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Investigating the inflammatory pathways involved in placental detachment in the mare

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Abstract

The mechanism of placental detachment in the mare has not been investigated in detail and as such remains poorly understood. Research in other species provides strong evidence that parturition and fetal membrane detachment from the endometrium requires involvement of the immune response. Research in horses has also suggested that movement of leukocytes in the umbilical vessels, and their attraction to fetal membranes at parturition, contribute to placental detachment.

The aims of this study were to 1) confirm whether a leukocyte "concentration gradient" exists between the equine neonatal umbilical artery and vein, and whether this gradient was associated with fetal membrane retention time 2) to investigate the expression of pro-inflammatory cytokines in the equine endometrium and fetal membranes at parturition using quantitative real time polymerase chain reaction (RT-qPCR) and 3) to localise pro-inflammatory cytokine proteins within the equine endometrial and fetal membrane tissues using immunohistochemistry to correlate with gene expression.

Data was collected from 33 spontaneously foaling mares and their foals on a single Thoroughbred stud farm in New Zealand. Umbilical artery and vein blood, fetal membrane and endometrial biopsy tissue samples were obtained.

The average fetal membrane retention time was 92 ($\pm$ 130) minutes. The median fetal membrane retention time was 47 minutes (IQR 30). There was a significantly higher number of total leukocytes, lymphocytes and neutrophils in the equine neonatal umbilical artery than the umbilical vein at parturition ($p < 0.001$). Fetal membrane retention time tended to decrease with an increase in umbilical leukocyte concentration gradient but did not reach statistical significance ($p = 0.098$). The mRNA expression of IL-1, IL-6 and IL-8 was significantly greater in the endometrium of foaling mares compared to control mares ($p < 0.001$). The mRNA expression of IL-8 was significantly higher in the fetal membranes of foaling compared to control mares ($p < 0.001$) whereas mRNA expression of IL-6 in fetal membranes was significantly lower between the two groups ($p< 0.001$). Whilst mRNA expression for IL-1 was lower in the fetal membranes of foaling mares compared to controls, the difference was not significant ($p = 0.16$).

This study has demonstrated the presence of an innate inflammatory immune response in the placenta of spontaneously foaling mares, suggesting the role of inflammation in the detachment of the fetal membranes from the endometrium. The results from this study have highlighted the need to further investigate the mechanism of placental detachment in the mare and also understand the pathophysiology behind abnormal fetal membrane retention.

Declaration

This is to certify that:

1. The thesis comprises only of my original work towards the Masters of Veterinary Science

2. Due acknowledgement has been made in the text to all material used, and

3. This thesis is fewer than the maximum 40,000-word limit in length, excluding tables, bibliographies and appendices.

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Cristina Maria Rosales

Preface

All scientific work described in this thesis was performed by the author at the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia and at the Oaks Stud, Cambridge, New Zealand.

Sample collection from animals as described in Chapter 2 was performed by the author.

Dr Natali Krekeler and Associate Professor Brett Tennent-Brown were involved throughout the project in the conception of it as well as manuscript composition.

The statistical analysis was performed with the assistance of Professor Mark Stevenson.

Parts of this thesis has been presented at the following symposium:
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Abbreviations

ANOVA	Analysis of Variance
DAMPs	Damage-associated molecular patterns
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horse radish peroxidase
ICE	IL-1- converting enzyme
IL-1	Interleukin-1
IL-1R	Interleukin-1 receptor
IL-6	Interleukin-6
IL-8	Interleukin-8
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
PAMPs	Pathogen-associated molecular patterns
PGE-2	Prostaglandin E2
PRRs	Pattern recognition receptors
RFM	Retained fetal membranes
RPL30	Ribosomal protein L30
RPL32	Ribosomal protein L32
RPS5	Ribosomal protein S5
RT-qPCR	Quantitative Real Time Polymerase Chain Reaction
SIRS	Systemic inflammatory response syndrome
TIMP	Tissue inhibitors of metalloproteinases
TLRs	Toll-like receptors
TNF-α	Tissue necrosis factor

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Chapter 1 - Literature Review

1.1 Introduction

The mechanism for normal detachment of fetal membranes from the equine uterus at parturition is not yet well understood. Furthermore, the causes for failure of placental detachment are also poorly comprehended. This is important because prolonged fetal membrane retention can lead to serious, and potentially fatal, complications such as metritis, endotoxaemia/systemic inflammatory response syndrome (SIRS) and laminitis (Reed et al., 2010, Rapacz et al., 2012). The intensive care required for secondary medical issues (metritis, SIRS and laminitis) incurs high costs and may lead to a delay in achieving a subsequent pregnancy. Currently, treatment strategies for retained fetal membranes are based on anecdotal rather than empirical evidence as a consequence of limited research.

Along with the health consequences, mismanagement of retained fetal membranes (RFM) can also have a negative impact on a mare's reproductive performance; manual removal of membranes and delayed administration of the uterotonic drug oxytocin was associated with an increase in the time from foaling to last day of service in a large group of heavy breed draft mares (Ishii et al., 1999). Both Thoroughbred and heavy draft breed mares that experience RFM beyond 3 hours have been shown to take longer to get in foal and therefore produce foals born later in the season (Ishii et al., 2013). In the same study it was shown that pregnancy rates at foal heat declined significantly after 3 hours of fetal membrane retention (Ishii et al., 2013). This becomes important, because in the equine racing industry there remains strong pressure to breed mares as soon as possible after foaling (i.e. the first oestrus period that occurs after foaling) to ensure that a foal is produced annually. In order to achieve similar pregnancy rates at foal heat compared to subsequent cycles, criteria have been developed to select those mares to be bred on foal heat to ensure optimal pregnancy rates; one of those criteria to optimize conception rates is to not breed mares that had retained fetal membranes (Hanlon et al., 2012).

Previous research in horses has suggested that components of the innate immune response, including leukocyte migration and activation, as well as collagen remodeling, play an important role in detachment of the fetal membranes (Volkmann, 2002, Oddsdottir et al., 2007). A dampened immune response and subsequent impaired leukocyte migration and pro-inflammatory cytokine release at the time of parturition may therefore be responsible for fetal membrane retention (Kimura et al., 2002, Benedictus et al., 2011).

This literature review seeks to describe the current understanding of the biological mechanisms underlying normal placental detachment at parturition. Given the limited number of studies undertaken in equids, the more extensive research performed in cattle and humans will also be discussed.

1.2 Equine Placentation

The equine placenta is classified as diffuse epitheliochorial with six tissue layers separating the fetal and maternal vascular systems. The formation of microcotyledons provides a large surface area for exchange of nutrients, waste and gases between the fetal and maternal systems and is thought to compensate for the large diffusion distance (Sharp, 2000). Extra-embryonic membrane development starts at 21 days of gestation. Around day 35, non-invasive trophoblast attachment to the endometrial luminal epithelial cells occurs via microvillous formation. Attachment is well established by day 40, due to expansion of the allantois, and coincides with

transformation from a choriovitelline to a chorioallantoic structure (Sharp, 2000, Allen and Stewart, 2001). From Day 50 to 60, the rudimentary villi begin to branch and the allantochorion expands until reaching maturity by day 80-85 (Sharp, 2000, Allen, 2001). By Day 120, the microvilli have become fully-functional microcotyledons interdigitated with the endometrium (Sharp, 2000).

A maternal and corresponding fetal artery provide vascular support to each microcotyledon with a capillary bed located on each side of the maternal-fetal interface to maximise exchange of nutrients, gases and waste products (Sharp, 2000). Equine placental development reportedly continues up to parturition; lengthening and branching of microvilli occurs throughout late-gestation and is maintained into the last two to three months of gestation, presumably to help meet requirements of the rapidly growing fetus (Macdonald et al., 2000). In humans, research has identified evidence that cellular senescence and ageing of the placenta occurs towards the end of gestation approaching labour. The increase in several ageing markers has been shown to correlate with the upregulation of pro-inflammatory factors, suggesting a relationship which enables the transition from the uterine quiescent phenotype to a state of inflammation at the onset of parturition, including fetal membrane detachment. (Cox and Redman, 2017). This is an area yet to be investigated in other mammalian species, including horses.

1.3 The innate immune response

The so called "innate" immune system lacks any form of memory and remains unchanged in its response irrespective of the provocateur. This system has been conserved throughout evolution and is found in both animals (vertebrates and invertebrates) and plants. It is often termed a "primitive" and "non-specific" defence mechanism, (Janeway and Medzhitov, 2002, Delves and Roitt, 2000, Mogensen, 2009). However, the innate immune system provides a rapid response to invasion or damage, allowing early recognition and an immediate response to invading pathogens as well as trauma and associated tissue damage (Bianchi, 2007). Furthermore, the innate immune system plays a vital role in the activation of the adaptive immune response (Iwasaki and Medzhitov, 2004). Dendritic cells, in particular, are key factors in the innate immune response that initiate T-cell activation and the transformation of naïve CD4+ T cells into T helper cells through antigen presentation via Major Histocompatibility Complex (MHC) molecule expression (Iwasaki and Medzhitov, 2004, Mogensen, 2009).

1.3.1 Components of the innate immune system

The innate immune system is divided into cellular and molecular components. The cellular component of the innate immune system consists of dendritic cells, phagocytic cells (macrophages and neutrophils), cells that release inflammatory mediators (basophils, eosinophils and mast cells) and natural killer (NK cells) (Delves and Roitt, 2000). The molecular component consists of cytokines, acute phase proteins and the complement system. Central to the innate immune response is the dendritic cell, whose major role is antigen presentation. Dendritic cell surface receptors are able to recognise the pathogen-associated molecular patterns (PAMPs) of invading pathogens or to detect endogenous danger signals (alarmins) resulting from damage of the host's tissues (Bianchi, 2007, Delves and Roitt, 2000). PAMPS and alarmins are considered subgroups of the damage-associated molecular patterns (DAMPs) since the downstream pathways following pathogen recognition and the host's ultimate response (a pro-inflammatory process) are similar (Bianchi, 2007).

Recognition of PAMPs and alarmins occurs by germ line-encoded pattern-recognition receptors (PRRs). Engagement of PAMPs or alarmins with PRRs initiates a signalling cascade within the cytosol that ultimately leads to activation of the transcription factor Nuclear Factor κB (NF-κB) with a subsequent increase in synthesis of pro-inflammatory cytokines and other inflammatory

molecules enabling a rapid response to an invading pathogen or tissue injury (Akira et al., 2006). The most well-understood family of the PRRs are Toll-like receptors (TLRs) which can be found both extra- and intracellularly on various cell types including dendritic cells, macrophages, B cells and some lines of T cells (Akira et al., 2006). Interleukin-1 receptor (IL-1R) is another PRR that has received much attention and plays a key role in the production of a pro-inflammatory response due to its function as a signalling receptor that is widely expressed (Bianchi, 2007, Feldmann, 2001). IL-1R binds both IL-1α and IL-1β and plays an important role in sterile inflammation; the binding of IL-1 to IL-1R induces the production of secondary pro-inflammatory cytokines IL-6 and TNF- α (Lukens et al., 2012).

1.3.2 Cytokines

Cytokines are small, non-structural glycoproteins that behave as chemical messengers between cells and regulate cellular activity (Dinarello, 2000). They are categorised as either pro- or anti-inflammatory, depending on the nature of their activating signal. Chemokines are a subcategory of pro-inflammatory cytokines with a primary role in attracting circulating leukocytes to damaged tissues (Turner et al., 2014). The cytokines interleukin-1β (IL-1β), IL-6, IL-8 and TNF-α have been shown to be upregulated in reproductive tissues during parturition (Bowen et al., 2002, Palm et al., 2013). In addition to activating neutrophil degranulation, IL-8 is an important chemoattractant (i.e., a chemokine) for neutrophils. The aim of the present study was to evaluate the role of these cytokines in normal placental detachment and therefore this review will focus on the action of these molecules.

IL-1β, IL-6 and TNF-α are pleiotropic cytokines in that they are produced by, and act upon, a variety of cell-types as well as producing a variety of responses depending on the cell-type upon which they act (Christiaens et al., 2008). The major source of pro-inflammatory cytokines, in particular IL-1β, IL-6, TNF-α and the chemokine IL-8, is macrophages (Feldmann, 2001, Turner et al., 2014). IL-1β, IL-6 and TNF-α share a common signalling pathway via type I cytokine receptors, whereas IL-8 signals via a G protein-coupled (Turner et al., 2014).

Acting synergistically with TNF-α, the primary role of IL-1β is to trigger gene transcription for additional pro-inflammatory mediators (Dinarello, 2000). IL-1β is primarily secreted by macrophages but can also be secreted by neutrophils, dendritic cells, B cells and hepatocytes (Turner et al., 2014). It is a potent pro-inflammatory cytokine existing as a cytoplasmic pre-cursor that is cleaved by IL-1- converting enzyme (ICE) following PRR stimulation prior to release from the cell (Feldmann, 2001). The primary target for IL-1β is other cells in the immune system including NK, T cells and B cells. IL-1β and TNF-α are also able to induce production of pro-inflammatory mediators through coordinated upregulation of type II phospholipase A2 expression generating prostaglandins and leukotrienes from arachidonic acid via cyclooxygenase and lipoxygenase enzyme activity, respectively (Dinarello, 2000). In addition, IL-1β (in concert with TNF-α) induces expression of endothelial adhesion molecules (Dinarello, 2000) and stimulates endothelial cell chemokine production to facilitate leukocyte adhesion to the endothelial surface and migration into the tissues (Feldmann, 2001). Together with chemokines, TNF-α and colony-stimulating factors, IL-1β stimulates leukocyte phagocytic activity (Feldmann, 2001). IL-1β is also an important link between the innate and adaptive immunes systems; Stimulation of CD4+ T cells by IL-1β directs differentiation of these cells into T helper cells (Turner et al., 2014).

Similar to IL-1β, IL-6 is also expressed by a range of cells including macrophages, fibroblasts and T helper cells. The primary targets for IL-6 are activated B cells and plasma cells. IL-6 promotes monocyte differentiation into macrophages, induces neutrophil apoptosis, is essential for maturation of B cells into antibody-producing plasma cells and for T cell activation and differentiation (Scheller, 2011, Kishimoto, 2010, Turner et al., 2014). Whilst IL-6 is generally

regarded as a pro-inflammatory cytokine, it is also recognised for possessing anti-inflammatory properties under certain conditions (Scheller, 2011). Two signalling pathways of this cytokine have been identified; activation of the classical pathway by IL-6 results in a regenerative or anti-inflammatory response, whereas activation of the trans-signalling pathway by IL-6 leads to production of pro-inflammatory mediators (Scheller, 2011). Whether the classical (regenerative/anti-inflammatory) or trans-signalling (pro-inflammatory) pathway is activated is determined by binding of IL-6 to a membrane-bound or soluble IL-6 receptor (IL-6R), respectively (Rose-John, 2012). The soluble form of IL-6R is generated by either shedding/proteolysis of the membrane-bound receptor or mRNA translation. Binding of IL-6 to the soluble form of its receptor activates local endothelial cells to secrete monocyte-attracting chemokines (e.g., MCP-1) and produces lymphocyte-selective cell adhesion molecules resulting in continuation of the pro-inflammatory state (Scheller, 2011, Rose-John, 2012).

TNF-α's main role as a pro-inflammatory cytokine, alongside IL-1β, is to induce the subsequent production of other pro-inflammatory cytokines as well as other non-cytokine inflammatory mediators via NF-kB pathways in other cells (Van Miert, 2002). Comparable to IL-1β, it is synthesised in precursor form and cleaved at the cell surface by TNF-α converting enzyme to create a soluble form of TNF-α (Turner et al., 2014). It is released principally from activated macrophages, but is also produced by NK cells, fibroblasts, T cells and mast cells (Turner et al., 2014). As highlighted previously, many pathways are shared with TNF-α and IL-1β regarding signal transduction, with virtually the same outcome. TNF-α is important contributor to the pro-inflammatory cascade - it induces production of other pro-inflammatory mediators (IL-6, IL-8, granulocyte-macrophage colony-stimulating factor and macrophage-inflammatory protein-1), initiates endothelial margination of leukocytes and directs extravasation and leukocyte movement into affected tissues and pyrexia (Van Miert, 2002). It also induces the production of acute phase proteins by the liver; however, this is driven by IL-6 as well (Taylor, 2015, Van Miert, 2002). However, in addition to stimulation of a pro-inflammatory response, TNF-α stimulated cells can also undergo apoptosis through activation of the TNF receptor's cytosolic 'death domain' which activates intracellular molecules co-ordinating programmed cell death (Dinarello, 2000, Feldmann, 2001).

1.3.3 Chemokines

The role of chemokines in the recruitment of leukocytes from the circulation into damaged or infected tissues (i.e., chemotaxis) is well known. In addition, these molecules are also involved in other biological or homeostatic processes including haematopoiesis, the adaptive immune response and immune surveillance (Moser et al., 2004, Turner et al., 2014). Expression of the pro-inflammatory chemokines is upregulated in inflammatory states, such as trauma, infection and neoplasia, and they interact with a wide variety of cells. Classification of chemokines is based on the location of cysteine moieties within the amino acid sequence; those two categories are α-chemokine (CXC) and β-chemokine (CC) (Van Miert, 2002). Functionally, the CC chemokines are chemotactic for monocytes, whereas CXC chemokines attract neutrophils and lymphocytes (Harada et al., 1994, Murdoch and Finn, 2000). IL-8 is the most widely recognised chemokine and belongs to the CXC subgroup. In response to an inflammatory stimulus, IL-8 can be produced by a wide variety of cells, including vascular endothelial cells, macrophages, T lymphocytes, neutrophils and hepatocytes (Harada et al., 1994). Release of IL-8 into the circulation can be induced by TNF and IL-1β. The main function of IL-8 is the recruitment of neutrophils to the location of injury or infection and their subsequent activation. Two receptors (CXCR1 and CXCR2) for IL-8 have been identified; however, binding of IL-8 to CXCR1 is the primary route resulting in chemotaxis (Turner et al., 2014). Chemokine binding to leukocytes' cell surface integrins enhances adherence to the endothelium and allows extravasation into the tissue (Murdoch and Finn, 2000). Simultaneously, chemokine binding activates leukocyte phagocytosis. Intracellular IL-8 signalling is initiated through activation of G protein-coupled receptors resulting in

activation of MAPK signalling (Turner et al., 2014). Activation of MAPK signalling ultimately leads to increased expression of adhesion molecules on the leukocytes' cell membrane and neutrophil degranulation/lysosomal enzyme release and phagocytosis through intracellular calcium release mechanisms (Turner et al., 2014). Upon IL-8 binding to neutrophils, CXR1 and CXR2 are internalised and undergo enzymatic degradation, followed by enzymatic degradation of IL-8 by lysosomal enzymes secreted by neutrophils. (Samanta et al., 1990).

The innate immune response is essential for survival and vital to combat both tissue injury and infectious disease. Once infection or injury has been overcome, downregulation of pro-inflammatory cytokine expression to return the body to a normal physiological state must occur. Without a balance of pro- and anti-inflammatory cytokines, the host's immune response to trauma or infection would result in a chronically inflamed state, and potentially catastrophic tissue damage. This has been highlighted experimentally and also in diseased states/inflammatory disorders affecting humans (Dinarello, 2007, Fernandez and Lolis, 2002, Turner et al., 2014). Anti-inflammatory cytokines exist to provide a balance to the pro-inflammatory response and to avoid an exaggerated response. Some cytokines are designated solely as anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-22, TGFβ), whereas others possess dual function, depending on pathway activation (e.g. IL-6 classical or trans-signalling) (Dinarello, 2007). The innate immune response is also controlled by suppressors of cytokine signalling; a prime example being the effect prostaglandins have on the production and activity of cytokines. Prostaglandin E2 (PGE2), for example, modulates and co-ordinates both pro- and anti-inflammatory responses and this molecule has received attention regarding its role in controlling both pathological and physiological inflammatory states (Ricciotti and FitzGerald, 2011, Keelan et al., 2003).

1.3.4 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of secreted and cell-surface bound proteolytic enzymes, also known as gelatinases, that degrade extracellular proteins. Within tissues they exist in pro-enzyme form, and once activated they remodel surrounding tissue by cleaving a wide variety of extracellular matrix molecules, including collagen (Sternlicht and Werb, 2001). MMP activity is negatively regulated by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), to ensure they accomplish their intended function but without excessive tissue degradation (Sternlicht and Werb, 2001).

MMPs have been located within fetal membrane tissue, and certain gelatinases have been demonstrated to have enhanced activity, contributing to fetal membrane rupture and placental detachment (Xu et al., 2002, Tsatas et al., 1999). Utilising zymography, proteolytic activity of MMP-2 and -9 has been investigated in term fetal membrane tissue of women. Studies have shown a general tendency for MMP-9 to significantly increase in post-labour tissues compared to non-labouring term and pre-term samples, being almost absent prior to labour (Xu et al., 2002, Maymon et al., 2000). Changes in MMP-2 activity appear to be less consistent, with some reporting a decrease in the active form of MMP-2 (Maymon et al., 2000) in fetal membranes following term parturition, whilst others have reported little to no change in the gelatinase's activity before and after labour (Weiss et al., 2007, Xu et al., 2002). Similar patterns have been reported in cattle; MMP-9 staining and mRNA expression within fetal membranes differed substantially post-calving compared to late gestation (Walter and Boos, 2001, Takagi et al., 2007) whereas MMP-2 showed either significantly higher expression in pre-labour fetal membrane tissue (Dilly et al., 2011) or demonstrated no significant change in mRNA pre- and post- labour (Takagi et al., 2007).

MMP behaviour in equine amniotic samples has revealed different results. In contrast to other species, a single study in mares at term discovered that whilst MMP-9 activity in amniotic fluid

increased towards the end of gestation, it was lower during foaling (Oddsdóttira et al., 2011). In this study, changes in MMP-2 activity were negligible and the same was the case for TIMP-2. The authors concluded that remodelling of the fetal membranes in equids occurs prior to rather than during parturition, suggesting that remodelling in equine reproductive tissues occurs earlier than in other species, e.g. humans and cattle. This was a single study, of small sample size, and whilst MMP activity in amniotic fluid was investigated this was not compared to or analysed alongside MMP activity in reproductive tissues. Therefore, whilst the information gained from the study provides some insight as to the role of MMPs, there is insufficient evidence here to provide a direct link between fetal membrane detachment and a local acute inflammatory response in mares.

Whilst MMPs were not investigated in the present study, mediation of extracellular matrix degradation within the uteroplacental unit by these proteinases is evident and therefore likely a part of the puzzle that is the mechanism of placental detachment (Xu et al., 2002, Tsatas et al., 1999, Oddsdóttira et al., 2011). For this reason, future research into the mechanism of placental detachment in mares warrants investigating the role MMPs play.

1.4 The Immunology of parturition and placental detachment

In mammalian pregnancy, the fetus is considered immunogenic/alloantigenic to the dam due to the expression of paternal antigens. Therefore, immunological tolerance towards the fetus by the maternal immune system must exist and be preserved in order for pregnancy to be maintained and to avoid abortion or pre-term delivery (Donaldson et al., 1994, Crump et al., 1987, Rapacz-Leonard et al., 2014). In equids, several studies have investigated this process, primarily focusing on early pregnancy, in particular immunological recognition of paternal-derived MHC 1 complex antigens (Crump et al., 1987, Donaldson et al., 1994, Donaldson et al., 1990, Sharp, 2000, Wang et al., 2018, Wang et al., 2013). Chorionic girdle cells (invasive trophoblast cells) are formed during early embryogenesis and surround the fetus. Around Day 36 to 38, these invasive trophoblast cells begin to invade and phagocytose the endometrial epithelial cells. They differentiate and hypertrophy to become endometrial cups (Sharp, 2000). The primary function of endometrial cups is the production of equine chorionic gonadotrophin, which stimulates the production of accessory corpora lutea of the ovaries, producing high levels of progesterone to maintain pregnancy (Allen and Stewart, 2001). In addition to their endocrine function, invasive trophoblast cells are also immunogenic. Paternally-derived major histocompatibility complex I (MHC I) antigen is expressed by these cells (Rapacz-Leonard et al., 2014, Donaldson et al., 1990) and maternal recognition of these antigenically foreign cells has been well-documented in mares (Crump et al., 1986, Donaldson et al., 1994). A maternal humoral immune response characterised by the generation of anti-paternal lymphocytotoxic antibodies occurs, leading to maternally-derived leukocyte destruction from day 70 and death of endometrial cups by day 100 (Sharp, 2000, Allen and Stewart, 2001). These antibodies are first detected within 10-14 days of endometrial cup development and have been documented to persist and maintain steady concentrations throughout the entire pregnancy (Antczak et al., 1982).

The maternal immunological recognition to paternal antigens is considered a physiological but localised immune response. It has been postulated that this process in early pregnancy educates the maternal immune response so that come parturition, the fetal membranes are rejected and passed in a timely manner (Rapacz-Leonard et al., 2014). In summary, pregnancy and parturition appear to both be immunological processes. Dysregulation can lead to complications during pregnancy and peri- or post-partum. An issue with this theory is how the fetus remains immunologically protected for the duration of the pregnancy, despite the generation and persistence of anti-paternal antibodies. Hormonal modulation and the shift to an anti-inflammatory state have been documented, characterised by an increased expression of anti-

inflammatory cytokines. Hormones of pregnancy, progestagens and oestrogens, are considered pertinent to this anti-inflammatory quiescent state (Robinson and Klein, 2012).

To date, there has been limited investigation into the mechanisms of detachment of the equine allantochorion from the endometrium at parturition. Therefore, the processes occurring at the time of parturition in equids are largely extrapolated from studies in other species, particularly cattle and humans. Previous studies in horses have mainly focused on possible causes of abnormal placental detachment leading to retention of fetal membranes. Inadequate uterine oxytocin receptor expression, abnormal rate of apoptosis within the fetal membranes, fibrosis or exuberant connective tissue and low serum calcium concentrations have all been postulated as causes of retained fetal membranes (RFM)(Rapacz-Leonard et al., 2015, Pazdzior et al., 2012, Rapacz et al., 2012, Sevinga et al., 2002a). However, some of these studies contained very small sample sizes ($n \leq 10$) and so results should therefore be interpreted with caution. In some cases, the membranes were forcefully removed which prevented evaluation of the tissues under natural circumstances. Additionally, in all studies the sample population was focused on only heavy breed or Friesian mares. Retained fetal membranes in these breeds is considered highly prevalent and may have other influences, such as genetics, making extrapolation to other breeds problematic.

1.5 Pro-inflammatory Cytokines in Uteroplacental Tissues

It is well documented in humans that parturition is associated with an upregulation of pro-inflammatory cytokines within the uteroplacental unit, and therefore parturition is considered an inflammatory process (Bowen et al., 2002, Norman et al., 2007, Haddad et al., 2006). Parturition revolves around a balance between endogenous endocrine and inflammatory systems, in particular progestagens, pro-inflammatory cytokines and prostaglandins. Prostaglandins are involved in each stage of parturition, and cytokines have been shown to play a role in the regulation of prostaglandin synthesis leading to uterine activation/contraction, fetal membrane rupture, cervical dilation and uterine involution (Christiaens et al., 2008). Therefore, without an appropriate and accurately-timed pro-inflammatory response in uteroplacental tissues, the process of spontaneous parturition at term would be disrupted and potentially contributing to complications such as premature delivery, failure of fetal membranes to rupture and retention of fetal membranes. Equine and human pregnancy and parturition share some similarities regarding endocrine function and immune responses (Conley, 2016). In particular the demonstration of pro-inflammatory cytokines in the endometrium and amnion of mares at parturition which correlates with those findings in human reproductive tissues during term labour (Palm et al., 2013, Bowen et al., 2002, Osman et al., 2003).

1.6 Cytokine involvement in the initiation of parturition

Activation of the pro-inflammatory cascade is typically associated with pathogen invasion and infection, initially detected by immune surveillance cells. However, this process also occurs as a physiological (rather pathological) process, referred to as sterile inflammation (Kobayashi, 2012). Such is the case for parturition in humans, characterised by an increase in pro-inflammatory cytokines and chemokines within the uterus and fetal membranes (Bowen et al., 2002). Research in this area has largely focused on the relationship between an elaborate pro-inflammatory response and premature birth in the absence of intrauterine infection (Keelan et al., 2003, Peltier, 2003). This is possibly due to incidental exposure to the fetal allograft which triggers an acute immune response. Subsequent cytokine and chemokine production by the maternal immune system eventually triggers parturition. Other research has questioned the role of placental cell senescence and premature cell death showing that the mechanisms behind idiopathic premature parturition is likely complicated and multifactorial (Cox and Redman, 2017). It is through similar, albeit less extreme, pathways that human parturition at term occurs. Whilst little evidence currently exists for this process in equids, based on recent research in

mares, the inflammatory component of parturition in horses may follow similar pathways to those identified in humans (Rapacz-Leonard et al., 2015, Palm et al., 2013, Osman et al., 2003, Xu et al., 2002).

Despite there being increasing evidence that parturition is an inflammatory process, there currently is no agreement as to what triggers labour and as to whether the presence of pro-inflammatory cytokines is the initial step, or a flow-on effect to an upstream trigger or event. The requirement for progestagens to maintain pregnancy and uterine quiescence is well known, and these hormones are considered an 'anti-inflammatory steroids' based on their interactions with the immune system (for example the inhibition of IL-6 production in fetal tissue and mononuclear cells) (Gotkin et al., 2006). Based on this knowledge, some consider that parturition is initiated by a 'progesterone withdrawal'- a rapid decline in progesterone concentrations or receptor affinity, permitting a pro-inflammatory cascade of events to occur (Peltier, 2003, Golightly et al., 2011). There is some evidence to suggest this occurs in equids, but confirmation has been problematic due, in part, to the difficulties in measuring progestagen proteins (Conley, 2016).

The role of pro-inflammatory mediators in equine parturition has been investigated in one study (Palm et al., 2013). That study demonstrated mRNA expression of pro-inflammatory cytokines within both fetal membranes and endometrium; however, no comparison was made in this study to mRNA expressions in these tissues prior to parturition. Another theory revolves around direct contact between the maternal and fetal systems; a 'sterile inflammatory response' occurs as the result of amniotic fluid carrying fetal DNA entering the uterine vessel circulation due to myometrial contractions. The fetal DNA acts as DAMPs that activate a maternal pro-inflammatory response (Kobayashi, 2012). Certainly, the enhanced expression of chemokines in response to cyclical myometrial stretch and other pro-inflammatory cytokines has been demonstrated but the evidence to support the link between myometrial contraction and initiation of an immune response to fetal DNA products remains to be thoroughly investigated (Hua et al., 2011, Kendal-Wright et al., 2010)

1.6.1 Cytokine expression and activity in fetal membranes and endometrium at parturition

Fetal membrane detachment from the endometrium is an essential step in normal parturition and signals the end of labour. There is extensive evidence from humans that parturition is a pro-inflammatory process supporting the suggestion that acute inflammation plays a pivotal role in the process of fetal membrane detachment. Pro-inflammatory cytokine upregulation has been extensively investigated in the context of premature rupture of membranes (Blank et al., 2008, Christiaens et al., 2008, Kumar et al., 2006). However, at the moment the mechanisms behind physiological membrane detachment from the endometrium in both humans and equids is not well understood due to research focused anatomically at the location of membrane rupture rather than the entire membranes. The author acknowledges the differences between the equine and human placenta; however, due to the lack of information regarding pro-inflammatory response in equine placental tissues at parturition and the similarities between these two species, the focus in this section will remain around the understandings of human fetal membrane detachment (Conley, 2016). Whilst it seems tempting to compare the mechanism of placental detachment between equids and bovines, the unique differences in placentation between these two species limits the usefulness of this information.

In humans, several biomarker studies have provided evidence that, at the feto-maternal interface during term labour, an inflammatory process is underway characterised by an influx of pro-inflammatory cytokines and chemokines into the fetal membrane tissue (Keelan et al., 2003, Osman et al., 2003, Haddad et al., 2006). Ageing of fetal membrane tissue and declining functional capacity occurs as a normal physiological process, coinciding with fetal maturation and readiness for birth at term (Menon, 2016, Cox and Redman, 2017). As a consequence of cellular injury,

senescent membranes release an array of mediators, including alarmins, prompting the transition to an inflammatory phenotype within the fetal membranes (Menon, 2016, Cox and Redman, 2017).

A significant increase in IL-6 and IL-8 mRNA expression was found within choriodecidual tissues of labouring women, with negligible increase in IL-1, whereas within amniotic tissues a significant increase in IL-1 and IL-8 was identified with little expression of IL-6 present (Osman et al., 2003). Upregulated mRNA expression of IL-1 and IL-6 in fetal membranes has been identified in labouring tissues by other researchers (Dudley et al., 1996, Norman et al., 2007). Unlike IL-1, IL-6 and IL-8, increased TNF-α expression has not been consistently identified or demonstrated in fetal membrane tissue (Dudley et al., 1996, Osman et al., 2003). The immunolocalisation of pro-inflammatory cytokines in labouring tissues has subjectively assessed their presence as well as identifying their cellular origin, complementing the changes in mRNA concentrations within these tissues (Young et al., 2002).

The results of these studies suggest that the upregulation of these pro-inflammatory cytokines in fetal membranes at the time of parturition indicates their contribution to placental detachment. It is proposed that this is achieved by enhancement of matrix metalloproteinase synthesis, as well as concurrent downregulation of TIMPS (Bowen et al., 2002, Christiaens et al., 2008). The increase in collagenase activity weakens the extracellular matrix (ECM) attachment between the membranes facilitating their detachment. In vitro assessment demonstrated that an increase in MMP-9 and decrease in TIMP-3 concentration in fetal membranes occurred in the presence of IL-1β and TNF-α causing collagen remodelling and reduction in fetal membrane tissue strength in a dose- and time-dependant manner (Kumar et al., 2006).

However, enhanced collagenase activity induced by pro-inflammatory cytokines may not be solely responsible for membrane detachment. Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 stimulate prostaglandin synthesis in uterine and placental tissues through the induction of COX-2 expression (Rauk and Chiao, 2000, Hansen et al., 1999, Mitchell et al., 1990). This increase in prostaglandin synthesis stimulates uterine contractions, contributing a mechanical mechanism for detachment as well as providing a positive feedback loop for producing more pro-inflammatory cytokines and MMPs within the tissues, primarily within the endometrium (Christiaens et al., 2008).

Overall, the induction of fetal membrane detachment appears to be due to the combination of a series of biochemical events and mechanical stretching, stimulating local tissue destruction and damage leading to separation of the membranes at parturition.

1.7 Leukocytes and Their Role in Placental Detachment

Infiltration of leukocytes to the uteroplacental unit appears to be an essential component of parturition as these cells are a rich source of pro-inflammatory cytokines and therefore considered largely responsible for the orchestration of the acute inflammatory response (Peltier, 2003, Young et al., 2002). Neutrophils are particularly important, not only as they are a major source of cytokines, in particular IL-8, but also due to their ability to degranulate, releasing a range of mediators and reactive oxygen species which directly affect surrounding tissues and recruit more leukocytes to the region of interest (Lacy, 2006).

Demonstration of leukocyte infiltration of fetal membranes appears difficult, and dependant on methodology. Entrapment of leukocytes within the fetal membranes, by demonstrating a significantly lower leukocyte count leaving the membranes via the umbilical vein than entering them via the artery, was found in a sample of spontaneously foaling mares (Volkmann, 2002). However, virtually no neutrophils were present in fetal membrane tissue sections examined

histologically. Similarly, human studies have also been unable to demonstrate an influx of leukocytes to the fetal membranes at labour despite demonstrating an increase in pro-inflammatory cytokine expression in them (Norman et al., 2007, Osman et al., 2003). Those authors postulated that the source of the cytokines may be from non-inflammatory cell types rather than leukocytes or that the study was underpowered. Alternatively, timing of recruitment and the rate of degradation of leukocytes in the fetal membranes may occur at different rates than the collection of cervical or membrane samples.

On the other hand, studies examining the chemotactic activity of leukocytes to the uteroplacental unit have shown that it is significantly increased in labour compared to pre-labour tissues (Gomez-Lopez et al., 2009, Gomez-Lopez et al., 2011). Significant inhibition of neutrophil chemotaxis to fetal cotyledon tissue and decreased neutrophil function was identified in cows who retained their fetal membranes following spontaneous parturition (Kimura et al., 2002). Similarly, there was an association in term cows that received exogenous corticosteroids and experienced RFM, with significantly reduced chemotactic activity of leukocytes to fetal cotyledon tissue noted. These results were compared to spontaneously-calving cows who did not retain their fetal membranes (controls) and corticosteroid-treated cows who did not retain their membranes (Benedictus et al., 2011). These results highlighted the necessity of a functioning innate immune response within fetal membrane tissue to allow for timely detachment.

1.8 Conclusion

The process by which normal placental detachment occurs appears to rely on an interplay between immunological, inflammatory and endocrinological processes, requiring a coherent and tightly-regulated sequence of steps. Currently, there is insufficient evidence for these mechanisms in horses. However, information provided from other species clearly advocates the involvement of the innate immune response, providing a platform to direct future study in equine research. Ongoing investigation of biological markers and understanding the role pro-inflammatory mediators such as cytokines and collagenases play, as well as more up-stream mediators and triggers will help clarify the process of fetal membrane detachment.

By understanding these pathways in horses, this knowledge can help investigation into what causes pathological conditions such as retained fetal membranes, and premature parturition due to placentitis, and enable the development of treatment or prevention of these conditions.

Aims

1. To determine whether a leukocyte concentration gradient exists between the equine neonatal umbilical artery and vein at parturition, and whether this gradient contributes to fetal membrane retention time

2. To investigate the expression of pro-inflammatory cytokines *IL-1, IL-6* and *IL-8* in the equine endometrium and fetal membranes at parturition using RT-qPCR

3. To localise pro-inflammatory cytokines within the equine endometrial and fetal membrane tissues using immunohistochemistry to correlate with their gene expression

Hypothesis

1. At parturition, there will be a higher concentration of leukocytes entering the fetal membranes via the umbilical artery than leaving them via the umbilical vein

2. Placental detachment is associated with the upregulated mRNA expression of pro-inflammatory cytokines *IL-1, IL-6* and *IL-8* in the endometrial and fetal membrane tissues of foaling mares compared to control mares

Chapter 2 - Materials and Methods

2.1 Sample Population

Data were collected from a total of 33 spontaneous foaling mares on a single Thoroughbred stud farm in the Waikato region (latitude 38° S, 175° E) of New Zealand during the 2013 breeding season. Sample collection was conducted in accordance with the guidelines provided by the University of Melbourne Animal Ethics committee.

Mares on this stud farm were kept at pasture all year-round in groups of 20 to 30 horses. At approximately 330 days of gestation, mares entered communal foaling paddocks, where they underwent continuous monitoring. Mares showing signs of impending parturition (udder development, waxing of the teats) prior to 330 days of gestation, or those with a history of premature parturition, were moved into these foaling paddocks earlier for continual monitoring.

At the commencement of second stage labour (indicated by rupture of the chorioallantoic membrane and release of allantoic fluid) mares were transferred to an individual, grassed foaling yard (approximately 60 × 40 m) for observation by a foaling attendant. Foaling attendants were responsible for the initial management of parturition and the resident veterinarian was only called if there was malpresentation, malposition or malposture of the fetus or if the foaling failed to progress after 20 minutes of second stage labour. At this farm, the usual practice was to leave the umbilical cord to break naturally. Mares received 10 IU oxytocin (Troy Laboratories, Glendenning, Australia), I/M every 4 hours if fetal membranes were retained for more than 4 hours. The mare's age, parity, length of gestation, comments regarding parturition, fetal membrane retention time, reproductive history and colostrum quality were recorded for both mare and foal, along with a 24-hour follow-up health check of the pair performed by the author.

2.2 Sample Collection

2.2.1 Umbilical Blood Collection

As soon as the foal was born and before the umbilical cord had broken, the umbilical artery, umbilical vein and urachus were identified. The umbilical arteries were identified by the presence of a pulse and relatively thicker wall compared to the thin-walled umbilical vein which bifurcates further away from the foal. Two millilitres (ml) of blood were collected from one of the umbilical arteries and from the umbilical vein approximately 10 to 20 cm distal to the foal's body wall. The blood was immediately stored into ethylenediaminetetraacetic acid (EDTA) vacutainer blood tubes (Beckton, Dickinson and Company, North Ryde, N.S.W, Australia) and stored at 4°C.

2.2.2 Fetal Membrane Examination and Collection

Immediately after the fetal membranes had been passed, they were collected and examined for any abnormalities. The vascular structure was evaluated (Chenier, 2011) and any observations were recorded. Two 40 mm x 40 mm allantochorion samples were taken from the middle third of the fetal and non-fetal horns each. Tissue samples were placed into a sterile container until further processing.

2.2.3 Endometrial Biopsy Collection

Once the fetal membranes had been examined and samples collected, the mare was restrained in the foaling yard (twitch used if necessary). An endometrial biopsy was then performed following standard procedure (Ricketts, 1975). Briefly, the mare's tail was wrapped and held out of the way,

the rectum was evacuated manually and the perineal area cleaned with dilute chlorhexidine wash. The biopsy forceps were introduced into the uterine lumen guarded by a gloved hand and once in place, the gloved lubricated arm was placed per rectum over the site where the jaw of the forceps were located. The jaws were turned in a horizontal orientation, with the gloved hand pressing gently downwards to obtain the endometrial biopsy tissue sample. The tissue sample was placed into a sterile container until further processing.

2.3 Sample Preparation

2.3.1 Umbilical Blood Samples

Approximately 0.5 ml of umbilical venous and arterial blood was aspirated from the EDTA tube and stored in RNAlater® (LifeTechnologies, Mulgrave, VIC, Australia). The remainder of the blood sample was stored at 4°C for leukocyte counts which were performed the following day. The samples of blood stored in RNAlater were repeatedly inverted over the following 24-hour period whilst stored at room temperature before storage at -20°C.

2.3.2 Fetal Membranes

One 10 mm x 10 mm sample each from the pregnant and non-pregnant horns was removed and stored in 10% buffered formalin at room temperature. A 5mm x 5 mm sample from the same tissue sample was stored in RNAlater® (LifeTechnologies, Mulgrave, VIC, Australia). The tube was inverted immediately to submerge the tissue in the storing reagent and repeatedly inverted over the following 24-hour period whilst the sample was stored at room temperature before storage at -20°C.

2.3.3 Endometrial Biopsy Tissue Samples

The endometrial biopsy sample was divided into two evenly-sized samples. One part was stored in 10% buffered formalin at room temperature. The remaining section of endometrial tissue was stored in RNAlater® (LifeTechnologies, Mulgrave, VIC, Australia). The tube was inverted immediately to submerge the tissue in the storage reagent and repeatedly inverted over the following 24-hour period whilst stored at room temperature before storage at -20°C.

2.4 Assessment of Umbilical Blood Samples

Using the blood stored in EDTA, leukocyte counts were obtained manually using the Unopette system (Beckton, Dickinson and Company, Auckland, New Zealand) following the protocol provided by the manufacturer. Briefly, after mixing the EDTA blood gently a sample of blood was collected via capillary action using the pipette provided. The outside of the pipette was cleaned with a Kimwipe (Kimberly-Clark, Mulgrave, Victoria, Australia) to remove excess blood that could affect the dilution. Pressure was applied to the reservoir and simultaneously the pipette inserted, followed by release of the chamber to draw the blood into the reservoir chamber. This was repeated two to three times to rinse the pipette of any blood. The solution was then mixed thoroughly by gentle inversion and left to stand for 10 minutes to allow lysis of erythrocytes. A haemocytometer counting chamber was used to perform the leukocyte count. The haemocytometer chamber was cleaned thoroughly with a Kim Wipe (Kimberly-Clark, Mulgrave, Victoria, Australia) and a cover slip placed over the location of the counting chambers. The pipette was withdrawn from the chamber and inverted to create a dropper. The chamber was swirled to thoroughly mix the blood/diluent mixture, the reservoir inverted and the first three to four drops of mixture discarded. The haemocytometer was charged with the mixture by gently squeezing the sides of the Unopette reservoir to fill the chamber properly (completely filled, with no air bubbles but not too much as to overfill the chambers) and then placed into a moist covered Petri dish for

10 minutes to allow leukocytes to settle in the same plane. Using a light microscope at 10X magnification the chamber was scanned to ensure even distribution of cells.
Leukocytes were counted in the four large outer squares (consisting of 16 smaller squares each). Cells were counted starting in the upper left large corner square, then right upper square, bottom right square and finally the left lower corner square. Cells that touched the upper and left lines were counted, those that touched a lower or right line were not included. In each of the large four squares, cells were counted in a 'zig-zag pattern'. The same procedure was carried out for the second counting chamber and the average number of total cells counted from both sides of the haemocytometer was used for calculations. The final cell count was reported as number of leukocytes per 10^9/L. Differential cell counts were obtained by microscopic evaluation of stained blood slides. Erythrocyte parameters were not measured.

2.5 Tissue Homogenisation and RNA Extraction

2.5.1 Tissue Homogenisation

Pregnant horn, non-pregnant horn and endometrial biopsy samples stored in RNAlater (LifeTechnologies, Mulgrave, VIC, Australia) were removed from storage at -20°C. Half of each sample was homogenised in 2 ml of Quiazol lysis reagent (Quiagen, Chadstone, VIC, Australia) using a Polytron homogeniser (IKA works, Selangor, Malaysia) and then divided into equal portions of 1 ml. The other half of the RNAlater sample (LifeTechnologies, Mulgrave, VIC, Australia) and one of the Quiazol aliquots was stored at -80°C as reserves.

2.5.2 RNA Extraction and Quantification

Automated RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Chadstone, VIC, Australia) and QIAcube (Quiagen, Chadstone, VIC, Australia) and the procedure carried out according to the manufactures' instructions. Briefly, 200 μL of chloroform was added to each lysed sample and centrifuged at 12,000 g for 15 minutes so that the upper aqueous phase could be harvested and transferred to a microcentrifuge tube. The rotor adaptors, spin columns and collection tubes were prepared and organised in the QIAcube depending on the number of samples, followed by placement of the microcentrifuge tubes in the shaker corresponding to the order of samples before the reaction bottle rack was placed in the machine. Each RNA sample pellet was resuspended in 80 μL of RNAse free water.

RNA concentration and purity were determined by spectrophotometry using a NanoDrop ND-1000 (ThermoFisher Scientific, Scoresby, VIC, Australia). Samples were selected for PCR analysis if the 260/280 ratio was greater than 1.95 and the 260/230 ratio was higher than 2.0.

2.6 cDNA Synthesis

An iScript cDNA synthesis kit (Bio-Rad, Gladesville, N.S.W, Australia) was used to complete reverse transcription of all RNA samples. The reaction was set up on ice. Four μl of iScript reaction mix and 1 μL of reverse transcriptase were added to the volume calculated from spectrophotometry to contain 1 μg of total RNA. RNase free water was then added to make a final volume of 20 μL. Sample tubes were centrifuged to ensure all reagents were combined and then incubated in a thermocycler (Bio-Rad, Gladesville, Australia) under the following conditions: 25°C for 5 minutes; 42°C for 30 minutes; 85°C for 5 minutes and a hold state at 4°C.

Once completed, samples were diluted with RNase-free water to a final concentration of 2 ng of total RNA per μL (5 μL of cDNA to 120 μL RNase-free water) and stored at -20°C for further analysis.

2.7 Polymerase Chain Reaction

2.7.1 Primer Selection

The oligonucleotide gene sequence design for IL-1, IL-6, IL-8, TNF- α, beta-actin and GAPDH were sourced from previous studies (Christoffersen et al., 2012), whereas specific primer pairs for the other three genes of interest (RPS5, RPL30 and RPL32) were designed using the NCBI primer blast website: (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastDescAd)

Where possible, primer pairs spanned at least one intron to prevent amplification of contaminating genomic DNA. This was not possible for RPL32 for the gene comprised of a single exon. All primers were commercially synthesised (GeneWorks, Hindmash, SA, Australia). Primer oligonucleotide sequences for each gene are provided in Table 1.

2.7.2 Reverse Transcription Polymerase Chain Reaction (rt-PCR)

The cDNA samples underwent reverse transcription PCR using a MyTaq™ Red Mix kit (Bioline, Eveleigh N.S.W, Australia). The reaction was set up on ice, with each 50 μL reaction prepared as follows; 10 μL 5X MyTaq buffer, 2 μL of each 10 μM primer (forward and reverse), 0.25 μL MyTaq Red DNA polymerase, 30.75 μL RNase-free water and 5 μL of cDNA product (equivalent to 10 ng of RNA). The PCR was run under the following conditions: incubation at 95°C for 1 minute; denaturation at 95°C for 15 seconds for 35 cycles; annealing at 55°C for 15 seconds for 35 cycles; extension at 72°C for 10 seconds for 35 cycles; hold at 4°C.

2.7.3 DNA Gel Electrophoresis

Gel electrophoresis was performed in a 2% agarose gel using TAE buffer (comprising of Tris base, acetic acid and EDTA) to prevent hydrolysis of the DNA. Once the gel rig was set, it was loaded into a horizontal electrophoresis system (Bio-Rad, Gladesville, N.S.W, Australia). Hyperladder II (Bioline, Eveleigh, N.S.W, Australia) was used for a DNA molecular weight marker and the gel was run at a range of 60-70V for 50-70 minutes depending on the size of the anticipated DNA fragments. When completed, the gel underwent nucleic acid staining with Gel Red (Bio-Rad, Gladesville, N.S.W, Australia) to allow visualisation of the DNA product in the gel.

2.7.4 Standard Curve Procedure for Quantitative Real Time Polymerase Chain Reaction (qPCR)

DNA purification was performed from the PCR reaction for each gene using the Wizard PCR DNA purification system (Promega, Alexandria, VIC, Australia) following the protocol provided by the manufacturer. Briefly, the DNA solution, purification buffer and resin were combined and then the DNA/resin mix manually filtered through a minicolumn system. The residue was removed by filtering 80% isopropanol through the same system. The minicolumn was then transferred to a new eppendorf tube and centrifuged. Nuclease-free water was then added to the system on a heat block at 37°C, followed by centrifugation of the tube to retrieve the DNA. Harvested purified DNA was stored at -20°C for further processing.

The ligation reaction was performed using the pGEM-T Easy Vector System kit (Promega, Alexandria, VIC, Australia) following the protocol provided by the manufacturer where 5 μL of 2x ligation buffer, 1 μL of pGEM-T Easy Vector, 1 μL of DNA ligase with 10 ng of purified PCR product were incubated overnight at 37°C.

Transformation with the ligation product (2 μL) was performed using α-select silver efficiency competent cells; efficiency ≥ 1 x 10^8 cfu/μg (Bioline, Alexandria, VIC, Australia) on ice for 20 minutes prior to a 42°C heat shock for 45 seconds. The cells were then incubated for 90 minutes

at 37°C in a shaking incubator (300 rpm). Following this, 100 μL and 50 μL aliquots were dispensed onto separate Mueller-Hinton (M. H.) agar plates containing the following: 100μg/ml ampicillin, 0.1M IPTG and 40μg/ml X-Gal before incubation overnight at 37°C.

Transformation colonies were screened based on colony colour (blue or white), which identified which contained the vector of interest. In order to prevent amplification of gene products not ligated in a plasmid, a PCR assay using a plasmid-specific M13 primer and either a gene specific forward or gene specific reverse primer was also performed. A colony from the M. H. agar plates was selected for each designated reaction, with the following reagents used: 1 μL M-13 primer, 1 μL of either forward or reverse primer (10 μM), 5 μL 10x PCR buffer, 1 μL dNTP mixture (10 μM), 1.5 μL $MgCl_2$ (50 μM), 0.2 μL Platinum Taq DNA polymerase and nuclease-free water to make a final volume of 50 μL. The following cycling protocol was selected: incubation at 94°C for 60 seconds; then for 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; hold at 4°C.

Several transformed colonies were selected and inoculated into M. H. broth which also contained ampicillin (50 μg/ml) and incubated at 37°C overnight in a shaking incubator (250 rpm). Purification of the plasmid was carried out using Pure Yield™ Plasmid Miniprep System kit (Promega, Alexandria, VIC, Australia) following the protocol provided by the manufacturer.

Correct identity of the plasmid insert within the transformed colonies was additionally confirmed through DNA digestion: 1 μg of purified plasmid DNA was digested with 1 μL of the restriction endonuclease EcoRI. Each reaction contained the following: 5 μl of 10X EcoRI buffer and water to a final volume of 50 μl, incubated in a water bath overnight at 37°C. Gel electrophoresis was performed in a 2% agarose gel using TAE buffer at 80V for 60 minutes. The size of the insert was compared to the DNA molecular weight marker and estimated accordingly to confirm its presence and identity.

The plasmid underwent sequencing at the University of Melbourne's Applied Genetic Diagnostics Laboratory (Department of Pathology, Melbourne, VIC, Australia). According to the plasmid DNA and the plasmid size, a dilution series in copy numbers of the target DNA sequence was provided. Standard curves ranging from 3x10 to $3x10^7$ copies per 5 μL were created for each individual gene within its plasmid vector, and stored at -20°C.

2.7.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Non-pregnant horn, pregnant horn and endometrial biopsy samples all underwent RT-qPCR, with all three samples from each mare analysed together in the same run. The RT-qPCR assay was performed using a Roto-Gene Q PCR machine (Qiagen, Chadstone, VIC, Australia). For each reaction the following reagents were included: 10 μL iTaq Universal SYBR Green Supermix (Bio-Rad, Gladesville, N.S.W, Australia), 1 μL forward primer (10 μM) and 1 μL reverse primer (10 μM), 3 μl RNase-free water and 5 μl of cDNA to make up a final reaction volume of 20 μL. For each machine run, a no-template control (5 μl RNase-free water) and a sequence of serially diluted DNA templates (standard curves) for each gene of interest were included and all samples were assayed in triplicate. The following cycling conditions were performed: activation at 95°C for 30 seconds; 40 cycles of denaturing at 95°C for 5 seconds and annealing at 60°C for 30 seconds. Melt curve analysis was performed between 65-95°C in 0.5°C increments to analyse each amplicon.

2.8 Immunohistochemistry

Formalin-fixed fetal membrane and endometrial tissues from foaling and control mares, as well as liver, kidney, lung and spleen samples from one control mare were trimmed into individual 5 mm samples and placed into processing cassettes, then re-submerged in formalin.

2.8.1 Paraffin Wax Embedding and Section Cutting

Following fixation, paraffin wax embedding of tissue samples was performed (Goldstein and Watkins, 2008). Briefly, using an automated system the tissue samples were dehydrated through a sequence of ethanol dilutions and then a series of xylene dilutions before being submerged in molten paraffin wax at 58°C and allowed to set in a fresh bath of paraffin. Paraffin-embedded tissue blocks were chilled on ice to facilitate cutting of thin sections. Blocks were trimmed to expose the tissue surface to the level where an appropriate section of tissue could be cut. Sections of 4 to 5 μm thick were cut with a microtome and section ribbons were transferred to a water bath (40-45°C) and separated. Tissue sections were then mounted onto adhesive-coated Polysine microscope slides (ThermoFisher Scientific, Scoresby, VIC, Australia) and dried overnight at 37°C.

2.8.2 Deparaffinisation

Slides containing the tissue section were placed in an incubator for either 5 minutes at 60°C or overnight at 37°C in preparation for de-waxing. Slides were then placed in xylene for 5 minutes and the solution changed three times at 5-minute intervals followed by placement in 1:1 xylene:100% ethanol solution for 5 minutes. Finally, slides were placed in 100% ethanol for 5 minutes for three changes for rehydration, then rinsed in tap water and transferred to distilled water to prevent drying.

2.8.3 Antigen Retrieval

The antigen retrieval technique used in this study involved high-temperature heating of tissue sections to enhance the immunohistochemical staining and antigen exposure. A sodium citrate buffer for antigen retrieval was made by adding 2.94 grams of sodium citrate (10 μM) to 1000 ml of distilled water. The pH of the buffer solution was adjusted by adding 200-600 μL aliquots of hydrogen chloride until a pH of 6.0 was reached. Finally, 0.5 ml of 0.05% Tween 20 detergent (Chem-supply, Gillman, S.A, Australia) was added.

The antigen retrieval buffer was heated for 10 minutes until it began to boil in a microwave-safe dish using the medium-high setting in a domestic microwave. Slides were then added to the dish for a further 15 minutes, taking care to ensure that the slides remained completely submerged and were not touching each other. Following the antigen retrieval process, slides were rinsed in cold tap water for 10 minutes, then placed in phosphate buffered saline (PBS) (100 ml of 10x PBS to 1000 ml distilled water). The edges of the slides were dried with paper towel to allow tissue sections to be encircled with a wax pen. The slides were then placed in a humidified chamber to prevent drying out.

2.8.4 Quenching

Endogenous peroxidases in the tissue sections were blocked using 3% hydrogen peroxide. Sufficient hydrogen peroxide was applied to each slide to cover the tissue section completely and left for 5 minutes in the humidified chamber. The slides were then rinsed in PBS buffer for 5 minutes.

2.8.5 Blocking

Slides were rinsed in fresh PBS buffer to remove any peroxidase residue and placed within the humidified chamber. To prevent non-specific antibody binding, 100 μL of 50% fetal calf serum (FCS) was applied to each slide to cover the tissue section completely followed by incubation in the closed humidified chamber for 60 minutes. Once finished, excess serum was removed off the slides by gently tapping the edge of the slide on absorbent paper.

2.8.6 Application of the primary antibody

Two primary antibodies were selected. A monoclonal mouse anti-equine IL-1β antibody (R&D Systems, Mineappolis, U.S.A) and a polyclonal goat anti-equine IL-6 antibody (R&D Systems, Mineappolis, U.S.A). The primary antibody was diluted in 1% BSA to a sequential series of concentrations during optimisation of this method, applied to each slide to cover the tissue section completely and incubated overnight at 4°C. The slides then were rinsed in PBS buffer three times for 5 minutes each. For IL-1, concentrations 1/20, 1/60 and 1/100 were used. For IL-6, dilutions of 1/20, 1/40, 1/80, 1/100 and 1/200 were used. 1% BSA was used as negative control.

2.8.7 Application of the secondary antibody

For IL-1β, a DAKO goat anti-mouse secondary polyclonal antibody (Agilent Technologies, Mulgrave, VIC, Australia) was used. For IL-6, a DAKO rabbit anti-goat polyclonal antibody (Agilent Technologies, Mulgrave, VIC, Australia) was used. The secondary antibody was applied to each slide to cover the tissue section completely and incubated for 30 minutes in a humidified chamber before being rinsed in PBS three times for 5 minutes each. For antigen detection, a horseradish peroxidase (HRP) conjugate VECTOR® NovaRED™ (Vector Laboratories, Burlingame, California, U.S.A) was used following the protocol provided by the manufacturer. Briefly, immediately before use, the substrate solution was prepared by adding three drops of Reagent 1, two drops of Reagent 2, two drops of Reagent 3 and finally two drops of Hydrogen Peroxide Solution to 5 ml of distilled water, mixing in between. Slides were incubated at room temperature and evaluated under a light microscope at 1-minute intervals to determine optimum staining time. Once completed, slides were rinsed with distilled water.

2.8.8 Counterstaining

Slides were briefly submerged in haematoxylin, rinsed in clean water then submerged 15 times in lithium carbonate, washed again in clean water before being taken through the dehydration process as per description in step 2. 9. 2 before a coverslip was applied with DPX mounting medium (Merk, Bayswater, VIC, Australia).

2.9 Selection of control mares

Four healthy full-term pre-partum mares without signs of impending labour (e.g. no distension of the udder, waxing or secretions from the mammary gland) were sourced from a local abattoir to serve as control animals. Mares were also screened for any gross signs of disease or poor health before slaughter. Immediately following slaughter, once the fetus was confirmed to be full term (based on overall physical development, appearance of hair coat, lack of flexural limb deformities and absence of domed forehead appearance) and there was no gross evidence of disease of either the dam, fetus or reproductive tissues, endometrial and fetal membrane samples were collected and prepared as per sections 2.3.2 and 2.3.3.

2.10 Statistical analysis

Collected data for peri-partum foaling information was recorded and transcribed into a commercial spreadsheet (Microsoft Excel 2013, Microsoft Corporation, Redmond, Washington, USA). Descriptive statistics were calculated for fetal membrane retention time and equine neonatal umbilical vessel leukocyte counts using Microsoft Excel Version 16.12. A two-sided Student's t test implemented in R (R Core Team, 2017; R Foundation for Statistical Computing, Vienna, Austria) was used to test the equality of the arterial and venous white blood cell counts.

Statistical analysis for each of the inflammatory cytokines within each group (foaling and control) and tissue type (endometrium and fetal membrane) was implemented in R (R Core Team, 2017;

R Foundation for Statistical Computing, Vienna, Austria). Differences in log transformed beta-actin, GAPDH, RPL30 and RPS5 cytokine gene concentrations in fetal and non-fetal horn (considered as a single group) and the endometrium for foaling and control mares were assessed using analysis of variance (ANOVA). Pairwise comparisons were carried out using Tukey's Honestly Significant Difference Test (Tukey, 1949) with P values adjusted to account for the multiple comparisons. An ANOVA and Tukey Honestly Significant Difference test was performed for the comparisons of pro-inflammatory cytokines IL-1, IL-6 and IL-8.

Table 1. Primer oligonucleotide sequences for cytokine amplification using reverse transcriptase PCR

Primer	Sequence 5'-3'	Sequence 3'-5'	Insert size (bp)	Reference/Accession Number
IL-1β	CAG TCT TCA GTG CTC AGG TTT CTG	CAT TGC CGC TGC AGT AAG T	84	Christoffersen 2012
IL-6	GGA TGC TTC CAA TCT GGG TTC AAT	TCC GAA AGA CCA GTG ATT TT	65	Christoffersen 2012
IL-8/CXCL8	CTT TCT GCA GCT CTG TGT GAA G	GCA GAG CTC AGC TCC GTT GAC	189	Christoffersen 2012
TNF-α	GGC CCA GAC ACT CAG ATC AT	TTG GGG GTT TGC TAC AAC AT	73	Christoffersen 2012
beta-actin	CGT GGG CCG CCC TAG GCA CCA	TTG GCC TTA GGG TTC AGG GGG G	243	Christoffersen 2012
GAPDH	GGG TGG AGC CAA AAG GGT CAT CAT	AGC TTT CTC CAG GCG GCA GGT CAG	418	Christoffersen 2012
RPL30	GGC CGT CCC GCA CCT AAG	ATG ACC AGT TTC GCT TTG CCT	166	XM_001491150.4
RPL32	TGG TCC ACA ATG TCA AGG AGC	TCG TCT ATT CGT TTT CTT CGC TGC	180	XM_001500029.4
RPS5	TGC CAT CAT CAA CAG TGG TCC	AGG TTT ATT GGG GCT GTG GTC G	301	XM_001495360

Chapter 3- Results

3.1 Descriptive Statistics Describing the Study Population

The average (± standard deviation) age of mares in this study was 9 ± 3.4 years. The average gestation length of mares was 353 ± 10 days. During this study period, 20 foals (60%) born were fillies and 13 (40%) were colts.

The average (± standard deviation) fetal membrane retention time was 92 ± 130 minutes. Median fetal membrane retention time was 47 minutes (25%: 25, 75%: 55, IQR 30, min: 5, max: 480). The percentage of mares that passed their fetal membranes per hour after foaling is shown in Figure 1. Overall, 85% of mares (28/33) passed their membranes within 2 hours of foaling.

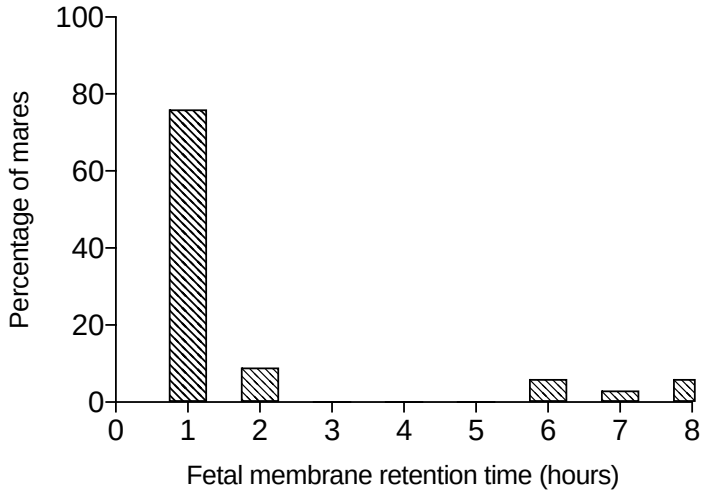


Figure 1. Percentage distribution of fetal membrane retention time (hours) after foaling for 33 spontaneous foalings on a single Thoroughbred stud farm in the Waikato region of New Zealand during the 2013 breeding season. Time categories include recorded intervals in the preceding hour (e.g. 1 hour includes all times < 1 hour).

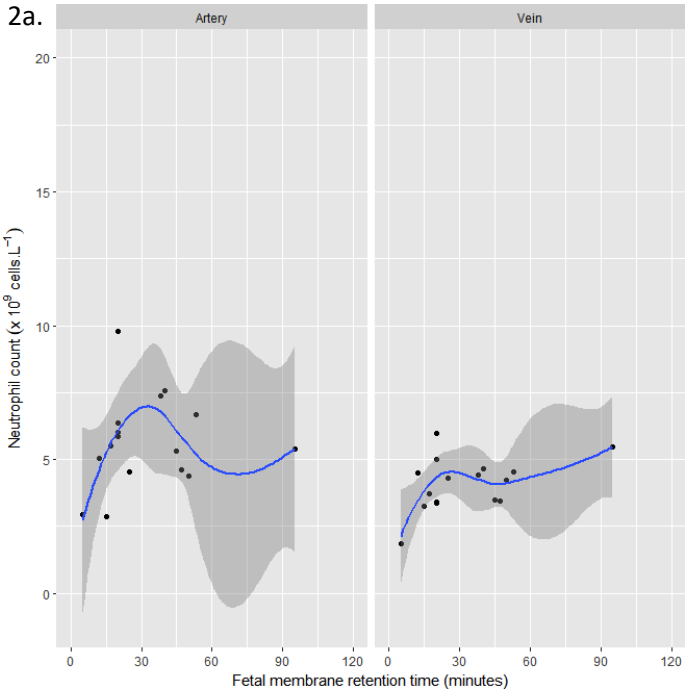
3.2 Leukocyte counts for umbilical artery and vein blood samples

Total nucleated cell count, neutrophil count and lymphocyte count from blood samples collected from the umbilical artery and vein of 16 foalings are shown in Table 1. There was a significantly higher number of leukocytes in the umbilical artery compared to the umbilical vein ($p < 0.001$). The numbers of neutrophils and lymphocytes was also significantly higher in the umbilical artery than in the umbilical vein ($p < 0.001$).

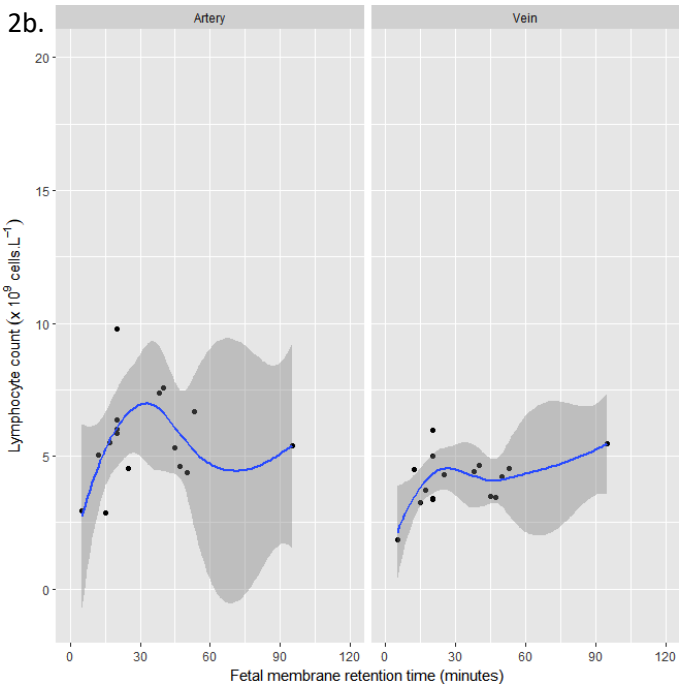
Table 2. Total nucleated cell counts, neutrophil and lymphocyte cell counts for equine neonatal umbilical artery and vein (mean ± SD). TNCC = total nucleated cell count.

Parameter	Umbilical artery ($x10^9$ cells/L)	%	Umbilical vein ($x10^9$ cells/L)	%	Difference ($x10^9$ cells/L)	P (paired t-test)	95% CI
Neutrophil	5.64 ± 1.68	62	4.10 ± 0.96	67	1.54	< 0.001	0.85-2.22
Lymphocyte	3.08 ± 1.03	34	1.83 ± 0.48	29	1.25	< 0.001	0.69-1.86
TNCC	9.04 ± 2.37		6.11 ± 1.21		2.93	< 0.001	1.95-3.90

2a.


Artery
Vein
Neutrophil count (x 10^9 cells.L^-1)
20
15
10
5
0
0
30
60
90
120
Fetal membrane retention time (minutes)

2b.


Artery
Vein
Lymphocyte count (x 10^9 cells.L^-1)
20
15
10
5
0
0
30
60
90
120
Fetal membrane retention time (minutes)

2c.

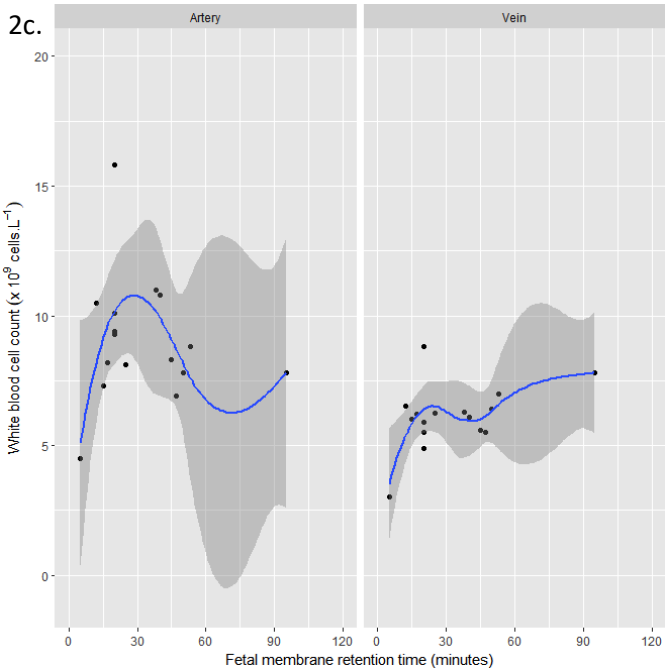


Figure 2. Scatterplots for a) neutrophil, b) lymphocyte and c) total white cell count as a function of fetal membrane retention time (mins) for each mare.

Arterial and venous white blood cell counts (total nucleated cell count, neutrophil count and lymphocyte count) for each mare were plotted as a function of fetal membrane retention time (in minutes), as shown in Figure 2. For every one unit (1 x 10^9/L) increase in difference between the arterial and venous total nucleated cell count fetal membrane retention time decreased by 5.2 (95% CI 1.1 to 11) minutes (p = 0.098). For each one unit increase in difference between arterial and venous neutrophil cell count, membrane retention decreased by 3 (95% CI 6.8-13) minutes (p = 0.52). For each one unit increase in difference between arterial and venous lymphocyte cell count, membrane retention time decreased by 10 (95% CI 0.3-20) minutes (p = 0.0559).

3.3 Housekeeping gene expression in Endometrial and Fetal Membrane Tissue Samples in Foaling and Control mares

Four housekeeping genes (HKG) were evaluated in this study; beta-actin, GAPDH, RPL50 and RPS5.

Beta-actin mRNA was constitutively expressed within the same tissue type (endometrium or fetal membranes) between the two groups (foaling vs control mares) (Table 2). Beta-actin mRNA expression between endometrium and fetal membranes within each group was, however, significantly different.

For the remaining three housekeeping genes evaluated, the level of gene expression between the two groups varied depending on the tissue type (Table 2). Whilst GAPDH mRNA expression was significantly different between endometrium of foaling and control mares (p <0.001) there was no significant difference in mRNA expression within fetal membrane tissue of foaling and control mares (p = 0.402). The mRNA expression of RPL30 between endometrium of foaling and control mares was significant (p = 0.001) but was not significant for fetal membrane tissue between the two groups (p = 0.933). RPS5 showed a similar pattern to GAPDH and RPL30 regarding expression within tissue type between groups; RPS5 mRNA expression was significantly different within endometrium tissue (p = 0.046) but not in fetal membrane tissue (p = 0.952).

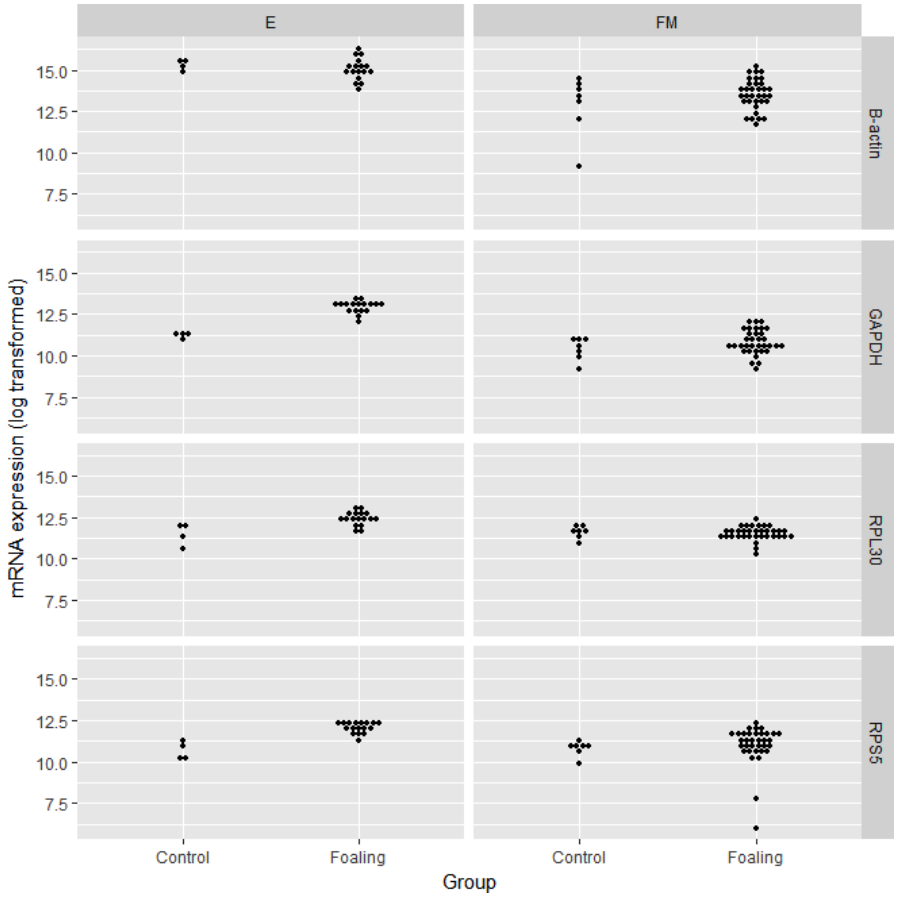


Figure 3. Dot plot for endometrium and fetal membrane mRNA expression of housekeeping genes beta-actin, GAPDH, RPL30 and RPS5 in foaling and control mares. Log-transformation was performed due to the highly-skewed data.

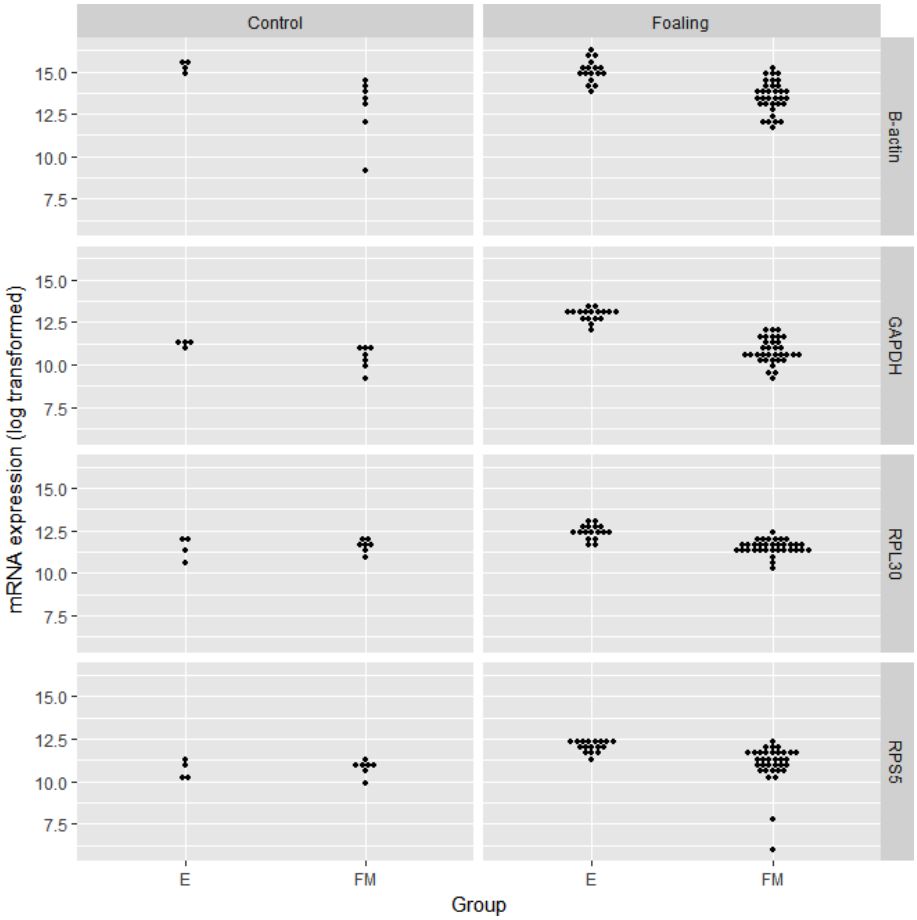


Figure 4. Log transformed mRNA expression of housekeeping genes beta-actin, GAPDH, RPL30 and RPS5 for foaling and control mares in endometrial and fetal membrane tissues. Log-transformation was performed due to the highly-skewed data.

The mRNA expression of beta-actin was significantly different between endometrium and fetal membranes for both the control and foaling group. In contrast, whilst GAPDH, RPL30 and RPS5 mRNA was constitutively expressed in the control group for fetal membrane and endometrial tissue, the mRNA expression of these genes in foaling mares was significantly different between tissue types.

Table 3. Difference in mRNA expression for housekeeping genes beta-actin, GAPDH, RPL30 and RPS5 within groups (foaling vs control) and between tissues (endometrium vs fetal membranes). The asterisks denote the categories for which beta-actin was not significantly different between groups within a tissue type. P values highlighted in bold denotes non-significant difference between categories. FM = fetal membrane; E = endometrium.

HKG	Comparison	Difference	Lower	Upper	Adjusted P value
Beta-actin					
	FM: Control-E: Control	-2.358	-3.974	-0.742	0.002
	E: Foaling-E: Control	-0.211	-1.644	1.222	0.980*
	FM: Foaling-FM: Control	0.654	-0.416	1.724	0.377*
	FM: Foaling-E: Foaling	-1.493	-2.259	-0.728	0.000
GAPDH					
	FM: Control-E: Control	-0.835	-1.842	0.173	0.138
	E: Foaling-E: Control	1.649	0.755	2.542	< 0.001
	FM: Foaling-FM: Control	0.397	-0.271	1.064	0.402
	FM: Foaling-E: Foaling	-2.087	-2.564	-1.609	< 0.001
RPL30					
	FM: Control-E: Control	0.136	-0.558	0.831	0.954
	E: Foaling-E: Control	0.930	0.314	1.546	0.001
	FM: Foaling-FM: Control	-0.104	-0.564	0.356	0.933
	FM: Foaling-E: Foaling	-0.898	-1.227	-0.569	< 0.001
RPS5					
	FM: Control-E: Control	0.075	-1.454	1.603	0.999
	E: Foaling-E: Control	1.371	0.016	2.727	0.046
	FM: Foaling-FM: Control	0.202	-0.810	1.215	0.952
	FM: Foaling-E: Foaling	-1.094	-1.819	-0.370	0.001

3.4 Combination of Fetal and Non-Fetal horn cytokine gene expression due to non-significance between groups

After controlling for the effect of group (control and foaling) there was no significant difference in the log mRNA concentration for IL-1β, IL-6 or IL-8 measured in fetal and non-fetal horn tissues ($p = 0.738$). Therefore, fetal and non-fetal horn tissues were combined to create one group - fetal membranes (FM) - for further analysis of cytokine gene expression.

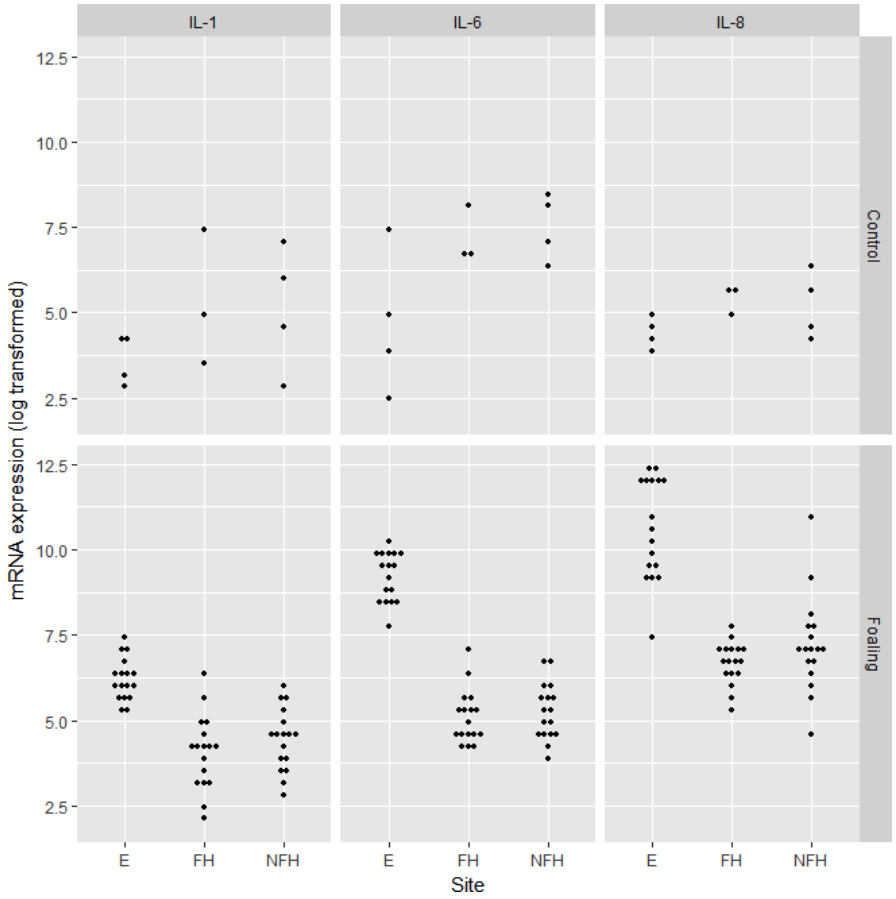


Figure 5. Dot plots showing log transformed mRNA expression of IL-1β, IL-6 and IL-8 for endometrium, fetal horn and non-fetal horn for foaling and control mares. Log-transformation was performed due to the highly-skewed data.

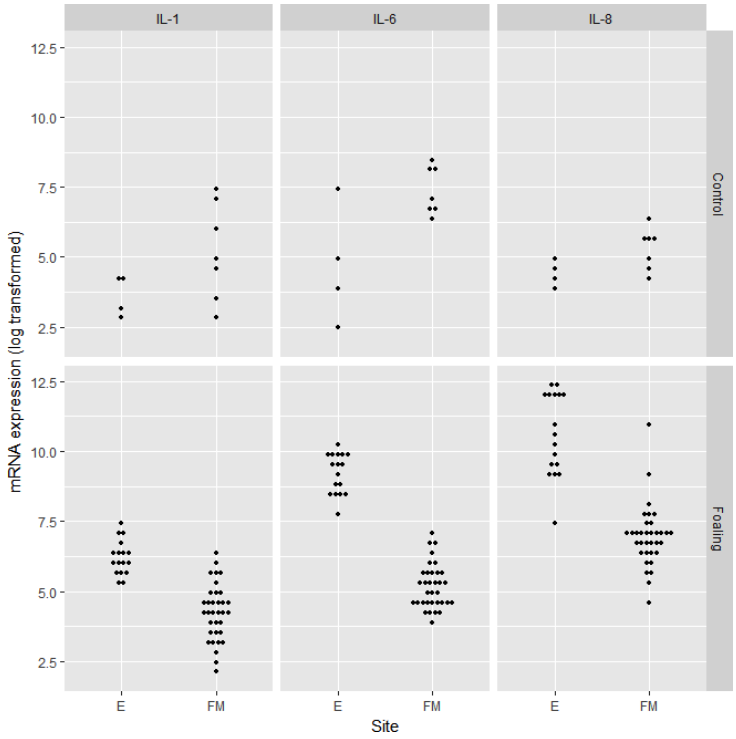


Figure 6. Dot plots showing the log transformed mRNA expression of IL-1β, IL-6 and IL-8 for endometrium and fetal membranes for foaling and control mares. Log-transformation was performed due to the highly-skewed data.

3.5 Comparison of Cytokine Gene Expression within tissue groups between Foaling and Control Mares

Quantitative PCR data measuring expression of genes coding for IL-1β, IL-6 and IL-8 were available for 20 foaling and 4 control mares.

Table 4. mRNA concentration in copies per 10ng for cytokines IL-1β, IL-6 and IL-8 for fetal membrane and endometrial tissue from each group. F = foaling; C = control.

mRNA concentration (copies per 10ng)			
	IL-1	IL-6	IL-8
Endometrium (F)	657	10045	66313
Fetal membranes (F)	248	758	3362
Endometrium (C)	42	449	79
Fetal membranes (C)	558	2194	242

Based on measurement of IL-8 mRNA, expression of IL-8 in endometrial tissue samples from foaling mares was more than 839 times greater than endometrial samples from control mares. Endometrial expression of IL-6 and IL-1β in foaling mares was 22 times and 15 times greater, respectively, than in control mares (Table 4). Overall, the difference in level of expression for all three cytokines in the endometrium was significantly greater for foaling compared to control mares (Table 4).

Table 5. Difference in mRNA expression for individual cytokines within groups (foaling vs control) and between tissue types (endometrium vs fetal membranes). FM = fetal membrane; E = endometrium.

Comparison	Site	Difference	Lower	Upper	Adjusted p-value
IL-1: Foaling vs IL-1: Control	E	2.59	0.83	4.34	< 0.001
IL-6: Foaling vs IL-6: Control	E	4.52	2.76	6.27	< 0.001
IL-8: Foaling vs IL-8: Control	E	6.31	4.56	8.07	< 0.001
IL-1: Foaling vs IL-1: Control	FM	-1.02	-2.24	0.20	0.16
IL-6: Foaling vs IL-6: Control	FM	-2.20	-3.42	-0.98	< 0.001
IL-8: Foaling vs IL-8: Control	FM	1.66	0.44	2.88	< 0.001
	Group				
IL-1:FM vs IL-1: E	Control	1.70	-0.66	4.05	0.27
IL-6:FM vs IL-6: E	Control	2.63	0.27	4.98	0.02
IL-8:FM vs IL-8: E	Control	0.99	-1.37	3.34	0.79
IL-1:FM vs IL-1: E	Foaling	-1.91	-2.76	-1.05	< 0.001
IL-6:FM vs IL-6: E	Foaling	-4.09	-4.94	-3.23	< 0.001
IL-8:FM vs IL-8: E	Foaling	-3.67	-4.52	-2.82	< 0.001

The differences between foaling and control mares in fetal membrane gene expression was not as large as for endometrial samples. Expression of IL-8 in the fetal membranes of foaling mares was 14 times that of control mares (compared to 839 times for endometrium). Fetal membrane expression of IL-6 was almost three times greater in control mares than foaling mares (compared

to the endometrium, where IL-6 expression was higher in foaling mares). Interleukin-1 expression in fetal membranes of control mares was double that of foaling mares (Table 4).

Genetic expression of IL-1, IL-6 and IL-8 was significantly greater in the endometrium of foaling mares compared to control mares ($p < 0.001$). The mRNA expression of IL-8 was significantly greater in fetal membranes of foaling mares ($p < 0.001$) and IL-6 concentration significantly lower ($p< 0.001$) in fetal membranes of foaling mares compared to control mares (Table 5). Whilst the mRNA expression for IL-1 was lower in foaling mare fetal membrane tissue compared to control mares, the difference was not significant (Table 5).

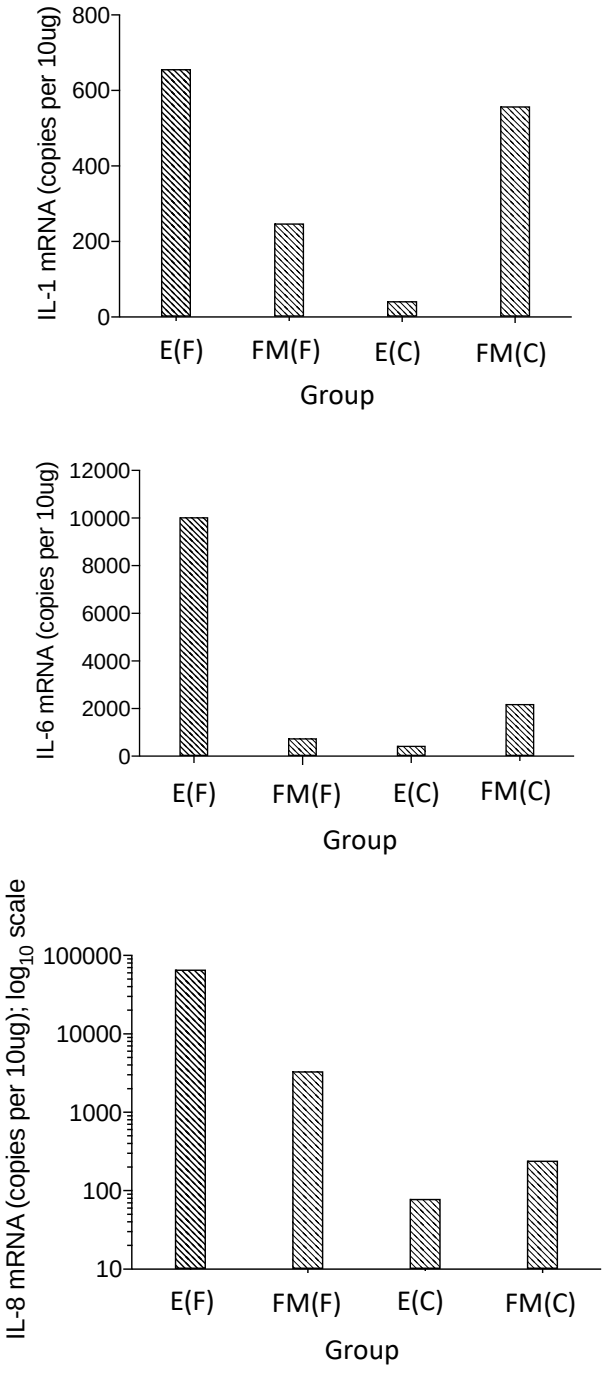


Figure 7. mRNA copy numbers of cytokines IL-1, IL-6 and IL-8 from endometrial and fetal membrane tissue in foaling and control mares.

3.6 Comparison of Cytokine Gene Expression between endometrium and fetal membranes in Foaling and Control mares

For foaling mares, expression of IL-1, IL-6 and IL-8 was significantly greater in the endometrium than in fetal membranes (Table 4). In order of magnitude, the greatest difference was for IL-8, then IL-6 followed by IL-1 mRNA expression (Table 3).

For control mares, mRNA expression of IL-1, IL-6 and IL-8 was higher in the fetal membranes compared to the endometrium. The difference in IL-6 mRNA expression between fetal membranes and endometrial tissue in control mares was significant ($p = 0.02$), whereas the difference in IL-1 and IL-8 mRNA expression between the two tissue types was not (Table 5).

3.7 Immunohistochemistry

Optimisation of an immunohistochemistry protocol using IL-1 and IL-6 primary antibodies was performed. Results for immunohistochemistry are not available due to being unable to produce sufficient staining to allow interpretation despite trouble shooting the protocol. This included trying numerous serial primary antibody dilutions for each antibody. Two different incubating conditions for the primary antibodies were also used; incubation for 1 hour at room temperature in a humidified chamber as well as 16 hours at 4°C, also in a humidified chamber. Kidney, lung, spleen and liver control tissues from one control mare were also used for immunohistochemistry, but with no success.

Chapter 4: General Discussion

4.1 The importance of understanding normal fetal membrane detachment

Until recently, the mechanism of normal placental detachment in horses had not yet undergone thorough investigation and as such remained poorly understood, with the majority of studies focused largely on the pathogenesis and treatment of retained fetal membranes (RFM) and placentitis (Rapacz-Leonard et al., 2015, Sevinga et al., 2002b, Ishii et al., 1999, Rapacz-Leonard et al., 2015, Leblanc et al., 2012). Two recent studies that investigated the role of inflammation within the equine uteroplacental unit were able to demonstrate the presence of both pro-inflammatory cytokines and MMPs but neither of these studies targeted the mechanisms of placental detachment directly (Palm et al., 2013, Oddsdóttira et al., 2011). Here in the present study, however, this was achieved; the role that pro-inflammatory mediators and leukocytes play in fetal membrane release from the equine endometrium under normal conditions has been uncovered. This evidence provides further avenues of investigation that can be explored to help clarify the physiology behind placental detachment in mares, and beyond that how abnormal placental detachment occurs.

4.2 The implications of abnormal fetal membrane detachment

The reason for prolonged fetal membrane retention in mares after foaling has been addressed from a mechanical, functional and immunological level. Unfortunately, these studies have predominantly been performed in heavy draft breeds, which reportedly have a higher incidence of RFM, rather than light breed mares (Rapacz-Leonard et al., 2015, Pazdzior et al., 2012, Rapacz-Leonard et al., 2015). It could be suspected that this high occurrence of retained fetal membranes in heavy or draft breed mares could have an immunological basis due a loss of genetic diversity (for example loss of paternal MHC 1 antigen) as a consequence of breeding selection (Sevinga et al., 2004). The loss of a proper maternal immune response to fetal allergens at parturition could result in a dampened immune response within the fetoplacental unit at parturition resulting in prolonged fetal membrane retention time. As such, information gained from these studies may not be suitable when addressing reasons for RFM in light breed mares, which make up a significant component of the equine breeding industry. The intention of this research was to ensure the mechanisms behind placental detachment in light breed mares were understood in order to provide a sound basis to investigate mechanisms of abnormal placental detachment, with Thoroughbreds considered an appropriate model. It is fully appreciated that the present study has not been able to expose all of this information, but it has still demonstrated that fetal membrane detachment is associated with an active pro-inflammatory immune response, characterised by an increase in pro-inflammatory cytokine expression and leukocyte movement to the fetal membranes at foaling. What remains to be investigated is the role of the innate immune response in cases of abnormal fetal detachment in mares. In this study, due to a low sample size of RFM and placentitis cases, analysis of these cases was not performed. In future studies, however, further investigation of these pathways will allow development of evidence-based prevention and treatment strategies, which will improve the overall health and welfare of mares and foals.

4.3 Establishing normal fetal membrane retention time in the mare

What is considered normal fetal membrane retention time in the mare, varies significantly within the literature. Some authors suggest as little as 30 minutes but it can range up to 6-12 hours (Sevinga et al., 2002a, Frazer, 2003, Frazer, 2011)., Currently, there is no evidence-based consensus as to what time after foaling therapeutic intervention for RFM should be instituted, nor

which treatment. This, in part, is likely due to disagreement over when retention time is considered abnormal. Delayed onset of treatment may reduce the chance of recovery from associated co-morbidities as well as potentially impacting reproductive performance.

A longitudinal study by the author examined fetal membrane retention time in a large group of Thoroughbred mares on one stud farm (Rosales et al., 2017). For this group of mares, 95% had expelled their membranes within 4 hours and almost 77% expelled their membranes within the first hour of foaling, with a median fetal membrane retention time of 40 minutes. Another study of Thoroughbred mares reported very similar results, with 96% of mares passing their membranes within 4 hours and a median fetal membrane retention time of 30 minutes (Ishii et al., 2013). The median fetal membrane retention time for mares in the present study was 47 minutes. Approximately 85% of mares expelled their fetal membranes in less than 2 hours post-foaling, as shown in Figure 1. Whilst membrane retention time in this study was slightly longer compared to the two previously mentioned studies, it was still less than one hour, despite a smaller sample size in comparison to the other studies. The data gathered from the larger studies were from different hemispheres, and therefore the two study groups likely vary in terms of genetics, management and environment. Despite this they still reported similar patterns regarding fetal membrane retention time. At this time, based on the present study's findings, it should be considered that abnormal fetal membrane retention time is beyond 4 hours and that the majority of mares will be expected to pass their membranes in under an hour of foaling.

4.4 Equine neonatal umbilical leukocyte concentration gradient and migration to fetal membranes

During gestation, the equine fetal and maternal circulation are in intimate apposition at the level of the chorioallantoic microcotyledon to allow exchange of gases, nutrients and waste products (Allen and Stewart, 2001). The umbilical vein brings nutrients and oxygenated blood from the placenta to the fetus whereas the umbilical arteries transport oxygen-depleted waste-carrying blood away from the fetus (Wang and Zhao, 2010). The present study demonstrated a significant difference in concentration of total nucleated cells between the equine neonatal umbilical artery and vein at parturition (Table 2). The significant difference in concentration of leukocytes between the umbilical artery and vein was due to a higher concentration of both neutrophils and lymphocytes leaving the fetus via the artery than returning via the umbilical vein. It is hypothesized that the unaccounted leukocytes were most likely deposited within the fetal membranes. Previous research has also shown that there is a concentration gradient of leukocytes between the equine neonatal umbilical artery and vein suggesting entrapment of leukocytes within the fetal membranes (Volkmann et al., 2002). No significant relationship between the concentration gradient of leukocytes between vessels and the time of fetal membrane expulsion was found by those investigators.

Similarly, this study was unable to demonstrate a significant correlation between fetal membrane retention time and the leukocyte concentration gradient. However, the results to do show a trend for an increase in umbilical leukocyte difference to decreased fetal membrane retention time, albeit by a short time. Both studies utilise a relatively small sample size, highlighting the importance of using a larger population to properly determine the finding's significance. Regardless, this result adds support to the theory that a larger concentration gradient of leukocytes may influence the duration of fetal membrane retention time. This could be postulated to be related to a strong local innate inflammatory response, and concurrent release of essential pro-inflammatory substances (such as cytokines and MMPs) leading to a more rapid detachment and subsequent expulsion of membranes after foaling.

It is evident in humans that spontaneous parturition requires an orchestrated response by the innate immune system and that leukocytes are critical to this immune response due to their

relationship with pro-inflammatory cytokines (Bowen et al., 2002, Norman et al., 2007, Keelan et al., 2003b, Young et al., 2002, Keelan et al., 2003a). Degranulation of neutrophils within tissues leads to secretion of other essential pro-inflammatory substances, including MMPs, which will ultimately lead to extracellular matrix degradation, such as the detachment between maternal endometrium and allantochorion at parturition (Lacy, 2006).

Of the chemokines, IL-8 is perhaps the most well-recognised due to its ability to recruit neutrophils to tissues (Turner et al., 2014). In cattle there is an association between retained fetal membranes and inhibited chemotaxis of neutrophils to the cotyledon as well as concurrent decreased neutrophil function (Kimura et al., 2002). The relationship between exogenous corticosteroid administration and a dampened immune response, characterised by reduced migration of leukocytes to the fetal membranes in cows has also been demonstrated (Kimura et al., 2002, Benedictus et al., 2011b). In the present study, mRNA expression of IL-8 was found to be significantly higher in foaling mare endometrium and fetal membranes compared with control mares. Therefore, the entrapment of leukocytes would appear to correlate with the increase in chemokine expression within these tissues at time of foaling. What remains to be determined, is if cytokine/chemokine production is maternal or fetal in origin. Unfortunately, due to the unreliability of housekeeping gene expression in this study, we were unable to determine in this study whether maternal or fetal cytokine/chemokine production is responsible for the attraction of leukocytes to the fetal membranes at parturition.

Localisation of leukocytes within the fetal membranes would further strengthen the argument that the separation of endometrium from fetal membranes is an inflammatory process. Several studies have attempted to localise leukocytes using various methods in the fetal membranes but without success (Volkmann et al., 2002, Norman et al., 2007, Keelan et al., 2003b, Keelan et al., 2003a). The reason for this is postulated to be due to rapid neutrophil degranulation following tissue extravasation. An inflammatory response is a co-ordinated step-wise process, and neutrophil degranulation ensures continuation of the response through activation of downstream pro-inflammatory mediators. In the case of placental detachment, neutrophil-derived pro-inflammatory substances, including MMPs, would lead to degradation of the extracellular matrix attachment between fetal membrane and endometrium. Failed activation along this pathway had been proposed as the reason why adhesion of the allantochorion to the endometrium occurred in a large proportion of mares from one study with retained fetal membranes compared to mares who did not retain their membranes (Rapacz-Leonard et al., 2015)

Importantly, timing of collection could influence whether leukocytes are present in tissues or not. In this study, the time difference between equine neonatal umbilical cord blood collection and tissue collection ranged from 20 up to 95 minutes due to the varying times of fetal membrane release. Even though localisation of neutrophils in endometrial and fetal membrane tissue was not undertaken in this study, it is understood that degranulation of these leukocytes would have likely occurred by the time tissues samples were taken, leading to steps such as ECM degradation and subsequent membrane release. This highlights the opportunity to further investigate the role of leukocytes in placental detachment, by attempting to localise these cells in reproductive tissues of mares who experience abnormal fetal membrane detachment compared to those who do not. Perhaps failure of neutrophil degranulation is one component for failure of the membranes to detach from the endometrium.

4.5 The problem with housekeeping genes

Housekeeping, or reference genes, maintain basic cellular function and theoretically are expected to be constitutively expressed at a constant rate irrespective of cell conditions (Eisenberg and Levanon, 2013). They are therefore considered valuable in molecular biology when assessing

mRNA expression, as they standardise against variations in quantitative expression of target genes. Whilst it has been generally assumed that housekeeping genes are ubiquitously expressed in tissues, and therefore considered 'controls' there is evidence to suggest housekeeping genes are not to be taken for granted, and selection may need to be more focused based on cell types and physiological states (Radonić et al., 2004).

The purpose of this study was to determine if mRNA expression of the three pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were upregulated in reproductive tissues of foaling mares compared to controls to confirm the hypothesis that placental detachment is an inflammatory process. The selected housekeeping genes were chosen based on previous research in equine reproductive tissues (Klein et al., 2011, Rapacz-Leonard et al., 2015, Marth et al., 2016, Palm et al., 2013). However, whilst these studies found that these housekeeping genes were stably expressed within the equine endometrium, none of them mention assessment for suitability in fetal membrane tissues. The present study is the first that the authors are aware of to investigate mRNA expression of the aforementioned housekeeping genes (beta-actin, GAPDH, RPL30 and RPS5) in post-foaling equine fetal membranes.

When comparing the same tissue type between the two groups (foaling vs control mares), beta-actin appeared to be the most stably expressed housekeeping gene of the four investigated. In this circumstance, it was therefore considered the only appropriate gene to use as a housekeeping gene for this part of the analysis. However, beta-actin was not stably expressed when comparing tissue types (endometrium vs fetal membranes) in either the foaling and control mare group. For the remaining three housekeeping genes, GAPDH, RPL30 and RPS5, none of these genes showed suitability as housekeeping genes due to their varied stability between and within groups.

As a result, unfortunately, a suitable gene for internal control was not found from the panel of four to allow comparison between fetal membrane and endometrial mRNA expression within each group. Two earlier studies utilising RT-qPCR both chose beta-actin as a housekeeping gene when comparing equine endometrial and fetal membrane tissues (Palm et al., 2013, Rapacz-Leonard et al., 2015). In one of those studies, beta-actin was selected on the basis of its stable expression within endometrium but the level of expression within fetal membrane tissue was not described; the second study extrapolated results from another study investigating gene expression in non-reproductive tissues (Zhang et al., 2009). Thus, while beta-actin may be a suitable housekeeping gene for analysis of equine endometrial tissue, there remains a knowledge gap as to what housekeeping genes might be suitable for evaluation gene expression in equine fetal membrane tissue.

The variation and suitability of housekeeping genes can potentially be influenced by the methodology used for both tissue storage and RNA extraction. This study utilised RNAlater as a non-toxic tissue preservative due to its ability to stabilise tissue RNA being equivalent to that of freezing samples (Mutter et al., 2004, Wang et al., 2018). From the two other studies assessing mRNA expression in equine fetal membrane and endometrial tissue, RNAlater was only used as a tissue preservative in one study (Rapacz-Leonard et al., 2015). Another possible explanation could be the duration of tissue storage, with potential RNA degradation within the tissues over time. However, in the present study, the time and conditions of storage were performed as per manufacturer's instructions in order to avoid the potential of tissue degradation.

Subject variation and breed differences in genetic expression within the tissues need to be considered to explain the differences in housekeeping gene expression observed in this study compared to previous studies. No studies comparing post-partum endometrial and fetal membrane gene expression in Thoroughbred mares are available. Of the two studies performed in equids, one investigated heavy draft breed mares which are notorious for a higher incidence of retained fetal membranes compared to light breed mares (Ishii et al., 2013, Rapacz-Leonard et al., 2015). The other study was performed in Shetland ponies (Palm et al., 2013).

Another, more plausible, reason for the discrepancy for housekeeping gene expression between endometrium and fetal membranes of foaling mares is the degree of viability between the two tissues at the time of collection. In this study, there was a significantly greater expression of all housekeeping genes in the endometrium of foaling mares compared to fetal membranes. By the time fetal membranes were collected after foaling, they would have been devoid of a blood supply and oxygen for some time, and therefore be considered dead or dying tissue, in comparison to the 'living' endometrial tissue. The expression of housekeeping genes can vary depending on the biological activity of the tissue, such as the cell cycle, stage of growth, or disease (Zhang et al., 2015). Selection of an appropriate housekeeping gene in this situation was complicated by the fact that the two tissues, endometrium and fetal membranes, existed in two different physiological states which very well could have influenced the results. This highlights the importance of evaluating genetic products at the transcriptional and protein level, which is the reason for performing immunohistochemistry in this study. While it was attempted to control for varying gene expression by using a fixed amount of total RNA in all reactions, it is possible that the amount of mRNA varied between tissue types.

Whilst evidence suggests that there appears to be a trend for a suitable housekeeping gene within the equine endometrium post-partum, this study is the first to compare gene expression between equine fetal membrane and endometrial tissue in light breed mares. These findings have highlighted the fact that there may be no such thing as a universal housekeeping gene, especially under such circumstances. Furthermore, it reinforces the understanding that selection of an appropriate housekeeping gene needs to be based on potential variables, such as physiological or pathophysiological processes, genetic influence and comparison of different tissue and cell types.

4.6 Pro-inflammatory cytokine expression in endometrial and fetal membrane tissue

Research has demonstrated that the immune system is involved in normal physiological processes within equine reproductive tissues; for example, the maternal humoral immune response that occurs against endometrial cups due to paternally-derived MHC I complexes in early gestation and activation of the innate immune response in the uterus shortly after breeding (Donaldson et al., 1990, Crump et al., 1987, Christoffersen et al., 2010). An immunological response has also been identified in equine amniotic fluid, allantochorion and endometrium at parturition (Oddsdóttira et al., 2011, Palm et al., 2013, Rapacz-Leonard et al., 2015). In humans, there is strong evidence demonstrating that parturition and fetal membrane rupture and detachment require a functional innate immune response (Osman et al., 2003, Bowen et al., 2002). Despite this, direct investigation of the immunological processes involved in placental detachment in the horse appears to have been overlooked, despite the strong evidence from the human literature that this is the case and the potential importance of the process in RFM.

For the purpose of analysis in this study, mRNA expression of cytokines in the fetal and non-fetal horn were combined as there was no significant difference in expression between the two locations. In the case of prolonged fetal membrane retention, especially for cases where only one horn was retained, the mRNA expression of cytokines could potentially vary between fetal and non-fetal horns. Only mares who retained their membranes for less than 4 hours were included in the analysis to represent the population of normal foaling mares. It would be postulated that if an intact innate immune response is required for timely detachment of fetal membranes, components such as cytokine expression, neutrophil function and chemotaxis would be hindered in mares with RFM. Due to the low numbers of mares with retained fetal membranes in this study (n = 4), comparison of cytokine expression between foaling and RFM mares was not performed. However, analysis with a larger RFM group could provide interesting information into the pathogenesis of retained fetal membranes focusing on the role of inflammation.

It has been established in humans that the upregulation of cytokines occurs in the placenta at the time of parturition, ultimately contributing to fetal membrane rupture and detachment (Keelan et al., 2003b, Osman et al., 2003, Keelan et al., 2003a). Significant increases in IL-8 expression have been repeatedly shown in choriodecidual tissues of women undergoing spontaneous labour, with the upregulated expression of IL-1 and IL-6 in the same tissues being less consistent between studies (Osman et al., 2003, Norman et al., 2007, Dudley et al., 1996). In comparison, a single study in horses showed that whilst IL-6 expression was greater in the endometrium compared to the allantochorion, IL-8 expression was no different between the two tissues (Palm et al., 2013). The discrepancies in pro-inflammatory cytokine expression patterns between studies can be attributed to a combination of each cytokine's role and timing as part of the inflammatory response, the method of tissue collection and also the method of evaluating cytokine activity (for example, subjective assessment using immunohistochemistry versus quantitative analysis with RT-qPCR).

In this study, as well as in human studies, TNF-α was either not expressed or found to be present in extremely low concentrations (Dudley et al., 1996, Osman et al., 2003). For this reason, RT-qPCR of TNF-α was not pursued here based on the poor qualitative PCR results. Similarly, IL-1 expression in both endometrium and fetal membranes of foaling mares was lower in magnitude compared to IL-6 and IL-8. The primary role of IL-1 and TNF-α is to induce subsequent production of other pro-inflammatory cytokines (Van Miert, 2002). This early role in the innate immune response could explain why expression of both cytokines is either low or inconsistent across studies. Whilst there was a significant difference in IL-1 expression between foaling and control endometrial samples, IL-1 expression between foaling and control fetal membrane samples was not significant. This could potentially be a result of the fact that IL-1 was no longer a major component of the immune response at that time point. Alternatively, expression of IL-1 in foaling fetal membrane tissue may be lower than that of endometrium because the tissue is in a different physiological state compared to the endometrium.

The results from this study have provided evidence for an innate inflammatory response occurring at parturition in mares and are consistent with those findings in human studies. The mRNA expression patterns for pro-inflammatory cytokines IL-1, IL-6 and IL-8 were different for endometrium and fetal membranes, especially so for the foaling mare group. For the endometrium, there was a significantly greater mRNA expression of IL-1, IL-6 and IL-8 in foaling mares compared to control mares. This was not the case for fetal membrane results, with only mRNA expression of IL-8 being significantly greater in foaling mares compared to controls. This uniform upregulation of the three main pro-inflammatory cytokines within the endometrium suggests a possible role of the maternal immune system in rejection of fetal membranes. It is also important to recognise the effect that tissues undergoing cell death (such as fetal membranes after foaling) might have on gene expression, as previously discussed. Nonetheless, this information therefore provides a new platform for further investigation and could direct which treatment strategies be appropriate in regards to abnormal placental detachment. For example, it is accepted that along with the role IL-1 and TNF-α play in recruiting other pro-inflammatory cytokines, they also play a role in the induction of prostaglandin synthesis via the arachidonic acid pathway as part of the innate immune response (Dinarello, 2007). From a clinical perspective, it raises the question regarding the use and benefit of non-steroidal anti-inflammatory (NSAID) drugs in the treatment of RFM; that being if fetal membrane detachment requires a functional localised innate immune response, and both IL-1 and TNF-α are essential to that response, will NSAIDs interfere with the inflammatory cascade and delay detachment of the membranes? The purpose of NSAIDs is founded on their ability to ameliorate the effects of endotoxaemia, which poses the question as to whether clinicians should be considering alternative options to deal with the effect of endotoxaemia such as the cationic antimicrobial polymyxin B (Barton et al., 2004).

In contrast to its upregulated expression in endometrial tissue, fetal membrane IL-6 mRNA expression was significantly lower in foaling mares compared to controls. Whilst IL-1 mRNA expression tended to be lower in the fetal membranes of foaling mares compared to controls this difference was not significant. These results correlate with the other study assessing pro-inflammatory cytokine expression in normal foaling mares which found a greater expression of IL-6 in the endometrium compared to fetal membranes (Palm et al., 2013). An explanation for the reduced expression of IL-6 in fetal membranes may be due to the fact that fetal membranes are considered dying tissue, which could be associated with reduced expression of various genes, as proposed in the previous discussion of housekeeping genes. Nonetheless, both studies have demonstrated that IL-6 and IL-8 are actively expressed within reproductive tissues during parturition. The expression of IL-6 in reproductive tissues during spontaneous parturition varies amongst studies. Whether or not IL-6 functions as a pro or anti-inflammatory cytokine in fetal membrane tissue at parturition also requires further clarification (Scheller, 2011). Based on the higher concentration of IL-8 expression in fetal membranes, and the fact that IL-6 is also responsible for IL-8 suppression and eventual transition from early neutrophilic to lymphocytic inflammation, it could also be hypothesized that at the time of tissue collection this transition had not yet occurred, hence the reduced expression of IL-6 within fetal membranes. Alternatively, IL-6 may be behaving as an anti-inflammatory cytokine, based on the findings in this study that mRNA expression in control mare fetal membrane tissue was significantly greater than that of foaling mares. However, these results need to be interpreted with caution in light of the housekeeping gene results as previously explained.

When examining cytokine expression between tissues in foaling mares, there was an overall significantly greater expression of all three cytokines in the endometrium compared to the fetal membranes. A possible explanation for this result may be rapid cell death and necrosis within the fetal membranes following the loss of blood supply at the time of umbilical cord rupture and detachment, leading to a change in molecular and genetic activity compared to endometrium. Another reason for this discrepancy could be that the maternal immune system mounts a response to the fetal membranes at the time of parturition. Therefore, there would be a higher degree of inflammation occurring within the endometrium directed against the fetal membranes at this time. As a maternal humoral immune response occurs during early gestation to fetal allergens, a similar response at the time of parturition could occur; the transition from a state of immune tolerance throughout pregnancy to an immunologically activated state for parturition (Rapacz-Leonard et al., 2014, Donaldson et al., 1992). How the fetus remains protected from the maternal immune system and an inflammatory quiescent state is maintained throughout pregnancy remains unknown.

The raw mRNA concentrations in both endometrium and fetal membranes of foaling mares was the highest for IL-8, followed by IL-6 with IL-1 mRNA concentration being the lowest. This variation could reflect each cytokine's role in the immune response, which is essentially a series of sequential events based on signalling pathways. Furthermore, whilst IL-1 and IL-6 share a common signalling pathway, chemokine IL-8 signals via a separate pathway (Turner et al., 2014). IL-1 is responsible for the expression of adhesion molecules and initial stimulation of chemokines, as well as responding to DAMPs through IL-R1 and so is functional towards the beginning of the inflammatory response (Bianchi, 2007). Interleukin 6, as previously discussed, has both anti- and pro-inflammatory properties depending on the signalling pathway activated, which could explain the discrepancy in its expression between reproductive tissues of foaling and control mares (Scheller, 2011). Nonetheless, as a pro-inflammatory cytokine IL-6 main roles involve neutrophil apoptosis, monocyte differentiation as well as B and T cell maturation/activation. It therefore would be most functional towards the later part of the immune response, following neutrophilic infiltration and degranulation which could explain why the concentration of IL-6 was so much lower than IL-8 in foaling mares at the time of tissue collection.

Whilst we were able to demonstrate an upregulation of pro-inflammatory cytokines in reproductive tissues at parturition in this study, in order to fully understand the mechanism of placental detachment in mares, the triggers of this innate immune response, and the subsequent actions that these chemical messengers induce, needs to be understood. Initiation of fetal membrane detachment could be due to direct contact between fetal and maternal systems, where the maternal immune system recognises fetal antigens as alarmins. The mechanical action of myometrial contraction during parturition could lead to transfer of fetal DNA into the maternal circulation, however it remains unknown whether the subsequent inflammatory response that contributes to placental detachment occurs that quickly. There is evidence to suggest otherwise, based on the pattern of MMP activity in equine amniotic fluid, indicating that extracellular matrix degradation may have begun prior to parturition (Oddsdóttira et al., 2011). Nevertheless, initiation of the inflammatory response directed by pro-inflammatory cytokines would result in accumulation of leukocytes within the tissues, subsequent release of other pro-inflammatory mediators such as MMPs which would ultimately lead to degradation of extracellular proteins. Failure of this to occur has been demonstrated histologically in mares that retained their membranes (Rapacz et al., 2012).

Towards the end of gestation, ageing of fetal membranes and associated decline in their function is considered a natural process (Menon, 2016). As a result, cell death within the fetal membranes could lead to the release of DAMPs (alarmins) with subsequent increase activity of pro-inflammatory cytokines due to triggering of the immune response following interaction with PRRs (Bianchi, 2007). Senescent cells themselves can also produce pro-inflammatory cytokines, leading the further attraction of other necessary inflammatory mediators (Menon, 2016).

Transition from quiescent state to active parturition through the functional removal of progesterone has also been postulated to ignite the inflammatory response. Following regression of endometrial cups, the allantochorion assumes the primary role of progestogen synthesis for the remainder of gestation (Conley, 2016). Progesterone is also recognised as an 'anti-inflammatory steroid', and therefore upon its removal a cascade of inflammatory events could ensue within the reproductive tissues (Peltier, 2003). To date, however, no direct correlation between the rapid decline of progestagens and increased activity of pro-inflammatory mediators within the fetal membranes has been described.

4.7 Immunohistochemistry to Identify Cytokines in Equine Endometrium and Fetal Membranes

Immunolocalisation of cytokines by colorimetric detection was attempted in this study in order to correlate genetic expression of cytokines and their presence and localisation within endometrial and fetal membrane tissue (Amsen et al., 2009). Unfortunately, despite attempting various techniques, we were unable to develop a protocol that localized cytokines within tissues. A similar protocol to those trialled in this study successfully identified cytokines in human reproductive tissue at parturition (Young et al., 2002).

Primary anti-equine antibodies IL-1 and IL-6 were sourced for this component of the project; however, an anti-equine IL-8 antibody suitable for immunohistochemistry purposes was not available at the time of the experiment. Potential causes for failure to identify the cytokines within the tissues might include the timing of collection relative to timing of expression of that cytokine. For example, certain cytokines, such as IL-1, are secreted in an inactive form, and the timing of sample collection may not have coincided with when the cytokine had been cleaved into an active form (Feldmann, 2001). In addition, different primary antibodies might be required for localising the active form of those cytokines (Amsen et al., 2009). Perhaps the availability and use of anti-equine IL-8 would have yielded more positive results in light of the fact that this cytokine of interest was expressed in far higher concentrations than IL-1 and IL-6 in both endometrium and

fetal membranes of foaling mares. The dilutions used for both antibodies could have been incorrect; the choices made in this study were based on personal experience and extrapolation from other species rather than studies performed in equine tissues. In addition, the retrieval method used in this study, although similar to another successful study in humans (Young et al., 2002), may not have been suitable for these tissues. For potential future investigations, research could focus on the use of enzyme linked immunosorbance assay (ELISA) to detect cytokines at a protein level (Amsen et al., 2009). Cytokine bead array has also been advocated as another way of detecting multiple cytokine proteins at once, making it a more cost- and time-efficient system (Elshal and McCoy, 2006).

4.8 Limitations and Future Directions

Whilst this research has provided insight into the presence of an immune response associated with fetal membrane detachment in normal mares at the time of foaling, there are several limitations to this study. First, the smallest sample size considered suitable for this study to obtain umbilical cord blood, fetal membrane and endometrial tissue from, based on power analysis, was 30 normal foaling mares (i.e. no incidence of dystocia, retained fetal membranes or placentitis). In total, samples were collected from 33 mares that foaled during the study period. Four of these mares experienced RFM and three mares that had placentitis; thus only 26 mares met the inclusion criteria. At least 6 control mares were also considered necessary based on power analysis, but over the duration of the study period only four late-gestation healthy pregnant mares were sourced. Ideally, mares enrolled as controls would have been those undergoing elective caesarean procedures and having not yet entered labour. However, this procedure is not commonly performed at the investigators' hospital. For this reason, mares undergoing slaughter were selected instead as it was thought this would be a more reliable source. For future investigations, however, collaboration with other institutions (both nationally and internationally) could provide access to a reasonable number of control samples for analysis.

In order to make comparisons of gene expression between groups, or between tissues within groups, a suitable housekeeping gene is required. In this study, none of the four housekeeping genes selected were stably expressed across both groups and tissues limiting interpretation of the RT-qPCR results for the genes investigated. This was unexpected given that all four of the housekeeping genes used in this study had been reported to be reliable in RT-qPCR analysis of equine reproductive tissues in previous investigations. This raises the question of what constitutes a suitable internal control under these conditions. Based on the results of the present study, we have speculated that the level of expression in fetal membrane tissues does not correlate with that of endometrium because the tissues are in very different physiological states, considering that the fetal membrane tissues are essentially dying or dead.

Whilst the results of this study allude to the role of an inflammatory response in placental detachment in mares, the physiological mechanisms and pathways involved requires further investigation. This understanding will allow clinicians to make an informative, evidence-based decision on prevention strategies and treatment of abnormal fetal membrane retention. Understanding the 'trigger' signal of the immune response, such as alarmins, within reproductive tissues that results in fetal membrane detachment could provide a means of therapeutic intervention in the pathological state, such as premature or delayed detachment. Whilst the difference in leukocytes between umbilical vessels at parturition is significant, the exact role of leukocytes in the mechanism of placental detachment is yet to be elucidated. Demonstrating the function, rather than the physical presence, of leukocytes within the placenta at parturition, may provide a better understanding on their involvement. The correlation of gene expression with the presence of inflammatory cytokines (i.e., translation of mRNA into proteins molecules) in reproductive tissues needs further attention. This could be achieved by employing methods such as ELISA or cytokine bead array as an alternative to immunohistochemistry. As yet, no research

using these techniques in equine reproductive tissues has been published. Perseverance with immunohistochemistry techniques should also be considered and should not yet be abandoned. Investigating the consequences of a pro-inflammatory response may also be an important component in understanding placental detachment, such as the activity and localisation of MMPs within tissues, which likely contribute to the physical detachment of allantochorion from endometrium.

Conclusion

The data provided in this study has highlighted that placental detachment in the mare is likely to involve an inflammatory response that is driven by the increased expression of pro-inflammatory cytokines in both endometrial and fetal membrane tissue.

As part of the local innate immune response during parturition, neutrophils and lymphocytes from the equine neonatal blood circulation make up the majority of leukocytes that are attracted to and sequestered within the fetal membranes during parturition. The chemotaxis of leukocytes is most likely due to the presence of IL-8 both within the endometrium and the fetal membranes. Subsequent degranulation of neutrophils, and release of pro-inflammatory mediators such as MMPs, is hypothesised to contribute to the detachment of the fetal membranes from the endometrium through extracellular matrix remodelling.

Research from humans and cattle has provided strong evidence that an orchestrated innate immune response and subsequent inflammatory cascade of events is required for fetal membrane detachment. Whilst the information from this study is fundamental to understanding the pathogenesis of abnormal placental detachment, and that inflammation clearly plays an important role in membrane detachment, ongoing research to further investigate the inflammatory pathways is indicated.

In conclusion, the findings of this study have provided valuable insight into the mechanism of placental detachment of the mare. The information gained here provides a platform for ongoing investigation into both normal and abnormal placental detachment in mares, and a basis to develop evidence-based treatment strategies for abnormal placental detachment.

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