support endogenous BMP expression to support the fully differentiated articular chondrocytic phenotype.

References
FINDING THE PATHOLOGY OF WHAT MAKES WOBBLERS Wobble

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The term ‘wobbler’ is not a very specific term but is useful generically to refer to horses that display (usually symmetric) spastic paresis and ataxia without other accompanying clinical signs. The term implies that the spinal cord has been damaged (has a myelopathy) but does not necessarily implicate any particular aetiology. This presentation aims to outline

1) a convenient practical approach to the dissection of wobblers in order to determine where the spinal cord has been damaged, and what is causing it.
2) The nature of the various causes of the spinal cord damage.

1. Dissection of wobblers: As the dissection will be influenced by the nature of the clinical diagnosis it is preferable to delay starting the dissection until the clinician has supplied the pathologist with clinical information, including the results of any radiography, and the clinical-based diagnosis. It is important to establish that ‘other’ signs are absent – i.e. no cranial nerve or other neurological signs.

Neck only – or the whole spine? If the neurologic deficit affects the forelegs it is fairly safe to assume that the lesion/s will be found in the neck or most cranial thoracic vertebrae – so the rest of the carcase and the thoraco-lumbar spine need not be examined. If the forelegs are not affected it means that if no macroscopic cord lesion is identified in the neck from C1 to T1 then the examination will need to be extended to include inspection of the whole spinal cord and vertebral canal.

Head and neck: Suggested protocol: Obtain CSF.

Brain removal: remove head, split parasagitally on the band saw, carefully remove brain and inspect it (external surfaces and cut surface); hold the two parts back together and check for asymmetries; examine the interior of the cranial cavity. Postpone putting the whole brain into formalin until the dissection of the neck is complete – so that if the diagnosis is still uncertain unfixed CNS can be preserved, e.g. deep frozen, in case required for microbiology at a future date.

Cervical spinal cord removal: Remove the large muscles from around the cervical vertebrae in order to palpate the articulations. Note any obviously enlarged or asymmetric articular processes. Make transverse bandsaw cuts across mid C2, C3, C4, C5 and mid C6 leaving the articulations at C1/2, 2/3, 3/4, 4/5 and 5/6 intact. Make a parasagittal cut from mid C6 to the level at which the neck was removed – usually the T1/T2 junction. With a no 11 scalpel and rat-tooth or Aliss forceps grasp the dura mater and cut the spinal nerves within the vertebral canal, turning the vertebra around to start each end until the cord can be pulled cleanly from the canal. Place the cord within the dura on a flat surface and open up the dura along the dorsal surface to expose the cord and spinal nerves. Very gently run a finger along the cord to assess whether there is a palpable compression site. Tie a label at the rostral end of each
segment identifying the site and drop into fixative. Insert a finger into the now empty vertebral canal and palpate for evidence of stenotic sites. If the carcase is fresh, and rigor mortis has not set in, passive movements of each articulation when a finger is within the canal can provide further tactile evidence of focal compression. The cord from mid C7 to the C7-T1 articulation can be removed through the laterally exposed vertebral canal. One advantage is that possible encroaching influences can be visualised directly – e.g., dosally or laterally encroaching synovial cysts.

Vertebral evaluation: To further assess the gross morphology of the cervical vertebrae, their size, shape and the 3-dimensional conformation of articulating surfaces it is useful to clean the vertebrae of soft tissues by boiling and bleaching with sodium perborate. Morphology and measurements are then entered on a record sheet.

Spinal cord histology: After a few days fixation a TS of cord is processed for histological review at each intervertebral junction, including any sites of palpable compression. At least one LS section is included. Hopefully the histology will confirm the gross assessment of a cord compression site.

Thoraco-lumbar spinal cord removal: Ideally this requires the use of a bandsaw, and preferably some technician help to remove the vertebral column from the carcase – by cropping away the ribs with tree-lopper style long handled cutters, or a necropsy chain saw. Once reduced to the vertebral column only the spinal cord can be removed in segments of 6-10 vertebrae by a parasagittal longitudinal vertebral cut and cord removal as before, the dura opened and the cord inspected and palpated.

2. The nature of the causes of spinal cord damage
The majority of clinical wobblers are found to have suffered focal spinal cord compression. An illustrated review of the common causes of the compression will be presented, embracing static and functional vertebral stenosis, synovial cysts and bursae associated with chronic arthropathy, traumatic lesions and a few examples of congenital malformations and tumours. Degenerative conditions causing wobbling are very rare in the UK e.g. Equine Degenerative Myelo-encephalopathy. Inflammatory CNS conditions usually show clinical signs other than ataxia and so would not be designated as wobblers. A possible exception is Equine Protozoal Myelitis, which has a broad spectrum of lesions and clinical signs, sometimes quite subtle. The few cases seen in the UK have been in imported horses.
CURRENT ADVANCES IN RESPIRATORY MEDICINE

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This paper is designed to address issues in the adult horse. It assumes that the horse has had an appropriate history collected and has undergone a thorough physical examination. Based on these the clinician is suspicious of respiratory disease. The paper will now discuss the applicability of a number of ancillary aids useful in the diagnosis of respiratory disease. At the end some treatment strategies will be addressed, particularly for infectious (bacterial) forms of respiratory disease.

Haematology and Serum Biochemistry
These may be useful and certainly may provide some insights into the duration and severity of the systemic effects of the disease, particularly in cases of pleural effusion. In acute cases there may be haemoconcentration due to stress and dehydration, neutropaenia with a degenerative left shift and the presence of toxic neutrophils, hypoproteinaemia (due to loss of protein into the pleural space) and prerenal azotaemia. With increasing chronicity the anaemia of chronic disease is more common, as are leucocytosis, hyperfibrinogenaemia and hyperglobulinaemia. Similar changes may be noted in horses with bacterial pneumonia.

Ultrasonography
Ultrasound is the method of choice for determining the presence of pleural effusions in the horse. Examination of the pleural space is a relatively easy skill to acquire. Either sector or linear array scan heads may be used to examine the pleural space/thorax. Although it is ideal to clip the hair prior to scanning, effective scans may be obtained either by using copious amounts of coupling gel or wetting the skin and hair thoroughly with alcohol or paraffin. You should become familiar with the normal echogenic motion as the visceral pleura rubs against the thoracic wall. The presence of fluid separating the visceral and parietal pleura should then become readily detectable.

Findings on ultrasound examination in horses with pleural effusion are variable according to the cause and severity of the problem. In horses with pleuropneumonia most fluid accumulations occur in the ventral thorax and displacement of the lung by the accumulated fluid is characterised by a relatively homogeneous space, which allows good penetration of the ultrasound waves to the deeper structures. When fluid occupies the midline of the thorax and aerated lungs float out toward the thoracic wall the volume of fluid present may appear less than is actually present. In this situation it is important to scan near the diaphragm as this will reveal fluid behind the peripheral lung surfaces. Fluid may be homogeneous or contain variable amounts of echogenic material. This can include blood, gas, lung parenchyma and fibrin. When pleuropneumonia becomes more chronic, dense strands of fibrin may occur. When the condition is more severe a fibrinoid reaction within the pleural space occurs with pleural fluid noted as isolated pockets. In some cases where large numbers of
anaerobic bacteria have colonised the pleural fluid small, bright echoes consistent with gas within the fluid, may be noted. Consolidation of the lung and pulmonary abscessation may also be noted when scanning. When scanning the thorax it is important to attempt to determine cardiac position, the presence of pericardial effusions or pericarditis, extrapulmonary abscessation and neoplasms. In general, in horses with pleural effusions secondary to neoplasia, the effusion is transudative in nature thereby being homogeneous and containing few, if any, fibrin strands.

**Transtracheal Wash**

The use of tracheal washes in horses enables you to acquire secretions from the trachea and lungs. There are three methods of obtaining a tracheal wash sample. First, the procedure may be performed during endoscopic examination via a catheter inserted through or directly via the biopsy channel of the endoscope. Samples obtained in this manner are adequate for cytology but are not appropriate if culture of the sample is anticipated, due to contamination. Recently, ‘guarded’ or insertion via an endoscope through the nasopharynx have been described for obtaining tracheobronchial samples. These catheters substantially decrease the chance of contamination of samples with oral bacteria but do not eliminate the possibility entirely. However, in a recent study conducted at the University of Sydney it was found that using this procedure, samples of similar quality to those obtained using the traditional transtracheal wash technique (contamination free) were obtained. **The traditional technique** for collection of samples from the lower airways for culture involves tracheal puncture. The transtracheal wash (TTW) technique allows aseptic sampling of the lower airways and bypasses contamination from the upper airway. This is important in samples from which bacterial culture and sensitivity testing is required as many bacteria that populate the normal horse's pharynx are pathogenic in the lower respiratory tract, and contamination of a tracheal wash with any of these organisms could be diagnostically confusing.

**TTW Sampling Technique**

**Guarded Aspiration Catheters**

In recent times a relatively non-invasive method for collection of samples from the trachobronchial tree involved the use of the double/triple-guarded catheter. These are often difficult to obtain unfortunately.

**Sample Processing**

A number of techniques for TTW have been described, but aseptic technique is important in them all. After collection the sample should be divided into two parts; one for cytology and one for subsequent culture if indicated. Then a decision must be made as to whether the sample will be processed immediately or if you expect a delay. Important cellular degenerative changes can occur in the period between sampling and processing. For this reason wet fixation is often advocated if a delay is to be anticipated. This entails adding an equal volume of 40-50% ethanol to the aspirate. This immediately prolongs degeneration, prevents maturation or phagocytosis and preserves cellular detail. If there is no delay in processing, or once you are back in your practice, a drop of the sample for cytology may be smeared onto glass slides and stained with Diff Quik® (Diff Quik; Labaids, Aus.) if the TTW is sufficiently cellular. An easy way to assess cellularity is to look for turbidity or flocculation. However, because many TTWs are relatively acellular, it is often
necessary to centrifuge the sample. This involves transferring your sample to a conical centrifuge tube, centrifuging 5-10 minutes at 1500 x g (g = force of gravity), decanting the supernatant fluid and then aspirating the mixed sediment to make cellular smears. Alternatively, cytocentrifuges may be used, if available, to concentrate cells from larger volumes of fluid onto a small section of the slide. The portion of the tracheal wash for culture and sensitivity should be processed rapidly to maximise accurate results. It is important to culture specimens rapidly as even non-bacteriostatic sterile saline solution is reported to kill some organisms, including *Streptococcus pneumoniae*. A decision should be made at the time of sample collection as to whether anaerobic bacteria could be involved in the respiratory pathology you are investigating. This is required as anaerobic isolation cannot be performed on samples not placed in the appropriate culture media that maintains viability of these bacteria. For example, the commonly used culture swabs within a plastic sheath are not suitable for anaerobic isolation. Various commercial transport media are available that are satisfactory for both aerobic and anaerobic isolation. Alternatively, if anaerobes are suspected, air can be dispelled from the syringe, the syringe capped with a new needle and sent to the laboratory promptly for cultivation. For culture of TTWs it is generally preferable to use agar plates rather than liquid culture media. Liquid culture media may allow some bacteria, present in low numbers in the sample, to grow overnight at the expense of other bacteria. Thus, it can be difficult to interpret the relative proportion and importance of bacteria present in these samples. If blood agar plates are used, the relative numbers and types of bacteria can easily be identified after 24 hours of incubation. Two percent Sheep Blood agar plates and Anaerobic Sheep Blood agar plates are suitable for tracheal wash samples. Gram stains of the original sample should be performed promptly and will aid early antibiotic selection. One should remember when reviewing slides that age of bacteria can alter stain retention and commonly Gram positive organisms can stain Gram negatively. In addition, the numbers of bacteria present as well as cellular density and presence of pyknotic cellular debris or stain precipitate can make interpretation of gram stains more difficult. Finally, the presence of bacteria, especially a mixed population, and cytological evidence of oral/pharyngeal contamination but no evidence of septic cellular changes, should be cautiously interpreted.

*Interpretation of Results of the TTW*

The cytology and bacterial isolates of tracheal washes obtained via an endoscope, guarded swabs or tracheal puncture have been reported. There are several important points regarding all of these techniques. First, there is a moderate to marked variation in the amount of saline flushed and retrieved with all of these procedures. Thus, the dilution factor for these samples is very variable. **Consequently most investigators suggest that total protein concentrations and total cell counts are not useful in the evaluation of tracheal washes.** In addition, there can be great variation in cellular distribution within and between smears from the same sample. Accurate counting of cells in clusters or in mucus plugs is not possible and a sample taken directly from an isolated pool of exudate in a trachea may look different to that obtained from flushing and aspirating a larger area of the same trachea. For these reasons, **many authors also question the validity of differential cell counts.** Finally, although the trachea does not have a normal flora, horses may harbour transient bacteria and fungi in this site. **If bacteria are seen in the absence of cytological evidence of inflammation, it is unlikely they are the cause of the horse’s respiratory ailment.**
Normal Cytological Findings
Cells from the lower respiratory tract include epithelial cells and inflammatory cells. In horses without lesions of the lower respiratory tract, the predominant cells in a TTW are ciliated and non-ciliated columnar epithelial cells and pulmonary alveolar macrophages (PAMs). Variable numbers of neutrophils and lymphocytes may also be found. A small amount of mucus is usually present. Samples vary in their cellularity but are usually less cellular than those from diseased airways.

Epithelial Cells: Ciliated columnar epithelial cells form a significant proportion of cells retrieved from normal horses and may be more numerous in aspirates obtained via the endoscope. When seen from the side, these long cells have a nucleus at one end with finely granular chromatin and sometimes a nucleolus, homogenous cytoplasm and well defined cilia at the end opposite from the nucleus. When low columnar or cuboidal epithelial cells from the small airways are seen on end, little cytoplasm is visible around the nucleus and the cells are frequently in cohesive clusters. Goblet cells are rare or absent in aspirates from normal horses. Their size varies according to their mucus content. They resemble ciliated columnar epithelial cells but are non-ciliated and they contain numerous azurophilic granules of mucus. Smaller squamous cells from parabasal layers may occasionally be seen in normal horses, although their presence could indicate squamous metaplasia. They have relatively large nuclei and, if in groups, may have intercellular bridges.

Macrophages: Pulmonary alveolar macrophages vary in size and shape but all have in common abundant, often foamy, cytoplasm and a vesicular nucleus. Absence of PAMs within a sample indicates the wash may not have retrieved adequate material from deep in the respiratory tract and hence may not accurately represent the lower airways. The PAM nucleus is often bilobed and cells may be bi- or tri-nucleate. It has been suggested that the numbers of multinucleated PAMs increases when there is extracellular debris or chronic inflammation. However, multinucleated macrophages may also be seen in normal horses and in humans are often seen in sputum with no evidence of significant inflammation. Percentage of PAMs reported in normal horses varies according to investigator with some reporting up to 48% and others up to 68% of cells.

Neutrophils: Variable numbers of neutrophils can be observed even in normal horses, but they are usually well preserved. However, as the lung is a normal site for removal of neutrophils, the presence of a few degenerate neutrophils is of no clinical significance. Several studies of normal horses and ponies have shown the percent neutrophils to range from 0 to 21. In contrast, a higher percentage of neutrophils (39, 21 or a range of 3 to 83) was found in two other studies of normal lungs using TTWs performed post-mortem and antemortem. In a study of 20 clinically healthy foals between one and six months of age, ten had more than 70% neutrophils in their TTW. Degenerate neutrophils were sometimes present and were more common when numbers of neutrophils were increased. However, their presence was not significantly associated with isolation of pathogenic bacteria. Thus, it is evident that the percentage of neutrophils in tracheal washes is variable, even in normal horses, and makes interpretation of increased numbers associated with inflammation difficult.
**Eosinophils:** Eosinophils are recognised by their characteristic granules or by multiple hollow spheres when degranulated. They may be seen in low numbers in clinically normal horses. Levels cited have usually been less than 3%. Because eosinophils are frequently unevenly distributed in a smear and often grouped in mucus, some clinicians prefer to classify whether they are rarely or frequently seen. It is important to look at several smears in their entirety, as cell differential counting in one area can be misleading.

**Lymphocytes and Plasma cells:** Lymphocytes have large nuclei relative to small amounts of cytoplasm. They may be difficult to differentiate from columnar epithelial cells seen ‘end on’ or small macrophages. Plasma cells are rarely seen in TTWs of normal horses.

**Mast cells:** Mast cells are often not quantified because stains commonly used on tracheal washes (Diff-Quik, Gram stain) usually do not identify mast cells of horses. If mast cells are suspected, slides should be fixed in alcohol then stained with the quick toluidine blue method. Mast cells are identified by their characteristic metachromatic staining granules. Studies in other species have demonstrated their important role in allergic respiratory disease but there is scant data specific for the horse. They are often present in low numbers in normal horses' tracheal or transbronchial aspirates.

**Others:** Erythrocytes are frequently present in tracheal washes from normal horses due to minor trauma to the epithelium during the procedure. Basophils are rarely seen in TTWs, but were reported in a few horses with chronic cough.

**Mucus:** Material obtained from the lower respiratory tract always contains some mucus. In Diff Quik stained smears, mucus appears as pink to light blue amorphous strands. Mucus may also appear as dark-staining tight spirals (Curschmann's spirals), which are inspissated mucus casts derived from small bronchioli. Curschmann's spirals can be found in samples from animals with prolonged and excessive production of mucus such as chronic lung disease.

**Micro-organisms:** It is common to find various bacteria, plant spores and fungal elements in TTWs. Their presence does not indicate infection and probably reflects the horse's environment. This is particularly true of horses housed indoors. If bacteria are seen in the absence of cytological evidence of sepsis, it is unlikely that they are the cause of the horse's respiratory ailment. Several studies have examined the bacterial isolates from TTWs of healthy horses. Only samples determined to be normal cytologically were included in these studies. Bacteria were isolated in a high percentage of horses in all studies, though generally in low numbers. A wide range of transient bacterial species were isolated including *Micrococcus* spp, nonhaemolytic spp., coagulase negative *Staphylococcus* spp., *Nocardia* spp., *Acinetobacter* spp, and *Bacillus* spp. More importantly, bacteria that have been associated with disease of the lower airways were also isolated in normal horses. Most frequently isolated were *Pasteurella* spp., *Klebsiella pneumoniae*, alpha haemolytic *Streptococcus* spp., *Actinobacillus* spp, *Pseudomonas aeruginosa* and a variety of anaerobic bacteria. Interestingly, *Streptococcus equi* subsp. *zooepidemicus*, while identified in tracheal washes from a large percentage of diseased horses, was isolated in very low frequency from the respiratory tract of
normal horses in all of the studies. Low numbers of fungi were also isolated from
and *Alternaria* spp. were found. *Alternaria* are easily recognised by their large
macroconidia and are frequently observed in TTWs. Interestingly, higher numbers of
horses kept at pasture were found to have positive cultures when compared to
racehorses in these studies. This suggests that, as in other species, age, exercise and
environment are important in determining equine mucociliary clearance function and
therefore bacterial presence in the lower respiratory tract. A recent study investigated
factors contributing to **coughing** (and poor performance) in racehorses. We found that
young horses were at greatest risk for coughing, with horses racing in the previous
week also at increased risk. Coughing was associated only rarely with increased
incidence of fever and inappetence. Endoscopic and cytological signs of inflammation
routinely accompanied coughing. However, there was no association between
coughing and seroconversion to EHV-1, EHV-4 or rhinovirus-1or 2. The majority of
coughing horses had **no** pathogenic bacteria isolated from the respiratory tract. In
addition, there were no characteristic haematological changes associated with
coughing, suggesting that these variables may often be over interpreted. Thus, it was
interpreted that many of the changes occurring in the respiratory tract were the result
of airway hyper-responsiveness, more than likely the result of exposure to
environmental allergens.

**Contamination of Tracheal Wash by Oral or Pharyngeal Material**
Occasionally, a tracheal wash may be contaminated with oral or pharyngeal material
and it is important for appropriate assessment of the tracheal wash to determine
whether contamination has occurred. This is particularly the case if the sample is
collected via an endoscope or with a ‘guarded swab’. Cytological features of oral or
pharyngeal contamination include the presence of squamous and superficial epithelial
cells and bacteria of various types, particularly *Simonsiella* spp. Squamous cells are
large rectangular cells, often without nuclei, that usually stain lightly basophilic, but
may also stain lightly acidophilic. They appear flattened and often have straight
borders with distinct corners. They also sometimes appear rolled up in cigar shapes.
Squamous epithelial cells may have bacteria adhered to their surfaces. Contaminating
bacteria from the upper respiratory tract are generally mixed flora; the presence of a
large variety of bacteria frequently suggests the sample has been contaminated.
*Simonsiella* spp. have a striking and characteristic appearance. These bacteria divide
lengthwise and therefore line up in a parallel row. They appear in smears as a single
large organism with parallel dark and light stripes. When *Simonsiella* spp. are seen
they provide extremely strong evidence of oral or pharyngeal contamination of the
tracheal wash. Care, however, should be taken if squamous epithelial cells are
observed as it may be difficult to distinguish pharyngeal squamous epithelial cells
from those originating from squamous metaplasia in the lower respiratory tract. They
also may be seen in tracheal washes retrieved from horses that are aspirating
secondary to transportation, strenuous exercise or laryngeal surgery. One should not
assume that squamous cells can only originate from the upper airway or be
insignificant, and if the cellular pattern and/or clinical signs suggest other possibilities
these should be investigated.

**Conditions Causing Abnormal Tracheal Fluid**
Controversy exists over the correlation of TTW cytology, bacterial isolation and the
presence of lower respiratory tract disease, with some investigators reporting good
correlation and others not. We have found the technique to be useful for evaluating lower airway disease, particularly bacterial pneumonia, providing one is cognizant of the dynamic state of airways and the rapidity with which the cellular characteristics can change. Care must also be taken in sample collection to qualitatively examine smears for cytological patterns of disease processes; you cannot expect an inadequate or poorly handled aspirate to accurately reflect disease processes.

**Acute and Chronic Inflammation:** One of the most common lesions detected by examination of material from the lower respiratory tract is acute (purulent or suppurative) inflammation. Neutrophils are the primary cells in acute inflammation and this is reflected by an increased percentage of these cells in inflammatory conditions. Although the percentage of neutrophils observed in normal horses is very variable one study of TTWs from 27 clinically normal horses with lungs that were histologically normal and 57 with respiratory tract disease suggested that more than 40% neutrophils was usually indicative of lower airway inflammation. Neutrophils collected from the respiratory tract of animals with acute inflammation often show evidence of degeneration such as karyolysis and cytoplasmic vacuolisation. These features are often used to indicate the possibility of sepsis in samples obtained from other sites of the body. However, in TTW samples, these features of degeneration are not pathognomonic for sepsis. Lavage samples, particularly those that are left in the collection saline for a long time, often contain vacuolated, karyolytic neutrophils. If, however, neutrophils are the dominant cell type in the sample, infection of the lower airways should be strongly considered and culture of the TTW sample should be performed. In addition to neutrophils, PAMs and columnar epithelial cells are usually present in acute inflammation. Columnar epithelial cells usually appear normal, but small clusters of hyperplastic epithelial cells may be found in acute inflammation. When inflammatory lesions persist, the ratio of neutrophils to PAMs decreases, in favour of increased PAMs. The relative numbers of PAMs and neutrophils determine the classification of the lesion, which can vary from subacute to chronic. However, be careful when using these terms to indicate the age of a lesion as they may be misleading. For example alveolar macrophage responses can be rapid and purulent inflammation can persist for a long time. Nevertheless, these classifications of inflammation may be useful because they help describe the appearance of the material obtained if not always the duration of the lesion.

**Bacterial Infections:** All inflammatory lesions should be examined for an aetiological agent. Bacterial infections are the most common cause of acute and chronic inflammation in horses and may be found intra- and extra-cellularly. It is necessary to differentiate bacteria from other small, round or elongated structures including mucus granules, mast-cell granules and precipitated stain. In mature horses with signs or histories of bacterial pneumonia, Gram positive bacteria are most frequently cultured including *Streptococcus* spp., and less frequently *Staphylococcus aureus*. Commonly isolated Gram negative bacteria include *Pasteurella* spp. *Escherichia coli*, *Enterobacter* spp., *Pseudomonas* spp. and *Klebsiella pneumoniae*. In addition to the above bacteria, in foals *R. equi* is an important isolate. Anaerobic bacteria that have been most frequently isolated include *Bacteroides* spp. and *Clostridium* spp. Mycoplasmas have been reported in a small percentage of normal and diseased horses. However, the significance of the presence of these bacteria in horses is controversial and their isolation requires the use of special media. When evidence of oral/pharyngeal contamination is present along with severe acute
inflammation, inhalation pneumonia must be considered as a possible cause of the pneumonia. In humans with chronic bronchitis and cystic fibrosis, colonisation and infection with bacteria is common and some of these bacteria, especially *Pseudomonas* spp. can stimulate mucin secretion and exacerbate airway obstruction. Whether this occurs in horses is unknown, but it is interesting that *Pseudomonas* is a frequent isolate from horses showing no signs of septic lung disease but having chronic excess mucus production. Recording sensitivity patterns may be helpful as it can reveal whether there are emerging resistance patterns in the bacteria isolated in your practice. Results of these tests may indicate that it is necessary to alter antibiotics commonly prescribed for respiratory infections in situations when culture and sensitivity are not feasible.

**Fungal and Protozoan Infection:** Fungi and protozoa may, on rare occasions, cause lower respiratory tract disease in horses. The most frequently identified pathogenic fungus from horses is *Aspergillus* spp. but *Cryptococcus neoformans* and *Histoplasma capsulatum* have also been isolated. The protozoa *Pneumocystis carinii* can cause pneumonia in immunosuppressed horses. These organisms can be identified cytologically in TTWs.

**Viral Infections:** Horses may be affected by a variety of respiratory viruses although recent evidence suggests that these infections are less frequent in horses in training than was previously suspected or elsewhere in the world. However, when they do occur, severe infections are often accompanied by secondary bacterial infection. However, tracheal washes are not significantly different in secondary bacterial pneumonia from those of primary bacterial pneumonia. Increased numbers of lymphocytes are occasionally found in people with viral pneumonia, and may also be seen in horses with viral infections. Inclusions have been reported in epithelial cells and macrophages in tracheal washes from both healthy horses and those with respiratory disorders. Inclusions are frequently used as an indicator of viral infections; however, they may be idiopathic due to deranged cell function or be part of non-specific cell degeneration and death.

**Chronic Obstructive Pulmonary Disease (COPD) or Recurrent airway obstruction (RAO) – ‘Heaves’:** This condition, which is seemingly rare in Australia (or at least not frequently manifest due to the large proportion of horses in Australia housed at least 12 hours a day in paddocks), is best diagnosed primarily using BAL fluid results rather than tracheal washes and thus will be discussed in more detail in that section.

**Eosinophilic Inflammation:** Increased numbers of eosinophils are seen in allergic bronchitis and lungworm migration in horses. Eosinophilic inflammation is usually accompanied by marked PAM proliferation. Larvae of *Dictyocaulus arnfieldi* are an unusual finding, but they may be found occasionally using the Baermann technique. Eosinophilic pneumonitis has been reported in horses that have been in contact with chickens. Probably some factor, such as feather dander, causes an allergic reaction in the lower airways.

**Exercise Induced Pulmonary Haemorrhage (EIPH):** Because of the distribution of EIPH, this pathology is usually best diagnosed with BAL and will be discussed in more detail in that section.
Neoplasia: Primary lung tumours are very rare in horses. In addition, TTW and BAL rarely facilitate diagnosis of primary lung neoplasia as they rarely exfoliate into the airways in horses. Additionally, these procedures are rarely of use in diagnosing tumours that have metastasised to the lungs of horses. For neoplastic cells to be retrievable by TTW/BAL, the tumour must have invaded the bronchi or bronchioli, and these structures can not be located peripherally or be blocked by mucus. Some metastatic lung tumours in people have been accurately diagnosed by examination of BAL, but similar reports have not occurred in horses.

Bronchoalveolar Lavage
Bronchoalveolar lavage (BAL) is a diagnostic procedure that has become relatively widespread in human pulmonary medicine over the past 10 to 15 years and its use in has increased dramatically in recent years. The aim of the procedure is to atraumatically instill fluid into the airways and to collect epithelial lining fluid and cells from the alveoli and distal airways when this fluid is aspirated. This technique is thought to better reflect the cell populations in the lower airways than tracheal washes and thus provides useful information with respect to the integrity and constituents of this site, especially in cases of chronic disease. However, it must be remembered that the BAL will only provide information about the portion of the lung sampled. It may not be indicative of the overall lung condition. As a result this technique may not be as useful as a transtracheal wash and pleural fluid analysis in horses with suspected pleural effusions. In contrast this technique has excellent application in a variety of other pulmonary disorders.

Comparison of the Relative Values of Tracheal Aspirates and Bronchoalveolar Lavage in the Diagnosis of Equine Pulmonary Disease

Advantages of Tracheal Aspirates:
- collection of tracheal aspirates is easier than BALs and rarely necessitates sedation, an advantage in horses competing under the rules of racing.
- tracheal aspirates collected by tracheal puncture are, unlike BAL fluid, free from nasopharyngeal bacterial contamination and are thus more suitable for bacteriological analysis
- tracheal aspirates contain secretions and cells originating from all areas of the lung and are therefore more suitable for detecting focal pulmonary lesions, especially of the cranio-ventral lung lobes. In contrast BAL fluid is harvested from localised lung segments; predominantly the caudo-dorsal lung lobes.

Advantages of Bronchoalveolar Lavage:
- BAL fluid cytology, unlike tracheal aspirate cytology, shows good correlation with pulmonary histopathology. There is little correlation between the cytology of TTWs and BAL fluid.
- BAL fluid cells have better morphology than those in tracheal aspirates, making it easier to identify cells and therefore perform a differential cell count. Identification of cells in tracheal aspirates is further hampered by the presence of strands of mucus and large numbers of degenerating cells.
interpretation of BAL fluid cytological analyses is frequently easier.

**BAL Technique**
This procedure is simple, often providing useful information with respect to the integrity and constituents of lower respiratory tract. BAL can be performed 'blind' or with a flexible fibre endoscope. When employing the endoscopic technique, I usually sedate the horse (xylazine at a dose rate of 0.2-0.4 mg/kg is suitable – but porphanol may be added – 0.2 mg/kg to add sedation, pain control and reduce the cough reflex) and the tip of the endoscope is passed into the lower respiratory tract and wedged in the smallest possible airway. With the 'blind' technique an equine BAL tube (I prefer the Bivona Inc., Gary, Indiana) is passed into the lower airway and wedged into position. Passage of the tube into the trachea is facilitated if an assistant extends the horse's head. Obviously the operator has little knowledge of which respiratory unit the lavage tube is wedged in although the right mainstem bronchus is normally intubated. Usually we instill 300 mL of saline is infused in 50 mL aliquots and aspirated immediately following infusion. Subsequent to collection, samples are observed for colour and subjected to cytological (total and differential nucleated cell counts) examination. Whatever technique is used, it should be standardised for your practice, and preferably according to accepted technique. This is required for meaningful comparison of data between individual animals within your practice as well as comparison to results achieved from studies both in Australia and overseas.

**Sample Processing**
The volume and colour of the recovered lavage fluid should be recorded. Specimens for cytological evaluation should be placed in EDTA tubes or fixed immediately in ethanol (add an equal volume of 40% ethanol). Cytological smears are usually prepared by cytocentrifugation using 0.1 mL of the sample at 1500 rpm for 5 minutes. However, cytocentrifuges are rarely available in practice and so a bench centrifuge may be used for 20-50 mL of BAL fluid at 1500 rpm for 5 minutes. The supernatant fluid should be poured off and a smear made of the remaining cell pellet and stained with Diff Quik®. Total nucleated cell counts can be done manually using the Unopette® microcollection system and the Neubauer haemocytometer. Automatic cell counters should not be used for counting cells as they may significantly underestimate the number of cells. The dilution of the pulmonary epithelial lining fluid with lavage fluid may be estimated using urea or albumin as endogenous markers of the variable proportion of these fluids. Cytological examination should include a differential cell count, morphologic description of cells and identification of pharyngeal contamination. For differential cell counts, 200-300 cells from a representative area of the Diff-Quik stained smear should be examined. A toluidine blue stain preparation can be used to detect the presence of cells with metachromatic granules (predominantly mast cells). The use of different stains for identifying variable cell types is important and should not be overlooked. Differential cell counts may be affected by the stain used. For example, significant overestimation of PAMs has been reported when using Wright's stained cytocentrifuged preparations compared to the same preparations stained with non-specific esterase. The latter is more accurate for macrophages and so a more accurate count is obtained. In humans, post-retrieval handling is recognised as affecting results and even when samples have been collected by experienced people using standardised technique, one multicentre study found 30% of 1588 BAL samples to be unsatisfactory because of a paucity of pulmonary alveolar
macrophages, an excess number of epithelial cells, mucopurulent exudate, degenerated cells and laboratory artifact either alone or in combination.

**BAL Results**

**Normal Horses:** Because of the variation of techniques described in the literature, the total cell and differential counts reported from normal horses have varied. However, when approximately the same volumes of fluid (between 200 and 500 mL) were used, total cell counts and differential cell counts are similar in normal horses.

**Reference Ratios (Median% and Range) of BAL Fluid (from McGorum and Dixon, 1994)**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>1.0 (0.7-4.0)</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>39.7 (20.0-51.3)</td>
</tr>
<tr>
<td>Macrophage</td>
<td>49.7 (36.0-74.3)</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>0.0 (0.0-0.7)</td>
</tr>
<tr>
<td>Mast Cell</td>
<td>9.3 (0.7-12.3)</td>
</tr>
<tr>
<td>Epithelial Cell</td>
<td>0.3 (0.0-1.7)</td>
</tr>
</tbody>
</table>

In general, total cell counts should be less than 500 x 10⁶ cells/L with most horses having approximately 300 x 10⁶ cell/L. The majority of cells are macrophages or lymphocytes (approximately 40-45% of each). However, care must be taken as epithelial cells that are ‘decapitated’ by trauma of processing or degeneration can be lymphocyte ‘look alikes’ and erroneously classified. The remaining cells include neutrophils, which are usually less than 5%, mast cells, epithelial cells (ciliated and non-ciliated bronchial cells) and eosinophils. The description of these cells has already been reported in the section for transtracheal wash. Care must be taken in identifying neutrophils in BAL samples as they can be pyknotic. In this instance they will appear as small, deeply staining cells with a tightly condensed nucleus. Care must be taken not to confuse these cells with lymphocytes.

Information is also available for BAL results from foals. There are age related increases in the total number of cells and macrophages recovered in foals. In a study of foals between 2 and 63 days of age, repeated lavages showed the proportion of macrophages declined from >80% to 55-82% and the percent of lymphocytes rose from approximately 5 to 13-30%. In individual foals, the percentage of cells, particularly neutrophils, varied with percentages varying from 1.5 to 18.

**BAL Fluid Cytology of Horses with Pulmonary Disease**

Additional information on BAL results are available for ponies with COPD, transport stressed horses and horses with various types of lung pathology. **However, it must be remembered that BAL samples do not necessarily reflect the whole lung; rather, they reflect only the changes in the epithelial lining fluid of the lung segment lavaged.** Unless the pulmonary disease or condition is diffuse, a BAL sample may ‘miss’ the affected area and lavage a normal area of the lung. This is particularly true for BALs in horses with moderated to severe pneumonia.
**BAL fluid Neutrophilia:** In BAL fluid, elevated neutrophil counts are considered if the level of neutrophils is >5%. Horses with signs of RAO always show a BAL fluid neutrophilia. Although not as common in Australia as in Europe and North America, RAO can occur due to a hypersensitivity and hyperirritability to inhaled irritants or allergens. Other cytological features observed in horses with RAO include an increased amount of mucus and a mixed neutrophil and macrophage exudate. Due to the chronic increased mucus production and the relationship to hypersensitivity, Curschmann's spirals and/or increased numbers of eosinophils may also be seen. Clusters of hyperplastic ciliated epithelial cells and increased numbers of goblet cells are recorded and infrequently, squamous metaplasia of epithelium is found. It should be noted, however, that while the increase in neutrophils is considered one of the markers for RAO, numbers of neutrophils can also increase in certain environments in non-‘heavey’ horses and ponies. One experiment exposed normal and ‘heavey’ ponies to a barn and demonstrated that normal ponies had an 8-fold increase in neutrophils, but the ponies with RAO had a significantly higher percentage increase. With this finding taken into consideration, BAL fluid neutrophilia is currently the most useful diagnostic indicator of equine RAO, being considerably more sensitive than clinical examination, arterial blood gas analysis and testing of pulmonary mechanics. Experimental infections with EHV-1 induce a marked but transient neutrophilia, with neutrophil ratios returning to normal within seven days post infection. It is probable that other respiratory viruses induce a similar transient neutrophilia. Bronchial epithelial cells with cytoplasmic and nuclear inclusions, resembling those seen in man with parainfluenza and herpes infections, have been reported in horses, but are not a consistent finding. BAL fluid from horses with bacterial bronchopneumonia and pleuropneumonia may have increased neutrophil percentages. The neutrophils may contain intracellular bacteria and occasionally be degenerate or toxic. However, the diagnostic value of BAL fluid cytological examination is often limited in these cases due to possible inadvertent collection of BAL fluid from an unaffected lung segment. In one report, even with the use of a guided fibreoptic endoscope, 50% of the horses lavaged had total cell counts, differential cell counts and cytologic examinations similar to that of normal horses indicating that normal lung was lavaged. In the remaining horses there was a significant increase in total cell counts ranging between 500 to 5000 x 10^6 cells/L. In horses in which cell counts were abnormal, the percent of neutrophils was greatly elevated to 10-91% of the cells obtained. A mild BAL fluid neutrophilia has been reported in racehorses exercising at higher intensities. The initiating cause of this inflammation is not known but may reflect increased exposure of small airways to inhaled irritants following increases in ventilation.

**BAL Fluid Eosinophilia:** Changes in the eosinophil numbers or mast cells have been inconsistent and difficult to interpret. Furthermore, this finding is uncommon and usually attributed to infection with *Dictyocaulus arnfeldi*.

**Haemosiderophages and Erythrocytes:** Free erythrocytes may be identified in BAL fluid collected shortly after pulmonary haemorrhage. This is commonly the result of EIPH or rarely iatrogenic haemorrhage during the procedure. Following pulmonary haemorrhage, erythrocytes are phagocytosed by pulmonary macrophages and their haeme pigments reduced to haemosiderin. Haemosiderin is easily recognised on preparations stained with any of the routinely employed haematological stains as intracytoplasmic amber, green or brown pigment granules, their colour being dependent on the age of the pigment. Haemosiderin may be more readily identified...
after specific staining with Perl's Prussian Blue stain, but this is rarely necessary. While the presence of haemosiderophages in BAL samples is currently the best indication of EIPH, these cells have been found in BAL samples in 90% of horses in training, indicating that EIPH occurs in virtually all racehorses. Thus the clinical significance of the presence of these cells is dubious. Haemosiderophages are cleared very slowly from the lungs and may be observed in BAL samples from horses that have not raced for several months. Therefore, haemosiderophages may not be a good indicator of recent episodes of EIPH. It is also interesting to note that haemosiderophages were found more frequently in BALs than in TTWs from the same horses.

**Epithelial Cells:** The presence of numerous bronchial epithelial cells in BAL samples indicates iatrogenic trauma and is of no clinical significance.

**Mast Cells:** There is currently little information regarding the significance of increased numbers of mast cells in BALs. Some studies have shown that normal horses and ponies can have very high mast cells ratios (>20%) with no clinical or laboratory evidence of pulmonary disease over a three year monitoring period. Other studies have found much lower ratios. It is possible that the difference in reported mast cell ratios is due to different techniques used to collect, process, stain and identify BAL fluid cells.

**Lymphocytosis:** The significance of alterations in BAL lymphocytes percentages is currently unclear. Furthermore, interpretation of lymphocyte percentages is complicated by the wide range demonstrated in normal horses. Decreased lymphocyte percentages and increased macrophage percentages have been recorded in horses with chronic coughing, the significance of which is unknown.

**Bacteriology:** Due to unavoidable contamination of the BAL catheter or endoscope with upper respiratory tract bacteria, bacteriological analysis of BAL is not reliable.

**Thoracocentesis**
This procedure is indicated when physical, ultrasonographic or radiographic examination suggests the presence of pleural effusion and/or thoracic neoplasia. As stated in the preceding material many disorders produce pleural effusion in horses, but pleuropneumonia and neoplasia are the most common. As the prognosis for horses with pleuropneumonia, particularly if diagnosed early, is often fair to good, and the prognosis for horses with thoracic neoplasia is grave, care must be exercised when interpreting the results of pleural fluid analyses. Thus, in order to offer an accurate prognosis and provide appropriate therapy, it is crucial that you diagnose the underlying cause of the effusion.

**Sample Collection**
In general, thoracocentesis should be performed in the ventral third of the thorax with care being exercised to avoid the heart. Although not imperative, ultrasound guided thoracocentesis is the optimal method. For initial sampling, if only a small amount of fluid is suspected, a 6-7.5 cm teat cannula or vascular catheter can be used. A needle longer than 5 cm is also suitable. Aseptic technique is important and therefore ideally an area of skin over the sixth, seventh or eighth intercostal spaces about 7.5-10 cm above the level of the olecranon should be clipped, shaved and aseptically prepared.
Local anaesthetic is infused subcutaneously and into the intercostal muscles, and sufficiently deep to include the parietal pleura (remember this is very sensitive!!). If a cannula is to be used a stab incision in the skin is made with a small blade (#15) and the cannula inserted cranial to the rib border to ensure the intercostal vessels and nerves, which course down the caudal aspects of the ribs, are not damaged. The cannula is advanced into the pleural space and fluid aspirated.

If there are large amounts of fluid present or if the fluid has a high concentration of fibrin material, larger bore catheters can be utilised. Blunt tipped thoracic drainage tubes, which are designed for human use, are ideal and are available in a variety of sizes. These may be sutured in place using an anchoring Chinese finger (transfixation) suture. Catheters may be plugged if repeated drainage is required or a condom attached to the end. The tip of the condom is cut off and this allows the condom to act as a one way valve. Thoracocentesis should be performed on both sides of the thorax as different bacteriological/cytological results can be found. The quantity and quality of fluid obtained during this procedure may provide valuable information. In the normal horse, little or no fluid is obtained from the thorax. In conditions where pathology exists, in particular pleuropneumonia, >25 L fluid may be drained from each hemithorax. In addition to fluid volume reflecting pathology, the colour of the fluid, degree of opacity, presence of fibrinous material and odour are all useful indicators of the severity of disease. With infectious causes of pleural effusion the fluid will often become more opaque, malodorous and contain fibrin clots. The presence of a foetid odour is often reflective of the presence of anaerobic bacteria.

**Sample Processing**

Only 1-2 mL of fluid are usually required for a total nucleated cell count, cytological examination, total protein measurement and bacteriological testing (if necessary). Part of the collected fluid should be placed in EDTA tubes for cytology and total protein estimations and the remainder used for culture and sensitivity. If thoracocentesis is performed bilaterally, fluid from both sides should be examined and cultured as although the mediastinum in the horse is usually fenestrated, differences between sides is common in horses with septic pleuritis. It should be noted that in foals less than four months of age, these fenestrations do not exist and there is division of the two pleural spaces. If no fluid is obtained, yet you suspect neoplasia of the pleural cavity, it may be helpful to lavage the pleural space with several hundred ml of warmed pH adjusted saline and then to aspirate and analyse the fluid. It does not adversely affect the patient if only a small portion of the volume instilled is recovered.

**Total nucleated cell counts** should be made on the pleural fluid using either the Unopette System or automated cell counters. If possible smears for cytology should be made. If the total nucleated cell count is less than 10 x 10^9/L, 10 mL of the sample should be centrifuged and a smear made of the sediment.

**Total protein content** is the only biochemical value routinely measured on pleural fluid. It is usually measured using a refractometer. If however, chylothorax is suspected, fluid should be submitted for triglyceride and cholesterol analysis.

**Glucose concentrations** may be of value for diagnosis of bacterial pleuritis, decreased concentrations occurring due to fermentation of the sugar by bacteria. Low glucose concentrations (<50% of blood glucose) may be reflective of septic fluid.
However, fluids with very high cell counts also may have low glucose concentrations due to consumption of glucose by the nucleated cells.

Aerobic and anaerobic culture of the pleural fluid should be performed as pleuropneumonia in horses commonly involves mixed infections of facultatively anaerobic and strictly anaerobic bacteria. Ideally, a portion of the sample should be aseptically introduced into liquid culture media that will support the growth of both aerobic and anaerobic bacteria (e.g. Signal Blood Culture Media - Oxoid Australia Pty Ltd). In addition, these media provide sodium polyanetholesulfonate (SPS), a polyanionic anticoagulant that acts to inhibit the bacteriostatic agents contained in pleural fluid. However, these bacteriostatic agents will inhibit growth of bacteria on blood plates, causing a falsely negative culture result if only blood agar plates are used for culture of pleural fluid previously placed in fluid media.

Interpretation of results of thoracocentesis

Normal Pleural Fluid: As with peritoneal fluid, normal pleural fluid is a dialysate of plasma, with low cellularity and total protein concentration. Normal horses have either minimal (2-8 mL) or no retrievable fluid. Their fluid is clear, pale, straw yellow in colour and odourless. However, one study on 18 clinically normal horses reported some had hazy fluid. Red tinged colouration can occur secondary to bleeding from the puncture site but this usually clears as more fluid is withdrawn. In normal horses total cell counts have usually been reported to be less than $4 \times 10^9$/L and total protein is usually less than 20 g/L. Although a range of cell counts of 0.8 to $12.1 \times 10^9$/L and total protein in the range of 2 to 47 g/L have been reported in clinically normal horses, only a small fraction of these horses were necropsied to be sure there were no pleural abnormalities.

Neutrophils are the predominant cell population in normal pleural fluid, with a range of 32-91% reported. Neutrophils that enter the thoracic cavity do not return to the bloodstream. Consequently ageing and cell death give rise to moderate hypersegmentation, pyknosis and leucophagocytosis by macrophages. Lymphocytes in pleural fluids are usually small to medium sized cells. Plasma cells and lymphoblasts are not found in normal thoracic fluid. Large mononuclear cells include macrophages, reactive macrophages and mesothelial cells. These cells are often difficult to distinguish morphologically and are usually grouped together and referred to as mononuclear phagocytes as they all have phagocytic potential. They appear as large cells, with moderate to abundant amounts of bluish cytoplasm and a variably sized and shaped nucleus. Reactive cells may have prominent cytoplasmic vacuolation and/or inclusions. Reactive (mitotic) mesothelial cells may have multiple nuclei, but can appear in normal pleural fluid. Rarely, eosinophils, basophils and mast cells may be found in pleural fluid.

Classification of Abnormal Pleural Fluid

If the volume of fluid in the pleural space is increased, an effusion is present. Effusions develop if pleural fluid is produced at a faster rate than it is removed. Pleural effusions are generally classified as: transudates, modified transudates, exudates or haemorrhagic effusions. Transudates are ascitic types of effusions with low cellularity and protein concentration. Causes of transudates include increased capillary and lymphatic hydrostatic pressure (e.g. congestive heart failure) and low plasma colloid osmotic pressure (e.g. hypoalbuminaemia). Modified transudates occur
when transudates are ‘modified’ by the addition of either protein or cells. Modified transudates are observed in a variety of non-specific conditions that are frequently difficult to diagnose. Exudation occurs if there is increased capillary permeability and compromised lymphatic drainage, resulting in fluid with increased nucleated cellularity and protein content. This type of effusion may be seen with inflammation (pleuritis) and possibly neoplasia involving the pleura. Finally, haemorrhagic effusions result from leakage of blood into thoracic fluid. Care must be taken when trying to classify a pleural effusion as either transudate, modified transudate, exudate or haemorrhagic effusions. Some conditions (e.g. chylous effusion, neoplasia) frequently don't fall neatly into one category or the other. Also, the characteristics of transudate and modified transudate tend to merge and these effusions may have common causes. Finally, because pleural fluid reflects the status of the thoracic cavity and its viscera, it may change as the course of disease progresses. Despite these shortcomings, the above four categories are clinically relevant in most cases and can be diagnostically valuable. A knowledge of the most common causes of these effusions is therefore very useful.

**Transudate:** These effusions have increased volume with low cellularity (<10 x 10^9/L) and total protein concentration (<25 g/L). Cytological findings are usually unremarkable, with neutrophils, lymphocytes and large mononuclear cells often present in normal proportions. Morphology of these cells is also usually normal, though occasional reactive cells may be noted, especially in long standing effusions. Transudates are uncommon in the thoracic cavity, accounting for 0-7% of effusions in published surveys. Causes include hypoalbuminaemia, congestive heart failure and chronic liver disease. Other clinical and laboratory results are usually required to differentiate these causes.

**Modified Transudate:** These pleural fluids have an increased volume, usually with a grossly normal appearance, a normal or slightly increased total nucleated cell count (5-15 x 10^9/L) and an elevated total protein concentration of 20-50 g/L. Neutrophils with normal morphology tend to be the most numerous cells present. Modified transudates are also uncommon for pleural effusions and are most likely to result from congestive heart failure and chronic hepatitis associated with increased venous or lymphatic pressure. Occasionally thoracic neoplasia may cause a modified transudate.

**Exudate:** Inflammatory exudates are the most frequent cause of pleural effusion in horses. They account for 53-91% of abnormal pleural fluids in published surveys. Exudates have increased cellularity and total protein concentration, usually with greatly increased volume.

**Haemorrhagic Effusion:** Haemorrhage in a pleural fluid sample may be the result of iatrogenic contamination at collection, haemorrhagic diapedesis or intrathoracic haemorrhage. In cases of iatrogenic haemorrhage, the specimen usually ‘clears’ of blood during collection and there is negligible erythrophagocytosis evident in the smear. Haemorrhagic diapedesis may be associated with pleuritis or neoplasia. Few cases of haemothorax in the horse have been reported and may occur following trauma or erosion of blood vessels by a neoplasm. The PCV and total protein concentration is usually lower than that of peripheral blood unless the haemorrhage was severe and recent. Few platelets are observed on smears of haemothoracic fluid.
and the fluid often does not clot. A haemolysed supernatant fluid and evidence of erythrophagocytosis suggests the haemorrhage was not recent.

**Specific Causes of Abnormal Pleural Fluid**

**Bacterial Pleuropneumonia:** The most common cause of exudates in the horse is bacterial pleuropneumonia or pulmonary abscessation. The right apical lung lobe and right hemithorax are most frequently affected as this is thought to be the most direct route that oropharyngeal bacteria take when tracking down the lower respiratory tree. Commonly degenerate neutrophils are observed in these pleural fluids, in particular evidence of karyolysis (nuclear degeneration). If degenerate cells are seen, the smear should be carefully examined for the presence of bacteria. If, however, total nucleated cell counts exceed 50 x 10^9/L, septic disease is most likely, even if only small volumes of fluid are retrieved and no bacteria observed. These fluids should always be cultured. It should be remembered, however, that total nucleated cell counts and total protein content of pleural fluid are not accurate indices for prognostication. Septic pleural fluid is cloudy, usually yellow, sometimes beige tinted or bloody, and may contain fibrin clots. A foul odour of the pleural fluid suggests necrosis and possible anaerobic infection. However, although a foul odour suggests the presence of anaerobes, absence of an odour does not eliminate their existence and non-odorous fluid should never be interpreted as meaning no anaerobes are present. Macrophages may be very active in septic exudates and reactive mesothelial cells are common. These cells may be difficult to differentiate from neoplastic cells as there is no clearly defined demarcation between benign and neoplastic mesothelial cells. Frequently an experienced veterinary cytologist may be required to identify these cells in fluid samples from horses with pleuropneumonia.

There are usually few (if any) lymphocytes in an acute exudative effusion but they are more frequently found in ongoing chronic inflammatory reactions or in resolving ones. Their morphology is usually normal; however, large atypical lymphoblastic cells may sometimes be present. Care must be taken not to confuse these cells with neoplastic lymphocytes. In cases of lymphosarcoma, large numbers of these cells are usually present. Plasma cells can be present in relatively high numbers in pleural fluid in response to chronic antigenic stimulation. Commonly isolated bacteria from cases of pleuritis/pleuropneumonia include *E. coli*, and species of *Bacteroides, Clostridium, Pasteurella, Pseudomonas, Staphylococcus* and *Streptococcus*. Isolation of anaerobes from horses with pleuropneumonia, either alone or in conjunction with facultatively anaerobic bacteria, is associated with a poorer prognosis. Not all infected horses yield positive cultures from their pleural fluid. Tracheobronchial washes may be positive in some of these cases, and so it is worthwhile culturing these samples as well in suspected cases of pleuropneumonia.

**Neoplasia:** Neoplasia involving the chest cavity may produce an effusion. Thoracic neoplasia accounted for 5-38% of pleural effusions in reported studies. A few points should be remembered when neoplastic effusions are suspected. First, intrathoracic tumours may not exfoliate cells into the pleural fluid. This would preclude diagnosis of these tumours by thoracocentesis. Pleuroscopy may be useful to visualise intrathoracic masses and pleural metastases in these cases. Tumours that obstruct lymph flow from the pleural cavity may produce a voluminous effusion characteristic of a modified transudate. Tumours involving serosal surfaces, especially those that
erode blood vessels, may cause haemorrhagic diapedesis or haemothorax. Necrosis or infection of a tumour may result in pleuritis. Such an inflammatory exudate may overshadow the presence of tumour cells and may be diagnosed as a bacterial pleuritis by mistake. Finally, reactive mesothelial cells may be mistaken for neoplastic cells, especially by inexperienced cytologists. Such cells are commonly seen in effusions, especially septic effusions. Neoplasms reported to cause thoracic effusions include lymphosarcoma, gastric squamous cell carcinoma (metastasised to the pleural cavity), adenocarcinomas, mesotheliomas and haemangiosarcomas.

**Chylothorax (rare):** Cloudy white, pale pink opaque or opalescent fluid suggests chylothorax which may be idiopathic or occur secondary to lymphatic duct rupture. The fluid will not clear with centrifugation but will clear when ether is added. In normal pleural fluid, lymphocyte counts are less than 0.675 x 10⁹/L. If a preponderance and increased number of small lymphocytes are found in pleural fluid, triglyceride concentration should be checked to determine whether it is a chylous effusion and not lymphocytic lymphosarcoma. Pleural fluid triglyceride concentration should be compared with serum concentration; an elevation in the former indicating chylothorax. Pseudochylous effusion can occur with chronic inflammation and cellular degeneration and is characterised by a high pleural fluid cholesterol concentration.

**Thoracic Radiography**
Thoracic radiographs may provide useful information in certain situations. However, in the horse with a pleural effusion, results of thoracic ultrasonography are likely to provide much more useful information from a diagnostic point of view, particularly in the initial examination. In horses with pleural effusion, radiographs should be only taken immediately following removal of as much fluid as possible from the pleural cavities to avoid “white out”.

**Some considerations for therapy of respiratory disease in horses**
**Parenteral antibiotics** are integral to the treatment of horses with pneumonia. It is important (if possible) that bactericidal rather than bacteriostatic antibiotics be used. From a bacteriological point of view pneumonia does not usually constitute a medical emergency. As a result, treating the horse supportively until appropriate samples for bacterial culture and sensitivity are obtained is a far superior practice to commencing ‘shot gun’ therapy in the absence of pertinent bacteriological data.

Once samples have been collected (if serious pneumonia is suspected ultrasound examination of both thoracic cavities and a tracheal wash are indicated), empirical antimicrobial therapy can be commenced until culture and sensitivity results come to hand. Penicillin and aminoglycoside antibiotics are usually the drugs of choice in pneumonia as they:

- are active against the vast majority of organisms encountered in this disease
- are bactericidal
- provide good plasma and tissue concentrations
- have a reasonably broad therapeutic index
- are relatively cost effective.

Selection of antibiotic therapy ‘in the dark’ or use of ‘shotgun’ antibiotic treatment
is prone to error as it may be difficult in many cases to predict the organisms involved.

Therapy can be commenced with gentamicin (6.6 mg/kg IV q24h) and crystalline penicillin (20,000 IU/kg IV or IM q6h) in horses showing signs consistent with acute bacterial disease (eg, pneumonia). Procaine penicillin (15-20,000 IU/kg (15-20 mg/kg) IM, q12h) is also a good choice to follow up initial therapy with crystalline penicillin.

Ceftiofur sodium (2-5 (or even greater) mg/kg, IV q12h) has been a popular choice in recent years, although some report that ~1-2% of horses receiving this drug have been reported to develop colitis.

Enroflaxicin - in general although having good activity against many of the organisms involved in pneumonia/pleuropneumonia, it is expensive and has poor activity against anaerobes.

Trimethoprim-sulpha combinations may be useful in low grade respiratory infections. But it must be remembered that these drugs are inhibited in the presence of purulent material. Of course they have the advantage of not causing problems when drug testing is conducted.

Similarly, tetracyclines may be used but they are bacteriostatic and will have limited activity against many of the more significant pathogens involved in complicated pneumonias.

Following the return of bacterial culture and sensitivity results the appropriate antibiotic selections can then be made.

As many cases will have anaerobic bacteria involved, the use of metronidazole (15 mg/kg PO q6h) should be strongly considered if the culture results indicate penicillin resistant B. fragilis. However, it should be noted that it is has been rare to culture penicillinase producing B. fragilis from horses with septic pneumonia in Australia. In addition, we have noted that some horses treated with metronidazole become inappetent when administered metronidazole per os. As a result some use this drug per rectum. Another perspective is that most anaerobes involved in this disease in Australia appear to be sensitive to penicillin and this is bactericidal and penetrates well into infected areas. This may not be the case with a variety of other drugs.

As suggested above a variety of other antimicrobial agents have been suggested for use in the management of pneumonic processes in horses. These include ceftiofur and other second and third generation cephalosporins including cefoxitin and cefotetan. The latter drugs have good efficacy against many anaerobes but are expensive. In addition, the cephalosporins are much less effective against streptococcal species than is penicillin. Fortunately the combination of two beta lactam antibiotics (e.g. penicillin and a cephalosporin) appears to be appropriate so that combination may be worthy of consideration. The efficacy of ceftiofur against beta lactam producing anaerobes remains to be determined so if ceftiofur is to be used the clinician should consider including penicillin also. So despite our best efforts to perform culture and sensitivity of organisms isolated from thoracic fluids obtained from horses with
pneumonia we still face a number of limitations when initiating antimicrobial therapy in these cases. In general our principles of antibiotic therapy are to:

- use penicillin in most cases as it is bactericidal with good serum and tissue concentrations achievable, penetrates well, is active against all beta haemolytic streptococci and many anaerobes and is relatively cheap and safe
- add gentamicin or ceftiofur if Enterobactereaciae are involved. Again these agents are relatively safe and free of side effects. Although there are some theoretical reasons why gentamicin may not be the most effective there is much evidence in the literature on humans to suggest the selection and efficacy of this agent in similar conditions
- add metronidazole to the combination if beta lactamase producing anaerobes are suspected. However, we are always wary of the potential for inappetence with this agent.

Supportive therapy. Anti-inflammatory therapy is also important in an attempt to limit the degree of debility inflicted by the disease. A variety of agents are readily available. Examples include flunixin meglumine, phenylbutazone and ketoprofen. Care should be exercised in horses with associated dehydration as the nephrotoxic potential of these agents is increased.

In cases where significant systemic manifestations of the disease exist (e.g. toxaemia, dehydration) fluid therapy may be indicated. The degree of volume contraction is reflected by physical findings (skin turgor, mucous membrane colour and capillary refill time) and appropriate clinicopathological measurements (PCV, TPP, urea, creatinine). Some horses will experience considerable losses of protein associated with the pleural inflammatory response. In these horses when the TPP falls below about 45 g/L infusions with at least 3-4 litres of plasma is often of benefit.

Following initial replacement of deficits, oral fluid supplementation can usually cover ongoing losses.

Mucolytics have been used in these types of cases. The efficacy of these agents is not clearly established. However, there is some evidence bromhexine may increase the concentration of antimicrobial agents in pulmonary secretions.

With the exception of ‘heaves’ there is no compelling evidence that most of the common pneumonic processes occurring in horses result in bronchoconstriction. As a result widespread use of these agents in affected horses may not be warranted. However, the bronchodilator clenbuterol may be useful due to its effects of stimulating mucociliary clearance as opposed to any particular bronchodilating effects.

Horses with environmentally induced airway inflammation (see accompanying paper by Jennie Hodgson) respond well to administration of anti-inflammatory agents (particularly steroids) by inhalation.

Good nursing care is also vital in the management of pneumonia. This includes limitation of stress, provision of a highly palatable and digestible diet and constant
surveillance for complications such as lung abscessation, anterior thoracic masses/abscesses, pulmonary infarction, bronchopleural fistulas, pericardial effusion and laminitis.
EPIDIDYMAL SPERMATOZOA

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Introduction
Castration, death or euthanasia of a valuable stallion results in loss of genetic potential. However upon death, viable gametes are often present in the epididymes that can be harvested to produce additional offspring. Alternatively, prior to gelding a colt it may be worthwhile to collect and freeze ejaculated spermatozoa and then epididymal spermatozoa at the time of castration. In the past few years, the potential to collect these gametes at the time of castration or at post mortem provides an option for owners of stallions to preserve their genetics.

Harvesting and Use of Epididymal Spermatozoa
The first reported pregnancy from the use of frozen-thawed stallion sperm was in a mare inseminated with epididymal sperm in 1957 (Barker and Gandier, 1957). Since then, little has been published on the fertility of epididymal sperm in horses. In sheep, however, it has been shown that the in vivo fertility of cauda epididymal ram sperm only achieved that of ejaculated sperm when it was deposited surgically in the region of the utero-tubal junction (Fournier-Delpech et al. 1979). Sperm require a period of plasma membrane maturation as they transit from the caput to the cauda epididymis. During this maturation process the potential for motility is acquired, albeit suppressed until the time of ejaculation. The reasons for the differences in fertility between epididymal and ejaculated sperm may include variations in cell surface characteristics and the lower progressive motility of epididymal sperm.

Handling epididymal spermatozoa
After castration, testes have been transported to the laboratory at either room temperature (Morris et al. 2002) and processed immediately or cooled to approximately 5ºC (Bruemmer et al. 2002; Neild et al. 2006) for up to 24h prior to processing. The spermatozoa were recovered using a retrograde flushing technique. Neild et al. (2006) observed better post thaw motility characteristics in the spermatozoa from testes transported at 5ºC during prolonged transport (24h).

Insemination with Frozen-thawed Epididymal Spermatozoa
In studies performed at the Equine Fertility Unit, Newmarket (UK), the epididymes of 22 colts ≥2 years of age were recovered at the time of standing castration. The testes and epididymes were transported to the laboratory at room temperature within 4 hours of castration. Sperm were then flushed from the cauda epididymis and vas deferens with skim milk diluent and processed for freezing. Each pair of epididymes provided between 10 and 30 doses (200 x 10⁶ per dose) of epididymal sperm.
The mean (± SD) total progressive motility of frozen-thawed epididymal sperm with or without prior exposure to seminal plasma was, respectively, 16.4 ± 5.1% (n=14) and 20 ± 4.4% (n = 11). Therefore, the simple addition of seminal plasma to epididymal sperm failed to improve its progressive motility to the level of frozen-thawed ejaculated sperm (30.2 ± 8.3%, n = 64). Examination of the capacitation status of the sperm by chlortetracyclin staining revealed that 38% of epididymal sperm were acrosome reacted after thawing in comparison with only 7% of frozen-thawed ejaculated sperm. This high proportion of spontaneously acrosome reacted sperm may be detrimental to the fertilizing capacity of the frozen-thawed epididymal sperm population.

A total of 117 mares were inseminated hysteroscopically with epididymal sperm using a fixed time insemination protocol described for ejaculated spermatozoa (Morris et al. 2003). When hysteroscopic inseminations were used to deposit 200 x10⁶ fresh epididymal sperm directly onto the utero-tubal papilla, 45% (9/20) of mares conceived (Morris et al. 2002). These conception rates were reduced substantially when mares were inseminated with similar numbers of frozen-thawed epididymal spermatozoa by either conventional (1/13, 8%) or hysteroscopic (9/51, 18%) insemination. When the frozen-thawed epididymal spermatozoa were processed through sperm TALP, 29% (7/24) per cycle conception rates were obtained after hysteroscopic insemination with only 5 to 10 x10⁶ spermatozoa. No mares conceived (0/9) when inseminated hysteroscopically within 6 hours post ovulation with 200 x10⁶ frozen-thawed epididymal sperm.

The in vivo fertility of epididymal spermatozoa appears to be lower than ejaculated sperm in the horse. This may be a reflection of the differences between stallions in their intrinsic fertility and their response to sperm freezing, as well as the variable fragility of the plasma membranes of sperm which have accumulated for varying periods of time during storage in the cauda epididymis. Further studies are currently underway to optimise the freezing diluents for epididymal sperm to improve its fertility. Based upon the low motility and the fragile plasma membranes of frozen-thawed epididymal sperm, it is recommended that mares be inseminated hysteroscopically by depositing the epididymal sperm onto the papilla of the utero-tubal junction within 6 hours prior to ovulation.

Use of Frozen-thawed Epididymal Sperm for ICSI

The most efficient use of epididymal sperm may be intracytoplasmic sperm injections. Research in this area has been limited. Recently, epididymal sperm was collected after standing castrations of 3-year old colts and frozen in pellets (Rosate et al. 2004). Sperm were later thawed and injected into oocytes that had been matured in vitro. Injected oocytes were placed in culture, and 39% of the oocytes cleaved. Although no blastocysts developed during the project, the study demonstrates the potential use of ICSI for assisted fertilization with epididymal sperm. Pregnancies have resulted from the transfer of oocytes injected with epididymal spermatozoa (EM Carnevale, Colorado State University, Equine Reproduction Laboratory, unpublished data 2005). Because minimal numbers of sperm are used for ICSI, this procedure may provide a future method to maximally use epididymal sperm after death of a valuable stallion.
Conclusions
Harvesting of spermatozoa from the epididymes of stallions after castration or death can be used to produce pregnancies. However, optimal methods to cryopreserve and use epididymal spermatozoa are still being investigated.

References
TREATMENT OF TRANSITIONAL THOROUGHBRED MARES WITH AN INTRAVAGINAL PROGESTERONE-RELEASING DEVICE

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The imposed start of the Thoroughbred breeding season of September 1st in the southern hemisphere commences at a time when many mares are still in the transition phase from winter anoestrous to normal, regular oestrous cycles. A variety of methods have been utilised in an attempt to advance the onset of normal oestrous cycles in transitional mares. The most effective method for shortening the transition period is through the use of controlled light exposure commencing several weeks prior to the start of the breeding season (Guillaume et al 2000). Unfortunately, controlled light exposure is labour intensive and therefore expensive, and as a result, only 40% of stud farms in a recent New Zealand survey used lights to manage their dry mares (Rogers et al, 2007). Other methods to reduce the duration of the transition period utilise pharmacological treatment such as GnRH (gonadotrophin releasing hormone) or its analogues (Hyland and Jeffcott 1988; McKinnon et al 1996), oral progestagens (Webel and Squires 1982; Wiepz et al 1988), progesterone administered parenterally (Alexander and Irvine 1991) or dopamine antagonists such as domperidone (Besognet et al 1997; McCue et al 1999). None of these methods have been shown to reliably advance the transition phase and many of them have been shown to only be effective when used in conjunction with light treatment (Daels 2006; Nagy et al 2000).

The off-label use of progesterone-containing intravaginal devices designed for cattle has been described (Jochle et al 1991). Generally, these devices have not gained widespread acceptance because clinically they are associated with obvious discomfort and marked vaginitis (Grimmett 1992). The intravaginal route offers a method of administering progesterone to mares that avoids the need for daily treatment. Cue-Mare® (Bioniche Animal Health Australasia, Victoria, Australia) is a recently developed intravaginal progesterone-releasing device specifically designed for mares. The device consists of two main components: the carrier body or “wishbone” is used to hold the treatments inside the vagina and two “pods” are attached to the arms of the wishbone which carry progesterone (1.72g, 10% ww) formulated into a soft, silicone matrix. Animal comfort and progesterone profiles during treatment with the device have been described previously (Grimmett et al 2002).

The efficacy of the Cue-Mare device for the management of 151 transitional mares was determined on three commercial Thoroughbred stud farms (two in the Waikato region of New Zealand, and one in the Hunter Valley region of Australia) during the 2004, 2005 and 2006 breeding seasons. Mares with a history of oestrous behaviour lasting for longer than 14 days and that had no ovarian follicles greater than 25mm in diameter detected during this period were selected for the study. None of the mares
had been previously exposed to light treatment in the season they were treated. Each mare was treated with an intravaginal device on Day 0. On Day 7 the reproductive tract of each mare was examined by ultrasound and the device was removed if a follicle of greater than 30mm in diameter was detected. If there were no follicles greater than 30mm in diameter present on day 7, the mare was re-examined 3 days later (Day 10). The reproductive tract of each mare was examined at 24 hourly intervals following device removal and hCG (human chorionic gonadotrophin, 1500 iu IV) was administered when a follicle of greater than 35mm was detected. Each mare was served naturally 24-36 hours after hCG administration. All mares were examined at 24 hourly intervals after service to determine the day of ovulation. Each mare was pregnancy tested 14-16 days after service.

Results of the trial are shown in Tables 1 and 2. Forty-one mares (27.2%) had the device removed on day 7, with the remaining 110 mares having the device removed on Day 10 (72.8%). Of the 151 treated mares, 128 (84.8%) of them were in oestrus within 5 days of device removal, the average interval from device removal to oestrus being 1.87 ± 0.85 days. Of the 128 mares that were in oestrus within 5 days of device removal, 113 (88.3%) ovulated within 7 days of device removal. Nine mares were not served because they were either booked to an injured stallion (3 mares) or the stallion to which they were booked had too many mares to serve on the day of ovulation (6 mares). Of the 104 mares bred within 7 days of device removal, 63 conceived (60.6%).

Table 1: Reproductive performance of transitional Thoroughbred mares treated with an intravaginal progesterone-releasing device for 7 or 10 days.

<table>
<thead>
<tr>
<th>No. of treatment days</th>
<th>No. of mares</th>
<th>Oestrous within 5 days* No. (%)</th>
<th>Ovulation within 7 days# No. (%)</th>
<th>Pregnancy rate@ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>41</td>
<td>41 (100)a</td>
<td>38 (92.7)a</td>
<td>60.7a</td>
</tr>
<tr>
<td>10</td>
<td>110</td>
<td>87 (79.1)b</td>
<td>75 (86.2)a</td>
<td>60.0a</td>
</tr>
<tr>
<td>Total</td>
<td>151</td>
<td>128 (84.8)</td>
<td>113 (88.3)</td>
<td>60.6</td>
</tr>
</tbody>
</table>

* From the time of device removal. Percentage is of the total treated.
# From the time of device removal. Percentage is of those in oestrus within 5 days.
@ For those mares served within 7 days of device removal.

a,b Values within the same column with different superscripts are significantly different (P<0.05)
Table 2: Reproductive performance of transitional Thoroughbred mares treated with an intravaginal progesterone-releasing device for 7 or 10 days by country.

<table>
<thead>
<tr>
<th>Country</th>
<th>No. of mares</th>
<th>Oestrous within 5 days* No. (%)</th>
<th>Ovulation within 7 days# No. (%)</th>
<th>Pregnancy rate@ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Zealand</td>
<td>118</td>
<td>99 (83.9)a</td>
<td>90 (90.9)a</td>
<td>60.0a</td>
</tr>
<tr>
<td>Australia</td>
<td>33</td>
<td>29 (87.9)a</td>
<td>23 (79.3)a</td>
<td>60.9a</td>
</tr>
<tr>
<td>Total</td>
<td>151</td>
<td>128 (84.8)</td>
<td>113 (88.3)</td>
<td>60.6</td>
</tr>
</tbody>
</table>

* From the time of device removal. Percentage is of the total treated.
# From the time of device removal. Percentage is of those in oestrus within 5 days.
@ For those mares served within 7 days of device removal.

aValues within the same column with different superscripts are significantly different (P<0.05)

Intravaginal treatment with a progesterone-releasing device for 7 or 10 days was effective in inducing oestrus and ovulation in transitional Thoroughbred mares. Mares that had a device inserted for 7 days were more likely to show oestrus (P<0.05) after device removal than mares that had the device removed after 10 days. This is to be expected because all treated mares were examined on Day 7 and the device was removed if a follicle of greater than 30mm was detected. This underlines the importance of examining treated mares on Day 7. There were no significant differences between the two countries in any of the reproductive parameters measured. The proportion of mares that ovulated within 7 days of device removal was lower for mares in Australia than for mares in New Zealand, however this difference was not significant (79.3% vs 90.9% respectively; P=0.17). The small sample size from Australia probably accounts for this difference, although other effects such as nutrition, body condition, and ambient temperature have all been shown to affect the duration and ovulatory response of the transitional phase in mares (Allen 1987, Carnevale and Ginther 1997). Whilst these parameters were not measured in the current study, it is possible that regional differences in these aspects could affect the response to treatment.

The intravaginal progesterone-releasing device used in this study (Cue-Mare) effectively induced oestrus and ovulation in transitional Thoroughbred mares without the need for previous light exposure. This treatment protocol offers a convenient and reliable method of managing transitional mares on commercial stud farms.

Acknowledgments:

Thank you to Dr. Allan Gunn, Gundy Veterinary Services, Arrowfield Stud, Scone, NSW for participating in this study.

References:


THE EFFECT OF DIETARY SUPPLEMENTATION WITH ANTIOXIDANTS ON FERTILITY PARAMETERS OF STALLIONS

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Across the species, considerable variability exists in the intrinsic fertility amongst males (Morris et al. 2001; 2003; Morris and Allen, 2002; Tucker, et al. 2007). Indeed, this variability may be further exaggerated when semen is cooled or frozen and thawed. Variability amongst stallions in the response of their semen to the freeze-thaw processes has been reported and is commonly cited as a major factor influencing the fertility of frozen semen (Voss et al.1981; Samper, 2001). To reduce this variability, and improve the quality of stallion semen, the management of the breeding stallion needs to be standardised. For the stallion to be in good health he needs to be well nourished, in optimal condition and stress needs to be minimised.

Sperm viability is dependent upon the integrity of the plasma membrane surrounding the cell and the chromatin structure within the nucleus of the cell. The sperm plasma membrane is a phospholipid bilayer which must remain intact until the process of capacitation commences prior to fertilization. The membrane then undergoes a “scrambling process” and the acrosome reaction occurs as the spermatozoon binds to the zona pellucida surrounding the oocyte (Cheng et al., 1996, reviewed by Colenbrander, 2005). The major component of this phospholipid bilayer is docosahexanoic acid (DHA), an omega-3 fatty acid (Harris et al. 2005). It has been shown in boars and humans that a high ratio of DHA: docosapentaenoic acid (an omega-6 fatty acid) is associated with high fertility (Conquer et al. 2000; Maldjian et al. 2005).

It is known that post-ejaculatory metabolic changes occur when semen is cooled or frozen, exposing the spermatozoa to high levels of ROS exposure if antioxidants are not present in sufficient amounts (Griveau et al., 1995). To minimise the detrimental effects of oxidation during semen processing, many researchers have added the antioxidants to semen diluents for different species, some of which include natural antioxidants found in semen such as hypotaurine, taurine and glutathione (Li et al. 1975; Kankofer et al. 2005). In the horse, the simple addition of ascorbic acid to the semen diluent can increase the percentage of membrane intact spermatozoa at 5°C (Aurich et al. 1997); the addition of catalase reduces the level of H$_2$O$_2$ and prevents DNA fragmentation (Baumber et al. 2000; 2003; Kankofer et al. 2005) and 2mM pyruvate was beneficial in maintaining sperm motility over 48h (Bruemmer et al. 2002). However, this approach does not prevent the oxidative damage which occurs during spermatogenesis and storage in vivo.

Spermatogenesis occurs within the anaerobic environment of the teste. Under these conditions spermatozoa are highly susceptible to damage from oxidation, caused by ubiquitous reactive oxygen species (ROS) produced from normal metabolism.

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involving mitochondrial respiration. It is known that animals which are very active produce far more ROS than sedentary ones, as they have a higher turnover of cellular and repair mechanisms coupled with a faster metabolic rate, in order to supply enough energy to meet their work load. Consequently, high levels of oxidative stress experienced by a competition stallion may lead to exposure of the reproductive tract to excessive levels of ROS which result in lipid peroxidation of the polyunsaturated fatty acids within the sperm plasma membranes and ultimately a reduction in fertility (Lenzi et al. 2002). At a cellular level, the toxic end products of lipid peroxidation result in membrane degradation, fragmentation of DNA and cell death. Superimposed on these destructive effects of oxidative damage are the observations in many species at an endocrine level, that chronic stress induces the production of glucocorticoids to the extent that testosterone levels may be reduced (Welch et al. 1982), which may also be detrimental to the process of spermatogenesis.

In a well controlled study in stallions, Janett et al. (2006) demonstrated the negative effects of repeated strenuous treadmill exercise on semen quality and freezability. They measured an increase in heart rate, plasma concentrations of cortisol, testosterone and lactate which were associated with an increase in acrosome defects and reduced sperm motility after freezing. Consequently, strenuous exercise results in an animal with elevated antioxidant requirements which, if excessive and not met by feed supplementation, can be detrimental to sperm production. Indeed, to protect the fragile population of spermatozoa in the aerobic testicular environment from the effects of oxidation, there are high levels of antioxidant vitamins and minerals concentrated in the testes (Volhra et al., 1973; Behne et al. 1982; Hansen and Deguchi, 1996). Antioxidant protection is afforded by vitamins E, C and A and the trace minerals selenium (Se), manganese (Mn), copper (Cu) and zinc (Zn), which can all be provided in the diet. The minerals, and in particular Se, are important in the formation of antioxidant enzymes, which are responsible for the safe removal of ROS from tissue.

Feeding tuna oils to boars has resulted in increased levels of sperm output as high intake of DHA altered the phospholipid structure of the sperm membrane and improved sperm quality (Rooke et al. 2001). In stallions, dietary supplementation with polyunsaturated omega-3 fatty acids resulted in an increase in daily sperm output and a higher percentage of morphologically normal spermatozoa. Dietary supplementation has also improved the quality of semen from “poor coolers” (Brinsko et al. 2005) and the freezability of semen from a stallion which had low sperm quality (Harris et al. 2005). However, increasing the levels of oxidation-vulnerable fatty acids within the membrane structure (Surai et al., 2000) increases the risk from ROS damage and consequently increases the requirement for membrane stabilization. In such a situation, high levels of vitamin E and Se supplementation may have beneficial membrane stabilizing properties.

There are high levels of Se concentrated within the testes and the spermatozoa, where it protects the sperm chromatin from decondensation by detoxifying peroxides. It is interesting to observed that Se deficient mammals have lower levels of Se within their testes, reduced numbers of spermatozoa within their ejaculate, reduced sperm motility and similarly reduced fertilization capacity (Surai, 2001). Selenium deficiency in stallion spermatozoa has been associated with abnormal sperm chromatin condensation and has been significantly correlated with a reduced foaling rate.
Bertelsmann et al. 2005). Surai (2001) also described increased percentages of morphological abnormalities which include head and mid-piece defects (McCoy and Weswig, 1969), which contribute to reduced fertilization potential in Se deficient mammals. Adequate levels of the Se-dependent antioxidant enzyme, glutathione peroxidase (GSH-px), a powerful antioxidant protectant for developing spermatozoa, have been associated with improved percentages of viable spermatozoa with robust mitochondrial mid-pieces.

The efficacy of feed-based antioxidant minerals is related to their chemical form. Research has shown that minerals chelated to small peptide proteins or expressed in yeast are more readily absorbed, stored and utilised in animals, as these are the forms found in nature, which animals have evolved to exploit. Inorganic minerals, especially Se and Cu, conversely have pro-oxidant effects in vivo, exacerbating the problems of oxidative stress.

In conclusion, the inclusion of anti-oxidants in the stallion diet may have beneficial effects on the quality and fertility of stallion semen and warrants further investigation.

REFERENCES:

CHECK IT BEFORE YOU CHUCK IT.  
THE ART AND SCIENCE OF PLACENTAL INSPECTION

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Diagnostic labs dealing with equine material have long realised that placental inspection is a vital part of a postmortem investigation to determine the cause of loss in equine abortion, stillbirth or neonatal death. It is strongly advised that studfarms encourage their staff to examine the placenta from all foalings routinely. On birth of a live foal there is a one-off, unique window of opportunity for stud personnel and/or a veterinary surgeon to carry out a quick but detailed inspection of all placental parts (amnion, cord and allantochorion) prior to disposal. If done conscientiously it yields useful information as to the normality or otherwise of the foal’s intra-uterine environment, which may help explain why the foal is defective at birth, or becomes so in some way in the first week of life. Completion of a placenta form for each foal’s placenta documents the findings for future reference, alongside reference measurements for placentas from ‘normal’ TBs. Digital photos can also be helpful.

It is recommended that the inspection is performed after spreading the placenta on a waist-high board or table with good lighting. Inspection is facilitated if the chorion is spread out in an F-shape, whichever side is outermost, and that both surfaces (villous and allantoic) are carefully examined, the amnion inspected and any contents noted (meconium in particular), and the total and amniotic cord lengths are measured after untwisting. Excessive cord twists, urachal dilatations, and the relative size and distribution of the umbilical arteries are quickly noted and the sites of implantation and term foetal location are recorded. The state of the free end of the ruptured cord is also noted. The integrity of the horn tips is checked in case part is retained. It is usually possible, by elevating the horn tips above the table, to determine whether the foal had occupied the left or right horn in late pregnancy.

Sampling from normal foals’ placentas is not carried out routinely but samples for bacteriology and/or histology are recommended if any site is perceived to be possibly abnormal. This presentation aims to provide an illustrated refresher course in (macroscopic) placental inspection, highlighting some of the abnormalities found in the placentae from ‘normal’ pregnancies.
THE PHARMACOLOGIC ACTIVITY OF DOMPERIDONE IN HORSES

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Domperidone is a benzamide molecule with primarily dopamine-2 receptor (D2R) antagonist activity. In human medicine it has been administered as an anti-emetic and prokinetic drug. In veterinary medicine it was originally investigated for use as a prokinetic in both horses and small animals. Understanding of the pharmacologic actions of domperidone has lead to its extensive use in equine reproduction and its investigation for other possible therapeutic strategies.

Dopamine-2 Receptors (D2R)

Dopamine receptors are widely spread throughout the mammalian body and have been linked to actions as diverse as modulation of mood and behaviour through to control of lactation. Like the dopamine-1 receptor, D2R is a G-protein-coupled transmembrane receptor, which is linked via adenylate cyclase to the control of K+ and Ca2+ channels, arachidonic acid release and many other cytoplasmic events. However, D2R is an inhibitory receptor, and the binding of dopamine results in decreased activation of the secondary messenger systems and the subsequent decrease of cellular events controlled by these secondary messengers.

Domperidone – Structure and Activity

Domperidone’s antagonist effect at the D2R is believed to be responsible for the pharmacologic effects reported in horses. Domperidone is excluded from the brain by the blood brain barrier, which is possibly a function of its active efflux by P-glycoprotein[1]. This is the likely reason that it has not been implicated in inducing extrapyramidal motor effects or causing sedation - common adverse effects from other D2R antagonists like metoclopramide.
Therapeutic Purposes

In the horse, domperidone has been investigated and used for several different purposes.

1. Induction of Lactation

Both domperidone and another D2R antagonist, sulpiride, have been successfully used for the induction of lactation in barren mares after oestrogen and progestagen priming\[^2-5\]. The induction of lactation in mares is often necessary when attempts to find lactating foster mares fail. Whilst induction of lactation may appear to be the most important component of the foster mare unit for initial survival of the foal, behavioural acceptance or adoption of the foal by the mare is often the limiting factor to success of any adoption event. It would appear that it is extremely important to also milk the mare at frequent intervals during domperidone administration to successfully induce lactation. However, as already discussed, induction of maternal behaviour is vital to getting the mare to accept an orphaned foal. Along with the priming of mares with oestrogen and progestagen administration, induction of maternal behaviour and the chance of foal acceptance appears to be increased by sedation and vaginal and cervical stimulation of the mare\[^3, 6\]. Adoption success rates appear to vary between investigators; however, induction of lactation would appear to be successful in most instances where mares have previously lactated. Investigators have commented that colostrum is not always produced by mares that are induced to lactate as a foster mother, so an alternate source of colostrum should be made available to the foal. In one study, 4 of 6 mares treated with an adoption/lactation induction program including daily administration of domperidone cycled normally after lactation was induced\[^3\].

2. Management of Vernal Transition (Induction of Ovulation)

Due to the mandatory establishment of the horses birthday as August 1\(^{st}\) (September 1\(^{st}\) for Standardbred horses) in the Southern Hemisphere, considerable pressure is placed on breeders to have foals born as soon after that date as possible to ensure that horses reach developmental targets with respect to sales and age related events. As mares are long day breeders, with the main regulator of reproductive activity being photoperiod, there can be significant periods at the beginning of the breeding season (when days are still relatively short) where mares will cycle in an irregular manner. The period between anoestrus and regular cyclical reproductive activity has been termed the vernal transition period and is now considered to be a result of inhibitory signals such as short day length on gonadotrophin and GnRH secretion\[^7\].

Domperidone has been used in transitional mares to induce ovulation, and thereby allow earlier covering of mares. As prolactin is responsible for increasing gonadotrophin receptors in developing follicles, domperidone’s effect of increasing prolactin may explain its ability to induce ovulation. Increased sensitivity to FSH and LH results and, consequently, follicular development and ovulation are induced.

A blinded, placebo controlled, investigation of the effects of domperidone administration on 47 thoroughbred mares considered to be either in anoestrus or vernal transition has been performed in Australia\[^8\]. Mares were not placed under
artificial lighting. Results indicated that administration of domperidone to mares with follicles 2 cm or larger resulted in 87% ovulating within 16 days of treatment initiation. In comparison, mares treated with an altrenogest/cloprostenol regime resulted in only 73% of mares with follicle size of 2 cm or more ovulating 17 to 21 days after treatment. Ovulation appeared more likely to occur if commencement of domperidone treatment coincided with a follicle of 2 cm or greater. No adverse effects were recognised with administration of daily doses up to 1200 mg/mare. Over 70% of mares treated with domperidone conceived. There were 15 (out of a possible 17) foals born to mares that had received domperidone. All foals were considered normal at birth and 12 hour post partum IgG levels from these foals showed no evidence of deficiency of collostral transfer.

In a separate communication there is reference to the use of artificial lighting for 14 days followed by daily sulpiride administration resulting in mares ovulating approximately 16 days earlier than mares only under artificial lighting\(^7\). Whilst this has not been investigated with domperidone, the common mechanism of action would suggest that similar results should occur.

3. Prokinetic Activity

Whilst there are references to the use of domperidone as a prokinetic treatment in horses with gastrointestinal disturbances such as ileus\(^9, 10\), there appears to be no published review of its efficacy in equine patients. A preliminary study using an experimental model of post operative ileus in ponies showed encouraging results when domperidone was administered at 0.2 mg/kg intravenously\(^11\). Gastrointestinal transit time, electromechanical activity and coordination of gastric and intestinal cycles were restored after administration.

The commonly proposed mechanism of action of domperidone in the gut is through its D2R antagonism, resulting in reduction of the inhibitory effects of dopamine on motility. Several studies in humans and laboratory species have demonstrated excessive gastrointestinal dopaminergic and adrenergic activity in post-operative ileus. Laboratory models showed domperidone successfully antagonises the inhibitory effects of dopamine on gastric motility\(^12\). However recent work in people indicated that domperidone was responsible for an increase in circulating motilin and somatostatin, possibly as a result of an effect at muscarinic receptors\(^13\). These two peptides play important roles in gastrointestinal contractility. Whether direct effects at D2R or the release of motilin and somatostatin as a result of a putative muscarinic receptor effect is responsible for changes in motility is unknown.

Though nausea and vomiting are not recognised in equine practice, domperidone can be expected to have an anti-emetic effect through its D2R activity at the chemoreceptor trigger zone. It appears that there is little effect on colonic motility related with domperidone administration\(^14\). There are no reports of increased GI motility from administration to mares for reproductive of lactation management (Jurox APVMA Pharmacovigilance Reports 2003-2006). Unfortunately, the currently available formulation of domperidone in Australia is an oral paste, which may make it unsuitable for treatment of animals suffering from gastric reflux and ileus. Direct or retrospective studies into the efficacy of domperidone for management of ileus may be warranted.
4. Diagnostic Tool for Diagnosis of Equine Cushing’s Disease

Cushing’s disease in the horse is almost always associated with functional adenoma development in the pars intermedia region of the pituitary gland. Along with characteristic clinical signs such as hirsutism and polyuria/polydypsia, pre-mortem diagnosis relies on laboratory investigation. Diagnostic tests for Cushing’s disease in the horse include evaluation of baseline plasma cortisol and ACTH levels and dexamethasone suppression tests. Some horses with histologically confirmed tumours of the pars intermedia may exhibit normal baseline cortisol and ACTH levels and interpretation of dexamethasone suppression tests can sometimes be misleading.

Recent work performed at Purdue University investigated the effect of domperidone on plasma ACTH levels in horses with pars intermedia tumours[15]. Whilst only a limited number of cases were investigated, horses that had histologically-confirmed tumours of the pars intermedia had plasma ACTH levels above reference ranges 4 hours post dosing and horses that did not have tumours had levels within the reference range. Exaggerated responses to domperidone administration were exhibited in horses with pars intermedia tumours, most likely as a result of antagonism of the inhibitory effects of hypothalamic dopamine binding to D2Rs. Interestingly, this is in contrast to a placebo controlled study performed in normal humans, which indicated that administration of domperidone resulted in blunted secretion of ACTH and cortisol in response to the stress of venipuncture[13]. A full understanding of the effects of domperidone on the hypothalamic-pituitary-adrenal axis may develop with future research.

5. Laminitis

There is currently no published peer-reviewed information about the use of domperidone for treatment or management of laminitis in the horse. However, a patent search revealed that there is an application lodged for the use of domperidone in the treatment and management of laminitis in the horse and other hoofed species[16]. It is proposed by the patent applicant that by virtue of an α-adrenergic antagonist action, domperidone can prevent lamellar detachment. The application claims that trials have indicated long term elimination of the signs of laminitis and 6 case examples indicating resolution of clinical signs of laminitis have been sited. Whether there is any scientific merit to the use of domperidone for laminitis will no doubt be clarified in the future by the presence (or lack of) peer-reviewed publications on this subject.

6. Prevention and Treatment of Fescue Toxicosis in Pregnant Mares

Though it is minor concern for horse studs in Australia and New Zealand, certain regions of the US have recognised for many years a syndrome of gestational and post-parturient problems in mares and foals that have been exposed to endophyte infected fescue grass. After exposure, pregnant mares exhibit a range of symptoms including prolonged gestation, agalactia, thickened and retained placentas and will often give birth to dysmature and weak foals. Death of the mare and foal may also occur.
Endocrine related findings in mares and foals exposed to endophyte infected fescue included decreased maternal plasma progesterone and prolactin concentrations and higher plasma oestradiol-17\(\beta\) concentrations than normal mares at similar stages of their gestation\[17\].

The ergot alkaloid, ergovaline, is considered one of the major contributors to the syndrome of fescue toxicosis, and has been shown to be directly responsible for decreased prolactin production through its D2R agonist activity. The mechanisms of activity involved in the other components of the toxicosis have not yet been fully elucidated.

Domperidone has been used successfully to treat and prevent the signs of fescue toxicosis, most likely as a result of its D2R antagonist activity and prevention of the effects of ergovaline. However, this only clearly defines the role of domperidone in reversing the effect of ergot exposure on maternal prolactin secretion by the pars distalis. The mechanism/s associated with increasing maternal and neonatal progestagen, maternal ACTH and cortisol levels are not yet fully understood, but are possibly associated with neuro-endocrine feedback loops between the hypothalamus, pituitary gland, gonad, placenta and adrenal gland.

The efficacy of domperidone in preventing and treating fescue toxicosis has been reported in two studies previously presented at AAEP conferences. The first study\[18\], looked at the comparative effects of domperidone administration in four groups of mares : – 1. not exposed to endophyte infected fescue; 2. exposed to endophyte infected fescue; 3. exposed to endophyte infected fescue and treated with domperidone up to parturition; and 4. exposed to endophyte infected fescue and treated with domperidone past parturition. Whilst their was no difference in gestational length amongst all four groups there were statistically significant improvements in mammary scores and prepartum progestagen and prolactin concentrations for mares receiving domperidone up to and past parturition compared to mares exposed to endophyte infected fescue and not receiving domperidone. There were no differences in milk composition noted amongst treatment groups. All foals from non-exposed mares and mares exposed and treated with domperidone survived up to 10 days post partum whilst only 50% of foals survived up to 10 days from endophyte exposed untreated mares.

In the second study by the same research group\[19\], the clinical effect of domperidone on fescue toxicosis in pregnant mares was investigated. Clinical observations from 1423 pregnancies considered at risk of fescue toxicosis were reviewed. Two main sub-populations of mares were identified with respect to time of initiation of treatment with domperidone: 1. a preventative mode group, where domperidone treatment was initiated around 10 to 15 days prior to due date and 2. a treatment mode group, where mares started treatment less than 10 days before due date of foaling. A third small population of mares that started treatment greater than 16 days prior to expected due date was also evaluated. When compared to historical positive controls, mares receiving treatment had 1. normal gestational lengths and udder development, 2. much lower incidence of agalactia, 3. better live foal delivery rates and, 4. a lower rate of placental retention. Owners and vets administering domperidone felt it was an effective therapy.
Conclusion

Domperidone is a benzamide molecule that is proposed to have antagonistic pharmacologic activity at the dopamine-2 receptor. Currently in Australia, domperidone is primarily used for management of the vernal transition period and in the induction of lactation in foster mares. There are, however, several potential expansions of use for domperidone in horses, which may develop in coming years. It should be noted that use of domperidone in horses for laminitis, treatment of fescue toxicosis, promotion of ovulation, parturition and lactation are all protected by patent, therefore only the licensed registered equine product (Jurox Domperidone Paste for Horses) can legally be used for these indications, even though some are off-label uses.

References


ASSESSMENT AND TREATMENT OF PLACENTITIS

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Placentitis is considered the single most significant cause of foetal loss in horses. Most studies indicate that placental dysfunction and infection account for approximately 30% of all equine abortions. In addition, placentitis and chronic placental dysfunction contribute significantly to the delivery of premature, septic or dysmature foals that require expensive intensive care to survive.

Mares with high risk pregnancies can be classified into 3 groups
1) Mares with a previous history of abnormal pregnancy, premature delivery or delivery of a septic foal.
2) Mares showing clinical signs of an abnormal pregnancy – premature lactation, vaginal discharge, perineal relaxation
3) Mares with a systemic illness that can affect the foetus: colic, prolonged surgery, severe lameness, chronic laminitis, systemic infection.

Many mares that have a high risk pregnancy show no clinical signs and it remain difficult to readily monitor these mares pregnancies.

The most common presentation of placentitis is an ascending infection through the cervix. The cause of the compromised cervical seal can be varied. Stress to the foetus or mare can induce alteration of progesterone synthesis and metabolism which may result in abnormal relaxation of the cervix. It is apparently common for normal mares to have intermittent relaxation of the cervix and the specific factors that determine an individual mare’s risk of developing placentitis are unknown. However, the frequency and duration of relaxation of the cervix and the anterior vaginal micro flora may be significant factors. Bacterial vaginosis is an important risk factor for women at risk for premature labour. Once infection has been established in the placenta, the severity of the inflammatory response is critical. There is activation of the placental macrophages with synthesis of a range of cytokines and prostaglandins. These inflammatory mediators adversely affect the foetal metabolism resulting in significant stress and induce an increase myometrial contractility. The infection may cross into the amnion and ultimately infect the foetus, which results in abortion or the delivery of a weak septic foal

Assessment of the High Risk Pregnant Mare.
The emphasis during the examination of a high risk pregnant mare will vary according to the category of the cause of the risk.

History and Physical examination:
It is important to obtain a detailed history of previous pregnancies, previous foalings, last date of service, the type of insemination required and the number of services or inseminations required to obtain a successful pregnancy. In addition, whether there was a high incidence of early embryonic loss is important information.

A complete physical examination should be conducted on all mares carrying a high risk pregnancy. Close inspection of the perineal region, vulval lips and the udder is critical. Any vaginal discharge, premature perineal relaxation or precocious mammary development is significant. A vaginal examination with a speculum is rarely indicated except when imminent
labour is anticipated. If there is a vaginal discharge, some of the purulent material maybe aseptically collected from the caudal vaginal without significantly disrupting the vestibular sphincter seal. Although this is far from ideal, the risk of significantly contaminating the anterior vagina and cervical region, and further contributing to ascending infection outweighs the benefits of a more invasive examination. Examination of the udder and mammary secretions can provide useful information about the readiness of the foetus for extra uterine life. A sudden marked rise in calcium concentration to greater than 12 mmol/L indicates that foetus is sufficiently mature for extra uterine life and that parturition is likely within 48 hours.

**Hormonal evaluation:**

**Progesterones:** After 180 days of gestation the foeto-placental unit is the sole source of progestagens to maintain pregnancy. The concentration of progesterone in the maternal circulation for mid-gestation to approximately 300 days of gestation is low. Frequently with placentitis there is abnormally elevated maternal concentration of progesterone. This is most likely due to dysregualtion of progestagen synthesis in the abnormal placenta and possibly excessive precursor secretion by the stressed foetal adrenal gland. Clinical signs of placentitis and low maternal progesterone, indicates a very poor prognosis for foetal survival.

**Oestrogens:** Between day 150 to 280 maternal concentration of oestrogen is highest and levels less than 500 ng/ml indicate severe foetal compromise and imminent abortion. After 280 days of gestation, interpretation of maternal oestrogen concentrations is limited and not well correlated with foetal survival.

Relaxin is produce by the placenta and is detectable in maternal circulation from 70 days of gestation. There is an excellent correlation between low relaxin concentration and foeto-placental compromise, and maternal relaxin concentration could be used to monitor high risk pregnancies. Unfortunately validated assays of equine relaxin are not available commercially in Australia.

**Ultrasonography**

Transrectal ultrasonography has been used extensively to monitor the development of placentitis and there are numerous papers defining the normal width and different stages of pregnancy. It is very important to obtain images for measurement in a consistent position. The probe should be positioned just cranial to the cervical placental junction and then moved laterally till the middle uterine artery is visible on the ventral aspect of the uterine body. The combined thickness of the uteroplacental unit is measured in 3 places and averaged. In addition to the thickness of the uteroplacental unit the presence of separation of the placental from the endometrium, the extent of folding or roughening of the surface and the cloudiness of the amniotic and allantoic fluid should be recorded. The normal uteroplacental unit is smooth and the placenta can not readily be distinguished from the uterus.

Transabdominal ultrasonography significantly increases the amount of the placenta that can be evaluated. In addition the size and viability of the foetus can be determined. There are several description of the extensive evaluation of the placenta and foetus to develop a biophysical profile using transabdominal ultrasonography. The ultrasound examination should be conducted methodically; starting on the ventral midline and moving cranially form the mammary gland to find the foetal thorax. Foetal movement and heart rate should be recorded as they are important parameters to assess viability. Stressed foals move very slowly or not at all and have an excessively low or high heart rate. The size of the foetus can assess by measuring aortic diameter. It is very important that the aorta is measured as close to the heart as possible and in a consistent plane. As least 3 measurements should be obtained and averaged. It is critically important to record the thickness of the uteroplacental unit, any separation of the placenta, excessive folding of the placenta and the cloudiness of the fluid. The foetal position can be variable until approximately 9 months of gestation, after then the
foetus should be in the anterioventral position. Many of the publications discuss measuring foetal fluids, unfortunately this parameter seems to be highly variable and therefore of limited use.

An ultrasound assessment is also very useful to assess the response to treatment. The thickening, degree of folding and roughening and cloudiness of the fluid will all improve significantly with successfully treatment. Mares with a high risk pregnancy that fail to have improvement in the ultrasonographic parameters, have a worse outcome than mares where improvement is recorded.

**Treatment:**
The primary aim of treatment is to improve the number of live, strong foals that are delivered. Delivery of a weak, small for gestational age foal that does not grow to match the performance expectations of the owners is a failure of treatment. Any associated disease must be treated effectively to reduce the stress on the compromised foetus.

Mares with clinical signs of premature delivery that are not specifically treated have a less than 30 % chance of delivering a live healthy foal. In an experimental model of ascending placentitis where treatment was not provided, all the mares aborted or delivered a weak premature foal. Treatment with antibiotics, anti-inflammatory drugs and altrenogest results in the significant improvement in foetal viability and foal health. During the past 2 years at SVH aggressive treatment has resulted in the delivery of a live foal for all mares in which treatment continued until delivery. Some of the foals from the most severely affected placentas were very small and are unlikely to be successful athletes. Approximately 30% of the foals were euthanized due to poor prognosis or poor size. Approximately 67% of the foals delivered were strong healthy foals that required no veterinary attention. Treatment of the late pregnant mare with clinical signs of placentitis will improve foal survival, unfortunately, if the foetus is small for gestational age when the clinical signs present, there is little that can be done to improve the foetal growth rate.

**Antibiotics.**
The most common antibiotic used to treat placentitis is Trmethaprim-sulphdiazine (TMPS). This antimicrobial is broad spectrum, bactericidal and has excellent penetration of the placenta, readily reaching therapeutic concentrations in amniotic fluid and the foetus. Recently, penicillin and gentamycin have been shown to effectively penetrate the pregnant uterus and reach therapeutic levels in the allantoic fluid. This provides and excellent alternative treatment choice if there is a failure the respond to TMPS.

**Anti-inflammatory drugs.**
The inflammatory response to infection contributes significantly to foetal stress and predisposes the high risk pregnancy to premature parturition. There is significant synthesis of prostaglandins in response to infection and these substance increase myometrial contractility. It is important to control the inflammatory response and nonsteroidal anti-inflammatory drugs should be administered to mares with a compromised pregnancy. Flunixin meglumine is very effectively at inhibiting prostaglandin synthesis in response to many different inflammatory stimuli, particularly bacterial toxins. Administration of 1.1 mg/kg bid of flunixin meglumine is recommend for all mares a risk of premature labour. Once the infection and inflammatory response is adequately controlled the dose can be reduced or the drug changed to oral phenyl butazone.

There is significant synthesis of cytokines by the placental in response to infection. Pentoxifylline (8.5mg/kg PO TID) is effective at reducing endotoxin induced cytokine synthesis by equine inflammatory cells. In addition the drug may improve oxygenation of the placental by improving blood flow.
Hormonal treatment
The use of altrenogest in high risk pregnancy is widespread. This seems counter-intuitive because there is an already elevated maternal progesterone level and further increasing progestagen concentration is illogical. However, 5 alpha pregane maybe very important in maintaining myometrial quiescence by inhibiting the formation of oxytocins receptors. This is supported by work in the mare, where 44 mg altrenogest given daily prevented abortion in mares that were give a prostaglandin analogue. IN addition, there is now compelling evidence in women a high risk of premature delivery that administration of 17 β OH-progesterone significantly improves outcomes and prevents premature delivery. It is recommended that, in addition to antimicrobial and anti-inflammatory drugs, altrenogest (44 mg BID PO) for 14 days is administered initially to mares at risk of premature labour. If there is an adequate response, the dose is reduced to 44 mg sid and maintained until 330 days of gestation. If there is no response, the dose is increased to 88 mg BID PO for 14 days, when the mare is reevaluated.

Ancillary treatment:
Tocyolytics – clenbuterol has been used to inhibit uterine contractility, infuriately the doses required to reduce uterine contractility result in significant adverse side effects and the drug is not recommended
Intranasal oxygen (improve foetal oxygenation), Vitamin E (antioxidant) and low dose aspirin (improve placental oxygenation) have all been administered to mares at risk of imminent premature labour. There is no data available to indicate the success of treatment.

Although aggressive treatment of the mare at high risk of premature parturition can improve foetal viability, frequently the foal that arrives is disappointing.

Prevention Monitoring Program
Because placentitis frequently has no clinical signs, a program to monitor mares with a poor breeding history has been established. Mares that have repeatedly aborted or produced weak premature/dysmature or septic foals were selected for monthly transrectal and transabdominal ultrasonography. The incidence of ultrasonographic abnormalities in these mares was over 70 %. The mares were treated according to the ultrasound findings with antimicrobials, anti-inflammatory drugs and altrenogest. The foaling rate over the previous 4 years for the most severely effected group of mares was 23 %. The monitoring program and treatment resulted in a foaling rate of > 90 % for theses mares. Only 5% the foals born required intensive veterinary care and the average size was the same as foals from normal mares. Early identification and treatment of mares with placentitis results in excellent foal viability and most importantly delivery of foal with the potential to meet the athletic expectations of the breeders.

Acknowledgments:
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Further Reading:


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A Newmarket perspective on Equine Abortions and Stillbirths

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In the UK, Newmarket is a centre of Thoroughbred breeding. The high density of brood mares and inter-stud movements dictates that disease surveillance is a priority. Most abortions and stillbirths are sent for examination immediately, primarily to screen for Equine herpesvirus infection but also for evidence of uterine inflammation or degeneration that might require treatment and surveillance in future pregnancy to prevent recurrence. A weekend pathology callout service ensures prompt investigation. The mare’s history (her health, breeding history, current pregnancy, birth details, recent travel and vaccinations) is important and sourced prior to commencement of the examination. The submitting vet receives a verbal and/or written Preliminary report on the foetus and the placenta on the same day as the postmortem. The Final report is generated after completion of microbiology and histology.

Necropsy Procedures: With the foetus on its right side, examination of the foetus commences with weighing, measuring and external assessment for congenital defects, meconium staining, trauma and resuscitation. The navel is palpated and the foetal abdomen and thorax opened and reflected. Prior to handling of the viscera, the microbiology samples are taken - bacteriology swabs from heart or liver, lung or stomach and cervical pole of the allantochorion. Additional swabs are taken from neonates (e.g. kidney, CSF, navel). Using disposable sterile instruments virology samples are then taken from liver, lung, thymus and spleen into virus transport medium VTM. The necropsy then proceeds, with examinations of abdominal and thoracic contents and the head is split longitudinally on the band saw so as to inspect the brain, cranial cavity, mouth and oro-pharynx. The limb joints are opened to check for haemarthrosis and the rib cage assessed for fractures and bruising. The placenta is then examined after a cervical pole bacteriology swab has been taken. It is now routine to take 4 samples of chorion into VTM for PCR examination for the presence of EHV-1 and EHV-4 DNA. Photographic records of unusual lesions are made. All the findings are recorded on a postmortem form.

Histology: The minimum set of tissues taken into fixative includes liver, spleen, lung, thymus, adrenal, amnion and four samples of chorion from the cervical star, body and both horns. Usually other samples are routinely harvested (kidney, brain and navel in neonates) as well as tissues with an abnormal appearance, including umbilical cord. Routinely an H&E stain is used: other specials are used to search for evidence of pathogens, e.g. fungal stains and immunostaining for Equine Herpesvirus.

Results of examinations in the UK, Newmarket in particular
In 2003 both the diagnostic labs in Newmarket published reviews of their findings of the causes of abortions, stillbirths and neonatal deaths over a period of years, based on abortions occurring from 3-4 month onwards. The results fall into two main categories
- non-infective, and infective where infections enter the closed environment of the uterus via the cervix and/or via the uterine wall.

**Non-infective:** 75.9%

**Umbilical cord abnormalities:** 38.8%
In general these are associated with cords that are longer than 80cm (95% range in normal foals 36-83cm.) Factors governing the length attained by the cord are not understood, but it is apparent that long-cordedness has a high risk. Foetal death occurs when umbilical vessels become damaged either from twisting or by looping around foetal parts, resulting in vascular compromise in the chorion - thrombosis and ischaemia being evident histologically. In the UK, this is the commonest cause of equine abortion, the foetus remaining inside the uterus for a variable period after death, so the tissues, although sterile, are autolysed when aborted. A condition, ischaemic necrosis of the cervical pole of the chorion, is also associated with long-cordedness, but the mechanism is uncertain.

**Twins and triplets:** 6%.
Prior to the advent of early pregnancy scanning and twin reduction this was our commonest cause of observed abortion in TBs. Much twin material was available providing the opportunity to define the different types of placental arrangement and events following death of one twin. Only the very largest breeds can maintain twin pregnancies to term. Triplet abortions are very rare indeed.

**Intra-partum stillbirths:** 13.7%
Contributory causes are relative or absolute foetal oversize, malpresentation (particularly carpal flexion) and unattended foaling.

**Neonatal infection unassociated with placentitis:** 3.2%
These included septicaemias, pneumonias and clostridial enterocolitis.

**Miscellaneous:** 14.2%
This diverse group includes lethal anomalies, foetal neoplasia, premature placental separation, fetal diarrhoea, body pregnancy, villus hypoplasia, amniotic rupture, postnatal trauma, neonatal maladjustment syndrome and maternal disease.

**No diagnosis:** 7.7%
These included decomposed and predated foetuses and very early abortions.

**Infective:** 16.3%

Abortions caused by EHV1 and EHV4 (6.5%)
It is the constant threat of outbreaks of ‘virus abortion’ and of paralytic herpes in the mares that motivates the equine industry to have abortions investigated. Rigorous procedures are invoked if EHV is suspected at postmortem and its confirmation or exclusion is a priority. The advent of PCR techniques has greatly assisted rapid turn-around, results being available on the same day. Other methods include rapid processing of tissues or frozen sections: the gold standard is immunoperoxidase staining of sections to visualise viral antigen in situ. Using these techniques it was possible to confirm that a form of EHV abortion previously suspected, does happen
i.e. in ‘atypical’ cases the placenta (and uterus) are infected, and a rapid expulsion of foetus and placenta occurs but the foetal tissues are negative. For this reason diagnostic procedures were changed so that placental as well as foetal tissues are now routinely screened for EHV. The ratio of EHV1: EHV4 in the diagnoses is 95:5. Typical signs (fresh foetus, premature placental separation, excess serosal fluids, pale liver foci, peri-renal oedema, pulpy thymic medulla, jaundice, lung consolidation) are not always present. Histological diagnosis is aided by finding intranuclear inclusion bodies.

**Bacterial and fungal placentitis (9.8%)**

The majority of placentitis cases become infected via the cervix with opportunistic organisms from the lower genital tract, there often being a demonstrable defect in its anatomical integrity. In only a few cases is the foetus itself infected. Some mares will suffer the same problem in subsequent pregnancies. The infection has often become chronic by the time abortion occurs and the chorion cervical pole is by then demonstrably thickened and discoloured, and the foetus undernourished. As a small number abort early in the infection and the infection is *not* apparent grossly this is the site swabbed for bacteriology, and chorion from the cervical ‘star’ is always taken for histology. In the UK placentitis affecting only the base of the horns is very rare and, at least in the Newmarket labs, leptosprirosis has not been identified as a cause of abortion although efforts have been made to screen for it.

In both viral and bacterial abortions, the endometrium itself becomes infected and may become focally infarcted. Not unexpectedly therefore on rare occasions shreds of sloughed discoloured necrotic endometrium may be found amongst the submitted placental tissues. Mare Reproductive Loss Syndrome (MRLS) and Equine Amnionitis and Foetal Loss (EAFL) are not recognised as entities in the UK but recent reports in the UK press that, following the recent mild winters, swarms of brown tail moth caterpillars (‘covered by tiny toxin-containing hairs’) have moved northwards up England provide a reminder that we should remain on the alert for such case material. Perhaps it is the mare’s endometrium and her alimentary tract which will provide essential clues as to the true pathogenesis of such phenomena.
PROGESTERONE – A REVIEW
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INTRODUCTION
Manipulation of the mare’s oestrus cycle by the use of prostaglandins to reduce progesterone levels by the luteolytic effect of prostaglandin and monitoring the progress of pregnancy by the measurement of progesterone levels has now become so routine that this may be a useful time to stop and take a look at various aspects of progesterone in order to see exactly where we are at and where we’re going. In this paper it is proposed to review the history of this compound and its relatives, and to review how the information has led us through a pharmaceutical maze leading to therapies for the maintenance of equine pregnancies.

A free encyclopaedia, known as the Wikipedia describes progesterone as a C-21 steroid hormone, which supports pregnancy and embryogenesis of humans and other species. Progesterone, it points out, belongs to the class "progestagens" and is the major naturally occurring human progestogen. On the other hand, it points out that progesterone should not be confused with the progestins, which are synthetically produced progestagens. A significant feature of progesterone, is that it does not dissolve in water and is poorly absorbed in the gut.

Progesterone was first isolated by Willard Myron Allen in 1929. Allen gave it the name progesterone, which he coined from the name PROGESTational STEROidal ketONE. Progesterone as with all steroids is derived from cholesterol via the hormone pregnenolone.

Progesterone is produced in the adrenals, gonads/brain/placenta

Functions of progesterone
- Converts endometrium to secretory stage to support pregnancy
- Immunosuppressive for acceptance of pregnancy
- Decreases uterine contractility
- Inhibits lactation during pregnancy (humans)
- Drop in progesterone initiates labour (humans)
- Neuroprotective
- Raises levels of epidermal growth factor-1
- Increases core temperature during ovulation
- Reduces smooth muscle spasm. Widens bronchi and regulates mucus.
- Increases thyroid function with various anabolic results
- Regulates oestrogen thereby preventing endometrial cancer.

Bioavailability
- Not water soluble. Poorly absorbed by oral ingestion

Progesterone is not the only progestogen identified in the mare, although it certainly is the most common. Ousey and workers described a number or progestagens and were particularly interested in those seen in the second half of pregnancy. These are illustrated here with their common names as well as their chemical structures and their normal abbreviation.
The use of progesterone for a short period followed by its withdrawal was used to modulate ovarian function in transitional oestrus by Loy and Swan in 1966 (2) and later by van Niekirk (1973) (3). It is thought that the progesterone suppresses L.H. release through GnRH suppression. Not long after this, a synthetic product became available and had the advantage that it could be administered orally. This progestogen, we now know as altrenogest or commercially regumate*, which has the other chemical name of allyltrombenolone. This compound is a synthetic C-21 steroid progestomimetic that belongs to the 19-nor-testosterone series. This compound was marketed to provide the same pendulum effect as progesterone on the transitional ovaries. Under the influence of a progestagens the reproductive tract would experience dioestrus-like conditions through l.h. suppression with the effect that on cessation of administration after eight to 10 days there would be an increase in GnRh and LH production and so the ovaries would respond by producing follicle formation and oestrogen production thus establishing the oestrus cycle.

Altrenogest

Reference: Canadian Food and Drugs Regulations

Altrenogest (Allyl-trenbolone) a synthetic trienic C21 steroidal progestomimetic belonging to the 19-nor-testosterone series. It has a similar structure and function to progesterone.

Liposoluble-enters target cells where it binds to specific receptors.
PROGESTERONE IN THE PREGNANT MARE

By the 1970’s observers had found that in the first few days of pregnancy progesterone levels rapidly increase and elevate from levels of around 1 ng per ml on the day of ovulation increasing to peak at about day 64 to around 15ng/ml followed by a gradual decline with reported levels of one to 2 ng per ml. from 180 to 300 days gestation. The last 30 days gestation show a slight rise to around 4 ng per ml. at five days prepartum, then a rapid drop at parturition (Holtan, Nettt and Estergreen (1975)). This work has being repeated by many other workers.


(It is worth noting here we have found some significant differences between different laboratory readings and between different E.L.I.S.A. kits so this is important to remember when comparing results. Where we have had unexpected results the samples have been tested in duplicate by two other laboratories, thus providing a quality control)

As already described the progestin altrenogest can be used to supplement protestagen levels in the circulation to support pregnancy. This is particularly when progesterone levels produced in these mares are deemed to be low or inadequate. In order to assess which mares might need treatment, over the last two or three years we have taken a blood sample from selected cases in which we feel may have a problem or because of their value the owners thought they warranted closer monitoring. The samples were taken in early in pregnancy at about 16 to 20 days. As can be seen from the results
here, which are samples taken from mares during the 2005 season, the majority fit in between 15 and 30 ng/ml. with levels of 10 to 15. ng/ml. being somewhat marginal. We therefore elected to supplement these lower levels with altrenogest.

This seem to work well, but it can be seen in this illustration there are a certain number of mares which have unexpectedly high levels and these are the ones which we are taking an extra interest in. Generally, mares which have multiple ovulations appeared to have levels of around 40 to 50 ng per ml. Some of the higher levels were from mares which appear to have single ovulations. What is of interest is that it appeared in the beginning that these were mares that were either old or had histories of uterine problems and foetal loss. This early impression will have to be sustained by accumulating greater numbers to establish statistical significance. Quite where this progesterone is coming from is unclear. It may be that it is coming from elsewhere in the body such as the pituitary-adrenal axis, which we know is also capable of producing progesterone. Does this mean that distressed reproductive system is sending signals, resulting in these high levels? If so it could be possible that the same thing is happening with the high levels of progesterone seen in compromised pregnancies of late gestation as described by Stawicki (4) and other workers.

Further study of these figures is required and study of the 2006 stud season pregnancies will be possible when we have the final outcome at the end of this coming foaling season. In the meantime, these figures are still to be collected, and their meaning can be no more than conjecture.

SUPPLEMENTATION WITH PROGESTINS
Given this discussion of high progestagen levels we can go on to consider the supplementation with progestins to support what we consider to be compromised...
pregnancies. The use of altrenogest at a dose rate of 12 1/2 mls per day has been used as the standard dose rate for some time. At first this supplementation was given in the first 120 to 150 days, during which period it was considered that high levels of progesterone were necessary and where was considered from these mares were suffering early foetal loss because of inadequacies of progesterone at this stage. This was largely by clinical assessment, and very little laboratory evaluation of progesterone levels was undertaken in the field. This is what we will describe as a traditional role, although it is a new enough regime to hardly become traditional. However, this itself has changed, and it is common enough now to see altrenogest used to support pregnancy to the end of gestation. This on a worldwide basis is controversial because the opinions vary. If I can quote David Ramey.

“There's no evidence that a regular dose of Regumate is important for maintaining pregnancy. It appears to have zero value, and there's no proven rationale for it.”

However, the opinions vary much to the contrary. We therefore have opinions on one hand to consider Regumate has no function in supporting pregnancy, and certainly not in later gestation and other opinions from the same area, that consider altrenogest is used as the panacea for pregnancy maintenance. Newer dose rates make the situation even more confusing. In fact more recently, we have been seeing prescription levels. at way, way higher dose rates than previously described. We have regimes appearing over four times those previously recommended per day. It is therefore important that some of this information and the rationale should appear in the literature so that it can be correctly evaluated. It is hoped that one of the products of this paper might be to stimulate some such discussions.

Over the last few years it has been reported that a substantial increase may be seen in later pregnancy when the pregnancy has been compromised, as in cases of placentitis. Following treatment it appears these high progestagen levels will drop and a subsequent rise can indicate a recurrence of the condition. Untreated, these mares subsequently abort. It is therefore been taken that these high levels of progesterone in later pregnancy is an indicator of placental compromise and that treatment is necessary.

Given these early indicators of unusual or extreme progesterone levels may be suggestive of a problem in the foeto-placental unit, we anticipate collecting more data over the next year and possibly next two. The following is a list of information we plan to accumulate in order to assess if this observation is real, and if so, what is the significance?

- Total number of mares in survey
- Total number of levels >80
- % mares early foetal loss in survey
- % early foetal loss for >80 group
- Age of mares in survey
- Age of mares in group
- ?group preselected as problem mares
We have already discussed, there is a chain of events including the increase then lowering of levels of progesterone prior to parturition. Comparisons have been made with other species including sheep and women and demonstrate that while the mare is similar in many ways she inevitably demonstrates some differences. These many events were well described by W.R. (Twink) Allen in the 9th Bain Fallon Proceedings (5), including the steady decline in oestrogens described by Cox (6) and by Raeside and Liptrap(7) The complicated interaction between progestagens including both progesterone and members of the 5αpregnane group and cortisols, oestrogens and prostaglandins make this event a study of its own.

SUMMARY
To summarize it appears that there is much more to know about the role of progestagens and how we might best use these hormones, particularly in later pregnancy. It also appears that is still much to know about placental endocrinology. To quote Ganjam et al, who in their summary on their paper on progesterone metabolism in 1975,(8) said “since there is no conclusive evidence that abortion in mares results from a deficiency of progesterone. and since the doses of exogenous progesterone which are normally given appear incapable of maintaining physiological levels by themselves the whole question of habitual abortion in mares needs re-evaluation.” Whereupon we may ask ourselves, how much progress have we made in the last 30 years?

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*Regumate. Allyl trenbolone
PLACENTAL SURVEY FROM FOALS BORN IN THE 2006 SEASON

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Introduction

In the horse, maternal size dictates foetal size, by limiting the area of uterine endometrium available for placental attachment (Allen, Stewart et al. 1998).

Records for 392 Thoroughbred foalings showed placenta weight at 5.8kg, equivalent to 11.5% of the foal’s birth weight (Kurtz Filho, Depra et al. 1996). Eighty placentae from Thoroughbred mares were collected (Oulton, Fallon et al. 2003). Mare age (mean 13, 5 to 24 years) and gestational age (mean 342.5, 312 to 362 days) were noted along with average foal weight (46.9kg ± 12.8 SD), allantochorion weight (3.8kg ± 0.95 SD) (Oulton, Fallon et al. 2003). Mean placental weight of Standardbred mares was 4.39kg ± 0.867 (Whitehead, Chenier et al. 2005; Whitehead, Foster et al. 2003).

There was a good correlation between maternal weight and foal weight, placental weight and foal weight, and placental area versus foal weight (Allen, Stewart et al. 1998; Oulton, Fallon et al. 2003; Wilsher and Allen 2000). When abnormal placentas were excluded there was a linear relationship (P<0.0001, r=0.867) between allantochorion weight and foal weight (Cottrill, Jeffers-Lo et al. 1991). Placental area was linearly related (P<0.0001, r=0.759) to foal weight with normal placentas and pregnancies (Cottrill, Jeffers-Lo et al. 1991).

Allantochorion area, weight and volume were highly correlated to birthweight (r=0.87, 0.84, 0.91, P<0.001) when between breed embryo transfer was used. The between breed embryo transfer involved Thoroughbred in Pony pregnancies and Pony in Thoroughbred pregnancies compared to Thoroughbred in Thoroughbred pregnancies and Pony in Pony pregnancies. However, when a mixed group (age and parity) of commercial Thoroughbreds was investigated, there was a weaker correlation between allantochorion area, weight and volume compared to birthweight (r=0.36, 0.39, 0.29) (Wilsher and Allen 2002; Wilsher and Allen 2003a; Wilsher, Ball et al. 1999).

Foal weight was highly correlated with gestational age (P<0.0001, r=0.847) (Cottrill, Jeffers-Lo et al. 1991). When placental area was divided by foal weight and related to gestational age, a highly significant correlation was seen (P<0.0001, r=0.83) (Cottrill, Jeffers-Lo et al. 1991). With advancing gestational age there is less placental area per gram of foal. After 250 days the placental area does not change as the foal’s weight increases (Cottrill, Jeffers-Lo et al. 1991). Longer gestations were seen in older mares (Oulton, Fallon et al. 2003). Longer gestations were observed with dietary restriction. The moderate restriction of nutrition between 2 groups of primigravid mares did not show any differences in placental characteristics or birthweights. The main difference was the group fed maintenance had a longer gestation (342 ± 2.9 days) than the overfed group (333 ± 2.6 days) (Wilsher and Allen 2003b).

Placental weight and birthweight were lower in primigravid mares (Whitehead, Chenier et al. 2005; Whitehead, Foster et al. 2003; Wilsher and Allen 2000); (Wilsher and Allen 2002; Wilsher and Allen 2003a; Wilsher, Ball et al. 1999).
The placenta was usually expelled 50 min after parturition but up to 4 hours was not associated with infertility (Kurtz Filho, Depra et al. 1996). The time taken to stand (74 min ± 38 SD) and suck (115 min ± 47 SD) have also been recorded (Oulton, Fallon et al. 2003).

Material and Methods
This retrospective study was conducted on a thoroughbred stud located in the Hunter Valley of New South Wales, Australia during the 2006 breeding season. The study involved all foals born live on the property from the beginning of June 2006 to the end of December 2006.

Each foaling was supervised by an experienced attendant. Data was collected and recorded for every foaling. This data included: Age of mare at parturition, parity of mare, gestation length, time to pass placenta, placental and foal weight, sex of foal, sire and if the mare was a resident or a visiting mare. At the completion of stage 3 labour the placenta was retrieved by the foaling attendant, placed into a bucket in its entirety, including the umbilical cord and amnion and was weighed to the nearest ½ kg after the weight of the bucket was subtracted.

After weighing the placentas they were laid out in an “F” configuration with the body of the uterus as the vertical arm and the two horns of the uterus as the horizontal arms of the “F” shape (Morresey 2004). All placentas were inspected for completeness and for the presence of any gross abnormalities with both the allantois (foetal side) and chorion (maternal side) being assessed. If the placenta was incomplete or pathology was noticed it was noted on the foaling records.

Weighing of the foal was achieved by the foaling attendant cradling the foal in their arms whilst standing on a set of scales. The attendant’s weight was then subtracted and the weight was recorded to the nearest kilogram.

Time of birth was defined as the expulsion of the foal from the mare at the completion of stage 2 labour. Time of passing the placenta was similarly defined as the expulsion of the placenta at the completion of stage 3 labour, thus allowing time to pass the placenta to be calculated.

Gestation length was calculated using the foaling date as recorded by the attendant compared to the last service date. These dates were confirmed against the official records of the Australian Stud book which was accessed via the website www.studbook.org using a registered log-in number and password. Age and parity of the mare was also obtained via the use of the website.

A “Resident” mare was defined as mares that spend the entire breeding season and gestation on the stud farm. “Visiting” mares are those that arrive on the farm for a short period of time in order to foal and then are returned back to their property of origin.

Results
Analysis of results was still ongoing at the writing of this manuscript. Results will be presented and discussed during the presentation.

References


FROZEN EMBRYO TECHNOLOGY FOR AUSTRALASIA

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Background
Embryo transfer involves the recovery of an embryo from a “donor” mare which is then transferred into the uterus of a “recipient” mare. Allen et al. (1982) developed the method of embryo recovery and transfer in the horse that has formed the basis of the procedure for the last 2 – 3 decades. The first equine embryo transfers were performed in the early 1970s, surgically in the U.K. (Allen and Rowson, 1972) and nonsurgically in Japan (Oguri and Tsutsuni, 1972). The possibility of transporting the embryos across international borders became a reality, when Allen et al. (1976) transferred equine blastocysts into rabbit oviducts in vivo and shipped these embryos within the rabbits to Poland where they were transferred in recipient mares and consequently produced foals. The ability to collect and ship embryos between different locations has many advantages, which include eliminating the need to have donor and recipient mares at the one facility and opening the market for the international exchange of genetics. The complete portfolio of any artificial breeding programme for livestock species (cattle, sheep) typically includes the ability to superovulate females and store multiple embryos in a frozen state for insurance against genetic loss, for export or sale or until a suitable recipient is available.

Some of the advantages of an embryo transfer programme include the ability to recover embryos from competing mares and produce the pregnancy in a recipient mare. In the USA, there are large recipient mare facilities that manage recipients and transfer embryos shipped from all over the country. Embryos can be shipped in Ham’s F10 (supplemented with serum) at 5°C for 6 – 32h prior to transfer and result in pregnancy rates similar to those obtained after the transfer of fresh embryos whether by surgical (Cook et al. 1989) or non-surgical transfer (Carnevale et al. 2000).

Embryo transfer in New Zealand
Embryo transfer is gaining acceptance in the Standardbred and Sport Horse breeding industry in New Zealand with more than 30 embryo transfers performed in 2004/2005 and more than 50 embryo transfers performed annually since then with commercially viable pregnancy rates. Currently, it is very expensive to import or export a horse between New Zealand and any other country. Therefore, the ideal way of expanding the Sport Horse gene pool in New Zealand would be to import and export equine embryos. To make this possible, either cooled or frozen embryos would need to be shipped into and out of New Zealand. It would be possible to ship cooled embryos within 24h between Australia and New Zealand with the appropriate import health standard. However, it takes a minimum of 4 days for genetic material to be shipped from Europe to New Zealand or Australia and to be processed by the relevant Customs authorities. Therefore, in order to gain access to the European market equine embryos would need to be frozen and shipped in liquid Nitrogen. However, the major limiting
factor for importing equine embryos into New Zealand or Australia is the lack of a
government approved import health standard for shipping equine embryos between
Australasia and Europe. This is in the process of being remedied in New Zealand and a
draft import health standard (Jan 2007) has been distributed for public consideration.
More recently an application has been made by EquiBreed Ltd (NZ) to develop an
import health standard to import embryos from Australia into New Zealand.

**Pregnancy rates from embryo transfer**

The most important factors that influence the pregnancy rates from embryo transfer
include the recipient management, embryo quality and method of transfer.
Traditionally, equine embryos were transferred surgically using a midline (Allen et al.
1976) or flank laparotomy approach (Squires et al. 1985) because the pregnancy rates
obtained after non-surgical transfer were poor. It is now possible to achieve pregnancy
rates in excess of 65% after the non-surgical transfer of fresh horse embryos
the pregnancy rates from non-surgical embryo transfer were enhanced significantly
with increasing operator experience. The non-surgical embryo transfer technique has
since been improved by refining the sterility of the procedure with the use of “Wilsher
forceps” to manipulate the cervix and avoid contamination of the uterus with vaginal
bacterial flora (Wilsher and Allen, 2003).

**Physiology of embryo freezing technology in horses.**

Successful cryopreservation of cells is dependent upon our ability to effectively
dehydrate the cells prior to freezing the intracellular components. The first foals from
frozen equine embryos were produced in Japan (Yamamoto et al. 1982). Horse
embryos may be frozen by one of two methods, either by equilibration or vitrification.
Conventional equilibration freezing methods involve the slow cooling of cells at -0.5
or -0.3°C/min using computerised equipment and traditional cryoprotectants such as
glycerol (Slade et al. 1985, Squires et al. 1989, McLellan et al. 2002) or ethylene
glycol (Hochi et al. 1996, Huhtinen et al. 2000, Morris, Wilsher and Allen,
unpublished data). These studies have shown that 50-80% of frozen-thawed equine
embryos < 300µm in diameter are viable after freezing by equilibration, slow thawing
and transfer.

The second method of freezing embryos is called “vitrification” which rapidly freezes
embryos such that ice crystals do not form and the cryoprotectant becomes “glass-
like”. Vitrification can be performed with simple, but elegant equipment. This
technique was first developed for horse embryos by Hochi et al. (1996) and has been
further refined by Eldridge-Panuska et al. (2005). These studies used a combination of
ethylene glycol, glycerol and various sugars and high molecular weight compounds in
the cryodiluents (reviewed by Carnevale, 2006). A variety of vessels have also been
tested for freezing or vitrifying embryos including straws, open pulled straws
(Oberstein et al. 2001) and cryoloops (Morris, Wilsher and Allen, Cryologic
Vitrification Method, Cryologic Pty, Ltd Australia). Currently, the most practical and
successful method is the vitrification of equine embryos in 0.25ml straws in a Calcium
and Magnesium free ethylene glycol media (VS1, VS2, VS3, ABTechnology, USA).
Again, these studies observed that embryo survival rates were higher for smaller
(<300µm) embryos than for the larger blastocysts. Furthermore, pregnancy rates were
higher for embryos that were transferred directly into the recipient immediately after thawing, than those obtained after a brief culture period prior to transfer (Eldridge-Panuska et al. 2005).

Until recently, the survival of horse embryos after freezing, thawing and transfer has been limited (<60%). The survival of embryos frozen by either method is governed by the size of the embryo and the synchrony of the recipient mare with the donor mare at the time of transfer. Equine embryos that are >300µm in diameter are difficult to freeze successfully. The equine embryo enters the mare’s uterus between 6 and 6.5 days after ovulation (Oguri and Tsutsumi, 1972, Battut et al. 1998). In some mares the embryo may enter the uterus as a morula at only 5.5 days after ovulation, but the equine embryo blastulates rapidly thereafter (Boyle et al. 1989). At 6.5d after ovulation, Battut et al. (1998) found that the average size of the embryo was 186µm at 6 days after ovulation and then rapidly enlarges to an average of 244 µm by 7 days after ovulation. With increasing time from ovulation until day of embryo recovery there is also an increase in the embryo recovery rates. Therefore, to maximize the embryo recovery rates, embryos are normally recovered from donor mares at 7-8 days after ovulation when they are typically >300µm in diameter. Consequently, to optimize the embryo recovery rates and viability after freezing, the uterus of the donor mare needs to be flushed as early as 6.5 days after ovulation to recover the embryo while it is <300µm.

It has been hypothesized that the poor survival of these larger embryos (>300µm) after either cryopreservation method is due to the large, fluid filled blastocoelic cavity and the low permeability of the glycoprotein embryonic capsule to cryoprotectants (Legrand et al. 2000). In order to increase the permeability of the capsule, Legrand et al. (2000) obtained pregnancies after pretreatment of large embryos with 0.2% trypsin and subsequent equilibration freezing whereas McLellan et al. (2002) found that trypsin pretreatment was detrimental to embryo survival.

In an experiment designed to increase the permeability of large embryos to cryoprotectants, Morris, Wilsher and Allen (unpublished data) investigated the effects of freezing large embryos by either equilibration in an ethylene glycol based media, very slowly, at -0.15°C/min (duration 3.8h) or vitrification in an ethylene glycol based media with prolonged exposure times to cryoprotectants, (8 min versus <5 min). Preliminary studies were performed to evaluate the degree of embryo shrinkage in response to different exposure times to the cryoprotectants prior to freezing. It was concluded that an average total exposure time to the cryoprotectants of 8 min was required for the >300µm embryos to shrink and effectively dehydrate prior to vitrification. A total of 31 embryos were frozen. Of the 24 embryos that were transferred, none of the embryos >300µm survived thawing and transfer and only 20% of the small embryos (n = 15) survived these extreme freezing protocols and resulted in pregnancies. These pregnancy rates are lower than those reported (50-80%) for embryos that were frozen more quickly with reduced exposure to the same cryoprotectants. Therefore, improving the permeability of the embryos to these cryoprotectants by increasing the exposure times to cryoprotectants is toxic to the small embryos and does not improve the survival of large embryos after thawing.
Conclusions
To date, the preferred method of freezing equine embryos remains the equilibration method in either glycerol or ethylene glycol. It is also possible to achieve satisfactory pregnancy rates after vitrification of equine embryos. Regardless of the method of cryopreservation, embryo survival is highest when embryos <300µm are frozen and therefore the embryos still need to be recovered on day 6 – 7 after ovulation at the expense of maximum embryo recovery rates.

References


THE EFFECT OF EARLY TWIN REDUCTION ON PREGNANCY RATES IN THOROUGHBRED MARES DURING THE 2006 SEASON

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Introduction


Prior to the use of ultrasonography, the rate of pregnancy loss due to twins was about 2% of recorded pregnancies (Bain 1969) and the major cause for pregnancy loss in Thoroughbreds representing almost 30% of submissions for abortion investigation (Jeffcott and Whitwell 1973; Whitwell 1980). With the introduction of ultrasound technology in the 1980s, that allows early visualisation of pregnancy and detection of twins (Chevalier and Palmer 1982), pregnancy loss due to twins is now the second highest cause of abortion (McKinnon, Voss et al. 1993). A recent survey in the UK found twins a cause of abortion in about 6% of submitted cases (Smith, Blunden et al. 2003). With early detection, the rate of twinning was about 3% (Chevalier and Palmer 1982). Later studies have found twinning rates to be higher and influenced by the mare’s reproductive status. Lactating (wet) mares have a twinning rate of 10% and barren (dry) mares have a twinning rate of 24% (Grimmett, Hills et al. 1998).

Reduction of multiple embryos to a singleton is best achieved during the mobility phase before the embryos fix into position at day 17 (Ginther 1986). The reduction of embryo vesicles to a single pregnancy is achieved with gentle pressure between thumb and fingers or manipulating one embryo to a horn tip to crush the vesicle (Allen 1992; Asbury 1987; England 1994; Ginther 1986). Alternatively, the ultrasound probe can be used to force the uterine horn against the cranial and lateral margin of the pelvis to destroy the vesicle (McKinnon, Voss et al. 1993). The mare should be examined 2 days later to assess the wellbeing of the remaining embryo (England 1994).

Some workers state that the success rate of manual twin reduction can be considered to be 100%. This is because the remaining embryo has the same probability of surviving to foaling as a singleton pregnancy of the same age (Ginther 1986). The success of manual twin reduction was later revised to 93% as 7% lost the pregnancy between reduction and foaling (Ginther 1992). A success rate of 92% was recorded including successful reduction of 3 sets of triplets (Morris and Allen 2002).

Loss of the remaining pregnancy is due to endogenous prostaglandin (PGF2α) release during handling of the uterus (Pascoe, Pascoe et al. 1987a). This release of PGF2α can be inhibited by premedicating with flunixin meglumine. However, if the embryo reduction was gentle there was no need for flunixin or progesterone support for the procedure (Pascoe, Pascoe et al. 1987a).

Position of the 2 embryos influenced success rate of manual reduction. Bilateral embryos at the time of reduction had 97% success, unilateral twins had 87% success and reduction of
twins when in the uterine body was 75% successful (Morris and Allen 2002). Only one mare of the 136 that had successful twin reductions to a single pregnancy lost her pregnancy after day 35, compared to the background pregnancy loss of 7% between day 35 and foaling (Morris and Allen 2002).

Multiple ovulations occurred 15% of oestrous cycles in mixed mare populations (Osborne 1966) and 34% of oestrous cycles in Thoroughbred mares (Newcombe 1995). Of pregnancies conceived with multiple ovulations, 47% had multiple pregnancies (Newcombe 1995). Triplet ovulations occurred 2% of the time with triplet pregnancies 1% of the time (Newcombe 1995).

Mares were more likely to conceive twins in the second half of the season, especially the 2nd last month of the breeding season (Jeffcott and Whitwell 1973). In contrast, other workers found that although multiple ovulations were more likely at the end of the season, twins were more likely at the beginning of the season (Morris and Allen 2002). The age of mares producing the majority of twins was either 4-6 years of age (likely maiden mares) or between 10-12 years of age (Jeffcott and Whitwell 1973). Mares were less likely to produce twins as they aged (Jeffcott and Whitwell 1973; Morris and Allen 2002). Although aged mares had almost double the rate of multiple ovulation compared to younger mares (24% vs. 13%); the lower pregnancy rate in the older mares lead to a lower twinning rate than in younger mares (3% vs. 7%).

Reproductive status influenced ovulation rates and twinning rates (Morris and Allen 2002; Pascoe, Pascoe et al. 1987b). Maiden mares (17%) and barren (dry) mares (13%) had a higher twinning rate than lactating mares (4%) (Pascoe, Pascoe et al. 1987b). Another study found that although barren mares had higher multiple ovulations (23%), when combined with lower pregnancy rates (46%) they had equal twinning rates to other mares (8%) (Morris and Allen 2002). Aborted mares had higher multiple ovulations (26%) that did lead to a higher twinning rate (24%) (Morris and Allen 2002).

The aim of this study was to investigate the hypothesis that early reduction of multiple equine vesicles has a detrimental effect on the continuing singleton pregnancy. Information on the incidence and timing of twin pregnancies was also collected. This was compared to the background of singleton pregnancies.

**Material and Methods**

Data was recorded from the 2006 breeding season and analysed. The population was 100% Thoroughbred. The oestrous cycles that resulted in pregnancy were investigated. Information collected was the date of ovulation, the number of ovulations, the number of pregnancies detected, the type of reduction technique used for multiple pregnancy reduction, the success of any reduction procedures for multiple pregnancies, and continued monitoring of pregnancy status.

Mares were examined in crushes with rectal palpation and ultrasonography using an Aloka SSD-500 with 5.0 MHz linear probe (Aloka Co. Ltd., Tokyo, Japan). Physical restraint in the form of a nose twitch or sedation (xylazine hydrochloride (Ilium Xylazil-100, Troy Laboratories Pty Ltd, Smithfield, Australia) and butorphanol hydrochloride (Dolorex, Intervet Australia Pty Ltd, Bendigo East, Australia) was used on nervous mares or those straining during examination that did not respond to physical restraint. During multiple pregnancy reduction additional drugs that were used on some mares included propantheline bromide (Propan B, Nature Vet Pty Ltd, Glenorie, Australia), flunixin meglumine (Flunix, Parnell Laboratories (Aust) Pty Ltd, Alexandria, Australia) and altrenogest (Regumate, Intervet Australia Pty Ltd, Bendigo East, Australia).
The use of these drugs was assessed on an individual basis. Manual twin reduction was performed between the thumb and forefinger without using the ultrasound probe. Mares with a nervous disposition or a lack of rectal relaxation were administered xylazine with or without butorphanol with further rectal relaxation achieved by propantheline administration. Those cases of manual twin reduction thought to require more handling than normal were premedicated with flunixin meglumine. Based on the length of the normal reduction procedure and previous history, the use of altrenogest was employed for varying periods. Blood progesterone was measured before withdrawing altrenogest therapy.

The position of the multiple embryo vesicles was recorded and the place where the vesicles were manually reduced was also noted. The data was recorded as bilateral when embryos were in separate uterine horns and one was manually reduced in its own horn. The term unilateral was used when both embryos were in the same horn either in close proximity to each other or separate but still in the one horn. Unilateral reductions were achieved by attempting to move one embryo away from the other but sometimes vesicle rupture occurred in close proximity to the other vesicle. Body reductions occurred when one or both embryos were in the body of the uterus and one vesicle was ruptured by moving it caudally to the cervix.

Mares were examined at approximately 15 days for initial pregnancy diagnosis and then follow up at 17 days for assessment of twin reduction success, 30 days and 45 days for pregnancy status. Data was recorded in Microsoft Excel (Microsoft Corporation) and arranged into categories for statistical analysis using chi-square analysis.

**Results**

The total number of mares in the population studied was 725 (142 maiden mares, 174 dry mares, 409 wet mares). The total number of oestrous cycles resulting in pregnancy was 748. The proportion of these that resulted in multiple pregnancies was 88 (11.8%). Table 1 shows the distribution of pregnancies over the mares of different reproductive status for the 2006 season. The number of multiple pregnancies in the maiden and dry mares was significantly higher (P<0.001) than the frequency of multiple pregnancies in wet mares. Of the 88 pregnancies that had multiple embryos, only one pregnancy had more than 2 embryos, with the one triplet pregnancy being 1.14% of all multiple pregnancies and 0.13% of all pregnancies.

Table 2 follows the pregnancy status until 45 days comparing the loss rate between the singleton pregnancies and the multiple pregnancies. There were no losses after manual reduction of twin pregnancies in the maiden or wet mares. There was a 6% loss rate in the dry mares after manual reduction of twin pregnancy leading to a success rate of 97% overall. The loss rate after manual reduction of multiple pregnancies was less than the singleton pregnancies in the maiden and wet mares. The dry mares that had a manual twin reduction experienced a greater pregnancy loss rate at 30 and 45 days, but this was not significantly greater than the singleton pregnancies in the other dry mares. Overall, the pregnancy loss rate after manual twin reduction was equal to the loss rate of singleton pregnancies (Table 2).

The number of oestrous cycles that had multiple ovulations is presented in Table 3. The higher number of oestrous cycles with multiple ovulations in the maiden and dry mares compared to the wet mares was highly significant (P<0.001). However, the rate of multiple ovulation per oestrous cycle was not significantly different between the groups of mares during the season (Figure 1).

The frequency of twin conception during the season significantly (P<0.01) dropped off in the maiden mares from 39% to 0% (Figure 2), stayed relatively level in the dry mares with a rise from around 15% to 33% at the end of the season (Figure 3) and didn’t rise above 10% for the season in the wet mares (Figure 4). Overall the frequency of twinning was significantly higher
(P<0.05) at the beginning of September (20%) but dropped to around 10% for the rest of the season (Figure 5).

The pregnancy rate between those oestrous cycles with one detected ovulation did not differ from those oestrous cycles with multiple ovulations (Table 4). The rate of multiple pregnancies was significantly higher (P<0.001) if there was multiple ovulations as compared to oestrous cycles with a single ovulation. Interestingly, the detection of a single ovulation did not rule out multiple pregnancies (Table 4).

Ovulation rate per oestrous cycle did not significantly vary with age (Table 5). The frequency of twinning was affected by age (Table 5). The 4 to 6 year age group had a significantly higher (P<0.05) twinning rate (22.4%) and the 11 to 15 year age group had a significantly lower (P<0.05) twinning rate (7.4%).

The influence of position of manual twin reduction and reproductive status is shown in Table 6. The immediate success of the twin reduction was higher in the maiden mares (100%) and wet mares (100%) compared to the dry mares (93.1%). Immediate success for bilateral (100%) and body (100%) twin reductions was higher than unilateral twin reductions (92.9%). Dry mares tended to have the highest pregnancy loss rate to 45 days after twin reduction (17.2%) compared to the maiden mares (3.7%) and wet mares (8.7%). Unilateral twin reductions tended to have a greater risk for pregnancy loss to 45 days (17.9%) compared to bilateral (3%) or body twin reductions (11.1%).

Discussion

The overall twinning rate of 11.8% in this study is in agreement with other studies (Grimmett, Hills et al. 1998). The increased twinning rate in maiden mares and dry mares compared to wet mares was also found by other authors (Pascoe, Pascoe et al. 1987b). Although the overall rate of ovulations per oestrous cycle was not different between the groups, the number of oestrous cycles that had multiple ovulations was significantly higher in the maiden and dry mares compared to the wet mares. This undoubtedly contributed to the higher twinning rate in these mares and oestrous cycles with multiple ovulations were more likely to have a multiple pregnancy (35.6% chance) than oestrous cycles with a single ovulation (1.3% chance).

Twinning rates were significantly higher in the first half of September (20%) compared to about 10% for the rest of the season. This was boosted by the significantly high twinning rate of 39% in the maiden mares in the first half of September. Other workers have also found twins are more likely at the beginning of the season (Morris and Allen 2002).

The age at that mares were more likely to produce twins has been recorded at either 4 to 6 years of age or 10 to 12 years of age (Morris and Allen 2002). This study also found significantly higher twinning rates in the 4-6 year age group (22.4%) but the 11-15 year age group had the significantly lowest twinning rate (7.4%). Differences in this study might be from inclusion of older mares in the age block.

The immediate success rate of manual twin reduction (97%) in this study is comparable with other authors (Morris and Allen 2002). Very few studies have looked at ongoing pregnancy loss and compared it to the background singleton pregnancies. Against a background loss rate of 7%, only 1 out 136 (0.74%) twin reductions were lost after 35 days to foaling (Morris and Allen 2002). In this study, pregnancy loss to 45 days after manual twin reduction (9%) was equal to pregnancy loss in the singleton pregnancies (9%). Although, pregnancy loss rates to 45 days after twin reduction in maiden mares (3%) and wet mares (7%) were lower offsetting the higher pregnancy loss rate to 45 days in dry mares after twin reduction (16%). The higher pregnancy loss to 45 days in the dry mares after twin reduction (16%) was not significantly higher than the loss rate to 45 days of dry mares with singleton pregnancies (9%).

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As previously reported (Morris and Allen 2002), the position of the embryos at the time of manual reduction appears to influence success rate. Bilateral twins have the highest success when reduced to a single pregnancy with this study (100%) being comparable to other work (Morris and Allen 2002) (97%). Unilateral twins show a lower success rate in this study (93%) as shown elsewhere (Morris and Allen 2002). The success rate of body twin reductions in this study (100%) was higher than elsewhere (Morris and Allen 2002) (75%). The difference might be due to differing techniques. Reducing body pregnancies by hand might be easier and less traumatic than with the ultrasound probe.

In summary, this paper shows that twinning frequency is more likely in maiden mares and dry mares than wet mares, especially at the beginning of the season. Mares between 4 to 6 years of age are more likely to have twins and mares 11 to 15 years are less likely. Twins are more likely to be conceived when there are multiple ovulations, but the detection of a single ovulation does not rule out a twin pregnancy. The success of manual twin reduction continues to be high with the ongoing pregnancy loss not any higher than singleton pregnancies in the majority of cases. Manual twin reduction in dry mares or in a unilateral position might have an increased risk for future pregnancy loss.

References


**Table 1.** Singleton and multiple pregnancies (%) during the 2006 season for mares of different reproductive status.

<table>
<thead>
<tr>
<th>Reproductive Status</th>
<th>Total Pregnancies</th>
<th>Singleton Pregnancies</th>
<th>Multiple Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maiden Mares</td>
<td>145</td>
<td>116 (80%)</td>
<td>29 (20%)</td>
</tr>
<tr>
<td>Dry Mares</td>
<td>181</td>
<td>150 (82.9%)</td>
<td>31 (17.1%)</td>
</tr>
<tr>
<td>Wet Mares</td>
<td>422</td>
<td>394 (93.4%)</td>
<td>28 (6.6%)</td>
</tr>
<tr>
<td>Total Mares</td>
<td>748</td>
<td>660 (88.2%)</td>
<td>88 (11.8%)</td>
</tr>
</tbody>
</table>

**Table 2.** Pregnancy loss (%) for the 2006 season for all pregnancies from 15 days, 17 days, 30 and 45 days divided into mare reproductive status.

<table>
<thead>
<tr>
<th>Mare repro stat</th>
<th>Maiden Mares</th>
<th>Dry Mares</th>
<th>Wet Mares</th>
<th>All Mares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan days</td>
<td>15  18  30  45</td>
<td>15  18  30  45</td>
<td>15  18  30  45</td>
<td>15  18  30  45</td>
</tr>
<tr>
<td>Singl</td>
<td>116 98% 110 95% 109 94%</td>
<td>150 99% 149 97% 146 91%</td>
<td>394 98% 386 95% 375 95%</td>
<td>660 98% 649 96% 631 96% 602 91%</td>
</tr>
<tr>
<td>Mult</td>
<td>29  29  29  28 97%</td>
<td>31  29  27 94% 87%</td>
<td>28  28  26 84%</td>
<td>88  86  84 97% 95% 80 91%</td>
</tr>
</tbody>
</table>

**Table 3.** The total oestrous cycles and the number and percentage of oestrous cycles that had multiple ovulations divided by mare reproductive status.

<table>
<thead>
<tr>
<th>Mare Status</th>
<th>Total Cycles</th>
<th>Cycles with Multiple Ovulations</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maiden</td>
<td>212</td>
<td>72</td>
<td>33.96</td>
</tr>
<tr>
<td>Dry</td>
<td>312</td>
<td>118</td>
<td>37.82</td>
</tr>
<tr>
<td>Wet</td>
<td>688</td>
<td>137</td>
<td>19.91</td>
</tr>
<tr>
<td>Total</td>
<td>1212</td>
<td>327</td>
<td>26.98</td>
</tr>
</tbody>
</table>
Table 4. The rate of multiple pregnancy as it relates to the ovulation rate in all mares for the 2006 season. (* indicates significance P<0.001).

<table>
<thead>
<tr>
<th></th>
<th>No. Cycles</th>
<th>No. Pregnant Cycles (%)</th>
<th>No. Single Pregnancies (%)</th>
<th>No. Multiple Pregnancies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Ovulations</td>
<td>883</td>
<td>537 (60.8%)</td>
<td>530* (98.7%)</td>
<td>7* (1.3%)</td>
</tr>
<tr>
<td>Multiple Ovulations</td>
<td>328</td>
<td>216 (65.9%)</td>
<td>139* (64.4%)</td>
<td>77* (35.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>1211</td>
<td>753 (62.2%)</td>
<td>669 (88.8%)</td>
<td>84 (11.2%)</td>
</tr>
</tbody>
</table>

Table 5. The rate of multiple ovulation and frequency of twinning for mares of different ages (* indicates significance P=0.04).

<table>
<thead>
<tr>
<th>Mare Age (yrs)</th>
<th>Ovulation Rate per Cycle</th>
<th>SD</th>
<th>Number of pregnancies</th>
<th>Number of multiple pregnancies</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 to 3</td>
<td>1.171</td>
<td>0.382</td>
<td>29</td>
<td>4</td>
<td>13.79</td>
</tr>
<tr>
<td>4 to 6</td>
<td>1.321</td>
<td>0.53</td>
<td>125</td>
<td>28*</td>
<td>22.4</td>
</tr>
<tr>
<td>7 to 10</td>
<td>1.273</td>
<td>0.47</td>
<td>161</td>
<td>29</td>
<td>18.01</td>
</tr>
<tr>
<td>11 to 15</td>
<td>1.246</td>
<td>0.448</td>
<td>162</td>
<td>12*</td>
<td>7.41</td>
</tr>
<tr>
<td>16 to 20</td>
<td>1.342</td>
<td>0.529</td>
<td>61</td>
<td>10</td>
<td>16.39</td>
</tr>
<tr>
<td>&gt; 20</td>
<td>1.786</td>
<td>0.802</td>
<td>6</td>
<td>1</td>
<td>16.67</td>
</tr>
</tbody>
</table>
Table 6. The position of manual twin reduction and its relationship to the success of the procedure and ongoing pregnancy loss.

<table>
<thead>
<tr>
<th>Mare Status</th>
<th>Reduction Position</th>
<th>15 days</th>
<th>18 days</th>
<th>30 days</th>
<th>45 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maiden Mare</td>
<td>Bilateral</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Unilateral</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Dry Mare</td>
<td>Bilateral</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Unilateral</td>
<td>10</td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>29</td>
<td>27</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>Wet Mare</td>
<td>Bilateral</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Unilateral</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>All Mares</td>
<td>Bilateral</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Unilateral</td>
<td>28</td>
<td>26</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>18</td>
<td>18</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>79</td>
<td>77</td>
<td>75</td>
<td>71</td>
</tr>
</tbody>
</table>

Figure 1. The rate of ovulations throughout the season for each of the mare groups for the first and second half of each month. Standard deviation bars are represented.
Figure 2. The frequency of twin pregnancies in the maiden mares for the first and second half of each month.

Figure 3. The frequency of twin pregnancies in the dry mares for the first and second half of each month.
Figure 4. The frequency of twin pregnancies in the wet mares for the first and second half of each month.

Figure 5. The frequency of twin pregnancies in all mares for the first and second half of each month.
LOW DOSE ARTIFICIAL INSEMINATION

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Background
Artificial insemination has been used successfully in the horse since 1322, when semen from an Arabian stallion was recovered from the vagina of a recently mated mare, transported in camel milk and deposited into the vagina of another mare to produce a foal (1). Since then, semen collection, handling and conventional artificial insemination techniques have been refined and described in detail (2,3).

In order to compensate for the variability in the fertility amongst stallions for fresh, chilled or frozen semen (4), the minimum recommended number of spermatozoa contained within a conventional insemination dose is generally >300 x 10^6 progressively motile spermatozoa for fresh (5,6,7,8,9,10) and > 200 x 10^6 progressively motile spermatozoa for frozen semen (3,9). It has been shown that conventional transcervical insemination of mares with ≤100 x 10^6 spermatozoa results in unsatisfactory pregnancy rates per cycle (10,11).

Despite the high numbers and specific concentrations of spermatozoa required for optimal fertility, it has been demonstrated that most of the ejaculate is effectively and rapidly evacuated from the uterus through the very relaxed, estrous cervix (12). Indeed, only a small proportion of the ejaculate remains on the uterine side of the papillae of each utero-tubal junction (13) and very few spermatozoa are observed within the oviduct at different stages of the estrous cycle in mares (14,15).

Knowledge of the presence of a sperm reservoir at the utero-tubal junction (13) and the low numbers of spermatozoa present in the oviduct that result in normal fertilization and pregnancy rates (16), enables us to exploit low numbers of spermatozoa from individual stallions. The advantages of reducing the distance the spermatozoa have to travel in the uterus by depositing them closer to the oviduct have been evaluated in sheep (17), pigs (18,19) and humans (20). Similarly, in mares, a number of methods designed to deposit spermatozoa closer to the site of fertilization have evolved in recent years, which include deep uterine insemination (21), hysteroscopic insemination (22), gamete intrafallopian transfer (23) and intrafollicular insemination (24) and have been reviewed in detail (25).

Deep uterine insemination.
Buchanan et al. (21) developed a deep uterine insemination technique to deposit low numbers of spermatozoa closer to the utero-tubal junction. Several different types of flexible pipettes (1,2) or the rigid disposable implant gun (3) have been used for this

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1 Minitube Universal Insemination Pipette and Gun. www.minitube.com
2 IMV Flexible Equine Catheter 007356,30 www.imv-technologies.com
3 Disposable Insemination guns, B6-3650, Continental plastic, USA. www.continentalplastic.com
insemination method. The insemination dose is typically loaded into either a 0.25 or 0.5ml straw, which is inserted into the pipette. Buchanan et al. (21) achieved 35% pregnancy rates in mares inseminated with 25 x10^6 sex preselected spermatozoa in either 1ml or 0.2ml by deep uterine insemination with a rigid pipette. In an attempt to improve the efficacy of the deep uterine insemination method, Nie and Johnson (26) inseminated mares with 1 x10^6 spermatozoa that had been filtered through a glass wool / sephadex column. Their pregnancy results were disappointingly low (7-19%) and may reflect the possibility that less than 1 x10^6 spermatozoa actually reached the utero-tubal junction.

**Hysteroscopic insemination**

The utero-tubal papilla, or the gateway to the oviduct, is readily visualised during hysteroscopy (22,27) and consequently, it is possible to use this technology to deposit small numbers of spermatozoa directly onto this papilla, close to the sperm reservoir in the mare (13). When the utero-tubal papilla ipsilateral to the ovary containing the dominant pre-ovulatory follicle is visualised, the catheter is extruded from the tip of the scope and the small volume of spermatozoa is deposited directly onto the papilla, without attempting to catheterise its os. Hysteroscopic insemination is typically performed once, at a single fixed time after induction of ovulation. For frozen semen (28), hysteroscopic insemination is performed at 32h after administration of human Chorionic Gonadotropin, (hCG; Chorulon, Intervet, Milton Keynes, UK).

Morris et al. (22) inseminated mares hysteroscopically with 10, 5, 1, 0.5, 0.1 or 0.001 x10^6 motile spermatozoa prepared for insemination after centrifugation through a discontinuous Percoll (Sigma-Aldrich) density gradient. High per cycle conception rates of > 60% were achieved in the mares inseminated with 10, 5 or 1 x10^6 spermatozoa. However, insemination doses with ≤ 0.5 x10^6 motile spermatozoa began to approach the limit of fertilization success. These doses represent 1/500th of the accepted minimum, recommended dose of 500x10^6 spermatozoa used for conventional uterine body insemination in the mare (3).

Hysteroscopic insemination of commercially available frozen-thawed semen may increase the potential number of doses of frozen semen from particular stallions. Nevertheless, variability in the fertility of frozen-thawed semen observed amongst commercially available stallions (4) remained evident when a volume of 0.5 – 1ml of frozen-thawed semen containing 50 – 100 x10^6 spermatozoa was deposited onto the utero-tubal junction hysteroscopically within 6h of ovulation and compared with the per cycle pregnancy rates obtained after conventional insemination with frozen-thawed semen from the same stallions in a commercial breeding programme (29). Morris et al. (30) demonstrated that similarly high pregnancy rates (> 55% per cycle) can be obtained after insemination of only 0.5ml (14 x10^6) frozen spermatozoa from two fertile stallions when inseminated only once, either hysteroscopically or conventionally, at 32h after induction of ovulation with human Chorionic Gonadotropin (hCG, Chorulon, Intervet, Milton Keynes, UK).

In an attempt to simulate the in vivo selection process for morphologically normal spermatozoa, some studies have processed fresh spermatozoa through density gradients to enhance selection of those with intact plasma membranes (22,31). In these
studies, this step was performed during the centrifugation process to concentrate the fresh spermatozoa for insemination. However, no beneficial effect of this treatment was observed on the fertility of low numbers of frozen-thawed ejaculated spermatozoa.

It has also been observed that the incidence of post breeding endometritis after hysteroscopic insemination is negligible. Indeed, only 1% of mares inseminated hysteroscopically with fresh spermatozoa (22) and 3.5% of mares inseminated hysteroscopically with frozen-thawed spermatozoa had ultrasonographic evidence of intra-uterine fluid accumulation after insemination (28). Therefore, it is apparent that this is a minimally invasive method of insemination in normal mares. However, Sieme et al. (32) observed a significant interaction between mare fertility and insemination technique, such that hysteroscopic insemination produced lower pregnancy rates in problem mares than normal mares and conventional insemination produced higher pregnancy rates in problem mares than normal mares.

Rigby et al. (33) compared the fertility of deep uterine with hysteroscopic insemination of 5 x10^6 fresh spermatozoa suspended in 200µl of diluent. There were no statistically significant differences observed in the pregnancy rates of the mares inseminated either hysteroscopically (13/21, 62%) or after deep uterine insemination (10/20, 50%). The deep uterine insemination method is more practical, less labour intensive and easier to perform than the hysteroscopic insemination method, which requires 2 or 3 people. This concept was reinforced when no differences in fertility were observed in mares inseminated either hysteroscopically or by conventional insemination with 14 x10^6 motile frozen-thawed spermatozoa in 0.5ml diluent at 32h after induction of ovulation (28). However, in this study, once the numbers of spermatozoa were reduced to 3 x10^6, hysteroscopic deposition of the spermatozoa directly onto the utero-tubal junction produced significantly better pregnancy rates rather those obtained by conventional insemination of low numbers of spermatozoa.

**Sex-sorted spermatozoa**

Since the production of the first live offspring from sex-sorted spermatozoa (34) there have been many developments in the fluorescence activated cell separation (FACS) procedures which separate X- and Y- chromosome bearing spermatozoa based on their difference in DNA content. This difference in DNA content is 3.7% in the stallion (35). Once sorting is completed the stallion spermatozoa is centrifuged to provide 20 – 67 x10^6 spermatozoa / ml for insemination (36,37).

The low number of spermatozoa available after sorting requires that this technology be combined with advanced methods of insemination. Pregnancies have been obtained from many different methods of insemination with sex-sorted spermatozoa including surgical oviductal insemination of only 50,000 spermatozoa (38), deep uterine insemination of 25 x10^6 spermatozoa (21) and hysteroscopic insemination of 5 – 20 x10^6 spermatozoa (31, 36, 33). Indeed, insemination of 50 x10^3 sex-sorted, X-bearing spermatozoa directly into the oviduct produced the first the foal from sex-sorted spermatozoa (39). When the insemination dose of spermatozoa approaches the limits of the estimated population of the sperm reservoir (22), then it has been shown that hysteroscopic insemination of a small volume of spermatozoa provides better pregnancy rates than deep uterine insemination (28,37). After hysteroscopic
insemination, it is possible to obtain satisfactory pregnancy rates with $5 \times 10^6$ fresh (31) or $20 \times 10^6$ stored (37) sex-sorted stallion spermatozoa. However, the pregnancy rates after hysteroscopic insemination of the sorted, frozen-thawed spermatozoa are less than 15% (36, 40) and significantly less than the pregnancy rate obtained with similarly small numbers of non-sorted, frozen-thawed spermatozoa (36).

The ability to achieve satisfactory pregnancy rates after hysteroscopic insemination with low numbers of fresh or stored, sex-sorted spermatozoa, but not with frozen-thawed, sorted spermatozoa suggests that physiological questions about the timing of insemination and the ability of these frozen-thawed cells to bind to the oviduct or oocyte at the appropriate time are yet to be answered.

**Oviductal insemination**

It is also possible to deposit extremely low numbers of spermatozoa directly into the oviduct. Carnevale et al. (41) achieved conception rates of 67% (12/18) after gamete intrafallopian transfer (GIFT) of an oocyte with $2 - 5 \times 10^5$ fresh spermatozoa. Similarly, there were no significant differences in the pregnancy rates observed in mares inseminated conventionally with $1 \times 10^6$ fresh spermatozoa (17/26, 65%) and in mares which were inseminated directly into their oviduct (12/22, 55%) with only $2 \times 10^5$ fresh spermatozoa (42). These studies also reveal that gamete transfer into the oviduct contralateral to the recipient’s impending ovulation can result in normal fertilization of the transferred oocyte. Furthermore, failure of the recipient’s own oocyte to undergo fertilization demonstrates that there is no significant retrograde transport of spermatozoa from the contralateral oviduct through the uterus and into the oviduct ipsilateral to ovulation.

The fertility of chilled (4/31, 13%) or frozen-thawed (1/12, 8%) spermatozoa when used in oviductal insemination is less than the fertility of the same dose of $2 \times 10^5$ fresh spermatozoa. It is speculated that the reduced fertility of chilled or frozen-thawed spermatozoa is due to the presence of extenders which may interfere with the binding of spermatozoa to the oviduct or oocyte (43).

**Conclusions**

Different methods of *in vivo* insemination techniques are constantly emerging to improve the fertility of stallions and types of spermatozoa (fresh, chilled, frozen, epididymal, sex-sorted) that are now available. Indeed, spermatozoa currently not capable of fertilization in conventional circumstances may become fertile if deposited at different sites within the reproductive tract and at different times with respect to ovulation.

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THE DEVELOPMENT OF SPERM SORTING FOR SEX-PRESELECTION OF STALLION SPERMATOZOA – AN UPDATE

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The ability to choose the sex of one’s offspring is a desire long held by society. While many techniques have been suggested for sex preselection, success has only been achieved since the development of modern flow cytometry allowing the accurate identification and sorting of biological cells. A modified flow cytometer is used to sort sperm cells into two categories (those bearing X and those bearing Y chromosomes) based on the difference in DNA content. This allows the insemination of X- or Y-bearing spermatozoa for the production of female or male offspring respectively. In comparison to other agricultural species such as cattle and sheep, the potential financial returns per pre-sexed offspring does not begin to compare with those of a pre-sexed foal. Nevertheless, when compared to species such as cattle, sheep and pigs, flow cytometric separation of X- and Y- bearing stallion spermatozoa remains in its infancy. Refinement of the sex-sorting procedure for stallion spermatozoa is still required before the technology can be commercially released to the general equine industry.

Significant developments in the sex-preselection of stallion spermatozoa have been made over the past decade since the birth of the first foal after the oviductal insemination of fresh, sex-sorted stallion spermatozoa (Schmid et al. 1998). Since this first breakthrough, foals of predetermined sex have been born following deep uterine insemination of fresh sorted spermatozoa (Buchanan et al. 2000), hysteroscopic insemination of fresh and frozen sex-sorted spermatozoa (Lindsey et al. 2002) and embryo transfer after hysteroscopic insemination of fresh sex-sorted spermatozoa (Morris 2004). Although the insemination of fresh sex-sorted spermatozoa has resulted in pregnancies (Lindsey et al. 2002), satisfactory pregnancy rates using frozen sex-sorted semen continue to elude researchers. A recent study by Clulow et al. (2006b) investigated techniques for the cryopreservation of sex-sorted stallion spermatozoa prior to its use in a large scale fertility trial in Australia. Although only one foal was produced after the insemination of frozen-thawed, sex-sorted stallion spermatozoa, the trial highlighted the importance of screening stallions for suitability for the low dose insemination technique. Morris and Allen (2002) demonstrated that some stallions are excellent candidates whilst others are not. This phenomenon was particularly evident in our field study where per cycle pregnancy rates of 35.5% were obtained after conventional AI of >500 x 10⁶ motile non-sorted frozen-thawed spermatozoa from the same stallions and mares. Furthermore, this study highlights that hysteroscopic insemination of low numbers of spermatozoa (sex-sorted or non-
sorted) from stallions with reduced fertility does not compensate for or overcome their limited fertility *in vivo*.

The function of sex-sorted stallion spermatozoa has been tested *in vitro* using a validated heterologous sperm-oocyte binding assay (Clulow *et al.* 2006a). Sex-sorted fresh and frozen stallion spermatozoa were capable of binding to bovine oocytes and undergoing the acrosome reaction *in vitro*. Furthermore, fresh sex-sorted spermatozoa appeared to bind better than fresh non-sorted spermatozoa during the initial co-incubation period, suggesting that sex-preselected fresh stallion spermatozoa have acceptable fertilising potential.

To conclude, although the techniques for the sex-preselection of stallion spermatozoa by flow cytometric sorting are further developed and refined, satisfactory *in vivo* fertility after insemination of sex-sorted frozen-thawed stallion spermatozoa has yet to be achieved. The high functional integrity achieved *in vitro* suggests that sex-preselected stallion spermatozoa have an acceptable fertilising potential, as seen in other species. Following the confirmation of *in vivo* fertility, the dream of sex-preselection in the horse breeding industry can become a reality and may even extend to procedures such as equine IVF using sex-sorted epididymal stallion spermatozoa.

**References**