EQUINE AMYLOID A IN THE PERIPARTUM AND NEONATAL PERIOD

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Preface

This study was conducted to provide new knowledge pertinent to the acute phase protein, amyloid A (AA) in the peripartum and neonatal period. Hepatic derived serum AA is an acute phase protein in the horse. Its serum concentration increases with inflammation or infection but its function has not yet been fully elucidated. We evaluated the specificity of serum AA as an indicator of neonatal sepsis elaborated various other influences on the serum concentration of this protein in foals. A mammary derived isoform of AA has been discovered and is believed to have protective effects in the neonatal intestine. We identified this protein in equine colostrum and milk and investigated its potential intestinal absorption in neonatal foals. We also investigated the effect of other parturient events including induction of parturition on AA concentration in mares. This study analyzed the effect of a new, safer method of induction of parturition in the mare on the neonatal acute phase response. Specific objectives of this research were to a) assess hepatic derived AA as an indicator for sepsis in the neonatal foal; b) quantify mammary derived AA in equine colostrum and milk; c) assess whether intestinal absorption of mammary derived AA could affect neonatal serum concentrations of the protein and d) assess whether induction of parturition affected neonatal serum concentrations of this protein.

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NOMENCLATURE

APP	Acute phase protein
AA	Amyloid A
CRP	C reactive protein
IgG	Immunoglobulin G
ELISA	Enzyme linked immunosorbent assay
IL	Interleukin
TNF	Tumor necrosis factor

CHAPTER I

INTRODUCTION

Assays for acute phase proteins are currently being developed in different domestic animal species as a tool to assess animal health. An acute phase protein has been defined as one whose plasma concentration increases (positive acute-phase protein) or decreases (negative acute-phase protein) by at least 25 percent during inflammatory disorders. This response is non-specific in that serum concentrations of these proteins change during inflammatory disorders of all kinds, infectious or non-infectious.

Research has focused, however, on the use of a hepatic-derived acute phase protein, serum amyloid A (AA), as an aid in the differential diagnosis of weak neonatal foals. It has been claimed that concentrations of this protein rise during bacterial infection to levels that are significantly higher than those reached during non-infectious inflammatory conditions.

It is questionable whether measurement of serum AA is truly a specific indicator of equine neonatal sepsis and needs further investigation. Many other non-infectious sources of amyloid A (AA) may complicate the picture. Recent research has also detected a mammary gland derived isoform of the acute phase protein, AA, in the colostrum and early milk of women, cows and ewes and in the colostrum of one mare. It has not been determined whether amyloid A is present in the colostrum and early milk of all mares. Foals absorb macromolecules from the intestine during the early post partum period, making absorption of mammary derived amyloid A from the colostrum likely to interfere with interpretation of serum concentrations of this protein in the diagnosis of sepsis. The mammary derived AA protein has been shown to have protective effects in the neonatal intestine of infants and calves. If it is present in large quantities in the mammary secretions of healthy mares, its function in the neonatal intestine and/or extra-intestinal

tissues in the neonatal foal also warrants investigation but is outside the scope of this study (see Chapter 4).

Other peri-partum factors may also affect the concentration of serum AA in neonatal foals. Induction of parturition in the mare has been traditionally avoided due to concerns about complications such as dystocia, retained placenta and birth of a compromised foal. However, there have been advances in the techniques used to induce mares to foal. If induction of parturition in the mare compromises the neonatal foal and causes an inflammatory response beyond that seen secondary to natural parturition, this should be detectable in the postpartum serum AA profile of the foal and may lead to elevation of the serum AA concentration for reasons unassociated with sepsis. Acute phase protein concentrations in neonatal foals that have been induced have never been examined to establish the validity of concerns about compromising foals by inducing parturition.

The purpose of these studies was to investigate the specificity of serum AA measurement as a indicator of sepsis in the neonatal foal; to document the presence of AA in the colostrum and early milk of a large group of mares; to investigate whether the neonatal serum concentration of this protein could be influenced by intestinal absorption of AA from colostrum and early milk and to investigate the influence of induction of parturition on serum AA concentration in the neonatal foal.

CHAPTER II

REVIEW OF LITERATURE

Acute phase proteins

The systemic inflammatory response follows tissue damage or infection. It involves changes in a number of organ systems, many distant to the site of tissue damage, in an attempt to restore homeostasis (1). Acute phase protein concentrations in the plasma rise as part of this response (1). The term "acute-phase" was first used in this context in 1941 to describe serum in which a protein was found that was not normally present (2). An acute phase protein (APP) has been defined as one whose plasma concentration increases (positive acute-phase protein) or decreases (negative acute-phase protein) by at least 25 percent during inflammatory disorders (3). The acute phase response is a non-specific systemic response and occurs with acute and chronic inflammation of any kind (3). Conditions that induce increases in plasma concentrations of acute phase proteins in humans include infection, trauma, surgery, burns, tissue infarction, immune-mediated disease, cancer, strenuous exercise, heatstroke and childbirth (3).

The pathophysiology behind the increased plasma concentrations of APPs in response to inflammation involves a cascade of interactions between intracellular messengers. Tissue damage leads to the release of cytokines from activated cells into the blood stream (1). Most cytokines have multiple targets and functions and they may be inhibited or enhanced by other cytokines (3). They may also be influenced by hormones and by cytokine-receptor antagonists and circulating receptors (3).

Cytokine messengers stimulate cells to synthesize inflammatory proteins. APPs are primarily synthesized in the liver (1). There are essentially two families of APPs. Type I APPs, serum AA and C-reactive protein (CRP), are induced by Interleukin 1 (IL1) and Tumor Necrosis Factor (TNF), and Type II APPs, fibrinogen and haptoglobin, are

induced by Interleukin 6 (IL6) (1). Once synthesized, the APPs are released into the circulation to exert their effect.

APPs are part of the innate immune response and have several important functions including homeostasis, opsonization and trapping of micro-organisms and their products, activating complement, bacterial phagocytosis, decreased thrombosis, decreased proteolysis and antioxidants, neutralizing enzymes, scavenging free hemoglobin and radicals, and in modulating the host's immune response (1,4). However, the function of each individual APP has not been clearly determined.

A delay of several hours is intrinsic to the cascade of interactions which lead to elevation of serum APP levels (5). In other words, the time interval from the initial damage and activation of cytokine-secreting cells to the initiation of hepatic APP synthesis is responsible for the significant lag phase required until elevated serum APP exacerbations become measurable. Therefore, multiple serial concentration measurements give a better picture of the ongoing inflammatory process than one single concentration measurement at the onset of illness.

The acute phase response is highly variable and non-specific; the increase in serum concentration of these inflammatory proteins depends on the severity of infection or tissue damage, the stage of the disease, the interaction of the cascade of cytokines and the individual response to the disease. Bacterial infection and severe cellular destruction stimulate greater responses than does viral infection (4). It is thought that assessment of an index of various positive and negative APPs over time gives a markedly more sensitive and specific indication of the extent and stage of the disease process than a single measurement of a single protein at one time point (4, 5). Once therapy has been

initiated and /or the immunological response of the host has successfully contained the initial insult, the stimulus for new synthesis of APP is eliminated. Thereafter, concentrations are mostly a function of the half-life of the proteins and return to base level (4) unless there is persistence of the insult, e.g. from disseminated abscesses in various organs.

Acute phase proteins in Veterinary Medicine

APP concentrations in different veterinary species are used as markers of animal health (6, 7). Health problems of pigs have been shown to be correlated with increases in haptoglobin, CRP and ceruloplasmin (6). In cattle, a strong reproducible rise in serum AA and haptoglobin is seen following experimental infection with bovine respiratory syncytial virus (6). APPs are also thought to be useful as a tool in evaluating health in calf herds (8). Physical stress has been demonstrated to significantly increase serum AA concentrations in calves without a significant difference in cortisol or aldolase concentrations. Therefore, serum AA is suggested as a sensitive variable for assessing physical welfare in calves (9). In cats, serum AA, alpha 1 acid-glycoprotein and haptoglobin are measureable APPs (10). Ceruloplasmin and CRP concentrations were shown to be elevated in dogs with babesiosis compared with unaffected dogs (11). However, as is the case in humans, the acute phase response in animals is highly variable between individuals and dependent on stage and severity of disease. In cattle, it is suggested that the ability to produce serum AA and haptoglobin may be an innate characteristic of the individual cow, because APP concentration responses to lipopolysaccharide differed significantly among cows (12). In a study in dogs, where CRP and ceruloplasmin were consistently elevated in leishmaniosis at the time of

diagnosis, the serum AA concentration of some of the dogs remained within the normal range (13). Indices of various APPs over time have been used in the assessment of health in cattle and pigs (14, 15).

Equine acute phase proteins

Fibrinogens, serum AA, alpha 1 acid glycoprotein (a1AGP), CRP, haptoglobin and ceruloplasmin have been proposed as APPs in horses (16). In horses, serum AA appears to be the major APP with the earliest response to inflammatory stimuli (17). Alpha 1AGP appears to be the next to rise (18), followed in succession by CRP (19), haptoglobin (20) and fibrinogen (21). These latter proteins are minor acute phase proteins in the horse. Ceruloplasmin is slow to rise in the serum following inflammatory stimuli and may be a delayed minor acute phase protein in the horse (22).

Equine Serum Amyloid A

The dynamics of serum AA in the horse have been described. The average serum AA concentration of normal adult horses (>18 months old) is 21 +/- 9 mcg/ml (16). After induced non-infectious inflammation, the serum AA concentration rises above baseline by 16 hours post inflammatory stimulus and peaks at 36-48 hours at 227 times baseline (23). Elective surgery in horses resulted in a 164-fold increase in serum AA concentrations by 24 hours post surgery, whereas non-elective surgery resulted in a 273-fold increase by 24 hours post surgery (24). Horses with equine influenza virus infection had a rise in serum AA concentrations over the first 48 hours with the concentration returning to baseline by 11-22 days in uncomplicated cases (25). Horses with bacterial infections such as *Streptococcus equi* abscesses had serum AA concentration increases of up to 800-fold (17).

There is a distinct overlap in the ranges of serum AA concentrations resulting from different types of inflammation; however, this is consistent with the nonspecific nature of the serum AA response (26). For this reason serum AA is deemed to be a sensitive but nonspecific marker for inflammation.

Serum amyloid A in neonatal foals

In normal neonatal foals, there is a mild increase in serum AA concentration in the period immediately after birth, and it remains relatively high for 1 week (16). Serum concentrations of APPs are known to increase several hundredfold in response to bacterial infection in human infants and are routinely used in human medicine in the differential diagnosis of sepsis in infants (27-31). Research has shown that the measurement of serum AA can be a useful diagnostic tool in the differentiation of sepsis from other conditions in neonatal foals (32).

Bacterial sepsis is a challenging diagnosis in neonatal medicine. Neonates of all species are at increased risk of developing infection probably due to the immaturity of the neonatal immune system. Although equine neonates are immunocompetent at birth, they are particularly at risk for infection given the epitheliochorial nature of equine placentation and the lack of placental transfer of immunity.

Sepsis occurs most commonly in the foal in the first 48 hours of life, and the modes of infection are intrauterine, oral, and respiratory, via umbilical cord or traumatic penetration of the skin (33). The organisms establish infection in the entry organ. Bacteria and their toxins then invade the blood stream causing damage to endothelium and the release of cytokine mediators that initiate the systemic inflammatory response and the production of acute phase proteins.

In a study measuring serum AA as an aid to differential diagnosis of infection in newborn foals, foals suffering from weakness, neonatal maladjustment syndrome, traumatic birth or meconium colic had significantly higher concentrations than did controls (32). In fact, clinical cases of infection had significantly higher concentrations than did the other groups. In another study, serum AA was compared with total leukocyte count, neutrophil count and fibrinogen as an aid in the management of infectious disease in the foal. Results indicated that serum AA might be an aid in the differential diagnosis of neonatal weak foals (34). Serum AA and fibrinogen concentrations on admission were higher in foals with bacterial infections than in foals with nonbacterial or uncertain diagnoses. Foals with positive blood cultures had markedly increased serum AA and fibrinogen concentrations and varying leukocyte concentrations.

Other sources of AA may complicate the interpretation of serum AA concentrations in neonatal foals. The placenta is capable of producing cytokines, such as TNF alpha and interleukins in response to hypoxia and endotoxin stimulation (35-38). Placental umbilical vascular disease results in increased IL6 and IL8 concentrations in human fetal circulation (39, 40). It is possible, therefore, that perinatal events, such as placentitis or placenta previ, could induce an inflammatory response in the fetal foal. Twins that are carried full term are uncommon in mares. However, when this does occur, the foals often share a large area of the placentation, which can cause compromise and potentially induce an inflammatory response.

As twin foals and foals from mares with placental disease are typically compromised and prone to secondary sepsis, it may be a moot point as to the source of the circulating AA;

however, it is useful to elaborate the various sources of neonatal AA production stimulation.

Acute phase proteins such as haptoglobin, a1AGP and ceruloplasmin have been demonstrated in the colostrum and milk from several species (41-43) and some have been demonstrated to cross the placenta (42, 44, 45). In the neonatal foal, large proteins are absorbed intact as functional proteins across the intestinal wall (46,47). Absorption is maximal at about 6 hours after the milk is ingested, but increased permeability to macromolecules persists for up to 24 hours. To date, however, there are no studies on the intestinal transfer of colostral or milk APPs in mares, and it seems likely that such colostral proteins are absorbed.

Amyloid A in the broodmare

A major acute phase response involving serum AA has been demonstrated from onset of labor to 96 hours post partum in normal women; however a wide range of concentrations are seen (48). In cattle, serum AA concentrations increase significantly from pre-parturient concentrations in the first 24 hours post partum, reaching a peak 24 to 48 hours after delivery (49). In the post-parturient mare, serum AA concentration peaks at on average 137mcg/ml 3 days post partum but a wide concentration range occurs (16).

An isoform of AA was recently identified in the colostrum and early milk from women, cows, ewes, sows and one mare (41). This colostral and milk isoform, called milk AA, is produced by healthy animals, synthesized in mammary gland cells not in the liver, expressed by mammary gland epithelial cells and secreted in abundance as part of a normal process (41).

In cows with mastitis, AA is also elevated (50). It appears that AA accumulation in mastitic bovine milk is the result of both local synthesis of AA and transfer of hepatically-derived serum AA to the milk due to increased permeability of the blood-milk barrier. In the mare, only one isoform of AA, the mammary derived isoform, has been detected in colostrum (A Weber, personal communication), which is not unusual given the infrequent occurrence of clinical mastitis in mares.

Colostrum has received renewed interest over recent years due to the many bioactive substances it contains (51-57). Bovine colostrum in particular is promoted for the treatment of many human conditions due to the high concentration of immuno-globulins, growth factors, cytokines, and nucleosides that are found in it (51). These hormones and regulatory factors in colostrum and milk may have roles in the development and maintenance of neonatal tissues including but not limited to the neonatal intestine (51-56).

Mares synthesize and secrete on average 5 liters of colostrum. There have been limited studies on the regulatory and growth factors in mares' colostrum or milk (57-60). The milk AA protein was demonstrated in the colostrum of only one mare (41). However, researchers believe that this milk AA can prevent necrotizing enterocolitis in human infants. Milk AA enhances the innate immunity of the human and bovine neonatal intestines by up-regulating mucin production and by preventing the attachment of pathogenic *E coli* to intestinal cells (61, 62). It is likely that similar functions may be attributed to this equine colostrum and milk protein with respect to the equine neonatal intestine.

Necrotizing enterocolitis

Necrotizing enterocolitis is an intestinal disorder that frequently affects compromised neonatal foals and has a high case mortality risk (63-66). Necrotizing enterocolitis (NEC) is a multifactorial problem, and also represents the most common gastrointestinal emergency in human neonates (67). Despite extensive research, its etiopathogenesis is not completely understood and this neonatal disease remains associated with high morbidity and mortality rates. The intestinal injury in this disease may be a consequence of prematurity, hypoxia, enteral feeding and bacterial colonization (68). Together these factors can result in an exaggerated inflammatory response, leading to ischemic bowel necrosis.

Human milk constituents, including milk AA, may decrease the incidence of NEC by decreasing pathogenic bacterial colonization, promoting growth of nonpathogenic flora, promoting maturation of the intestinal barrier, and ameliorating the pro-inflammatory response (69).

Unfortunately, many equine neonatologists promote the withdrawal of enteral feeding from compromised neonates to avoid overloading the intestine with rich ingesta which may promote bacterial colonization. Withdrawal of enteral feeding in compromised foals, however, may result in restriction of access of the beneficial components of colostrum to the patients that are in most need of them. Unfortunately, there has been limited research in this field in the mare.

Induction of parturition

Induction of parturition with its associated complications has the potential to produce elevated serum AA concentrations in neonatal foals. Traditionally there were few

clinical indications for induction of parturition in the mare, because it has been associated with increased incidence of peri-partum complications such as dystocia and with unfavorable outcome in foals (70-72). However, it has recently been proposed that induction of parturition in mares at term with low doses of oxytocin is a safe and reliable way to induce parturition (73). Three main methods are described for induction of parturition in mares using oxytocin, prostaglandin F2 alpha and dexamethasone. Oxytocin is generally considered the drug of choice in that small doses of oxytocin (2.5–10 i.u.) are effective at successfully triggering parturition at term, and higher doses (40-120 i.u.) used to induce birth in previous studies are unnecessary and potentially dangerous to the fetal foal (73, 74). Low doses of 2.5-5 i.u. cause a slow progression toward delivery; higher doses evoke a more rapid delivery response. However, this low dose method of induction can override the physiologic events involved in normal parturition without considering fetal maturity (73). Therefore, parturition must be pending when the mare is induced.

Calcium concentrations in the mammary secretions have been shown to be useful in predicting full-term gestation and are commonly used to predict impending parturition (75). When the pre-foaling mammary secretion calcium carbonate equals or exceeds 200 ppm, there is a 51% probability that spontaneous foaling will occur within 24 hours, an 84% probability that it will occur within 48 hours and a 97% probability that foaling will occur within 72 hours (73). When a concentration of 300-500 ppm is reached, the majority of mares will foal within a short period of time (24 hours); however, not all mares reach this concentration before foaling. Administration of low doses of oxytocin to full term mares with calcium concentrations >200 ppm in the mammary gland secretions

for at least 24 hours is now considered an effective method of induction of parturition (73).

Several studies have examined various parameters in the neonatal foal with regard to induction of parturition (71, 75-77). In one early study describing the induction of mares with a synthetic prostaglandin, four of eleven foals from mares that were induced did not survive the first 24 hours (71). Although much is now known about how inducing drugs affect the mare, and methods of induction have been refined from earlier protocols which were associated with violent expulsion of a frequently premature foal, we are unaware of any published studies that directly address the influence of induction of parturition on the production of an inflammatory or acute phase response in the foal.

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CHAPTER III

SPECIFICITY OF SERUM AMYLOID A CONCENTRATION AS AN INDICATOR OF SEPSIS IN FOALS

Abstract

It is questionable whether serum AA concentration is truly a specific indicator of equine neonatal sepsis, therefore needing further investigation. Serum AA concentration was measured in a population of 19 neonatal foals every 12 hours from birth until 72 hours post partum. Blood samples and intensive clinical monitoring in the post partum period were used to divide the foals into those with evidence of neonatal infection, those with no evidence of infection but evidence of severe inflammatory disease and those that were normal. The serum AA concentrations were evaluated to assess the specificity of this measurement in the differential diagnosis of neonatal sepsis in the foal. Specificity at a threshold concentration of 200mcg/ml was found to be 60 per cent, positive predictive value (PPV) only 57 per cent and negative predictive value (NPV) was 82 per cent. When the threshold concentration was increased to 500mcg/ml, the specificity increased to 80 per cent, the PPV increased to 75 per cent and the NPV increased to 86%.

Introduction

Neonates of all species are at an increased risk of developing infection. This is particularly true for neonates born to high-risk dams. Conditions that place mares in a high-risk category include: fescue toxicosis, equine Herpes virus 1 infection, twin pregnancy, colic and endotoxemia, hydrops, abdominal hemorrhage, Caesarian-section, malnutrition, systemic illness, trauma, chronic illness or administration of medications, placentitis and induction of parturition (1). Other conditions that can indicate compromise of the neonatal foal include dystocia, premature placental separation (red-bag birth), prematurity/prolonged gestation and fetal diarrhea. Failure of passive transfer of antibodies leads to increased risk of bacterial infection in the foal, as maternal immunoglobulins do not cross the placenta (2).

Bacterial sepsis is probably one of the most common diagnostic challenges in neonatal medicine (1). Early identification is vital so that appropriate treatment can be initiated, but there is no single test that confirms sepsis early in the postpartum period. The diagnosis of sepsis is generally made on clinical signs and suggestive hematology. However, clinical signs of bacterial sepsis in neonates are variable and non-specific with much overlap in signs between septic neonates and neonates with other conditions (1), making identification of septic neonates difficult. The reporting of results of blood culture can take days and frequent false negative and false positive results are obtained (3).

Previous studies have examined various markers of sepsis and have developed a sepsis score to predict sepsis in foals (4). This sepsis score, using a prevalence of about 50%, had a sensitivity of 93 per cent, a specificity of 86 per cent, positive predictive value of

89 per cent and negative predictive value of 92 per cent for the diagnosis of sepsis in compromised neonatal foals. Numerous parameters were involved in determining the score including the history and the clinical and laboratory findings. The sepsis score has greater sensitivity and specificity for infection, with fewer false positive and false negative values, than any of the parameters taken individually (4). However, a recent evaluation of the sepsis score found it to have a sensitivity of only 67% and a negative predictive value of only 55% (5). In this study, abnormal neutrophil cytology had a strong association with sepsis. This is supported by another study describing the clinico-pathologic features of sepsis in foals, where the most useful white blood cell parameters were neutropenia, band neutrophils (greater than 0.2 X 10(9)/liter) and toxic changes in the neutrophil population (6). Neutrophil counts of < 2000 cells per ml suggest early overwhelming infection.

Sepsis occurs most commonly in the foal in the first 48 hours of life and the modes of infection are intrauterine, oral, respiratory and via navel cord or traumatic penetration of the skin (1). Microorganisms establish infection in the entry organ. The bacteria and their toxins subsequently invade the blood stream causing damage to endothelium and the release of cytokines that initiate the systemic inflammatory response and the increased production of acute phase proteins (APPs). In human infants, the APP C-reactive protein (CRP) is used to support a diagnosis of sepsis (7-9). In one study, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of CRP for proven sepsis and localized infection at a cutoff point > or = 500 mcg/dl were 100 per cent, 94 per cent, 91.6 per cent and 100 per cent respectively (7).

Serum AA is an APP which is present in measurable quantities in normal equine neonatal serum (10). The primary source of serum AA in the circulating plasma pool is endogenous production from hepatic cells in response to inflammatory stimuli, particularly IL6 (11). Recent work has supported the use of serum AA concentrations in the differential diagnosis of sepsis in neonatal foals as its concentration appears to increase dramatically in response to bacterial infection (12). In that study, foal serum AA concentrations greater than 200 mcg/ml were strongly correlated with sepsis whereas lower values (20-200mcg/ml) were suggestive of a non-infective acute phase response to trauma or pre-maturity (12).

However, the acute phase response is by definition non-specific in that non-infectious processes which cause tissue damage can elicit a similar acute phase response. Several non-infectious disease processes lead to pathological changes in foals and present with a clinical picture similar to that of sepsis. These include hypoxic-ischemic disease and neonatal isoerythrolysis where hypoxic tissue damage can be considerable. Clinically, it is frequently difficult to differentiate these conditions. In addition, it is likely that extraneous sources of AA such as colostral AA protein could affect the serum concentration of AA in the neonatal foal. Colostral macromolecules of molecular weight 19,000 – 58,000 can be absorbed in neonatal calves and piglets (13, 14). Serum AA has a molecular weight of 11,400 – 12,500 in different species (15). Milk amyloid A, an isoform of AA demonstrated in the colostrum of cows, sheep, humans and one mare has a molecular weight of 12,800 (16).

Our hypothesis was that the measurement of serum AA using a threshold concentration of 200mcg/ml is not a specific test for the differential diagnosis of sepsis in the neonatal

foal. It is likely that other extraneous sources affect serum AA concentrations, and that some non-infectious inflammatory conditions of neonatal foals are severe enough to elicit an acute phase response similar to that seen with sepsis which would render the use of serum AA concentration a less specific test for the differential diagnosis of sepsis. Increasing the threshold concentration to 500mcg/ml may increase the specificity of the test. The purpose of this study was to examine the dynamics of the AA protein in neonatal foals with infectious and non-infectious inflammatory disease to evaluate the sensitivity, specificity, positive predictive value and negative predictive value of the measurement of this protein in the differential diagnosis of sepsis in neonatal foals.

Materials and Methods

Animals

The study was conducted between January and July 2003. Nineteen pregnant mares in residence at the Oklahoma State University College of Veterinary Medicine (OSU CVM) Ranch were used. The 19 neonatal foals of the mares were intensively monitored during the post-partum period. Sixteen mares were Thoroughbreds and 3 were Quarter Horses (age range, 4 to 23 yr).

The low-dose oxytocin protocol (17) was used to induce parturition in the mares. Calcium concentration in the mammary secretions was used to predict proximity to parturition. Sampling was initiated 1 wk prior to expected foaling date or if the mare displayed udder development. Samples were taken daily at 0800 until the calcium concentration began to rise and then twice daily at 0800 and 1800. When the calcium concentration was >200 ppm in two samples 24 h apart, parturition was deemed imminent and the mare could be safely induced.

Before induction, the mares' tails were wrapped, their perineum was washed with soap and water, and they were placed in stalls bedded with clean straw. Parturition was induced with 2.5 i.u. oxytocin (VEDCO, St Joseph, MO, USA) per dose given IV at 20-min intervals until second stage labor was observed.

Physical examination of the mare and foal was done immediately following parturition and peri-partum complications were recorded. At 12, 24, 48, and 72 h postpartum, physical examination of the 19 foals was performed. Sequential complete blood counts in the foals at 24, 48 and 72 h were utilized to assess health status. Serum IgG

concentrations in foals at 24 h of age were measured using a commercially available ELISA (SNAP Foal IgG Test, IDEXX Laboratories, Inc. Westbrook, MN, USA). Because there is no definitive pre-mortem test for sepsis in foals, foals were retrospectively allocated to 3 different groups; Group 1: completely normal; Group 2: evidence of inflammatory disease but no suspicion of sepsis; and Group 3 strong suspicion of sepsis. A normal foal was defined as one with a normal gestational length and a normal placenta (i.e. no gross pathological changes), stood within 2 hours and nursed within 3 hours of parturition, had a serum IgG concentration measurement > 4 g/l at 24 hours, a neutrophil count within the normal range (4000 and 8,000/ml) at 24, 48 and 72 hours and presented with no clinical signs of disease within the first 3 days. Conditions which would have excluded foals from the normal group included a maternal history of fescue toxicosis, equine herpesvirus 1 infection, twin pregnancy, colic and endotoxemia, hydrops, abdominal hemorrhage, Caesarian-section, malnutrition, systemic illness, trauma, chronic illness or administration of medications or placentitis, or premature separation of the placenta, prematurity/prolonged gestation, fetal diarrhea, dystocia, failure of passive transfer of antibodies or abnormal neutrophil counts. Parameters which assigned foals to the septic group included neutropenia with or without band neutrophils and toxic changes and clinical evidence of infection such as fever, depression, petecchial hemorrhages, localized heat, pain and swelling, or other evidence of systemic infection.

Samples for serum AA analysis

Blood samples were collected into evacuated plain glass tubes from each neonatal foal prior to colostrum ingestion and at 12, 24, 36, 48, 60, and 72 h postpartum. The foals were gently and carefully restrained to prevent traumatic venipuncture and generation of an inflammatory response associated with the venipuncture site. Where possible, umbilical cord blood was substituted for the pre-colostral sample to prevent unnecessary stress associated with venipuncture of the newborn foal. Blood samples were stored at 4 ^{0}C until a clot had formed; they were subsequently centrifuged at 699 x g for 5 min and the serum was separated and frozen at –20 ^{o}C for later analysis.

Serum AA concentrations

All samples were analyzed for serum AA concentration using Tridelta´s Phase™ Range SAA assay (Tridelta Limited, Maynooth, Co Kildare, Ireland). This assay was previously described in studies that measured serum AA concentrations in clinically normal and diseased horses [16, 18]. Data are expressed as mcg/ml. On the first plate, samples were measured in duplicate and thereafter once only. The minimum detectable concentration of serum AA was 5 ng/ml. The intra-assay coefficient of variation was 7.7 and 5.3% at mean serum AA concentrations of 74 and 128 µg/ml respectively. The inter-assay coefficient of variation was 10.8 and 8.7% at mean serum AA concentrations of 81 and 134 µg/ml respectively.

Statistical analysis of data

The natural log of serum AA, was modeled using a mixed model, fitted using the Mixed procedure in SAS® v 9.1 (SAS Institute Inc., 2003). A backward selection procedure was used to determine an appropriate model; factors remaining significant ($P < 0.05$)

were retained in the model. The repeated measurements within a foal at different times were examined by including foal as a random effect. The significance of the random effect was tested by comparing a model excluding the random effect using a likelihood ratio test. Disease, time and the two-way interaction between these variables were fitted as fixed effects. Time was checked for linearity by the inclusion of quadratic terms and by comparing models that included log of time, with time as a categorical variable and time as a continuous variable. This analysis was then repeated including only the results from the abnormal foals. Suspicion of sepsis, time and the two way interaction between these variables were fitted as fixed effects.

To differentiate between abnormal foals which were considered septic and abnormal foals suffering from another inflammatory disorder, sensitivity (the probability of the test being positive when disease is present), specificity (the probability of the test being negative when disease is absent), positive predictive value (PPV) (the probability of having the disease if the test was positive) and the negative predictive value (NPV) (the probability of not having the disease if the test was negative) were calculated for the use of serum AA concentration in neonatal foal serum over the first 72 hours of life.

Results

Subjects

Nineteen mare-and-foal pairs were included in the study. One mare was foaled by elective Caesarian section. Parturition was induced in 11 mares. Seven mares foaled spontaneously before the 24-h sample was taken. One mare foaled prematurely (317 d) but the foal was healthy, and no complications developed. Regarding normalcy and partition induction, of the 11 mares with induced parturition, 7 had normal foals and 4 had abnormal foals.

Allocation to Groups

All foals had serum IgG concentrations > 4g/L at 24 h. Nine of the 19 foals were designated normal (according to our definition) and allocated to the normal group, Group 1. Ten foals were excluded from the normal group and were allocated further based on suspicion of sepsis.

Of the 10 abnormal foals, 5 foals were without signs suggestive of sepsis and were allocated to Group 2. Of these five foals, one was premature but was otherwise normal and did not require treatment. One had an abnormal neutrophil count (3000-4000 cells/ml) but was not treated and developed no further complications. Another foal took longer than 3 hours to nurse; however, it did not develop any further complications and the IgG concentration was >8g/l at 24 hours. One foal was delivered following dystocia but did not develop any clinical or laboratory findings suggestive of disease or subsequent complications. Finally, one foal suffered from progressive bilateral hind-limb paresis that became apparent 3 days after Caesarian section delivery. This foal was

eventually euthanized and diagnosed on post mortem with ischemia in the lumbo-sacral spinal cord.

Of the ten abnormal foals, five were considered to have a high index of suspicion of sepsis and were included in Group 3. These five foals developed neutrophil counts <2000 and were treated with ceftiofur sodium (5 - 10 mg/kg BID IV) for 3-5 days. Three of these foals did not develop clinical signs consistent with sepsis and did not require further intervention. One more foal was slow to nurse having suffered premature placental separation at birth. None of these four foals developed subsequent complications. The fifth foal developed clinical signs consistent with sepsis including fever, severe depression and mucosal petecchiation. Placentitis was detected in the dam's placenta at birth. The foal was intensively treated with broad-spectrum antibiotics (potassium penicillin and amikacin), hyper-immunized plasma and anti-inflammatory drugs (ketoprofen) and survived.

Normal and abnormal foals and their serum AA concentrations

At birth, the 9 normal foals had serum AA concentrations in the previously described range (i.e. < 20 mcg/ml). Their serum AA concentration ranged from 0.6 to11 mcg/ml (mean 6 +/- 3.2) (Table 1). Over the first 72 hours, however, the serum AA concentrations of these normal foals varied over a wide range (e.g. at 48 hours they ranged from 4 to 197mcg/ml (mean 43.2 +/- 62)), but these concentrations remained below the previously described septic range (> 200mcg/ml) although 6 of them were in the range indicative of inflammatory disease (i.e. >20mcg/ml).

Within the group of 10 abnormal foals a wide range of conditions and serum AA concentrations were observed (Tables 2, 3). Although the five foals in Group 2 did not

have clinical or laboratory findings suggestive of sepsis, 2 of them developed serum AA concentrations in the range previously described as indicative of sepsis (i.e.> 200mcg/ml) (Table 2). One of these was approximately 5 hours old before it nursed. It was otherwise clinically normal and had a normal IgG at 24 hours. Serum AA concentration peaked at 407mcg/ml at 36 hours, well within the described range for septic foals. Colostral concentration of AA in this foal's dam was 967 mcg/ml. The other foal developed bilateral hind-limb paralysis after Caesarian section delivery and was diagnosed on post mortem with ischemia in the lumbo-sacral spinal cord, but there was no post mortem evidence of sepsis. That foal did not develop an abnormal neutrophil count and besides initial depression, attributed to hypoxia after C-section delivery, no other clinical findings suggestive of sepsis were observed at any time. However, its serum AA concentration peaked at 560 mcg/ml at 36 hours.

Of the 5 foals that did develop clinical or laboratory findings suggestive of sepsis (Group 3), four foals had serum AA concentrations that peaked in the exceedingly high range of (500 – 1050 mcg/ml) (Table 3). However, one had a serum AA concentration that peaked at 191mcg/ml, slightly below the range previously described for sepsis. The peak concentration of serum AA concentrations in these foals occurred at different times consistent with the non-specific acute phase response. In one foal, the peak occurred at 24 hours and lasted to 60 hours at which time it began to decrease. In the other foals, the peak occurred at 48 to 60 hours and then began to decrease. In most cases, the decreased neutrophil count preceded the rise in serum AA concentration.

Statistical analysis

In the analysis of all of the foals, the random effect of foal was not significant ($p = 1.00$). The fixed effects, (disease and time), were both significant ($p<0.001$), and the 2-way interaction was significant ($p < 0.01$) (Fig. 1). Time, fitted as a categorical variable, gave a better fitting model compared to when fitted as a continuous, quadratic or logistic variable. The least-square mean serum AA for abnormal foals was significantly larger ($p< 0.05$) than for normal foals with means of 66.3 and 13.3 respectively. The least-square mean serum AA for normal foals was significantly lower ($p< 0.05$) than for abnormal foals after 12 hours (see Table 4).

In the analysis of the 10 abnormal foals, the random effect of foal was not significant ($p = 0.206$). The fixed effects, suspicion of sepsis status and time, were both significant ($p<0.001$), however, the 2-way interaction was not significant ($p = 0.457$). The least-square mean serum AA for septic foals was significantly larger ($p< 0.05$) than for non-septic foals with means of 123.3 and 35.0, respectively.

Taking the 10 abnormal foals into account when comparing Groups 2 and 3, and using the serum AA threshold concentration of > 200mcg/ml for the differential diagnosis of sepsis in compromised foals, the sensitivity of the serum AA results was 80 per cent and the specificity was 60 per cent.

Previous studies which evaluated prognostic and diagnostic variables in neonatal foals used a prevalence of 30-50 % for sepsis in abnormal (hospitalized) foals (19-20). Using a prevalence of neonatal sepsis of 40 %, the PPV and NPV of using serum AA concentration greater than 200 mcg/ml for the differential diagnosis of sepsis in neonatal foals were 57 % and 82 % respectively. If the serum AA concentration threshold for the

diagnosis of sepsis were increased to 500 mcg/ml, the sensitivity and specificity would both be 80 %, additionally the PPV and NPV would be 73% and 86% respectively.

Discussion

Of the 19 foals included in this study, only 9 were considered completely normal by the definition described in the methods section. One might question the extremely high number of complications in this study. The definition of normality that was used, however, was strict; foals that were considered abnormal in this study might have gone undetected in an environment where complete blood counts are not performed routinely every day, and foals are not intensively monitored.

Of the 10 abnormal foals, 5 had < 2000 cells per ml at some point over the 72 hours. These were considered to have clinical or laboratory findings suggestive of sepsis in accordance with a study describing the clinico-pathologic features of sepsis in foals, where the most useful white blood cell parameters were neutropenia, the presence of band neutrophils (greater than 0.2 X 10^9/liter) and toxic changes in the neutrophil population (6). However, only one foal showed clinical signs which were suggestive of sepsis i.e. fever, depression and petecchial hemorrhages.

As in the human infant, there is no definitive pre-mortem test for sepsis in the neonatal foal. In human infants, acute phase protein measurements are used to indicate sepsis (21-25). Recently serum AA was proposed as an aid in the diagnosis of sepsis in neonatal foals (12). In our study, there was a significant difference in the serum AA concentration between the normal foals (Group 1) and the abnormal foals (Groups 2 and 3) after 12 hours similar to that previously described (10). Serum AA concentrations clearly differentiate between groups of normal and compromised foals in the neonatal period. In addition, there was a significant difference in serum AA concentrations between compromised foals with a high index of suspicion of sepsis and those in which a non-

infectious problem was present. The change in serum AA concentration over time between these groups, however, did not differ.

It is suggested that serum concentrations of AA greater than 200mcg/ml are consistent with a diagnosis of sepsis in the compromised neonatal foal, and that concentrations of 20-200mcg/ml are consistent with non-septic inflammation. Without a way to definitively diagnosis sepsis in foals it is difficult to comment on the sensitivity of this test. However, we can make an observation on the specificity of the test for the foals in this study. Two of five abnormal foals with no clinical or laboratory evidence of sepsis had serum AA concentrations in the range associated with sepsis when a serum AA threshold of 200mcg/ml was used. In human infants, it is believed that in cases of perinatal asphyxia and infection equivalent increases in serum levels of inflammatory mediators are found (26). Similarly we found an extremely high serum AA concentration in the foal with spinal ischemic necrosis and no clinical, laboratory or post-mortem evidence of sepsis. This foal was delivered by Caesarian section and most likely suffered ischemia during the delivery. However, this finding lends further support to the argument that serum AA is not a specific indicator of sepsis. A second foal had a serum AA concentration in the septic range but did not have any clinical or laboratory evidence suggestive of sepsis, however, the concentration of AA in the colostrum of this foal's dam was exceedingly high (data not shown, see chapter IV). Therefore, colostral AA may be absorbed in the early post partum period of increased macromolecular absorption in the neonatal foal. We have shown a significant correlation exists between peripartum colostrum AA concentration in the dam and post-partum serum AA concentrations in their foal approximately 48 h after birth (27). Alternately, the mild post-partum colic in this mare

may have been caused by a peri-partum event that may also have lead to a post partum increase in serum AA concentration in the neonatal foal.

The specificity of serum AA concentrations for the differential diagnosis of sepsis using a threshold concentration of 200 mcg/ml was 60%, the PPV (i.e. the probability of having the disease if the test was positive) at a prevalence of 40% was only 57 per cent and the NPV (i.e. the probability of being disease free if the test is negative) was 82 per cent. When the threshold concentration was raised to 500 mcg/ml, the specificity of the test increased to 80 per cent, PPV increased from 60 per cent to 73 per cent and NPV increased to 86 per cent. While the usefulness of using serum AA in the differential diagnosis of sepsis in compromised foals increases by raising the threshold of the test, it still does not approach the usefulness of the acute phase protein CRP in the diagnosis of neonatal sepsis in human infants (sensitivity 100 per cent, specificity 94 per cent, PPV 91.6 per cent and NPV 100 per cent) or using the sepsis score in foals (sensitivity 93 per cent, specificity 86 per cent, PPV 89 per cent and NPV 92 per cent).

We suggest that as an indicator of sepsis in compromised neonatal foals, the threshold concentration of serum AA indicative of sepsis should be raised to 500mcg/ml and it should be interpreted carefully in the light of clinical and laboratory findings. In addition, investigation of the measurement of CRP as an indicator of sepsis in compromised foals should proceed.

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Table 1. Group 1 foal serum AA concentrations (mcg/ml)

Mare	Comments	Foal serum						
		0 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
17	Normal	7.6	8.1	43.5	23.4	24.4	14.5	12.1
25	Normal	7.8	9.5	12.8	12.7	13.7	9.0	10.6
27	Normal	6.3	13.2	19.2	20.4	22.2	13.5	12.1
28	Normal	11.0	16.8	20.7	21.8	18.3	15.4	16.8
29	Normal	4.5	55.4	94.0	96.0	81.6		30.5
30	Normal	8.7	6.6	12.4	11.0	15.0	15.1	
31	Normal	4.3	15.1	20.3	61.7	197.4	53.5	29.1
32	Normal	0.6	6.7	19.5	17.1	13.1	11.7	9.2
33	Normal	2.8	34.6	5.5	10.9	3.8	0.1	4.3
	Mean	6.0	18.4	27.5	30.5	43.3	16.6	15.6
	SD	3.2	16.3	27.0	29.0	62.0	15.7	9.5

Table 2. Group 2 foal serum AA concentrations (mcg/ml)

Mare	Comments	Foal serum						
		0 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
12	C-section, hypoxia, ischemia	5.1	58.0	45.3	557.9	418.2	82.8	102.5
19	Mildly decreased neutrophils	7.8	24.4	74.7		60.3	52.3	31.7
36	Premature	0.6	12.6	43.4	39.5	28.2	11.4	32.8
37	Maternal colic, slow to nurse	2.3	323.8	262.4	406.8	373.0	259.7	239.8
38	Mild dystocia	7.0	5.0	4.5	6.0	5.7	54.5	5.0
	Mean	4.6	84.8	86.1	252.5	177.1	92.1	82.3
	SD	3.1	135.1	101.7	272.8	201.0	97.1	95.1

Table 3. Group 3 foal serum AA concentrations (mcg/ml)

Mare	Comments	Foal serum						
		0 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
15	Placental separation, slow to nurse, neutrophil < 2000 cells/ml	3.9	22.5	133.8	185.3	191.1	129.3	68.1
16	Neutrophils < 2000 cells/ml	10.9	10.3	14.3	48.4	412.3	1042.0	851.1
17	Neutrophils < 2000 cells/ml	3.7	69.8	315.2	432.2	510.3	415.2	221.9
24	Mild dystocia; neutrophils < 2000 cells/ml	6.0	28.9	215.5	363.2	457.5	622.2	395.0
34	Placentitis, clinical sepsis, neutrophils < 2000 cells/ml	2.1	124.5	744.6	744.6	744.6	744.6	723.4
	Mean	5.3	51.2	284.7	354.7	463.1	590.7	451.9
	SD	3.4	46.7	279.8	264.9	198.8	343.5	330.4

Table 4. Least square means and 95% confidence intervals

Time	Normal			Abnormal			
	LSM	Lower	Upper	LSM	Lower	Upper	P-value
0	4.7	2.0	10.8	3.9	1.7	8.5	0.737
12	14.1	6.1	32.6	33.3	15.1	73.8	0.142
24	20.2	8.7	46.7	83.7	37.8	185.2	0.016
36	22.7	9.8	52.5	156.5	67.7	361.5	0.002
48	22.7	9.8	52.6	170.6	77.1	377.5	0.001
60	8.2	3.4	20.0	168.2	76.0	372.3	<0.001
72	13.2	5.4	32.0	116.7	52.7	258.4	<0.001

Figure 1: Mean serum AA concentrations over time for normal and abnormal foals

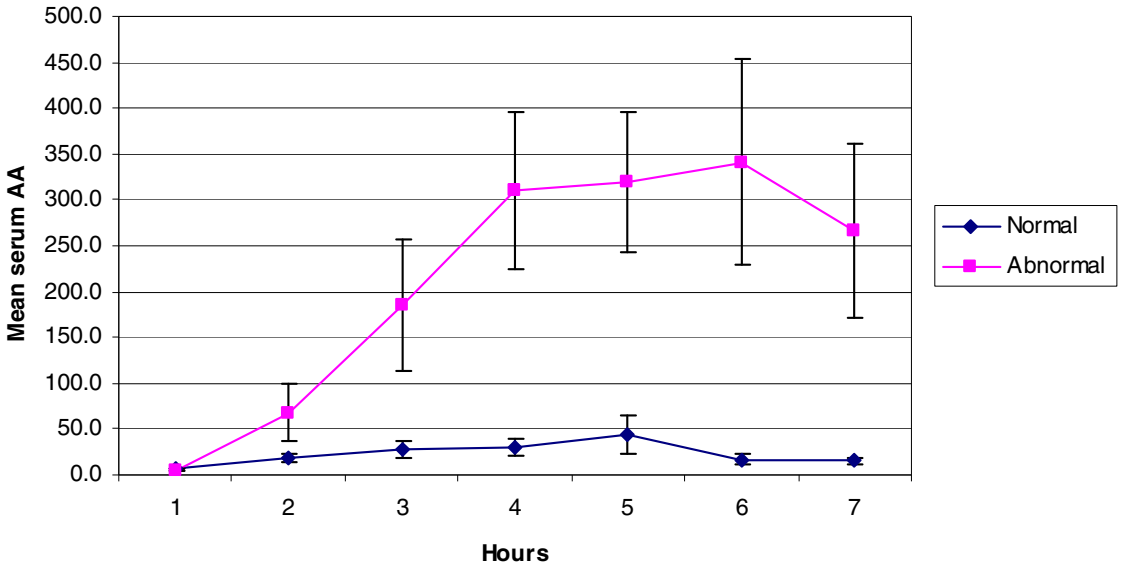
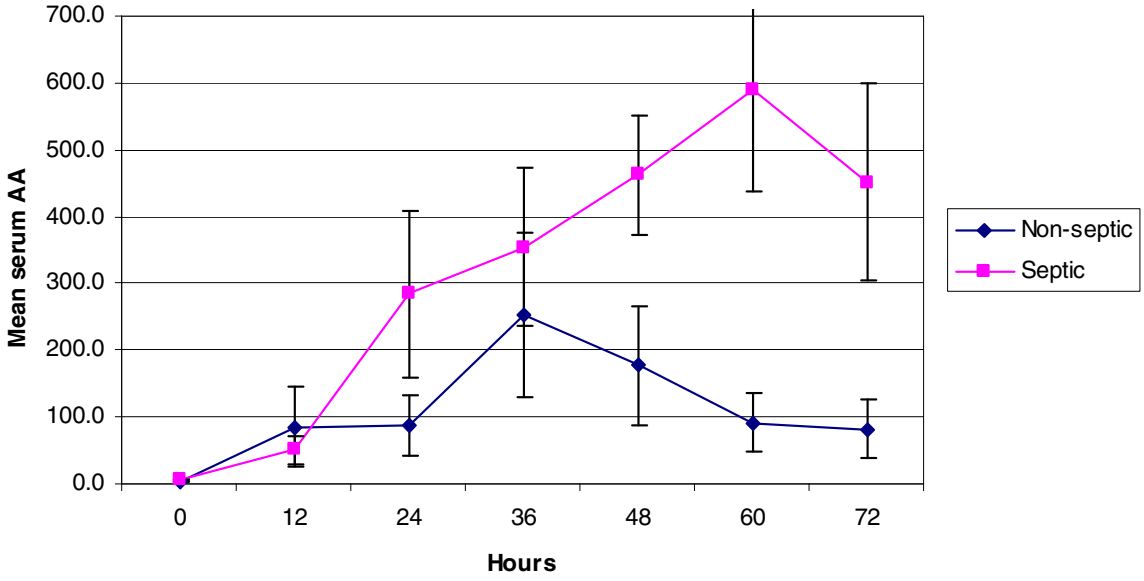


Figure 2: Mean serum AA concentrations over time for abnormal foals



CHAPTER IV

AMYLOID A IN EQUINE COLOSTRUM AND EARLY MILK

Abstract

The objective of this study was to investigate the presence of amyloid A in equine colostrum and milk. Thirty-eight peripartum mares were included in the study. Amyloid A (AA) concentrations were measured in peripartum maternal serum and colostrum and in early milk samples and found in all samples. Mean maternal serum concentrations of AA at parturition were consistent with previous studies, although the variation was wide. Colostrum and milk AA concentrations were consistently higher than peripartum maternal serum AA concentration. There was no correlation between serum and colostrum AA concentrations at parturition. There was no significant association found between the factors examined: age, breed, farm of residence, length of gestation, clinical abnormality or induction of parturition and the mare serum AA concentration at parturition. There was no effect of age, breed, farm of residence, or clinical abnormality on the concentration of AA in the colostrum or milk. For every 1 day increase in gestation there was an incremental fall in AA concentration in colostrum and milk. Mares that were induced to foal had a significantly lower AA concentration in colostrum and milk compared with mares that were not induced.

We conclude that AA is consistently present in equine colostrum and early milk at higher concentrations than in the maternal serum at parturition. Milk AA has been shown to protect the human infant intestine against infectious disease. Further studies are necessary to investigate the dynamics and the function of this colostral and milk protein and to assess whether equine colostral and milk AA has a protective function in the equine neonatal intestine.

Introduction

Serum amyloid A (AA) is an acute-phase protein that increases in response to inflammatory stimuli in horses (1). Serum AA concentration in normal pregnant and non-pregnant mares has been previously published (1); 6-7 year old mares had average serum AA concentrations of 21.24 mcg/ml; 8-10 year old mares had average concentrations of 28.49 mcg/ml; 11-15 year old mares had average concentrations of 21.04 mcg/ml; 16-20 year old mares had concentrations of 26.58 mcg/ml and mares older than 21 years had average concentrations of 30.33 mcg/ml . In the same study, pregnant mares 2 days pre-partum had serum concentrations of 23.81 mcg/ml and one day post-partum had serum AA concentrations of 51.83mcg/ml. Three days post-partum mean serum AA concentrations had increased to 136.78 mcg/ml indicating that parturition is an inflammatory event for the brood mare.

Acute phase proteins have been detected in the colostrum and milk of many species (2-4). An isoform of AA was recently demonstrated in the colostrum and early milk of women, cows, ewes, sows and one mare (3). This colostral and milk isoform of AA is called milk amyloid A and is synthesized extra-hepatically in mammary gland cells. It is produced by healthy animals, expressed by bovine mammary gland epithelial cells and secreted in abundance as part of a normal process in response to prolactin (5). In cows, the concentration of this protein is particularly high in colostrum but declines rapidly to reach low concentrations by 3 days post-partum (3). Besides being synthesized and secreted in normal colostrums Lipopolysaccharide and *Staphylococcus aureus* lipotechoic acid stimulate synthesis of milk AA (5, 6), and cows with mastitis have very high concentrations of AA in the milk. The function of this protein in relation to the diseased tissue has not been elucidated; however, the

protein is used as an early marker of mastitis in cows (6-10). Recently, serum AA accumulation in mastitic milk was shown to result from both local synthesis of AA (milk AA) and hepatic-derived AA (serum AA) that gains access to the milk due to increased permeability of the blood-milk barrier (9). However, a pathogen must be present in the mammary gland for AA from the serum to accumulate in milk (11).

Amyloid A was previously detected at a high concentration (704 mcg/ml) in the colostrum of one normal mare (3). When AA was affinity purified from that sample and sequenced, only one isoform (milk AA) was detected, and it was concluded that equine colostrum contained only milk AA (A Weber, personal communication), at least in normal lactating mammary glands. Clinical mastitis is uncommon in mares, so it is not unusual that only the mammary-derived amyloid A is present in mares' colostrum and milk. The concentration of milk AA in the mare was particularly high (704 mcg/ml) in comparison with that in bovine colostrums (267 mcg/ml) (3) and in previously reported equine serum at parturition (23.8 mcg/ml) (1). The concentration of this protein in early milk in that mare was not determined. To date, no further work has been done to elaborate the presence and concentration of this protein in equine colostrum or milk.

Recent research has focused on the many beneficial factors in colostrum and milk of a number of species, which promote both intestinal development and the development of other neonatal tissues (12-18). In humans milk AA has been shown to have protective effects in the infant intestine (19, 20). Given its protective effects it is appropriate for this protein to be delivered in high concentrations to the developing neonatal intestine. Milk AA functions in preventing the attachment of pathogenic bacteria to the intestinal wall and in the up-regulation of local protective factors such

as mucins (19, 20). Due to these protective effects, the milk AA protein has been promoted as a deterrent in the development of necrotizing enterocolitis in human infants (20). Necrotizing enterocolitis also frequently affects compromised neonatal foals and has a high case mortality risk (21-24). Unfortunately, there has been limited research in this field in the mare (25-28). It is important that the beneficial components in mare colostrum and milk are identified as they have been in other species.

The purpose of this study was to determine the concentration of milk AA in colostrum and early milk in a large group of broodmares and examine the variables that might affect those concentrations.

Materials and Methods

Animals

The study was conducted between January 2003 and July 2004. Thirty-eight pregnant mares, 30 in residence at the Oklahoma State University College of Veterinary Medicine ranch and 8 pregnant mares in residence at the Oklahoma State University Animal Science facility were used for this study. Eighteen mares were Thoroughbreds, 1 was Hanoverian and 19 were Quarter Horses, with an age range of 4 to 23 years old. There was no evidence of clinical mastitis in any mare. Careful records were kept in the peri-partum period with regard to clinical conditions, induction of parturition and complications of parturition.

The low-dose oxytocin protocol (29) was used to induce parturition in the mares. Calcium concentration in the mammary secretions was used to predict proximity to parturition. Sampling was initiated 1 wk prior to expected foaling date or if the mare displayed udder development. Samples were taken daily at 0800 until the calcium concentration began to rise and then twice daily at 0800 and 1800. When the calcium concentration was >200 ppm in two samples 24 h apart, parturition was deemed imminent and the mare could be safely induced. All eight of the OSU Animal Science Department mares were allowed to foal spontaneously. Before induction, the mare's tail was wrapped, her perineum was washed with soap and water, and she was placed in a 15 x 25 ft. maternity stall bedded with clean straw. Parturition was induced with 2.5 i.u. oxytocin (VEDCO, St Joseph, MO, USA) per dose given IV at 20-min intervals until second stage labor was observed. Physical examination of the mare was done immediately following parturition and peri-partum complications were recorded.

Samples

Colostrum samples were collected in plain plastic tubes from each mare at parturition. Milk samples were obtained in plain sterile plastic tubes at 12, 24 and 48 hours post partum from 30 mares. The colostrum and milk samples were promptly refrigerated and then stored in a freezer at –20^{o} C. Blood samples were collected in plain glass tubes from each mare at parturition. Blood samples were immediately refrigerated until a clot had formed; then they were centrifuged at 2500 rpm for 5 minutes, and the serum was separated and frozen at –20^{o}C for later analysis.

Analysis of colostrum, milk and serum amyloid A

All samples were analyzed for serum AA concentration using Tridelta´s Phase™ Range SAA assay (Tridelta Limited, Maynooth, Co Kildare, Ireland). This ELISA as has been previously described for colostrum and serum from several species including the mare (3, 30). Data are expressed as mcg/ml. On the first plate, samples were measured in duplicate and thereafter once only. The minimum detectable concentration of serum AA under the assay was 5 ng/ml. The intra-assay coefficient of variation was 7.7% and 5.3% at mean serum AA concentrations of 74 and 128 mcg/ml respectively. The inter-assay coefficient of variation was 10.8% and 8.7% at mean serum AA concentrations of 81 and 134 mcg/ml respectively.

Statistical analysis

Data were analyzed to determine whether age, breed, farm of residence, gestation length, clinical status or induction of parturition affected the concentrations of the protein in the mares' serum, colostrum or milk.

Correlation between mare serum and colostrum concentration at parturition was assessed using Pearson correlation (Microsoft Excel). Statistical analyses of the

association of the various factors with serum, colostrum and milk AA concentrations were performed using SAS® v 9.1 (SAS Institute Inc., 2003).

For serum AA, the natural log of AA was modeled using a general linear model, fitted using the GLM procedure in SAS. A forward selection procedure was used to determine an appropriate model; factors remaining significant ($P < 0.05$) were retained in the model. The variables tested as fixed effects were: age, breed, farm of residence, gestation length, clinical examination (normal/abnormal) and induction of parturition (Yes/No, note a c-section was classified as No).

For colostral and milk AA, the natural log of AA was modeled using a mixed model, fitted using the Mixed procedure in SAS. A forward selection procedure was used to determine an appropriate model; factors remaining significant ($P < 0.05$) were retained in the model. The repeated measurements for a mare at different times were dealt with by including mare as a random effect. The significance of the random effect was tested by comparing a model excluding the random effect using a likelihood ratio test.

The variables tested as fixed effects were: time, age, breed (Hanoverian/QH/TB), farm of residence, gestation length, clinical examination (normal/abnormal) and induction of parturition (Yes/No, note a Caesarian-section was classified as No). Time was checked for linearity by the inclusion of quadratic terms, and comparing models that included log of time, time as a categorical variable and time as a continuous variable.

Results

Thirty-eight mares were included in the study. Information on age, breed, farm of residence, gestation length, induction of parturition and clinical findings are presented in Table 1.

The mare serum, colostrum and milk AA concentrations are presented in Table 2. The mean concentration of this protein in the colostrum was 154 +/- 174 mcg/ml; in 12 hour milk was 296 +/- 246 mcg/ml; in 24 hour milk was 315 +/- 229 mcg/ml and in 48 hour milk was 332 +/- 252 mcg/ml. One mare delivered prematurely at 317 days (#36); the foal was clinically normal, but the mare's serum AA concentration was relatively high compared to previously published values (61 mcg/ml). One mare suffered mild dystocia i.e. one leg folded back at the knee (#38); however this was quickly corrected, and the foal delivered promptly. Serum AA was also relatively high in this mare (58 mcg/ml). One mare had no abdominal contractions and was assisted to foal (#24). Her serum AA remained low (20.5 mcg/ml). One mare suffered placentitis (#34) and the foal was presumed septic but this mare's serum AA remained low (5.7 mcg/ml). One mare suffered mild post partum colic which resolved without treatment (#37). This mare's serum AA concentration was high (136 mcg/ml), and her colostrum AA concentration was also particularly high (967 mcg/ml). One mare suffered atrial fibrillation for 12 hours post partum which converted spontaneously to a normal rhythm (#1). Her serum AA concentration was similar to previously published normal values (32mcg/ml) (1).

Two mares retained the fetal membranes (#13 and # 20) and one mare had placenta previ (red bag delivery) (#15); however, the serum and milk AA concentrations in these mares were not particularly high (Table 2). One mare underwent elective

Caesarean section due to an intra-pelvic mass (#12). Although pre- and post-surgical serum AA concentrations in this mare differed greatly (from 4.3 to 291 mcg/ml), there was no difference between the colostrum and milk concentrations of AA in this mare from those in the rest of the group.

Statistical analysis – There was no correlation between mare serum AA concentration at parturition and colostral AA concentration in this group of mares. In the analysis of AA in the mare serum at parturition none of the examined variables age, breed, farm of residence, gestation length, induction of parturition or clinical examination were significant ($p>0.05$).

In the analysis of AA in colostrum and milk the random effect of mare was significant ($p < 0.05$). The within mare variation was 23%. The variables time ($p<0.001$), gestation length ($p < 0.05$) and induction of parturition ($p < 0.05$) were significant. The other variables, breed, age, farm of residence and clinical examination were not significant. The two-way interaction of time x induction was not significant. Gestation length was included in the model as a continuous variable. For every 1 day increase in gestation length there was a fall in log AA by a factor of 0.98. When AA was compared at each of the time categories, the mean AA at time 0 was significantly lower ($p<0.001$) than at all other times. The mean AA concentrations at all other times were not significantly different.

Discussion

The mean serum AA concentration in pregnant mares at parturition in this study (36 mcg/ml) is slightly higher than that presented in a previous study (23.8 mcg/ml) (1); however, the variation in maternal serum concentration in this study was wide (Table 1). The population of horses described in the previous study included only Thoroughbreds and Arabians. This study included 18 Thoroughbreds, 19 Quarter Horses and one Hanoverian. When breed effects were examined, there was a tendency ($p=0.08$) towards lower serum concentrations of this protein in Thoroughbreds (average concentration 20.2 mcg/ml) than in Quarter Horses (average concentration 52.5 mcg/ml) although breed difference was not significant (Table 3). This may explain the lower concentration seen in the previous study which included primarily Thoroughbreds. The concentration seen in the serum of the Hanoverian mare (5.6 mcg/ml) was similar to that seen in many other mares.

There was no significant association found between the age of the mares and the concentration of serum AA at the time of parturition. This infers that the production of this protein is not related to the health or otherwise of the mammary gland and suggests that it may have more to do with providing large quantities of this protein to the neonate no matter the age of the mare. No association was found between the farm of residence of the mares and their serum AA concentrations at parturition. This is not unexpected, because management of the mares on the two farms and their health statuses were not dissimilar. Although the only mare that foaled prematurely did have a high serum AA concentration compared with the mean (69.1 mcg/ml vs 36 mcg/ml) an association between the length of gestation and the maternal serum AA concentrations at parturition could not be inferred.

There were a variety of clinical abnormalities detected in some of the mares. One mare had placentitis, one had slight postpartum colic, two suffered mild dystocia and one had transient atrial fibrillation for 12 hours post partum. One mare underwent elective Caesarian section at full term and predictably its serum AA concentrations increased markedly after surgery (from 4.3mcg/ml to 291mcg/ml in 12 hours). Two mares retained the fetal membranes and one mare suffered placenta previ (red-bag delivery). However, no association was found between clinical abnormality and maternal serum AA concentration at parturition. Perhaps these conditions were localized enough not to cause a systemic response; or if they were severe enough to cause a systemic response, we were sampling at the beginning of the 16 hour lag period after inflammatory stimulus before a rise in serum AA is detectable. Sequential serum samples from the 72 hours after parturition would be needed to draw any meaningful conclusions about the association between the health of the mare at parturition and the concentration of serum AA in her serum. No association was found between induction of parturition and serum AA in the mare at parturition. This is not surprising as induction was performed at parturition, and the lag period after an inflammatory stimulus before serum AA concentrations rise in the serum is about 16 hours.

Recent work has detected a mammary-derived isoform of AA in a number of species including one mare (3). The mare's colostrum had an AA concentration of 704 mcg/ml. In this study we demonstrated the consistent presence of AA in the colostrum and early milk of a large number of mares (Table 2). The concentration of AA in colostrum and milk was found to be considerably higher than the concentration in the serum of each mare at parturition. Therefore, the AA in the colostrum and milk

samples in this study may be mammary derived and not derived from serum AA or a combination. This is consistent with the previous study which, by affinity purification and sequencing, revealed only one isoform (milk AA) in the sample of mare's colostrums that was examined (3). In bovine mastitic milk, both serum and milk isoforms of AA are present in the milk (9). There was no evidence of mastitis in the mares in this study. In addition, and as found in cattle, there was no correlation detected between serum AA and colostral AA concentrations at parturition.

In healthy bovine milk, AA concentrations were minimal by day 2-3 post-partum (3); equine milk AA concentrations, on the other hand, remained consistently high in this study until at least 48 hrs post partum. In fact, the concentration of this protein in the milk after 12 hours was significantly higher than that in colostrum. This suggests a possible important role for this protein in the equine neonatal intestine similar to that found in humans and cows (19, 20). A conserved four-amino-acid motif (TFLK) contained within the first eight N-terminal amino acid residues of this protein is thought to be responsible for its beneficial effects in the intestine (19). This protein protects the infant intestine by enhancing the innate immunity of intestinal cells (19, 20). It up-regulates production of the intestinal mucin, MUC3, in infant intestinal cells, which provides a protective barrier in the gut (19, 20). Human milk AA also has a species-specific effect in decreasing the attachment of pathogenic organisms to human neonatal intestinal cells (20). If, as in other species, the equine isoform of AA has protective effects in the neonatal intestine, it may be appropriate to have a higher concentration of this protein in the milk from which it will not be absorbed after gut closure.

Age, breed and farm of residence did not appear to influence the concentration of AA in the colostrum or the early milk. Nor did clinical abnormalities present affect these concentrations. Although prolactin, lipopolysaccharide and *Staphylococcus aureus* lipotechoic acid are known to stimulate the production of this mammary-derived protein in other species (5,6), it is not known what stimulates production of this protein in the equine mammary gland. In any event none of the mares suffered clinical endotoxemia nor did any mare display clinical evidence of mastitis; therefore, the stimulus for milk AA production in this study was most likely physiologic.

Gestation length did appear to be associated with colostrum and milk AA concentration. Three hundred and forty two days is the recognized normal gestation length in mares; however, mares have been known to foal naturally from 320 to 380 days. For every one day increase in gestation length, the log of AA in the colostrum and milk decreased by a factor of 0.98. This may be due to increased volume of milk present in the mammary gland causing a dilution effect on the locally produced protein, or other physiologic reasons may account for this effect.

Induction of parturition with the low dose oxytocin protocol was also associated with lower baseline concentrations of AA in the colostrum and milk, although the change in concentration over time in induced and non-induced mares was similar. It is unknown what effect, if any, oxytocin would have on the production of this mammary derived protein, beyond inducing milk let down and diluting the concentration of AA protein in the milk. However, as the foals begin to nurse and endogenous hormone is released, the effect of the miniscule amounts of this hormone required to induce parturition (10 - 20 i.u.) would be obscured.

Many equine neonatologists promote the withdrawal of enteral feeding from compromised neonates to avoid overloading the intestine with rich ingesta which may promote bacterial colonization. Withdrawal of enteral feeding in compromised foals, however, may result in restriction of access of the beneficial components of colostrum and milk to the patients that are in most need of them. Recent studies have focused on the benefits of colostrum to neonates and the vast array of beneficial components therein that are involved in the protection, growth, development and maturation of the neonatal intestinal and immune systems and other tissues (12-18). There has been limited research into the biological factors present in mares' colostrum and milk (25-28). The milk AA protein has previously been detected in the colostrum of one mare (3). We now propose that AA protein is present not only in colostrum but also in the early milk of all mares and is likely to have an important function in the protection of the neonatal intestine. This protein has been proposed as a prophylactic treatment for the prevention of gastrointestinal diseases such as necrotizing enterocolitis and infectious diarrhea in infants (20), conditions that frequently affect compromised neonatal foals (21-24).

We concluded that AA is present in equine colostrum and early milk. Colostral/milk AA most likely represents equine milk AA, a mammary gland-derived acute phase protein identified in bovine, equine, ovine and human colostrum that has been shown to enhance the innate immune system in the human infant intestine. Given the relatively high concentrations of the milk AA protein detected in this study in equine colostrum and also in early milk (which contrasts starkly with bovine milk), further investigation into the intestinal and systemic effects of this protein and other colostral

factors in the neonatal foal are warranted to determine the function of colostral proteins in the health and development of the equine neonate.

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Table 1. Age, breed, gestation, induction, farm of residence and clinical examination data for all mares

Number	Age	Breed	Gestation	Induction	Clinical Exam	Farm
#1	16	QH	339	No	AF for 12hr	CVM Ranch
#2	12	QH	338	No	Normal	CVM Ranch
#3	22	QH	327	No	Normal	CVM Ranch
#4	9	TB	322	Yes	Normal	CVM Ranch
#5	13	TB	354	No	Normal	CVM Ranch
#6	13	QH	354	No	Normal	CVM Ranch
#7	16	QH		No	Normal	CVM Ranch
#8	12	TB	349	No	Normal	CVM Ranch
#9	5	TB	354	Yes	Normal	CVM Ranch
#10	12	Han	356	Yes	Normal	CVM Ranch
#11	10	TB	366	No	Normal	CVM Ranch
#12		QH		C-section	Elective C Section	CVM Ranch
#13	5	QH	353	No	Retained placenta	An. Science
#14	8	QH	343	No	Normal	An. Science
#15	4	TB	342	Yes	Red bag	CVM Ranch
#16	8	QH		Yes	Normal	CVM Ranch
#17	7	QH	335	No	Normal	CVM Ranch
#18	13	QH	342	No	Normal	An. Science
#19	14	TB	351	No	Normal	CVM Ranch
#20	8	QH	345	No	Retained placenta	An. Science
#21	4	QH	338	No	Normal	An. Science
#22	23	QH	375	No	Normal	An. Science
#23	15	QH	338	No	Normal	An. Science
#24	8	TB	343	Yes	Normal	CVM Ranch
#25	6	TB	339	Yes	Normal	CVM Ranch
#26	17	TB	331	Yes	Normal	CVM Ranch
#27	12	TB	343	Yes	Normal	CVM Ranch
#28	14	TB	353	No	Normal	CVM Ranch
#29	6	TB	337	No	Normal	CVM Ranch
#30	20	TB	337	Yes	Normal	CVM Ranch
#31	15	QH		Yes	Normal	CVM Ranch
#32	6	TB	337	Yes	Normal	CVM Ranch
#33	10	TB	344	Yes	Normal	CVM Ranch
#34		QH	338	No	Placentitis	CVM Ranch
#35	21	QH	335	No	Normal	An. Science
#36	9	QH	317	No	Normal	CVM Ranch
#37	11	TB	339	No	Mild colic postpartum	CVM Ranch
#38	4	TB	340	Yes	Mild dystocia	CVM Ranch

Table 2. AA concentrations of maternal serum, colostrum and milk (mcg/ml)

Number	Serum	Colostrum	Milk 12 hr	Milk 24 hr	Milk 48hr
#1	32.2	134.8	439.1	593.7	
#2	69.7	489.2	618.8	688.0	789.4
#3	1.1	441.5	830.9	875.0	922.3
#4	16.0	166.0	907.5	692.6	518.9
#5	17.9	96.1	460.6	401.7	493.2
#6	65.0	173.3	417.6	517.2	452.4
#7	387.7	156.7	683.4	549.1	429.1
#8	0.1	191.6	536.5	464.2	531.9
#9	1.2	285.7	550.6	286.8	307.3
#10	5.6	242.1	307.3	755.3	719.2
#11	1.9	253.8	302.2	230.1	365.6
#12	4.3	21.5	56.9	134.5	72.3
#13	7.7	76.2	90.6	169.5	76.6
#14	6.2	56.2	260.6	224.8	101.1
#15	3.0	13.1	82.0	39.1	125.8
#16	36.0	68.4	145.6	145.8	131.0
#17	6.3	90.2	127.9	231.0	159.3
#18	8.7	42.4	43.8		173.1
#19	11.0	85.6	214.0	140.7	179.6
#20	6.2	30.9	66.2	231.2	285.4
#21	52.2	300.8	133.2	210.7	369.2
#22	8.0	47.7	6.2	261.5	399.5
#23	155.5	191.5	509.0	347.2	754.1
#24	20.5	125.6	280.5	177.5	
#25	11.3	95.8	110.4	155.5	121.0
#26	8.2	29.4	74.2	161.6	104.7
#27	8.3	41.7	165.1	62.9	75.1
#28	20.4	93.0	226.6	107.1	41.0
#29	4.9	76.8	190.2	156.8	520.6
#30	14.7	35.8	48.2	115.4	86.1
#31	41.5	205.0			
#32	5.8	57.5			
#33	25.3	58.7			
#34	5.7	81.6			
#35	34.6	76.2			
#36	69.1	79.4			
#37	135.6	966.7			
#38	58.0	154.3			

Table 3. AA concentrations of maternal serum at parturition in Thoroughbreds and Quarter Horses (mcg/ml)

TB	Serum AA		QH	Serum AA
Number			*Number*	
#4	16.0		#34	5.7
#5	17.9		#35	34.6
#8	0.1		#36	69.1
#9	1.2		#31	41.5
#11	1.9		#1	32.2
#15	3.0		#2	69.7
#19	11.0		#3	1.1
#24	20.5		#6	65.0
#25	11.3		#7	387.7
#26	8.2		#12	4.3
#27	8.3		#13	7.7
#28	20.4		#14	6.2
#29	4.9		#16	36.0
#30	14.7		#17	6.3
#32	5.8		#18	8.7
#33	25.3		#20	6.2
#37	135.6		#21	52.2
#38	58.0		#22	8.0
			#23	155.5
Mean	20.2		Mean	52.5
SD	31.7		SD	89.5

CHAPTER V

ABSORPTION OF COLOSTRAL AND MILK AMYLOID A IN THE EQUINE NEONATAL INTESTINE

Abstract

The objective of this study was to investigate the possibility of neonatal intestinal absorption of the colostral and milk amyloid A (AA) protein. Nineteen mare-and-neonatal foal pairs were included in the study. AA concentration was measured in peri-partum maternal serum, colostrum and early milk samples and in foal serum samples from birth to 72 hours postpartum. Mean maternal serum concentrations of AA were consistent with previous studies, although the variation was wide. AA was found in all colostrum and milk samples and concentrations were consistently higher in the colostrum and milk than in the peri-partum maternal serum. Foal mean serum AA concentrations during the post partum period were consistent with those previously described. Colostral AA concentration was significantly correlated with serum AA concentration in normal foals at 48 and 60 hours post partum.

Introduction

Acute phase proteins (APPs) have been detected in the colostrum and milk of many species (1-3). An isoform of amyloid A (AA) was recently demonstrated in the colostrum and early milk of women, cows, ewes, sows and one mare (2). This iso-form of amyloid A, detected in colostrum and milk, called milk amyloid A, is synthesized extra-hepatically in mammary gland cells. It is produced by healthy animals, expressed by bovine mammary gland epithelial cells and secreted in abundance as part of a normal process. In that study the mare colostrum had an AA concentration of 704 mcg/ml. Concentrations of AA in the colostrum of healthy cows ranged from 89 to 484mcg/ml and decreased to almost undetectable levels by day 4 post partum. Milk concentrations of this protein in the mare were not examined in that study.

It has been shown that acute phase proteins in the colostrum of other species are absorbed into the neonatal circulation (3-5). In the neonatal foal large proteins are absorbed intact as functional proteins across the intestinal wall (6, 7). Absorption is maximal at about 6 hours after ingestion but increased permeability to macromolecules persists for up to 24 hours. To date there are no studies on the intestinal transfer of colostral or milk APPs in mares; however, it seems likely that such colostral proteins are absorbed.

Sepsis occurs most commonly in the foal in the first 48 hours of life and the modes of infection are intrauterine, oral, respiratory and via navel cord or traumatic penetration of the skin (8). The organisms set up infection in the entry organ. The bacteria and their toxins invade the blood stream, causing damage to endothelia and the release of cytokine mediators that initiate the systemic inflammatory response and the increased production of acute phase proteins. In a study describing the clinico-pathologic features of sepsis in

foals, the most useful white blood cell parameters were neutropenia and the presence of band neutrophils (greater than 0.2 X 10^9/liter) and toxic changes in the neutrophil population (9). Neutrophil counts of < 2000 cells per ml suggest early overwhelming infection. Recent work has supported the use of serum AA concentrations in the differential diagnosis of sepsis in compromised neonatal foals as its concentration appears to increase dramatically in response to bacterial infection (10). In that study, foal serum AA concentrations greater than 200 mcg/ml were strongly correlated with sepsis whereas lower values (20-200 mcg/ml) were suggestive of a non-infective acute phase response to trauma or pre-maturity (10).

If AA is present in high concentrations in colostrum and milk of mares and absorption of colostral AA occurs in foals in the first 24 hours of life, it could interfere with the use of the serum isoform of this protein as an indicator of sepsis in foals, as current assays do not distinguish the different isoforms. The purpose of this study was to determine whether absorption of colostral AA in the equine neonatal intestine occurs.

Materials and Methods

Animals

The study was conducted between January and July 2003. Nineteen pregnant mares in residence at the Oklahoma State University College of Veterinary Medicine (OSU CVM) Ranch were used. Sixteen mares were Thoroughbreds and 3 were Quarter Horses (age range, 4 to 23 yr). There was no evidence of clinical mastitis in any mare. Careful records were kept in the peri-partum period with regard to clinical conditions, induction of parturition and complications of parturition. The 19 neonatal foals of the mares were intensively monitored during the post-partum period.

The low-dose oxytocin protocol (11) was used to induce parturition in a number of the mares. Calcium concentration in the mammary secretions was used to predict proximity to parturition. Sampling was initiated 1 wk prior to expected foaling date or if the mare displayed udder development. Samples were taken daily at 0800 until the calcium concentration began to rise and then twice daily at 0800 and 1800. When the calcium concentration was >200 ppm in two samples 24 h apart, parturition was deemed imminent and the mare could be safely induced.

Before induction, the mare's tail was wrapped, her perineum was washed with soap and water, and she was placed in 15 x 25 ft. maternity stall bedded with clean straw. Parturition was induced with 2.5 i.u. oxytocin (VEDCO, St Joseph, MO, USA) per dose given IV at 20-min intervals until second stage labor was observed.

Physical examination of the mare and foal was done immediately following parturition and peri-partum complications were recorded. At 12, 24, 48, and 72 h postpartum, physical examination of the 18 foals was performed. Sequential complete blood counts

in the foals at 24, 48 and 72 h were utilized to assess health status. Serum IgG concentrations in foals at 24 h of age were measured using a commercially available ELISA (SNAP Foal IgG Test, IDEXX Laboratories, Inc. Westbrook, MN, USA).

For the purposes of examining the influence of AA in the colostrum and milk on serum concentrations of AA in the foal, the foals were divided into two groups, normal foals and abnormal foals. A normal foal was defined as one who had an unassisted natural or induced full-term birth and a normal placenta (i.e. no gross pathological changes), stood within 2 h and nursed within 3 h after parturition, had an IgG > 4 g/L at 24 h, a neutrophil count within the normal range (4 to 12 x 10^9 cells/L) at 24, 48 and 72 h, and had no clinical signs of disease within the first 7 d. Foals from mares that experienced dystocia, premature placental separation or placentitis were not included in the group of normal foals; we speculated that these conditions could affect endogenous serum AA production in the foal secondary to hypoxia. Only foals whose peri-partum period was closely monitored and who could beyond doubt fulfill all of the above prerequisites of normality were included in the investigation of the likelihood of intestinal absorption of this colostral protein. This was in order to remove any influence of increased endogenous hepatic-derived AA production (secondary to inflammatory stimuli) on serum AA concentrations and to isolate the influence of colostral AA, on foal serum AA concentrations.

Samples

Blood samples were collected into evacuated plain glass tubes from each mare at parturition and from each neonatal foal prior to colostrum ingestion and at 12, 24, 36, 48, 60, and 72 h postpartum. The foals were gently and carefully restrained to prevent

traumatic veni-puncture and generation of an inflammatory response associated with the venipuncture site. Where possible, umbilical cord blood was substituted for the pre-colostral sample to prevent unnecessary stress associated with venipuncture of the newborn foal. Blood samples were stored at 4 ^{o}C until a clot had formed; they were subsequently centrifuged at 699 x g for 5 min and the serum was separated and frozen at –20 ^{o}C for later analysis.

Colostrum samples were collected in plain plastic tubes from each mare at parturition. Milk samples were obtained in plain plastic tubes at 12, 24 and 48 hours post partum from 30 mares. The colostrum and milk samples were promptly refrigerated and then stored in a freezer at –20 ^{o}C.

Serum AA concentrations

All samples were analyzed for serum AA concentration using Tridelta´s Phase™ Range SAA assay (Tridelta Limited, Maynooth, Co Kildare, Ireland). This assay has been previously described in studies that measured colostrum and serum AA concentrations in clinically normal and diseased horses (12,13). Data are expressed as mcg/mL. On the first plate, samples were measured in duplicate and thereafter once only. The minimum detectable concentration of serum AA under the assay was 5 ng/ml. The intra-assay coefficient of variation was 7.7 and 5.3 at mean serum AA concentrations of 74 and 128 mcg/ml respectively. The inter-assay coefficient of variation was 10.8 and 8.7% at mean serum AA concentrations of 81 and 134 mcg/ml respectively.

Statistical analysis

The natural log of serum AA, was modeled using a mixed model, fitted using the Mixed procedure in SAS® v 9.1 (SAS Institute Inc., 2003). A backward selection procedure

was used to determine an appropriate model; factors remaining significant ($p < 0.05$) were retained in the model. The repeated measurements within a foal at different times were dealt with by including foal as a random effect. The significance of the random effect was tested by comparing a model excluding the random effect using a likelihood ratio test. Disease, time and the two-way interaction between these variables were fitted as fixed effects. Time was checked for linearity by the inclusion of quadratic terms, and comparing models that included log of time, time as a categorical variable and time as a continuous variable. Correlation between mare serum and colostrum AA concentration at parturition and milk AA concentration over the first 48 hours and foal serum AA concentration during the first 72 hours of life in normal foals was assessed.

Results

Animals

Nineteen mares and foals were included in the study. One foal was delivered by elective Caesarian section. Parturition was induced in 11 mares. Seven mares foaled spontaneously before the 24-h sample was taken. One mare foaled prematurely (317 d) but the foal was healthy and no complications developed.

Allocation to groups

Of the 19 foals included in the study, 9 were allocated to the normal group. Abnormalities in the other foals included C-section delivery, prematurity, placentitis, premature placental separation, dystocia, slowness to nurse, laboratory abnormalities – subnormal neutrophil counts, clinical suspicion of sepsis,

Normal and abnormal foals and their serum AA concentrations

The mare serum and colostrum AA concentrations and the foal serum AA concentrations of the normal foals are presented in Table 1. The 9 normal foals had serum AA concentrations in the previously described range at birth (i.e. from 0.6 to11 mcg/ml) (Table 1). Over the first 72 hours, however, the serum AA concentrations of these normal foals varied over a wide range (e.g. at 48 hours they ranged from 4 to 197 mcg/ml). Nonetheless, none of these normal foals had serum AA concentrations in the previously described septic range (> 200 mcg/ml) although a number of them would have been considered to be in the range of inflammatory disease (20-200 mcg/ml).

Statistical analysis

In the analysis of the whole group of 19 foals the random effect of foal was not significant. The fixed effects, disease and time were both significant ($p<0.001$) and the 2 way interaction was significant ($p < 0.01$). Time fitted as a categorical variable gave a better fitting model compared to when fitted as a continuous, quadratic or logistic variable.

A significant positive correlation was detected between AA concentration in the colostrum and in the serum of normal foals at 48 hr ($r=0.86$, $dof=7$, $p<0.01$) and 60 hr ($r= 0.83$, $dof=7$, $p<0.01$) post partum. No correlation was detected between the serum AA concentrations in the mare at parturition or the AA concentration in the milk from 12 hours and AA concentrations in the serum of normal foals at any time.

Discussion

The mean and median AA concentrations in the maternal and foal serum at parturition in this study support previous studies (14, 15) although the variation in maternal serum sample concentration was wide.

In this study, to minimize the effect of increased endogenous (hepatic) production of serum AA on the relationship between AA concentrations in colostrum and milk and subsequently in foal serum, only foals that could be unequivocally confirmed normal were included in the investigation. There was a significant difference in the serum AA concentration between the normal foals and the abnormal foals after 12 hours as has been previously described (10). Therefore, it was reasonable to exclude the abnormal foals from the assessment of the influence of colostral and milk AA on foal serum AA in the post-partum period.

A positive correlation between colostrum and foal serum AA concentrations was evident at 48 and 60 hours post-partum in the normal group of foals. In these foals, no inflammatory stimulus except the parturition process, common to all the foals, was thought to be present. It may be appropriate, therefore, to attribute this correlation to absorption from the intestine as has been shown to occur in other species (3-5). No correlation was detected between milk AA from 12 hours and subsequent foal serum AA concentrations at any time. Increased permeability to macromolecules persists for up to 24 hours. Correlation of the milk AA between 12 and 24 hours and subsequent foal serum AA concentrations may have been masked by the mild post-partum inflammatory response seen even in normal foals (14).

We concluded that colostrum AA was absorbed in normal foals. However, given the low number of normal foals in this study this finding warrants investigation in greater detail. Further work is indicated to determine whether colostral acute phase proteins are absorbed in the neonatal foal.

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Table 1. AA concentration in mare serum and colostrum and in the serum of normal foals (mcg/ml)

Mare no.	Mare serum	Colostrum	Foal serum						
	0hr	0hr	0hr	12hr	24hr	36hr	48hr	60hr	72hr
#25	11.3	95.8	7.75	9.53	12.84	12.67	13.70	9.00	10.58
#26	8.2	29.4	7.61	8.11	43.51	23.45	24.43	14.54	12.12
#27	8.3	41.7	6.34	13.18	19.15	20.35	22.20	13.54	12.14
#28	20.4	93.0	11.02	16.78	20.69	21.82	18.31	15.43	16.78
#29	4.9	76.8	4.49	55.37	93.98	95.98	81.60		30.52
#30	14.7	35.8	8.74	6.62	12.38	11.02	15.00	15.14	
#31	41.5	205.0	4.27	15.11	20.28	61.66	197.39	53.53	29.15
#32	5.8	57.5	0.57	6.73	19.54	17.08	13.14	11.66	9.20
#33	25.3	58.7	2.79	34.57	5.50	10.92	3.78	0.08	4.27
Mean	15.6	77.1	6.0	18.4	27.5	30.5	43.3	16.6	15.6
SD	11.9	53.5	3.2	16.3	27.0	29.0	62.0	15.7	9.5

CHAPTER VI

INDUCTION OF PARTURITION IN MARES AND THE NEONATAL ACUTE PHASE RESPONSE

Abstract

The objectives of the present study were to determine whether induction of parturition in mares at term with low doses of oxytocin (2.5 i.u. IV every 20 min) affected the incidence of peri-partum complications or inflammatory responses in the neonatal foal. Parturition was induced in 11 of 26 mares and the remainder foaled spontaneously. Serum concentrations of amyloid A (AA; an acute phase protein) were measured (with a commercial ELISA) from 0 to 72 h postpartum in 18 of the neonatal foals. The incidence of dystocia and premature placental separation was higher in induced mares (2 of 11 and 1 of 11 versus 0 of 15 and 0 of 15, respectively), whereas retained fetal membranes were more common in spontaneous foalings (2 of 15 versus 0 of 11). When abnormal foals were excluded (to decrease the influence of endogenous serum AA elevations), serum concentrations of AA increased to the same extent over time in foals with induced versus spontaneous parturition; foals with spontaneous parturition had a mean serum AA concentration of 7.8 mcg/ml at birth that increased to a maximum of 58.9 mcg/ml at 36 h; foals with induced parturition had a mean serum AA concentration of 5.4 mcg/ml at birth, that increased to a maximum of 41.4 mcg/ml at 48 h. Baseline serum AA concentrations were lower in induced foals. We conclude that inducing parturition with low doses of oxytocin in mares at term did not affect (relative to spontaneous parturition) the temporal dynamics of serum AA concentrations in the normal foal in the first 72 h of life. However, the induction procedure may lead to complications during parturition that, if not detected early, could result in the development of an inflammatory response in the neonate.

Introduction

Traditionally it has been considered that there are few clinical indications for induction of parturition in the mare (1), as it has been associated with an increased incidence of peri-partum complications such as dystocia, premature placental separation and with unfavorable outcomes in foals (1-3). Induction of parturition, however, has a number of potential benefits with regard to mare management, including controlled attended foaling, decreased incidences of rectal trauma and still-born foals, and optimized foal outcomes (3).

The three main methods of inducing parturition in mares are oxytocin, prostaglandin $F_{2\alpha}$ and dexamethasone. Oxytocin is generally considered the drug of choice (4); induction of parturition in mares at term with low doses of oxytocin was safe and reliable, as long as parturition was impending (4). Small doses of oxytocin (2.5 to 10.0 i.u.) effectively triggered parturition in mares at term, whereas higher doses (40 to 120 i.u.) were unnecessary and potentially dangerous (5). Calcium concentrations of the mammary secretions are useful in predicting full term gestation (6) and are commonly used to predict impending parturition. Administration of low doses of oxytocin to full-term mares with calcium concentrations >200 ppm in the mammary gland secretions for at least 24 h is considered an effective method of inducing parturition (4). However, the effects on neonatal foals following this induction protocol have not been thoroughly investigated.

Several studies have examined various parameters in the neonatal foal with regard to induction of parturition (2,7-9). In one early study describing the induction of mares with a synthetic prostaglandin, four of eleven foals from induced mares did not survive to 24 h (2). Although much is now known about how inducing drugs affect

the mare and methods of induction have been refined from earlier protocols, we are unaware of any studies that directly address the influence of induction of parturition on the production of an inflammatory or acute phase response in the foal.

An acute phase protein is a protein with plasma concentrations that increase or decrease by at least 25% during inflammatory disorders (10). Inflammation causes the release of cytokines and other inflammatory mediators into the blood. These mediators act on hepatocytes that are stimulated to synthesize and release acute phase proteins into the circulation (10). This response can be enhanced by glucocorticoid production (11). In the horse, serum amyloid A (serum AA) is a major acute phase protein that appears to increase soon after inflammatory stimuli (12). Serum AA was greatly elevated (up to five times normal concentrations) in mares 48 h post-partum, indicating that foaling is an inflammatory process (12). An isoform of serum AA, milk AA, was recently discovered in the colostrum and early milk of cows, sheep, mares and women (13); this protein is synthesized in the mammary gland (not in the liver) and secreted into colostrum and milk.

In two previous reports (12,14), serum AA concentrations in neonatal foals were 23.0 $\pm$ 10.7 and 22.2 $\pm$ 7.6 mcg/ml respectively at 0 h, and were 27.7 $\pm$ and 26.7 $\pm$ 16.6 mcg/ml at 72 h (n=12 in both studies). Serum AA concentrations in neonatal foals increased rapidly in response to inflammatory stimuli, e.g. hypoxic insult, meconium impaction and sepsis (14,15). In neonatal foals, it was proposed that serum AA concentrations from 20 to 200 mcg/ml were indicative of inflammatory disease, whereas concentrations >200 mcg/ml were indicative of sepsis (15).

The objectives of present study were to determine whether induction of parturition in mares at term with low doses of oxytocin affected the incidence of peri-partum

complications or the inflammatory response in the neonatal foal. In that regard, we documented peri-partum complications associated with induction of parturition and compared serum AA in newborn foals following induced versus spontaneous parturition.

Materials and Methods

Animals

The study was conducted between January and July 2003. Eighteen pregnant mares in residence at the Oklahoma State University College of Veterinary Medicine (OSU CVM) Ranch and eight pregnant mares in residence at the OSU Animal Science Department were used. The 18 neonatal foals of the OSU CVM Ranch mares were intensively monitored during the post-partum period. Sixteen mares were Thoroughbreds and 10 were Quarter Horses (age range, 4 to 23 yr).

The low-dose oxytocin protocol (4) was used to induce parturition in a number of the OSU CVM Ranch mares. Calcium concentration in the mammary secretions was used to predict proximity to parturition. Sampling was initiated 1 wk prior to expected foaling date or if the mare displayed udder development. Samples were taken daily at 0800 until the calcium concentration began to rise and then twice daily at 0800 and 1800. When the calcium concentration was >200 ppm in two samples 24 h apart, parturition was deemed imminent and the mare could be safely induced. All eight of the OSU Animal Science Department mares were allowed to foal spontaneously.

Before induction, the mares' tails were wrapped, their perineum was washed with soap and water, and they were placed in stalls bedded with clean straw. Parturition was induced with 2.5 i.u. oxytocin (VEDCO, St Joseph, MO, USA) per dose given IV at 20-min intervals until second stage labor was observed.

Physical examination of the mare and foal was done immediately following parturition and peri-partum complications were recorded. At 12, 24, 48, and 72 h postpartum, physical examination of the 18 foals was performed. Sequential

complete blood counts in the foals at 24, 48 and 72 h were utilized to assess health status. Serum IgG concentrations in foals at 24 h of age were measured using a commercially available ELISA (SNAP Foal IgG Test, IDEXX Laboratories, Inc. Westbrook, MN, USA).

For the purposes of examining the effect of parturition on the inflammatory response in the foal, the foals were divided into two groups, normal foals and abnormal foals. A normal foal was defined as one who had an unassisted natural or induced full-term birth and a normal placenta (i.e. no gross pathological changes), stood within 2 h and nursed within 3 h after parturition, had an IgG > 4 g/L at 24 h, a neutrophil count within the normal range (4 to 12 x 10^9 cells/L) at 24, 48 and 72 h, and had no clinical signs of disease within the first 7 d. Foals from mares that experienced dystocia, premature placental separation or placentitis were not included in the group of normal foals; we speculated that these conditions could affect endogenous serum AA production in the foal secondary to hypoxia.

Samples for serum AA analysis

Blood samples were collected into evacuated plain glass tubes from each mare at parturition and from each neonatal foal prior to colostrum ingestion and at 12, 24, 36, 48, 60, and 72 h postpartum. The foals were gently and carefully restrained to prevent traumatic veni-puncture and generation of an inflammatory response associated with the venipuncture site. Where possible, umbilical cord blood was substituted for the pre-colostral sample to prevent unnecessary stress associated with venipuncture of the newborn foal. Blood samples were stored at 4 ^{0}C until a clot had formed; they were subsequently centrifuged at 699 x g for 5 min and the serum was separated and frozen

at –20 ^{o}C for later analysis. Colostrum samples were collected from each mare at parturition.

Serum AA concentrations

All samples were analyzed for serum AA concentration using Tridelta´s Phase™ Range SAA assay (Tridelta Limited, Maynooth, Co Kildare, Ireland). This assay has been previously described in studies that measured serum AA concentrations in clinically normal and diseased horses (13,16). Data are expressed as mcg/ml. On the first plate, samples were measured in duplicate and thereafter once only. The minimum detectable concentration of serum AA under the assay was 5 ng/ml. The intra-assay coefficient of variation was 7.7 and 5.3 at mean serum AA concentrations of 74 and 128 mcg/ml respectively. The inter-assay coefficient of variation was 10.8 and 8.7% at mean serum AA concentrations of 81 and 134 mcg/ml respectively.

Statistical analysis

Fisher's Exact tests (two-tailed) were performed to detect difference in incidence of peri-partum complications in the induced and naturally foaling mares. Student's t-tests were used to detect differences between induced and spontaneous parturition mares for concentrations of serum AA in the serum samples and colostrum at parturition.

Correlations between the mare serum and colostrum AA concentrations at parturition and between these concentrations and serum AA concentrations in normal foal serum over the first 72 h of life were calculated. Only the normal foals were included in this analysis to eliminate the potential effect of other inflammatory stimuli on foal serum AA concentration.

To analyze the differences in foal serum AA concentrations in induced and non-induced foals, the natural log of serum AA was modeled using a mixed model and fitted using the Mixed procedure in SAS® v 9.1 (SAS Institute Inc., Cary, NC, USA). A backward selection procedure was used to determine an appropriate model; factors remaining significant ($P < 0.05$) were retained in the model. The repeated measurements within a foal at different times were handled by including foal as a random effect. The significance of the random effect was tested by comparing a model excluding the random effect using a likelihood ratio test. Method of foaling, time and the two-way interaction between these variables were fitted as fixed effects. Time was checked for linearity by the inclusion of quadratic terms, and comparing models that included log of time, time as a categorical variable and time as a continuous variable. An assessment of the goodness-of-fit was obtained by examining residuals. Comparisons of means were adjusted using the Tukey adjustment for multiple comparisons.

The analysis was initially performed on the entire group of 18 foals and then limited to the group of normal foals (to eliminate the potential effect of other inflammatory stimuli on an acute phase response).

Results

Induction

The eight OSU Animal Science Department mares were allowed to foal spontaneously. Parturition was induced in 11 OSU CVM Ranch mares. Seven OSU CVM Ranch mares foaled spontaneously before the 24-h sample was taken, making a total of 15 mares in the study that foaled spontaneously. One OSU CVM Ranch mare foaled prematurely (317 d) but the foal was healthy and no complications developed.

Peri-partum complications in the mares

Dystocia occurred in 2 of the 11 mares that had parturition induced. In one mare, the fetus presented normally but the mare did not have abdominal contractions and she required assistance. In another mare, the foal had unilateral carpal flexion that was quickly corrected and the foal was delivered without delay. One mare (spontaneous parturition) had transient postpartum colic that resolved without treatment. Placentitis was present in one mare with spontaneous parturition and premature placental separation occurred in another mare in which parturition was induced. Two mares that foaled spontaneously retained the fetal membranes.

The incidence of dystocia was higher in induced mares (2 of 11 mares versus none of 7 mares). No other difference in incidence of peripartum complications was detected.

Foal outcome

All foals had serum IgG concentrations > 4g/L at 24 h. Nine of the 18 foals were designated normal (according to our definition) and the other nine were designated abnormal. Parturition was induced in seven and four of the mares that had normal and abnormal foals, respectively.

Abnormalities observed in foals from induced mares

Two foals suffered dystocia. One of these cases of dystocia involved unilateral carpal flexion (easily corrected and the foal had no further complications). The other dystocia involved uterine inertia. This foal had a neutrophil count < 4 x 10^9 cells /L from 12 to 72 h and was slow to nurse. Two other foals from induced mares had neutrophil counts < 4 x 10^9 cells /L from 24 to 72 h; one of these suffered premature placental separation and was also slow to nurse.

Abnormalities observed in foals from spontaneous parturition mares

One foal was born at 317 d gestation (considered a premature birth); however, the foal was healthy and showed no evidence of pre-maturity. One foal from a mare with placentitis developed clinical signs consistent with sepsis and had a neutrophil count < 4 x 10^9 cells/L at 24 and 48 h. Two other foals had neutrophil counts < 4 x 10^9 cells /L between 24 and 72 h, but no other abnormalities were detected. One additional foal was slow to nurse, but its laboratory data were normal.

Maternal serum and colostrum AA concentrations

Mean maternal serum AA concentration at parturition was 29.4 mcg/ml. Mean colostrum AA concentration at parturition was 122.3 mcg/ml. There was no difference in the change in colostrum and milk AA concentrations over time between mares that were induced to foal and mares with spontaneous parturition. However, mares that were induced to foal had lower baseline concentrations of this protein.

Foal serum AA concentrations

In all 18 OSU CVM Ranch foals, mean foal serum AA concentration at birth in the group of foals from mares that were induced to foal was 6.0 mcg/ml (Table 1). The concentration increased gradually to a maximum of 139.7 mcg/ml at 72 h. Mean

pre-colostral foal serum AA concentration in the group of foals from mares that were not induced to foal (n=7) was 4.6 mcg/ml (Table 1); it increased gradually to a maximum of 290.1 mcg/ml at 36 h.

When the analysis was limited to the group of normal foals, with no other source of serum AA stimulation (n=9), mean foal serum AA concentration at birth in the foals from mares that were induced to foal (n=7) was 5.4 mcg/ml (Table 2). The concentration increased gradually to a maximum of 41.4 mcg/ml at 48 h. Mean pre-colostral foal serum AA concentration in the group of normal foals from mares that were not induced to foal (n=2) was 7.8 mcg/ml (Table 2). The concentration increased gradually to a maximum of 58.9 mcg/ml at 36 h.

Correlation between maternal and foal AA concentration

In the group of nine normal foals, there was no significant correlation between maternal serum or colostrum AA concentration and foal serum AA concentration at parturition. However, there was a positive correlation between colostrum AA concentration at parturition and foal serum AA at 48 h ($r=0.86$, $dof=7$, $p<0.01$) and 60 h ($r=0.86$, $dof=7$, $p < 0.01$).

Effect of induction on serum AA concentrations in foals over time

In the whole study population of 18 foals, the random effect of foal was not significant. There was a difference ($p<0.001$) between the two groups of foals with regards to method of foaling and time of sampling, however the two-way interaction was not significant. Time fitted as a categorical variable gave a better fitting model compared to fitting as a continuous, quadratic or logistic variable. The least-square mean serum AA for non-induced foals was significantly larger than for induced foals with means of 54.8 and 18.4 mcg/ml respectively (Table 3). The least-square mean

serum AA was lower at 0 h compared with at 12 h ($P < 0.005$) and at 0 h compared with all other times ($P < 0.0001$); there was no significant difference in the least-square mean SAA at any other times.

When only normal foals were included in the analysis, the random effect of foal was again not significant. There was a difference between the two groups of foals with regards to method of foaling and time of sampling ($P = 0.003$ and $P<0.001$ respectively), however the two-way interaction was not significant. Time fitted as a categorical variable gave a better fitting model compared to fitting as a continuous, quadratic or logistic variable. The least-square mean serum AA for non-induced foals was significantly larger than for induced foals with means of 26.9 and 12.0 mcg/ml respectively (Table 4). The least-square mean serum AA was lower at time 0 h compared to time 12 h ($P< 0.02$), at time 0 h compared with 24, 36 and 48 h ($p< 0.001$) and at time 0 h compared with time 72 h ($p<0.03$). There was no significant difference in the least-square mean serum AA at any other times.

Discussion

This study supported the findings of a previous report (4) that the administration of low doses of oxytocin in a full term mare with impending parturition (documented by rising calcium concentration in the mammary secretions) is an effective method of induction of parturition.

Peripartum complications

Peri-partum complications were noted in both groups of foaling mares. There was a significantly higher incidence of dystocia in the induced mares compared those with spontaneous parturition (2 of 11 vs 0 of 15, respectively). Fortunately, in both cases of dystocia, the attending veterinarian quickly resolved the problem and delivered the foals. The foal of the mare with inadequate uterine contractions was slow to nurse for the first time, suggesting mild hypoxic cerebral damage. It is not known if this was due to the dystocia (that was of short duration), placentitis or premature placental separation (although there were no indications that the latter two conditions were present). Decreased neutrophil concentrations, detected between 24 and 72 h in this foal, were consistent with in utero compromise. Otherwise there were no untoward consequences in this foal and its IgG concentration was within the normal range by 24 h. The foal of the other mare that suffered dystocia suffered no further untoward consequences.

Of the 11 mares that had parturition induced, one experienced premature separation of the placenta (red-bag delivery). None of the 15 mares that foaled naturally experienced this abnormality. Premature placental separation was one of the more frequently observed complications of an earlier study in which parturition was induced with high doses of oxytocin and this was attributed to manual manipulation

of the fetus (3). In the present study, the fetus was not manually manipulated during parturition, except for the cases of dystocia in which manipulation occurred only after the placenta had already ruptured at the cervical star. Induction of parturition was not significantly associated with the premature placental separation in this study; however, the occurrence rate (1 in 11) in induced mares seemed higher than that seen in the normally foaling mare population. In a report from the University of Kentucky Livestock Disease Diagnostic Centre over a 2 y period from greater than 1300 fetuses and/or placentae examined per year, only 12 or 13 cases of placental separation (about 1%) were recorded (17). The actual incidence of this condition is not known but, in the authors' experience, is far less than 10% of the normally foaling population. The foal that suffered placental separation was slightly slow to nurse and an abnormal neutrophil count was detected from 24 to 72 h. The severity of these abnormalities suggests that in utero placental compromise due to placentitis or separation may have occurred, but hypoxic damage to organs secondary to placental separation during parturition could not be excluded, despite immediate detection and correction of the abnormality as the chorio-allantoic membrane reached the vulvar lips and prompt delivery of the foal.

There was a higher incidence of retained fetal membranes in the group of mares that foaled naturally although this difference was not significant. This may be explained by the action of oxytocin. Oxytocin stimulates uterine smooth muscle contraction acting via a typical class 1 G protein-coupled receptor (18). In mares, oxytocin is used as an uterotonic agent, in the treatment of delayed uterine clearance and retained fetal membranes. It stimulates contraction of the uterine smooth muscle which is

believed to aid fluid clearance and release of the fetal membranes from the endometrium.

There was no increased incidence of uterine rupture, uterine prolapse, failure of passive transfer, or poor outcome in foals in the group of induced mares.

Maternal AA concentrations

Mean maternal serum AA concentrations were similar to those previously described (12). AA has been previously detected in the colostrum of one mare at a concentration of 1,704 mcg/ml (13). There was a wide range of AA concentrations detected in the colostrum of mares in this study (13 to 967 mcg/ml). Hepatic production of acute phase proteins is stimulated under the influence of inflammatory cytokines (10). Milk AA expression is up-regulated in human and bovine mammary gland cells in response to acute phase stimulants such as lipopolysaccharide and the lactational hormone, prolactin (19,20). Currently there is insufficient knowledge regarding the stimulus for colostrum and milk AA synthesis in the mare to explain the range of colostrum AA concentrations. Further work is warranted to elucidate the dynamics of mammary derived acute phase proteins in the mare.

There was no difference in serum AA concentrations at birth between the groups of induced and spontaneously foaling mares. There is a lag period of 16 h after inflammatory stimulus before serum concentrations of AA rise above normal (21). If induction of parturition had an effect on serum AA concentration it would not be apparent until at least 16 h after induction. Maternal serum AA concentration was not measured at this time. Mares that were induced to foal had lower baseline AA concentrations in the colostrum and milk although the change in concentration over time was not different. The dynamics of colostrum AA production are not clearly

understood; therefore, it is unknown how long it would take for any potential effect of induction on colostrum AA concentration to take effect. This needs to be investigated in more detail.

Foal AA concentrations

Serum AA concentrations in the neonatal foals in this study were consistent with previously reported values (Table 1) (12,14). In normal foals, there is a mild increase in serum AA concentrations early in life (12,14). As the stimulus for hepatic derived AA production is the release of cytokines from damaged tissue, this mild increase in foal serum AA concentrations in normal foals in the early neonatal period is likely attributable to mild intra-partum tissue damage enhanced by glucocorticoid release as part of the birth process. The possible etiology of intra-partum fetal tissue damage might include normal events such as separation of the placenta from the endometrium, rupture of the chorio-allantois and tearing of the umbilicus. As the placental blood continues to supply the fetus until the umbilicus separates, cytokines released during these events could potentially reach and stimulate fetal hepatic cells. The effects might be localized enough to produce the mild inflammatory response evidenced by the slight raise in normal foal serum AA after birth, but would not produce the serum AA concentration increase evident with systemic disease.

Correlation of maternal and foal AA concentration

The lack of correlation of maternal and fetal serum AA concentrations at birth supported the acknowledged minimal transport of proteins, such as AA, across the equine placenta (22) and the presence of individual endogenous inflammatory response systems in the dam and fetus. In addition, inflammatory cytokines, which might stimulate acute phase protein production, do not cross the endotheliochorial

placenta in humans (23) and are unlikely to cross the more complex epitheliochorial equine placenta from dam to fetus.

The correlation between colostrum AA concentration and normal foal serum AA concentration at 48 h suggested intestinal absorption of the colostral protein in the postnatal period of macromolecular absorption in the foal. Although the colostrum intake was not quantified, all foals nursed adequately and had IgG concentrations within an acceptable range by 24 h, indicating adequate colostral absorption. Foal neonatal intestinal cells are thought to be non-selective in the uptake of macromolecules during this period (24,25) therefore it is not unreasonable to suggest that absorption of this colostral protein does occur.

Peri-partum complications and foal serum AA concentration

We inferred that the induction of parturition may predispose to dystocia and an increased inflammatory response in the foal. One of the two foals that experienced dystocia developed a high serum AA concentration (Mare P). This foal was not mal-positioned but it required assistance because the mare did not have adequate uterine contractions. This foal developed a low neutrophil count from 24 to 72 h; whether this was attributable to the dystocia and subsequent hypoxic tissue damage and inflammation or to in utero events was unclear. The foal that was mal-positioned (unilateral carpal flexion) did not develop a high serum AA concentration, nor was its neutrophil count outside the normal range at any observed time. Perhaps the dystocia was more severe in the first case; however, in neither case was the birth prolonged as the dystocia was corrected quickly and the foals delivered.

Induction of parturition and foal serum AA concentrations

This study examined the inflammatory response in the foal over the first 72 h of life following induction of parturition with low doses of oxytocin in the term mare. To eliminate the effect of extraneous inflammatory events of serum AA concentrations in the population of foals in the study, in addition to assessing the whole study population of foals, we also assessed the subpopulation of normal foals with no extraneous inflammatory conditions, as a more accurate way to isolate the effect of induction on the inflammatory response.

Foals from mares in which parturition was induced did not exhibit an increased inflammatory response beyond that which results from the natural birthing process, either in the whole group of foals or in the sub-population of normal foals. The effects of differences in the birth process on foal serum AA would not be evident until at least 16 h post partum given the lag period (21). Therefore the response was measured over the first 72 h of life. There was no difference in the response over time between the two groups of foals, i.e. the change in serum AA concentration over time in both groups was not significantly different.

There were lower baseline serum AA concentrations over time in the foals that were induced. It is difficult to explain why the induced foals had lower baseline serum AA concentrations measurements than the foals that were not induced. Perhaps this could be attributed to absorption of colostral AA, as maternal colostrum concentrations of AA were lower in induced than non-induced mares.

The mares in this study that foaled naturally did so before they could be induced, i.e. once their milk calcium concentration reached 200 ppm but before two samples 24 h apart could be collected. Although the relationship between fetal cortisol production

and parturition is not yet clear in the foal, perhaps there was a higher concentration of circulating fetal corticosteroids in these foals leading to enhanced serum AA production. Indeed perhaps serum AA concentrations in the post partum foal could be an indicator of "readiness for birth". Comparison of serum AA concentrations at birth in premature/dysmature and mature foals should be assessed in the future to determine the effectiveness of this inflammatory protein as a marker of maturity.

It is unclear whether oxytocin may have some direct effect on the production of serum AA. Oxytocin receptors have been identified in many tissues, including the kidney, heart, thymus, pancreas, and adipocytes (26). In addition to the well known effects of oxytocin on uterine contraction and milk ejection oxytocin may also modify blood pressure and heart rate both through effects within the CNS and through effects in other organs, such as the heart, blood vessels and kidney (27). Oxytocin may have as yet unrecognized effects on the parturient foal.

It should be borne in mind, also, that the increase in serum AA concentrations in normal foals after natural birth may have protective effects in the neonatal foal. The exact functions of serum AA in the circulation have yet to be elucidated although they are proposed to be involved in lipid metabolism/transport, induction of extra-cellular-matrix-degrading enzymes, and chemotactic recruitment of inflammatory cells to sites of inflammation (11). Therefore the lower serum AA concentrations in induced foals may have a detrimental effect on neonatal viability. Further study is necessary to elaborate these findings.

In conclusion, induction of parturition at term with a low-dose oxytocin protocol was associated with a higher incidence of dystocia, but with a lower incidence of retention of fetal membranes. It did not induce an inflammatory response in the foal over time

in excess of that in foals with spontaneous parturition. This method of induction ensured an observed parturition and provided a controlled environment whereby potential problems and complications were readily identified and corrected. It must be emphasized that this induction protocol should followed strict indications for induction of parturition and that experienced personnel should be available to detect and correct any complications.

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Table 1. Concentrations of amyloid A in the serum and colostrum of all 18 mares and in the serum of their foals (mcg/ml)

Mare	Parturition	Mare serum	Colostrum	Foal serum					
		0 h	0 h	0 h	12 h	24 h	36 h	48 h	72 h
A	Spontaneous	4.9	76.8	4.5	55.4	94.0	96.0	81.6	30.5
B	Spontaneous	69.1	79.4	0.6	12.6	43.4	39.5	28.2	32.8
C	Spontaneous	135.6	966.7	2.3	323.8	262.4	406.8	373.0	239.8
D	Spontaneous	20.4	93.0	11.0	16.8	20.7	21.8	18.3	16.8
E	Spontaneous	6.3	90.2	3.7	69.8	315.2	432.2	510.3	221.9
F	Spontaneous	5.8	81.6	2.1	124.5	744.6	744.6	744.6	723.4
G	Spontaneous	11.0	85.6	7.8	24.4	74.7	-	60.3	31.7
Mean		36.2	210.5	4.6	89.6	222.1	290.1	259.5	185.3
SD		49.4	333.5	3.7	110.4	256.2	287.4	287.0	256.0
H	Induced	14.7	35.8	8.7	6.6	12.4	11.0	15.0	-
I	Induced	8.2	29.4	7.6	8.1	43.5	23.4	24.4	12.1
J	Induced	11.3	95.78	7.8	9.5	12.8	12.7	13.7	10.6
K	Induced	8.3	41.7	6.3	13.2	19.2	20.4	22.2	12.1
L	Induced	25.3	58.7	2.8	34.6	5.5	10.9	3.8	4.3
M	Induced	5.75	57.5	0.6	6.7	19.5	17.1	13.1	9.2
N	Induced	41.5	205.3	4.3	15.1	20.3	61.7	197.4	29.1
O	Induced	58.0	154.3	7.0	5.0	4.5	6.0	5.7	5.0
P	Induced	20.5	125.6	6.0	28.9	215.5	363.2	457.5	395.0
Q	Induced	36.0	68.4	10.9	10.3	14.3	48.4	412.3	851.1
R	Induced	3.0	13.1	3.9	22.5	133.8	185.3	191.1	68.1
Mean		21.1	80.5	6.0	14.6	45.6	69.1	123.3	139.7
SD		17.5	59.2	2.9	9.9	67.2	110.3	170.1	277.2
Prob.[a]		0.4	0.35						

[a]Prob. = probability of a difference between spontaneous and induced parturition.

Table 2. Concentrations of amyloid A in the serum and colostrum of mares of normal foals and in the serum of the foals (mcg/ml)

Mare	Parturition	Mare serum	Colostrum	Foal serum					
		0 h	0 h	0 h	12 h	24 h	36 h	48 h	72 h
A	Spontaneous	4.9	76.8	4.5	55.4	94.0	96.0	81.6	30.5
D	Spontaneous	20.4	93.0	11.0	16.8	20.7	21.8	18.3	16.8
Mean		12.7	84.9	7.8	36.1	57.3	58.9	50.0	23.6
SD		11.0	11.5	4.6	27.3	51.8	52.4	44.8	9.7
H	Induced	14.7	35.8	8.8	6.6	12.4	11.0	15.0	-
I	Induced	8.2	29.4	7.6	8.1	43.5	23.5	24.4	12.1
J	Induced	11.3	95.78	7.7	9.5	12.8	12.7	13.7	10.6
K	Induced	8.3	41.7	6.3	13.2	19.2	20.4	22.2	12.1
L	Induced	25.3	58.7	0.6	6.7	19.5	17.1	13.1	9.2
M	Induced	5.75	57.5	2.8	34.6	5.5	10.9	3.8	4.3
N	Induced	41.5	205.3	4.3	15.1	20.3	61.7	197.4	29.2
Mean		16.4	74.9	5.4	13.4	19.0	22.4	41.4	12.9
SD		12.8	61.5	3.0	9.9	12.0	17.9	69.1	8.5
Prob.[a]		0.7	0.7						

[a]Prob. = probability of a difference between spontaneous and induced parturition.

Table 3: Least square means and 95% confidence intervals of serum amyloid A concentrations (mcg/ml) for the entire group of 18 foals. (LSM for spontaneously delivered foals was different from that of induced foals ($p < 0.001$). LSM at time 0 was different from time 12 ($p < 0.005$) and from all other times ($p < 0.0001$))

Variable	**Value**	**Least-square mean**	**95% confidence interval**	
			Lower	**Upper**
Method	Spontaneous	54.8	36.4	82.4
	Induced	18.4	13.3	25.5
Time	0	4.7	2.5	8.8
	12	23.7	12.8	44.0
	24	48.0	25.9	89.1
	36	61.3	32.4	116.1
	48	66.9	36.1	124.1
	72	46.4	24.6	87.6

Table 4. Least square means and 95% confidence intervals for serum amyloid A concentrations (mcg/ml) for the nine normal foals. (LSM for spontaneously delivered foals was different from that of induced foals ($p < 0.003$). LSM at time 0 was different from time 12 ($p < 0.02$), from times 24 to 48 ($p < 0.001$) and from time 72 ($p < 0.05$))

Variable	**Value**	**Least-square mean**	**95% confidence interval**	
			Lower	Upper
Method	Spontaneous	26.9	17.2	42.2
	Induced	12.0	9.4	15.4
Time	0	5.9	3.4	10.0
	12	17.6	10.3	30.2
	24	25.3	14.7	43.3
	36	28.4	16.6	48.6
	48	28.4	16.6	48.7
	72	16.1	9.2	28.4

VITA

Vivienne Elizabeth Duggan

Candidate for the Degree of

Doctor of Philosophy

Thesis: EQUINE AMYLOID A IN THE NEONATAL AND PERIPARTUM PERIOD

Major Field: Veterinary Biomedical Sciences

Biographical:

Vivienne Duggan trained as a Veterinary Surgeon at the Veterinary College of Ireland, University College Dublin, graduating with a Bachelor of Veterinary Medicine in 1999. She subsequently spent 4 years pursuing further training in Equine Internal Medicine with an internship and residency at Oklahoma State University, Stillwater, Oklahoma, USA, interspersed with a year in general practice in Ireland with O'Scannaill Veterinary Associates, Ashbourne, Co Meath. While at Oklahoma State in 2002 she began a PhD in Veterinary Biomedical Sciences and completed the degree in December, 2006.

In 2004 she became a board certified specialist with the American College of Veterinary Internal Medicine – Large Animal. She subsequently worked in specialist equine practice in Ireland and New Zealand. She returned to Ireland permanently in January 2006 to take up a post as a Large Animal (Equine) Medicine Lecturer at the School of Agriculture, Food Science and Veterinary Medicine, University College Dublin where she is presently based.

Name: Vivienne Elizabeth Duggan Date of Degree: December 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EQUINE AMYLOID A IN THE NEONATAL AND PERIPARTUM PERIOD

Pages in Study: 119 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences

Scope and Method of Study:

AA concentration was measured in serum from 38 brood mares and 7 control non-pregnant, non-lactating mares at parturition and also in colostrum and milk samples every 12 hours from 0 to 48 hours post partum. AA concentration was measured in neonatal foal serum from 19 of the broodmares every 12 hours from birth until 72 hours post partum. The foals were divided into those with evidence of infection or severe inflammatory disease and those that were normal.

Findings and Conclusions:

Specificity of serum AA concentration as an indicator of sepsis in neonatal foals was found to be 60 per cent. AA was present in all colostrum and early milk samples. There was no effect of age, breed, farm of residence, or clinical abnormality on the AA concentration in colostrum or milk. Increased gestation length and induction of parturition were associated with decreased AA concentration in colostrum. Colostral AA concentration was positively correlated with serum AA concentration in normal foals. Inducing parturition did not affect (relative to spontaneous parturition) the temporal dynamics of serum AA concentrations in the normal foal in the first 72 h of life.

ADVISER'S APPROVAL: G Reed Holyoak