2008

The relationship between pro-inflammatory cytokines, prostaglandins, and fetal hypothalamic-pituitary-adrenal axis activation in mares with infective pre-term delivery

Sara K. Lyle
Louisiana State University and Agricultural and Mechanical College, slyle@lsu.edu

THE RELATIONSHIP BETWEEN PRO-INFLAMMATORY CYTOKINES, PROSTAGLANDINS, AND FETAL HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ACTIVATION IN MARES WITH INFECTIVE PRE-TERM DELIVERY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the
Department of Veterinary Clinical Sciences

by
Sara K. Lyle
B.A., Duke University, 1980
D.V.M., University of Florida, 1985
M.S., University of Florida, 1991
December 2008

ACKNOWLEDGEMENTS

The author would like to thank her major professor, Dr. Dale L. Paccamonti, and the members of her graduate committee, Drs. Bruce E. Eilts, Robert A. Godke, David W. Horohov, and Jill R. Johnson, for guidance and support during the research. The author would also like to thank the members of the committee and the Dean's Representative, Dr. John E. Chandler, for critical review of the manuscript and for providing recommendations on its improvement.

The author is extremely indebted to Dr. Jeremy D. Hubert, for his tireless efforts, perseverance, and Zimbabwen ingenuity. Without his input, support, and friendship, it is unlikely that the laparoscopic approach would have been successfully developed. His presence at the Veterinary School is sorely missed. Special thanks also go to Drs. Daniel Burba and Colin Mitchell, who cheerfully stood in for Dr. Hubert on several occasions.

The author would like to thank C.T. Rashid and Dr. Amanda Adams, and the entire Immunology Research group of the Gluck Equine Research Center of the University of Kentucky for welcoming her into their laboratory. Their assistance and resources for analysis of soluble cytokines and cytokine expression is deeply appreciated. The intellect, inquisitiveness, and camaraderie of the graduate students, post-doctoral fellows, and technicians in the section are impressive. Special thanks go to Dr. and Mrs. David Horohov, for hosting the author during visits to Lexington.

Sincere thanks are also offered to the Equine Health Studies Program, and its former director, Dr. Rustin M. Moore, and the interim director of research, Dr. Susan C. Eades, with funding for the studies. Special thanks also go to Dr. Moore for his mentorship and advice during grant writing.

The author would like to thank Dr. Donald H. Schlafer for his breadth of knowledge of reproductive pathology and host-pathogen interactions. He graciously interpreted the histopathology slides and provided stimulating discussions on things known and unknown concerning the placenta. Thanks are also extended to Dr. Robert C. Causey, for kindly providing the Streptococcal inoculates and performing pulsed-field gel electrophoreses on isolates. The author would also like to thank Drs. Laura Gentry and Donald Thompson, and Parker Pennington for assistance with the cortisol assays.

The *in vivo* trials were extremely labor intensive, and would not have been possible without the help of many individuals, including Catherine Koch, Mike Keowen, Frank Garza, Gil Barker, Oscar Johnson, Dr. Jenny Liford, Amy Bell, Jeffrey Cardinale, Ben McMath, Jeanne Pitre, Brenna Hanly, and the Veterinary Science Farm crew.

The author would like to thank her family and friends for their support, encouragement, and patience during the years of research. Many have sacrificed much and endured the process with charity and kindness.

This dissertation is dedicated in part to the ponies, which although at times could be challenging, also provided many moments of humor. Above all, we endeavor to improve the health and welfare of the horse.

Finally, the author would like to dedicate this dissertation in memory of her parents, Clifford and Julia Lyle, who never placed boundaries on a young girl's aspirations, and in honor of her long-time friend and unwavering mentor, Dr. Atwood C. Asbury, who has been a constant source of encouragement and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Ascending placentitis is a significant cause of abortions, stillbirths, and perinatal loss in horses. A technique for laparoscopic-guided catheterization of the allantoic space was developed and utilized in an experimental model of streptococcal infective pre-term delivery in pony mares. Mares received either 1 x 10^7 CFU live *S. zooepidemicus* (n=3), 5.1 x 10^8 CFU live *S. zooepidemicus* (n=1), 1 x 10^7 heat-killed *S. zooepidemicus* (n=3), 1 mL sterile PBS (n=3). Sham control mares did not receive a transcervical inoculation (n=3). One mare not instrumented with an allantoic catheter received 5.1 x 10^8 CFU live *S. zooepidemicus*. Mares with spontaneous abortion had significantly increased CTUP compared to mares in which delivery was induced. There was not a significant effect of infection within the allantoic space on CTUP. Intrauterine infection increased the expression of IL-1β, IL-18, IL-15, and IFN-γ in a site-dependant manner. Spontaneous abortion also increased the expression of IL-1β, IL-18, IFN-γ, and iNOS in a site dependant manner. Soluble TNF-α was detected in only a few samples of fetal fluids. The concentrations of PGE_2 and $PGF_{2\alpha}$ in fetal fluids were increased within 24 h of delivery in mares with spontaneous abortion or intrauterine infection. Increased cortisol concentrations were observed in fetal fluid in some mares with infection or with histologic inflammation of the chorioallantois. None of the fetal fluids from mares induced to deliver or without inflammation of the chorioallantois had increased cortisol concentrations. This data suggests that the equine fetal adrenal gland less than 295 d may be capable of response to stimuli. Based on these findings, the following sequence of events leading from intrauterine infection to infective pre-term delivery is proposed. Following infection of the chorioallantois, IL-1β, IL-18, IL-15, and IFN-γ are upregulated in a site-dependant manner. IL-1β causes increased PGHS-2 (COX-2) expression, resulting in increased PGE_2 and $PGF_{2\alpha}$ production, and ultimately labor. IL-1β may

also accelerate fetal hypothalamic-pituitary-adrenal axis (HPAA) activation, thereby promoting precocious *in utero* fetal maturation. The eventual outcome of pre-term labor, i.e., neonatal survivability, will depend on the degree of HPAA activation at the onset of labor.

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Overview of Placentitis

Placentitis has been reported to be responsible for 9.8 [1], 19.4 [2], 24.7 [3], and 33.5% [4] of abortions, stillbirths, and perinatal loss in horses. Bacterial infections were responsible for 53% of these losses; *Streptococcus equi* subsp. *zooepidemicus*, hereafter referred to as *S. zooepidemicus*, was isolated in 28% of these cases [3]. Other bacteria frequently identified were *Escherichia coli*, *Leptospira* spp., gram-positive filamentous rod (subsequently named as *Crosiella equi*), *Pseudomonas* spp., *S. equisimilis*, *Enterobacter* spp., *Klebsiella* spp., α-hemolytic streptococci, *Staphylococcus* spp., and *Actinobacillus* spp. [3]. The discrepancy in the reported incidences of placentitis lies in a considerably higher occurrence of umbilical cord problems (38.8%) in the survey population [1], compared to 4.5% [4] and 3.4% [3] in other populations. The localization of placentitis to the cervical star was present in 95% of cases, supporting the argument that ascension of aerobic bacteria through the vagina and cervix is the most frequent route of infection [5]. Clinically, mares may have a vaginal discharge, show udder development, prenatally lactate, and deliver a premature or dead foal. Empirical treatment for placentitis includes progestins to maintain uterine quiescence, broad-spectrum antibiotics to eliminate bacterial infection, and anti-inflammatory agents to prevent prostaglandin synthesis. Although these agents should address the basic problems of infection-induced prostaglandin synthesis that leads to uterine contractions and fetal expulsion, the efficacy of these regimes is often poor [6]. There are two major areas where the basic knowledge of the pathophysiology of equine placentitis is lacking: (1) the endocrinological events associated with loss of myometrial quiescence and (2) the temporal relationship between the inflammatory cascade, increases in

prostaglandin synthesis and release, and activation of the fetal hypothalamic-pituitary-adrenal axis.

1.2 The Equine Placenta

The equine placenta is characterized as diffuse, chorioallantoic, epitheliochorial, villous (microcotyledonary), and adeciduate [7]. Although there does not appear to be a relationship between the structural anatomy of the placenta and its steriodogenic activities, a brief review of the equine placenta is useful to the topic of placentitis.

1.2.1 Embryonic Events

The equine embryo enters the uterus on day 6 as a morula or early blastocyst. At this stage the outer covering is the zona pellucida, which thins and sheds around day 8 [8,9]. The equine embryonic capsule begins to form within the first days of intrauterine life and ruptures or retracts by day 21; its precise function is still unclear (review [10]). Ginther provides a most precise description of the development of the extraembryonic membranes in the mare [11]. At day 9 the blastocyst has a single layer of trophoblast (ectoderm) cells forming the blastocoele, and a single layer of endodermal cells, arising from the inner cell mass, beginning to encircle the blastocoele (the eventual yolk sac). The exact day on which formation of the bilaminar yolk sac is complete is unknown, but probably is around day 11 [11]. Invasion of a mesodermal layer (originating from the embryonic disc) between the trophoblast and the endoderm begins on day 14, gradually encroaching towards the mesometrial pole. Blood islands within this mesodermal layer form, enlarge, coalesce, and eventually connect with channels within the embryo proper (vitelline-embryo circulation). At the leading edge of the mesoderm, a prominent vein begins to develop (sinus terminalis). The three-layered yolk-sac wall (ectoderm, mesoderm, and endoderm) that extends from the embryo and the sinus terminalis is the trilaminar

omphalopleure. The bilaminar omphalopleure is the remaining two-layer wall of the yolk sac. As the trilaminar omphalopleure continues to expand, the bilaminar omphalopleure contracts to the pole opposite the embryo proper, and later becomes the site of umbilical cord attachment to the fetus. Just after the mesodermal layer begins its invasion between the ectoderm and endoderm, amniotic folds begin to form, folding beneath the embryonic disc, and eventual fusing to form the amniotic sac. At the margins of the amniotic folds an exocoelom forms within the mesodermal layer, the outer layer of which becomes the chorion (ectoderm and mesoderm). Following fixation on day 16, the embryonic disc assumes an antimesometrial orientation leaving the yolk sac mesometrial. The transition from a yolk sac to an allantoic sac begins with the emergence of the allantois from the primitive hind gut on day 21, which forms a cup beneath the amnion (within the exocoelom) and embryo proper by day 25, and continues to expand as the yolk sac regresses through day 40. As the allantois expands it fuses with the chorion to become the chorioallantois (or allantochorion) [11]. A unique feature of the equine placenta is the endometrial cups, which develop from trophoblast cells from the chorionic girdle (area of chorion between the developing allantoic sac and the regressing yolk sac) invading into the endometrium ([12], review [13]).

1.2.2 Attachment and Microcotyledon Formation

The process of attachment of the chorioallantois to the endometrium is an extremely prolonged process compared to other domestic animals. Although one might consider the invasion of the trophoblastic cells of the chorionic girdle equivalent to placental attachment, such attachment is temporary and ends when invasion is complete (day 36 to 38) [14]. Microcotyledons develop from primary villi, whose first appearance has been reported to range from day 40 to 61 [15,16]. Interdigitation of the microvilli and the maternal epithelium begins at

this stage, and by day 100, the microvilli have branched and fused with adjacent villi, thereby forming a microcotyledon [16].

1.2.3 Umbilical Vasculature

The equine umbilical cord is comprised of allantoic and amniotic segments. The allantoic segment is lined with endoderm contiguous with the allantoic membrane, and is composed of two major arteries. One artery supplies the gravid horn and cranial uterine body and the other artery supplies the non-gravid horn and the remainder of the uterine body. At the periphery, umbilical veins tend to parallel the umbilical arteries, until converging into two umbilical veins, which converge into a single umbilical vein while still within the allantoic segment of the umbilical cord. The amniotic segment of the umbilical cord is comprised of two umbilical arteries, one umbilical vein, and the urachus ("allantoic duct"), and the regressed yolk sac stalk. An ossified remnant of the yolk sac is commonly identified at delivery of the fetal membranes [17,18]. Umbilical veins carry oxygenated blood ("arterial") from the placenta to the fetus, while umbilical arteries return the poorer oxygenated blood ("venous") from fetus back to the placenta.

1.3 Myometrium

Since loss of myometrial quiescence and the initiation of labor is a key event in pregnancy loss due to placentitis, understanding myometrial function is crucial to elucidating the mechanisms involved with pre-term labor and identifying potential targets for interventional strategies. Investigations on the ultrastructure, biochemistry, and signaling events of equine myometrium are scarce. The following discussion is based on findings from myometrial function in other species, general smooth muscle physiology, and, when available, findings from the mare.

1.3.1 Cellular and Intracellular Organization

Myometrium is classified as unitary smooth muscle, also known as syncytial or visceral smooth muscle, meaning that a large mass of muscle fibers contract simultaneously as a single unit. Due to the aggregation of muscle fibers into bundles and the adherence of cell membranes at multiple points, contractile force generated in one muscle bundle is transmitted to adjacent bundles. The presence of gap junctions further contributes to the coordinated contraction of muscle fibers. In the majority of species, including the horse, myometrial fibers are organized into two layers, each with distinct phenotypic and biochemical properties that are maintained *in vitro* [19]. Myometrial cells are typically spindle-shaped. Their size (300 to 600 μm in length by 5 to 10 μm in diameter) and number vary with the degree of uterine distension and stage of pregnancy [20]. The outer longitudinal muscle layer shortens the length of the uterine horns during contraction, and the inner circular muscle layer, compresses the diameter of the horns during contraction. Differences in response to contraction-stimulating agents exist. Circular muscle from mid-gestational pregnant, rat myometrium spontaneously contracted upon exposure to norepinephrine; while longitudinal muscle did not. The circular muscle layer's response to catecholamines was inhibited by isoproterenol, and declined with approaching parturition [20,21]. Differences in *in vitro* spontaneous contractility due to stage of estrous cycle and myometrial layer were recently described in equine myometrium [22]. The frequency of contractions was increased in estrous circular muscle compared to longitudinal muscle, while area under the curve and time occupied by contractions were greater for longitudinal muscle during diestrus. These findings lead to the hypothesis that circular muscle contractions during estrus are crucial to uterine clearance mechanisms and that longitudinal contractions during diestrus are key to embryo mobility prior to day 16 [22]. In contrast, no spontaneous contractility

of either muscle layer was observed in myometrium collected from mares under general anesthesia during pregnancy [23], or from mares 18 h following insemination [24] , presumably due to either anesthetic agents or the presence of propranolol in the Krebs solution used to bathe the muscle strips. Under these experimental conditions [24] the length-tension relationships of the two layers did not differ and the response to contractile agonists were similar. Clearly, differences in the experimental design of these two studies make direct comparisons difficult, but it is likely that differences in contractile properties exist between circular and longitudinal equine myometrium.

1.3.2 Innervation

Postganglionic sympathetic fibers in the mare arise from the caudal mesenteric ganglion and plexus, and travel to the uterus via the hypogastric nerves and pelvic plexus. Parasympathetic innervation comes via the pelvic nerves from the sacral area [25]. The density of nerve fibers (noradrenaline, dopamine, and acetylcholine) of sheep myometrium decreases during pregnancy from day 15 through day 105 of gestation [20]. A similar pattern was seen with human myometrium, where 30% of nerve varicosities ("synapses") from nonpregnant, nonparous women were classified as adrenergic, 53% as cholinergic, and 17% as indeterminate, while nerve varicosities were only rarely observed at term [26]. Bae et al. [27] described the immunohistochemical distribution of adrenergic and peptidergic nerve fibers in the uterus and cervix of cycling mares. Nerves immunoreactive for the catecholamine-synthesizing enzymes tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DβH) were present in all regions of the uterus, with very large numbers of fibers identified in both the longitudinal and circular layers of the myometrium. Stage of the estrous cycle had no difference on the intensity of staining or distribution of TH and DβH within the uterus. Immunoreactive staining for the peptidergic

nerves containing the neuropeptides neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and substance P (SP) were also examined. Moderate numbers of NPY-IR fibers were present in the myometrium. Only very small numbers of VIP-IR and CGRP-IR fibers were present, while no fibers with staining for SP were identified. The adrenergic and peptidergic innervation of the pregnant uterus was not investigated, but presumably a similar reduction in nerve varicosities reported in other species occurs in the mare. If there is a functional consequence of this reduction and whether alterations in innervation occur with infective processes of the uterus and placenta are unknown. An alternative explanation is that the number of nerve varicosities is unchanged during gestation, but as hypertrophy and hyperplasia of the myometrium increases with advancing pregnancy, the relative number of nerve varicosities per myometrial cell declines.

1.3.3 Contractile Proteins and Cytoskeleton

The contractile apparatus of myometrium is composed of thin, thick, and intermediate filaments, dense bodies, and ion channels.

Thin actin filaments – Monomeric actin (globular or G-actin; 42.5 kDa) is polymerized into filamentous structures (F-actin), which in turn organize into a double-alpha helix to form the thin actin filaments (60 Å diameter) of myometrium. Several proteins (tropomyosin, caldesmon, and calponin) are also associated with the alpha helical coil. Three main actin isoforms (α, β, and γ) have been shown to be expressed in myometrium. Alpha-actin is invariant throughout pregnancy in the mouse and rat [28,29], but γ-actin localization changes significantly during gestation from predominant staining in the longitudinal layer to intense staining in the circular layer, suggesting that a change in the contractile apparatus is a key event to preparing the myometrium for parturition [29].

Intermediate filaments and microtubules – Intermediate fibrils (100 Å diameter, intermediate filaments) and microtubular networks (25 Å) are composed of desmin, a member of the vimentin-like superfamily, and are thought to provide cytoskeletal support and help regulate cell shape by connecting cytoplasmic dense bodies with plasmalemmal dense plaques [30]. Intermediate fibers may play a role in the cellular transmission of active and passive force, as demonstrated by a significant reduction in the active force per cross-sectional area in smooth muscle (urinary bladder and vas deferens) in a transgenic desmin null mouse model [31]. Their functional role in myometrium is unknown, although intermediate filaments were identified in gene clusters activated at term mouse myometrium (compared to the murine genome [32]), and in those mediating microtubule polymerization and microtubule-based movement in late pregnant (96% gestation) guinea pig myometrium (compared to the human genome [33]).

Dense Bodies – Dense bodies (so named because of a high affinity for electron-dense substances) are located within the cytoplasm and at the plasmalemma (1.2 μm long, up to 0.3 μm wide) and are referred to as "dense bands" or "plaques". They are composed of α-actin and are the site of attachment for thin actin filaments and intermediate filaments, and have been suggested to function as a primary link between the contractile apparatus and the cytoskeleton [34]. Some putative myometrial dense plaque proteins (α5 integrin, focal adhesion kinase, paxillin, heat shock protein (hsp) 27, and extracellular signal-related (ERK) kinase 2) are upregulated in rat myometrium at term [35-38].

Myosin-containing (thick) filaments – A thick filament subunit is composed of two heavy chains and four light chains. Each heavy chain (MHC, 220 kDa) has a globular head domain and an α-helical coiled-coil rod domain. The two rod domains are in parallel arrangement and coil around one another into a left-handed super helix, leaving the globular heads at one end of the rod. A

regulatory light chain ($MLC_{20,}$ 20 kDa) and an essential light chain ($MLC_{17,}$ 17 kDa) are associated with each globular head of a MHC. Although the exact organization of myosin filaments is the subject of debate, multiple subunits (hexameric groups of two MHCs and four MLCs) are associated in an anti-parallel fashion, with the globular heads exposed at opposite poles for interaction with thin actin filaments [39]. A single myosin filament (150 Å diameter) is located equidistant between two dense bodies and overlaps the many actin filaments emanating from dense bodies [30]. Myosin filaments also possess sidepolar cross-bridges, which hinge in opposite directions on opposing sides of the myosin filament for attachment to actin filaments. The cycling (time from attachment, to release, and subsequent reattachment) of cross-bridges is extremely slow compared to skeletal muscle, but the length of attachment is much longer. This translates into a greater force of contraction at reduced energy expenditure [40]. An additional feature of smooth muscle is the "latch" mechanism, whereby full force of contraction is maintained with only minimal excitatory signal needed compared to that required for initiation of contraction [41].

Ion channels – Fast and voltage-gated sodium channels, calcium channels, and potassium channels have been described in myometrial cell membranes. During pregnancy the Na^+ ion is the main determinant of the resting membrane potential, which in turn establishes the degree of membrane polarization. An increase in the Na^+ current densities was observed approaching term gestation [42], and a Na^+ gradient was important for the regulation of myometrial contraction [43]. L-type voltage-dependent Ca^{2+} channels (nifedipine-sensitive) are thought to be responsible for oxytocin (OT)- and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions and increases in intracellular calcium concentration in pregnant myometrium [44,45]. While L-type Ca^{2+} channels may be involved with agonist stimulated myometrial contraction, there is no evidence that these

channels are upregulated in term myometrium. Blockade of T-type Ca^{2+} channels by mibefradil indicates that this channel may also be important for OT-induced contractions [46].

1.3.4 Myometrial Contraction

Contraction of the myometrial smooth muscle cells begins with an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Four Ca^{2+} ions bind with the calcium binding protein calmodulin (CAM, 17-kDa) resulting in a conformational change in CAM, allowing Ca-CAM to interact with myosin light chain kinase (MLCK, 130-160 kDa). The MLCK phosphorylates Ser_{19} of MLC_{20} (regulatory chain), increasing the actin-activated Mg-ATPase activity of myosin, and its interaction (initiation of a cross-bridge cycle) with actin [40,47]. (See Figure 1.1) Substances ("contractile agents", "uterotonins", "uterotonic agonists") which increase $[Ca^{2+}]_i$, either by entry through receptor-mediated Ca^{2+} channels or release from intracellular stores will increase MLCK activity leading to contraction.

The principle agonists of myometrial contraction are oxytocin and prostaglandins ($PGF_{2\alpha}$ and PGE_2), which exert their effects through specific receptors on the cell membrane. Other agents such as endothelin-1, bradykinin, platelet-activating factor, and cholinergic agents increase MLC_{20} phosphorylation either by increasing $[Ca^{2+}]_i$ or decreasing cAMP activity [20]. The role of corticotrophin releasing hormone (CRH) as an indirect uterine stimulant in primates has received considerable attention. It is produced by the placenta (syncytiotrophoblast and intermediate trophoblasts) in response to cortisol, IL-1β, PGE_2, and OT, and it stimulates increased prostaglandin H synthase-2 (PGHS-2), PGE_2, and $PGF_{2\alpha}$ production by the placenta (review [48]). CRH production has not been documented in other species, and was shown to have extremely limited ability to cross the rat placenta from the maternal circulation [49]. Relaxation of smooth muscle occurs by dephosphorylation of MLCK by a type 2A phosphatase

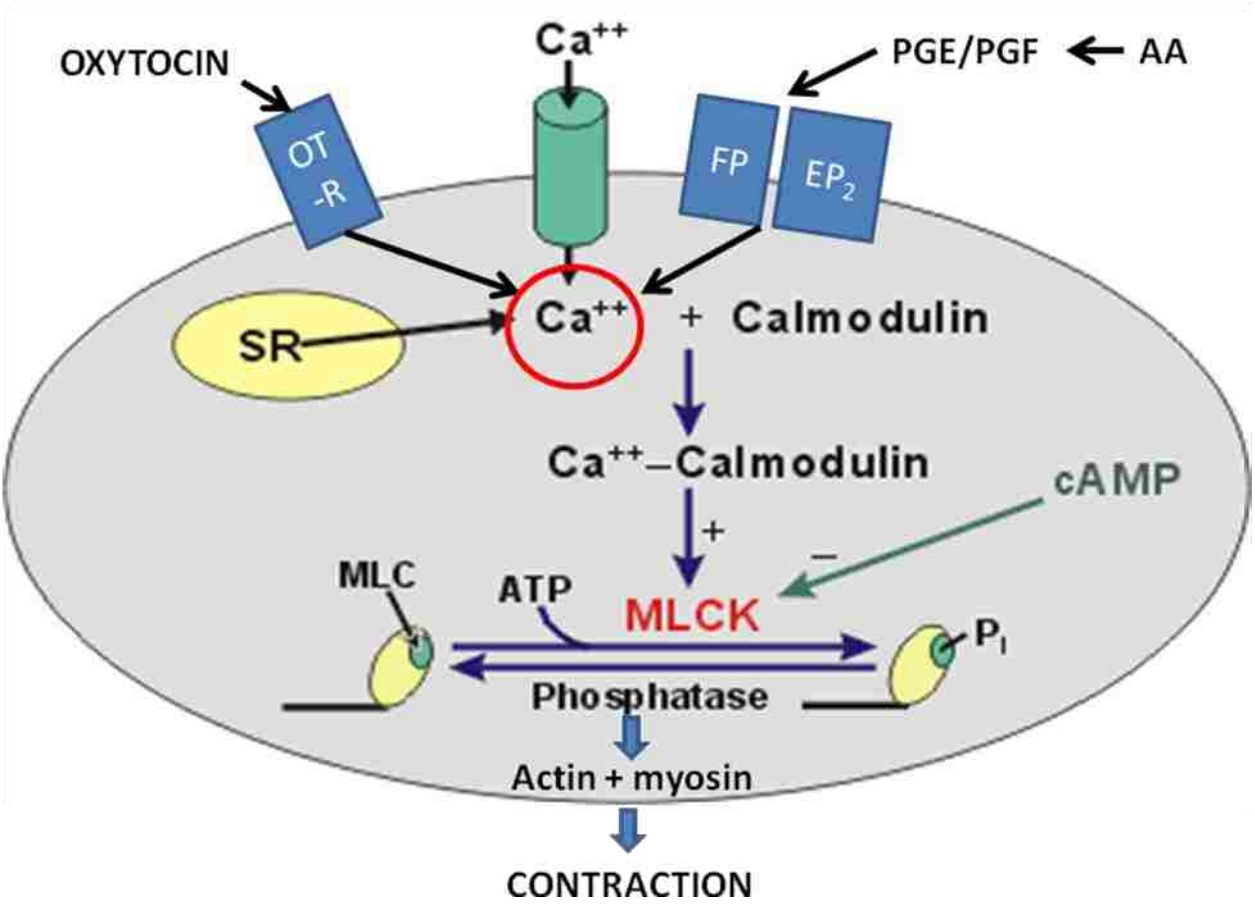

Figure 1.1 Biosynthetic pathways involved with smooth muscle contraction. Adapted from Challis et al. (2000) [48]. Abbreviations: oxytocin receptor (OT-R), AA (arachidonic acid), prostaglandin E_2 (PGE), prostaglandin $F_{2\alpha}$, (PGF), sarcoplasmic reticulum (SR), cyclic adenosine monophosphate (cAMP), adenosine triphosphate (ATP), myosin light chain (MLC), and myosin light chain kinase (MLCK).

[50], or by reduction of MLCK activity. Reduction of $[Ca^{2+}]_i$ reduces MLCK activity by dissociation of calcium from CA-CAM.

1.3.5 Stimulatory Agents

1.3.5.1 Oxytocin

Oxytocin is a nonapeptide (OT; 1007 Da; sequence: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2) synthesized by the supraoptic and paraventricular nuclei of the hypothalamus, and is stored bound to neurophysin 1 within dense-core vesicles of the posterior lobe of the pituitary

gland. The principle physiologic actions of OT are involved with contractions of the myometrium, epididymis, vas deferens, and myoepithelial cells within the mammary gland. OT causes direct stimulation of myometrium by increasing intracellular Ca^{2+} concentrations and MLC_{20} [51]. Indirect myometrial stimulation also occurs through increased prostaglandin release from the deciduas [52,53].

The oxytocin receptor (OTR) is coupled to several G proteins [54] and is dramatically increased near parturition [48], as demonstrated by a four-fold increase in *in vitro* myometrial sensitivity to OT and a ten-fold increase in OT-receptors in the 24 h preceding parturition in the rabbit [53]. Interestingly, oxytocin may not be essential to labor, as evidenced by failure of OT antagonists to prevent labor in rats [55] and the normal initiation of parturition in OT-deficient mice [56]. While OT is undoubtedly involved with normal parturition, these studies suggest that labor can occur in the absence of signaling through the OTR. The *in vitro* resting tension, contraction amplitude, and contraction frequency of equine myometrium displayed a dose-dependent response to OT, although at higher doses of OT, the amplitude and frequency began to decline. There was no effect of gestational age or presence of progestagens (progesterone, 5α-DHP, ββ-diol, βα-diol, or 20α-5P; see Table 1 for nomenclature) either prior to addition of OT or at the time of OT addition on the response to OT [23]. Although it was concluded that progestagens are ineffective at controlling agonist stimulated myometrial contraction *in vitro*, the myometrial preparation was not separated by layer, possibly masking significant effects.

1.3.5.2 Prostaglandins

Prostaglandins are 20-carbon unsaturated fatty acid enzymatic derivatives of membrane phospholipids containing a 5-carbon ring. Prostaglandins are mediators of a wide variety of physiologic processes including platelet aggregation, renal function, bronchial reactivity,

vascular reactivity, and maintenance of gastrointestinal mucosal integrity, to name a few. Prostaglandins also have many key roles in reproduction including ovulation, gamete transport, implantation, luteolysis, ejaculation, cervical dilation, and myometrial contraction.

1.3.5.3 Prostaglandin Synthesis

Isoforms of phospholipase A_2 catalyze the release of unesterified arachidonic acid from membrane phospholipids and are broadly categorized into cytosolic phospholipases (30-110 kDa, with no catalytic requirement for Ca^{2+}) and secretory phospholipases (13-18 kDa, which require millimolar Ca^{2+} concentrations for catalysis) [57]. Type-IV cytosolic phospholipase A_2 ($cPLA_2$-IV) is increased at term compared to pre-term in guinea pig cervix [58], human amnion [59], human myometrium [60], and in ovine endometrium [61]. In sheep and women there is no change in myometrial $cPLA_2$ during gestation and labor [60,61]. Type-IIA secretory PLA_2 ($sPLA_2$-IIA) has been shown to either have no change [60] or to be increased in lower uterine segment of human myometrium near term and in labor [62].

Prostaglandin H Synthase – Arachidonic acid is metabolized to prostaglandin H2 (PGH_2) by prostaglandin H synthase 1 (PGHS-1, 70-71 kDa), also known as cyclooxygenase-1 (COX-1), which is a constitutively expressed enzyme. Prostaglandin H synthase-2 (PGHS-2, 72-24 kDa), also known as cyclooxygenase-2 (COX-2), can be induced by hormones, cytokines, growth factors, tumor promoters, and bacterial endotoxin. PGHSs catalyze both *bis*-oxygenase conversion of arachidonic acid to PGG_2 and the peroxidase conversion of PGG_2 to PGH_2 (review, [63-65]).

PGHS-1 and PGHS-2 expression was localized to human amniotic epithelium and mesoderm [66,67] , while the chorion leave was found to either express both PGHS-1 and PGHS-2 [67,68] or only PGHS-1 [66]. In most studies, the expression of PGHS-2 has been

shown to increase with labor [66,69] and the activity of PGHS-2 (measured as the ability of the microsomal fraction of chorionic tissue to convert arachidonate to PGE_2) was significantly greater in chorion from term spontaneous labor patients compared to chorion from term prior to labor patients [69]. A gestational age dependent increase in PGHS activity was observed after 35 to 37 weeks of human pregnancy (term < 41 weeks), and was highly correlated with PGHS-2 mRNA levels, but not PGHS-1 mRNA levels [68]. Similar to human fetal membranes, PGHS-1 and PGHS-2 mRNA were expressed in cotyledonary tissues from ewes at 40 d to term; and while no change in the relative abundance of PGHS-1 was observed during this period, the relative abundance of PGHS-2 mRNA increased sevenfold between 40 to 85 d and 140 to 145 d. No significant change in PGHS-2 was observed between ewes not in labor at 140 to 145 d and ewes in labor [70]. To more critically evaluate the changes occurring near term, the changes in PGHS-2 expression and production were compared in ewes not in labor (140 to 145 d), in early labor (143 to 149 d), and in active labor (145 to 149 d) in relationship to fetal PGE_2, maternal PGFM ($PGF_{2\alpha}$ metabolite), and PG receptor subtype expression. Although an observed trend of increasing PGHS-2 mRNA was non-significant, there was a significant increase in PGHS-2 protein in myometrium from ewes in labor compared to ewes not in labor. Fetal PGE_2 increases followed the pattern of increased placental PGHS-2 mRNA (progressive increase towards labor), while maternal PGFM did not increase significantly until the onset of labor. No change in PGE_2 or $PGF_{2\alpha}$ receptor subtype with respect to onset of spontaneous labor was identified [71]. Taken together these studies confirm an increase in PGHS-2 in fetal and maternal tissues from women and ewes at term. Comparable studies have not been carried out with tissues from equine pregnancies.

Specific synthases and isomerases convert PGH_2 to the primary prostaglandins. Both cytosolic and membrane-bound PGE synthases (cPGES, mPGES) have been described.

Prostaglandin E Synthase – Cytosolic-PGES is the glutathione (GSH)-dependent terminal enzyme in the PGHS-1-mediated biosynthesis of PGE_2, and is identical to p23, a hsp90-associated protein [72]. Membrane-bound PGES (mPGES, also referred to as microsomal PGES) is also GHS-dependant; and two isoforms (mPGES-1 and mPGES-2) have been identified [30]. Microsomal-PGES-1 is coupled to a PGHS-2-mediated pathway leading to the production of PGE_2 and its expression is increased with lipopolysaccharide (LPS), IL-1β, and TNF-α [73,74]. Microsomal-PGES-2 was initially isolated from bovine heart, is constitutively expressed, is not homologous with the PGHS S-transferase family, and is thought to be coupled with PGHS-1. All three isoforms have been detected in human myometrium [75,76], with no change in the expression of cPGES in lower uterine segment (LUS) with labor, but an increased expression of mPGES-2 in LUS with labor [76]. Similar to women in labor, upregulation of myometrial and fetal membrane mPGES, but not cPGES, was seen following LPS-induced labor. There was no difference in time to delivery between wild-type mice and mPGES-1 (-/-) mice, most likely due to upregulation of mPGES-2 in mPGES-1 knockout mice [77]. No changes in relationship to spontaneous or dexamethazone-induced labor were seen in PGES in sheep, and PGES actually declined in endometrium, myometrium, cervix, amnion, and placentome, compared to gestational-age matched controls [78,79]. The reasons for the differences seen between women and sheep are not entirely clear, but location of sampling and selection of primers are possibilities. A single site of the mid-section ovine uterus was sampled (horn not specified, but presumably gravid), so potential differences in expression of PGES at different sites within the myometrium are possible. The designs for oligonucleotide primers for PGES used in the study

were based on the previously reported bovine sequence for inducible PGES (membrane-associated), for which the homology with cPGES in that species has not been reported [80]. Another possibility is that upregulation of PGES is not coupled with PGHS-2 in ovine gestational tissues as was shown with IL-1β treatment of human trophoblast cells [81]. Bovine endometrium and myometrium (day 16 of diestrus) have been shown to possess PGES (Western blot and immunohistochemistry), and although the antibovine-PGES was generated from a primer sequence based on human mPGES, the *in vivo* activity was more similar to that described for cPGES, since interferon-τ (IFNτ; the embryonic signal for maternal recognition of pregnancy in this species) caused no change in PGES or PGHS-1 expression, while PGHS-2 was upregulated [82]. It should also be noted that confusion in the literature exists over the designation of the three isoforms. Soorana et al., [75] suggested that PGES-1 investigated was cytosolic, when in fact, according to the Genbank reference number, it was membrane-associated.

Prostaglandin F Synthase – Prostaglandin $F_{2\alpha}$ is produced by one of three pathways (review, [83]). (See Figure 1.2)

(1) PGE 9-ketoreductase (9-KPR, 36,000 kDa), present in cytosolic fractions, converts PGE_2 to $PGF_{2\alpha}$ in the presence of NADH/NADPH in a reversible fashion, and has been isolated from human [84] and bovine [85] term placentas, and bovine endometrial epithelial cells [86]. Very little has been reported on the roles of 9-KPR in reproductive tissues. Increased 9-KPR activity was seen with retained bovine fetal membranes, whether pre-term or term, with higher activity in caruncular tissue than cotyledonary tissue [87]. Down-regulation and activity of 9-KPR due to IFNτ has been observed in bovine endometrium [86], which suggests 9-KPR has a key role in the signaling events of maternal recognition of pregnancy in this species. Reduced

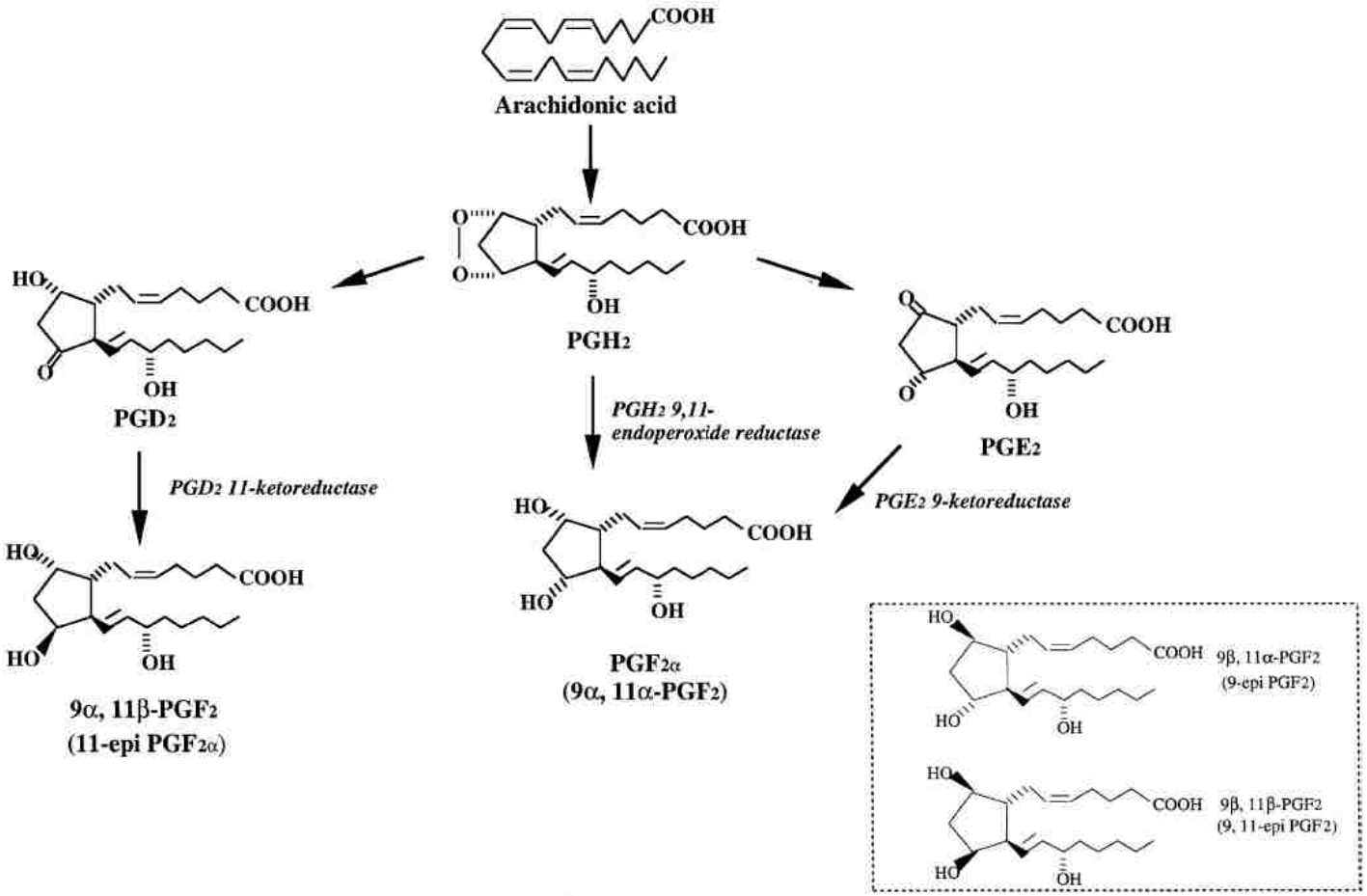

Figure 1.2 Pathways of PGF synthesis. Adapted from Watanabe (2002) [83].

activity of 9-KPR due to diethylamine NONOate (NOC-18, a nitric oxide donor), and increased activity due to aminoguanidine (an iNOS inhibitor) was seen in term human chorioamnion [88]. Further investigations are needed to determine if 9-KPR is increased in fetal and uterine tissues at term compared to pre-term, and whether *in vitro* incubation of endometrial epithelial cells with TNF-α or IL-1 causes increases in 9-KPR activity.

(2) PGD 11-ketoreductase (36,666 kDa) converts PGD_2 to 9α-, 11β-PGF2α (ll epi-$PGF_{2\alpha}$), requires NADPH as a cofactor, and has been isolated predominately in lung and liver [83]. PGD 11-ketoreductase was designated by Watanabe [83] as PGFSI (lung isozyme) and PGFSII (liver isozyme). PGFS (isozyme not specified) was detected in cycling and pregnant gilt myometrium; and although OT increased the *in vitro* production of $PGF_{2\alpha}$, there was no detectable change in the amount of PGFS [89].

(3) PGH 9-, 11-endoperoxide reductase converts PGH_2 to $PGF_{2\alpha}$, also in the presence of NADH or NADPH. Two PG endoperoxidases have been reported. The first was a 16,500 kDa glutathione- (GSH) dependent microsomal enzyme isolated from sheep seminal vesicles that did not utilize PGD_2 in the production of $PGF_{2\alpha}$ [90]. The second was a 33,000 kDa, cytosolic PG endoperoxidase isolated from *Trypanosoma bruci*, and although it was designated PGFS, it is considered distinct from mammalian PGFSs [91].

The effect of cytokines on prostaglandin production is well documented in a variety of tissues. Interleukin-1β (IL-1β) has been shown to increase prostaglandin production by cultured human amnion cells [92,93] and myometrial cells [94]; and while the administration of an IL-1β receptor antagonist prevented IL-β-induced labor in mice [95] it was unable to prevent delivery in endotoxin-induced pre-term labor [96]. The observed response to direct stimulation with IL-6 has been less consistent, varying from causing an increase in $cPLA_2$ expression by myometrial cells [75], to having no significant effect on PGE_2 production by human myoctyes [94].

1.3.5.4 Prostaglandin Metabolism

The enzyme 15-hydroxy prostaglandin dehydrogenase (PGDH) metabolizes the primary prostaglandins to biologically inactive metabolites [97], and due to its localization to trophoblast cells of the chorion, has been hypothesized to act as a barrier preventing the access of primary prostaglandins to the endometrium and myometrium [48]. PGDH is upregulated by progesterone (P_4), synthetic progestagens, and interleukin-10 (IL-10), and is down-regulated by glucocorticoids (endogenous or exogenous), IL-1β, tumor necrosis factor α (TNF-α), and CRH (review, [48]). A relative deficiency of PGDH activity was identified in a subset of human patients with idiopathic pre-term delivery [98]. The site of PGDH localization is somewhat different in the mare; PGDH was localized to maternal epithelium and interstitial cells in the microcotyledons of the equine placenta between 150 and 280 d, and to the maternal epithelium, maternal interstitium, and trophoblast cells by day 300 [99]. Altered expression or activity of PGDH due to infection in equine tissues, although not documented, would contribute to increased concentrations of $PGF_{2\alpha}$ and PGE_2 available to the myometrium.

1.3.6 Electromyographic Studies

The pattern of myoelectrical activity preceding parturition varies among species. In sheep during the second half of gestation, bursts of EMG activity occurred at 54.7 minute intervals and lasted for an average of 6.7 min. The frequency of activity increased in the 24 h preceding parturition, but the duration of bursts decreased [100]. Similar to sheep, no diurnal pattern of EMG activity was seen with goats [101], pigs [102], and cows [103,104]. In primates, a diurnal pattern of EMG activity was observed with a greater percentage of activity occurring during night and early morning hours [105-107]. Two patterns of activity have been described in primates. Contractures are approximately 5 minutes in duration and occur 2 to 6 times per hour. Contractions are 1 minute in duration, but occur up to 30 times per hour and with a greater force

of contraction than contractures. The switch from contractures to contractions occurs spontaneously at term, usually at the onset of darkness, over a variable number of days preceding labor [107]. The pattern of EMG activity in the mare is most similar to that of the primate, in that a diurnal pattern of activity exists with spontaneous parturition [108,109]. Interestingly in both the mare [108] and the goat [101], a period of quiescence (2 to 4 h and 8 to 12 h, respectively) was seen immediately prior to delivery. Relaxin was suggested as the cause of this phenomenon, possibly due to its ability to increase cAMP, leading to a lowering of $[Ca^{2+}]_i$ and a reduction in MLCK in myometrial cells. An increase in myoelectrical activity at night (spike bursts) was not seen in mares with experimentally induced placentitis [109], suggesting that pathways leading to spontaneous labor are altered with infection.

1.4 Gestational and Peripartal Endocrinology of the Mare (Control of Labor)

1.4.1 Maternal Progestagens

Progestagens are C-21 steroid hormones that are responsible for maintaining uterine myometrial quiescence during pregnancy. The biosynthetic pathway for their production is illustrated in Figure 1.3. Species such as the cow, rabbit, pig, dog, rat, and goat are dependent on P_4 from the corpus luteum for pregnancy maintenance, although placental contribution of progesterone during late gestation has been demonstrated in the cow and rat (review, [110-112]). In ewes, placental secretion of progesterone is sufficient to maintain pregnancy during the last two-thirds of pregnancy since ovariectomy after day 50 does not result in abortion [113]. Progesterone concentrations decline in all of these species approaching the end of gestation, with a precipitous drop immediately prepartum [111]. Primates are similar to sheep in that the placenta is the major source of progesterone during the last half of gestation. Serum P_4 in luteectomized baboons was unchanged from controls, and administration of the antiestrogen

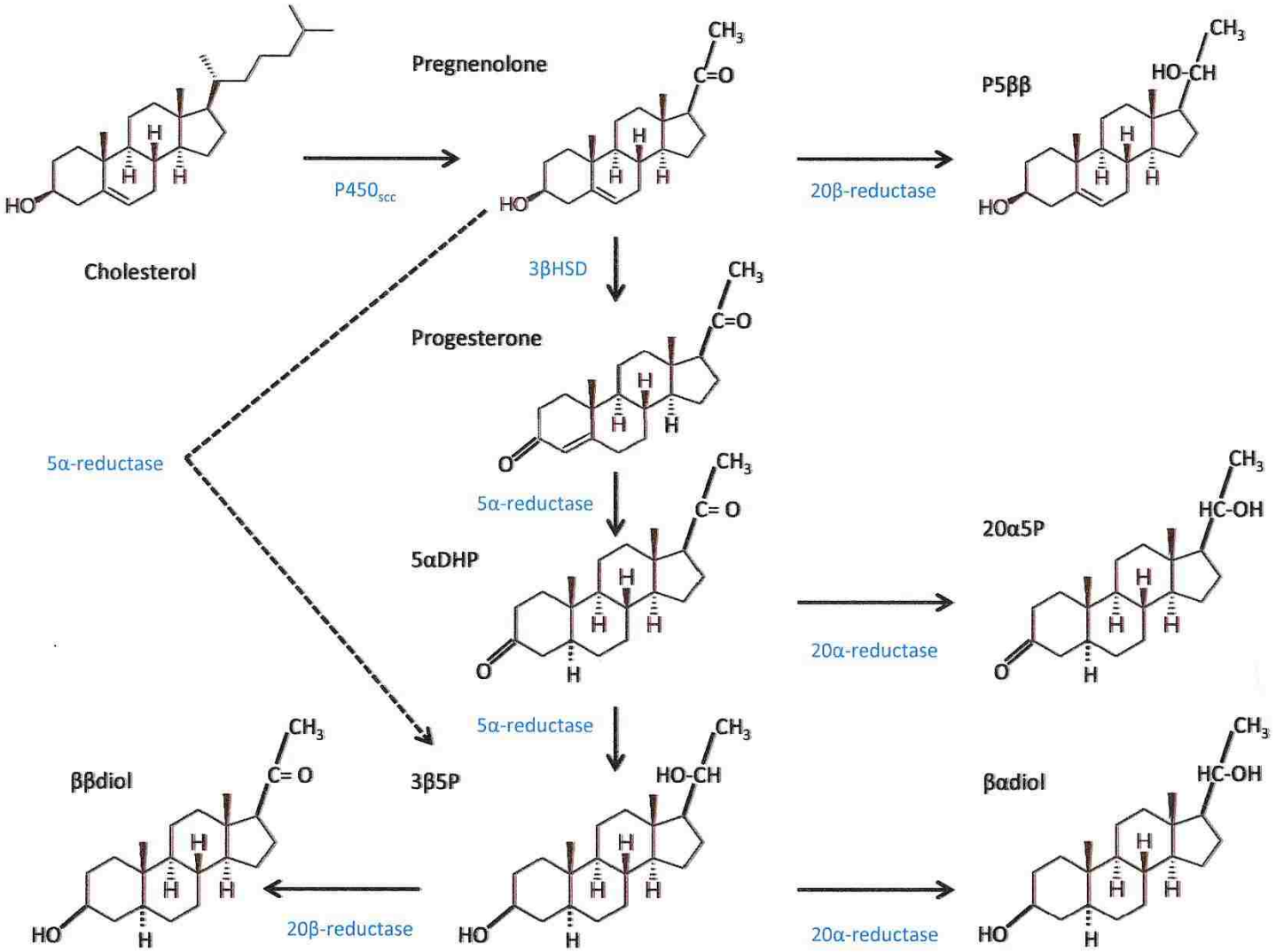

Figure 1.3 Biosynthetic pathways of progestagens in the pregnant mare. Adapted from Chavette et al. (1995) [114].

ethamoxytriphetol (MER-25) from day 130 to term (180 d) caused a 50% reduction of serum P_4 in intact animals, while producing an 80% reduction in luteectomized animals, indicating that the placenta is the predominant source of P_4 [115].

What is interesting is the concept that progesterone withdrawal, at least at the level of the maternal circulation, is not a prerequisite in the control of labor in primates [116]. Progesterone concentrations were measured in maternal and fetal blood, and in amniotic fluid, in long-term catheterized rhesus macaques from animals receiving epostane, an inhibitor of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) which converts pregnenolone (P_5) to P_4, alone or P_4 implants prior to the administration of epostane. Epostane alone caused a 75% reduction in maternal P_4 within 24 h and produced delivery within 48 h. Progesterone supplementation prevented alterations in maternal and fetal progesterone concentrations and uterine activity during the administration of epostane, but upon removal of the P_4 implant, delivery occurred within 48 h. A third group of animals received P_4 implants on day 146 to 148 of gestation (term gestation 167 d). These animals had markedly elevated maternal P_4, yet delivered spontaneously at term. Post-partum P_4 concentrations in this group were 8.13 ± 0.84 ng/mL, falling to 0.81 ± 0.13 ng/mL after implant removal [117]. Clearly, there are signaling events leading to labor that can override the effect of P_4 on myometrial quiescence.

In the mare, ovarian progesterone is responsible for maintenance of myometrial quiescence for roughly the first third of gestation; metabolites of progesterone produced by the fetoplacental unit assume this role thereafter. Holtan et al., [118] ovariectomized pony mares at different stages of pregnancy and revealed the following pattern: pregnancies less than 50 d resulted in abortion in all mares, 9 of 20 mares between 50 and 70 d aborted, and all mares over 140 d maintained pregnancy to term. The fetal gonad was subsequently hypothesized as a source of progestagens; however, this is not the case as was demonstrated by the failure of fetal

gonadectomy between 197 to 253 d of gestation to cause a reduction in maternal progestagens [119]. Progestagen profiles of the mare during late gestation have been studied extensively, with variable results depending on the type of assay used. Using an assay with a detection limit of 4 ng/mL, Short [120] found no measurable progesterone in jugular vein samples in mares from 120 to 310 d of gestation, but was able to measure progesterone in cord blood (3.8 to 6.3 ng/mL) of fetuses at parturition. Other early studies using competitive protein-binding assay or radioimmunoassay (RIA) found rising concentrations of progestagens (immunoreactivity with progesterone) during late pregnancy with maximums of 20 [121], 12.8 [122], and 10 ng/mL [123] on the day preceding foaling. Although a precipitous decline in progestagens began just prior to foaling, the progestagen concentration at the time of parturition was 11.3 [122] and 5 ng/mL [123], and was 3.3 ng/mL for progesterone [122]. This pattern is distinctly different from that observed in the aforementioned domestic animals.

In order to identify and quantify specific progestagens, Holtan et al., [124] used gas chromatography-mass spectrometry (GC/MS) to evaluate samples from maternal blood samples in normal (n=10) and abnormal (n=4) pregnancies, and from fetal and maternal blood samples in chronically catheterized animals or during general anesthesia (n=11). The systematic names of the progestagens are listed in Table 1. Progesterone, 5α-DHP, and 3β-5P had parallel increases during early pregnancy; but after day 200 P_4 was undetectable in most mares, while 5α-DHP and 3β-5P remained elevated to term. Other pregnanes were detectable after 60 d of gestation; 20α-5P and βα-diol were higher than all other pregnanes from day 170 to term. During the last 30 d of gestation 20α-5P increased 4- to 6-fold, reaching a maximum concentration (656.4 $\pm$ 210.7 ng/mL, range of 358 to 1063 ng/mL) 2 d prior to foaling. Twelve to twenty-four hours prior to

Table 1.2 Systematic names and abbreviations of progestagens.

Systematic name	Abbreviation
5α-Pregnane-3,20-dione	5α-DHP
4-Pregnene-3,20-dione	P_4
3β-Hydroxy-5-pregnen-20-one	P_5
3β-Hydroxy-5α-pregnan-20-one	3β-5P
20α-Hydroxy-5-pregnan-3-one	20α-5P
5-Pregnene-3β,20β-diol	P5ββ
5α-Pregnane-3β,20β-diol	ββ-diol
5α-Pregnane-3β,20α-diol	βα-diol
20α-Hydroxy-4-pregnen-3-one	20α-OH
20β-Hydroxy-4-pregnen-3-one	20β-OH

foaling a decrease in 20α-5P and βα-diol occurred, and by 24 h post-partum all pregnanes were undetectable. Pregnenolone, P5ββ, 3β-5P, and ββ-diol were significantly higher in the fetal artery than all other vessels (fetal or maternal). The predominant steroid in the fetal vein was 5α-DHP, and its concentration was higher than that of the fetal artery. Progesterone was higher in the fetal vein than fetal artery, and was detectable in only 3 of 19 maternal venous samples and no maternal artery samples. The concentration of 20α-5P was higher in the maternal vein than the maternal artery and was only detected in 1 of 8 fetal artery samples. Venous plasma samples from four mares with abnormal pregnancies were also evaluated by GC/MS and RIA-P_4. A transient increase in 20α-5P and RIA-P_4 was detected following endoscopically-induced placental separation in one mare; this mare went on to carry to term with a progestagen pattern similar to that of normal pregnancies. A mare with vaginal discharge that delivered with dystocia had increases in 20α-5P and RIA-P_4 from 30 d until 4 d prior to delivery. An EHV-1 infected mare delivered a dead fetus at 320 d having a profile that was a gradual decline in 20α-5P and RIA-P_4 during the week preceding the abortion. A mare with premature udder development delivered a premature foal at 294 d with a pattern of declining 20α-5P and RIA-P_4 in the 20 d preceding delivery. It was suggested [124] that abnormal pregnancies are associated with a

pattern of decreasing progestagens; however, this conclusion is based on a small sample size with a single observation from a specific type of abnormal pregnancy.

The possible site of 5α-DHP production in pregnant horse and pony mares during the last six weeks of gestation was investigated using a specific ELISA for this progestagen metabolite [125] . Samples were also obtained from four pooled samples collected at 20 to 30 minute intervals from a maternal artery and uterine vein, and umbilical artery and vein from a pregnant mare under general anesthesia at 254, 280, 284, or 300 d of gestation. At the end of the anesthetic period, the fetus, placenta, and endometrium were collected to evaluate the ability of each tissue to convert [^{3}H P_5] and [^{14}C P_4] to various progestagens as measured by thin-layer chromatography (values were expressed as the mean $\pm$ SEM percentages of added radioactivity found in TLC). There was no significant difference in the 5α-DHP concentration between pony and horse mares prior to 30 d prepartum, and both groups had increases during the last month which peaked at 3 d prepartum; however, during the last 30 d, the concentration of 5α-DHP in thoroughbred mares was higher than in pony mares at the majority of sampling points. Umbilical and venous plasma concentrations of 5α-DHP were higher than those of the umbilical and uterine artery. Chorioallantoic membranes surgically collected from day 254 to 300 were able to convert P_5 to P_4 (47.5 $\pm$ 9.0%), 5α-DHP (2.6 $\pm$ 1.2%), and 20α-DHP + 20β-DHP (4.1 $\pm$ 1.7%), and to convert P_4 to 20α- and 20β-DHP (7.7 $\pm$ 2.0%), and to 5α-DHP (2.9 $\pm$ 1.2%). The endometrium was able to convert P_5 to P_4 (11.0 $\pm$ 3.0%) and 5α-DHP (7.8 $\pm$ 2.4%); but the P_4 production was lower than that of chorioallantois, while the 5α-DHP production was higher than that of chorioallantois. There was negligible production of 5α-DHP from P_5 or P_4 by fetal tissues. Based on the venous-arterial (V-A) differences and the conversion of P_5 and P_4 to 5α-DHP by fetal membranes, it was concluded that maternal 5α-DHP is derived from endometrial metabolism of P_5 and/or P_4. Unfortunately, these conclusions are based on samples from a single

mare at each gestational age evaluated, and were obtained at the end of an anesthetic period of at least two hours duration.

Ousey et al., [126] catheterized the fetal aorta and vena cava, umbilical vein, uterine vein, and maternal aorta at three stages of gestation [180 to 220 d (n=5), 260 to 280 d (n=3), and > 300 d (n=4)] to measure umbilical and uterine plasma flows and progestagen concentrations (GC/MS). Antipyrine steady-state diffusion techniques were used to determine the uterine and umbilical flow rates, and were converted to plasma flow rates based on maternal and fetal hematocrits. Using Fick's principle (the rate of diffusion is proportional to the difference in concentration), uteroplacental uptake (UPU) and output (UPO) of the progestagens P_5, P5ββ, P_4, 3β-5P, 5α-DHP, ββ-diol, βα-diol, and 20α-5P were calculated. The following equations were used to calculate the uptake or output by the uterus or placenta:

$UPU_{umbilical\ circulation} = Flow_{umbilical} \times [A_{fetal} - V_{umbilical}]$ plasma

$UPO_{umbilical\ circulation} = Flow_{umbilical} \times [V_{umbilical} - A_{fetal}]$ plasma

$UPU_{uterine\ circulation} = Flow_{uterine} \times [A_{maternal} - V_{uterine}]$ plasma

$UPO_{uterine\ circulation} = Flow_{uterine} \times [V_{uterine} - A_{maternal}]$ plasma

where [A – V] = arterial – venous, or [V – A] = venous – arterial plasma concentration difference across the respective circulations. Significant uteroplacental uptakes from the umbilical circulation of P_5, P5ββ, 3β-5P, ββ-diol, and βα-diol; and significant uteroplacental outputs of 5α-DHP to the umbilical circulation during the second half of gestation were detected. Uteroplacental output of P_4 and 20α-5P to the umbilical circulation was also detected after 260 d gestation. There was no significant uteroplacental uptake from the maternal circulation of any of the progestagens. Significant uteroplacental outputs of 5α-DHP, βα-diol, and 20α-5P to the maternal circulation were found in all groups, with significantly higher concentrations of 20α-5P and βα-diol in pregnancies >300 d compared to pregnancies 180 to 220 d. There was a 5- to 10-

fold increase in the net output of 20α-5P (predominately to the mother) after 300 d, which most likely accounts for the increase in total progestagens seen near term in previous studies [121-124,127]. Based on the net uteroplacental uptakes and outputs, the main precursors for 5α-DHP and 20α-5P appeared to be P_5 and 3β-5P. The rate of uptake of these two progestagens increased 5-fold between mid and late gestation. It was concluded that the source of fetal P_5 and 3β-5P during the last half of gestation is difficult to explain, since fetal gonadectomy fails to reduce maternal progestagens [118] and fetal adrenal activity of $P450_{scc}$ was not detected until near term [114], although $P450_{scc}$ has been localized to the fetal adrenal during late gestation [128]. Although the full complement of enzymes involved in progestagen biosynthesis have not been identified in uteroplacental tissues, the equine term placenta has been shown to possess 3β-hydroxysteroid dehydrogenase (3β-HSD, Figure 1.3) activity [129].

If the assumption that the fetoplacental unit is the source of progestagens during the last half of pregnancy in the mare is correct, then processes that adversely affect the health of the fetus or placenta would be expected to affect progestagen production and peripheral maternal concentrations. Following induced placental separation between 228 and 262 d of gestation, jugular venous progestagen concentrations (RIA-P_4) were increased (defined as a value 2 s.d. outside of the mean established for that individual prior to induced separation) in 4 of 7 pony mares within 4-11 d of the procedure [127]. Increases were not seen until 42 d after the procedure in a fifth mare. Five of the placentas were available for examination; 4 of which had gross placental pathology. Three of the four placentas with pathology were from mares with increased progestagen concentrations. Four mares delivered live foals (3 induced deliveries) with a mean gestational age of 323 d. The three aborting mares delivered between 9 and 28 d (1 induced delivery) after the procedure. Placental histopathology was not performed. Repetitive embolization (112 to 128 d of gestation, term 144 to 151 d) of the uterine arterial circulation in

chronically catheterized pregnant ewes produced a significant increase in fetal arterial cortisol, with labor occurring during or immediately after embolization in 66% of ewes. Maternal progesterone exhibited an extremely variable pattern; however, when the output of progesterone was compared, there was a statistically significant increase in progesterone concentrations in embolized ewes compared to control ewes [130]. Similarly, comparison of plasma progestagen concentrations (RIA-P_4) from 25 abnormal equine pregnancies (n=25; criteria: RIA-P_4 > 10 ng/mL and < 308 d of gestation) to a group of control pregnancies revealed that 16 had increased progestagens, 24 had abnormal placentas, and 18 live foals were produced, of which 11 were abnormal based on postnatal behavior [127]. Unfortunately, only gross examination was used to determine placental abnormalities, and the types of placental pathology varied from avillous, edema at pole (although not defined by the authors, this is presumably cervical), twins, or necrotic; and more than one classification was present in many instances.

Subsequently Ousey et al., [131] used GC/MS and RIA-P_4 to evaluate plasma progestagens from normal and abnormal pregnancies with the aim of identifying specific progestagens present and their ontogenic profile in 14 mares with concurrent premature pregnancy termination. Mares with placentitis (n=7) had elevated (> 99.8% CI) concentrations of P5ββ, ββ-diol, βα-diol, and 20α-5P compared to control mares. Compromised pregnancies due to non-inflammatory placental lesions (avillous placenta, placental edema) or maternal causes (colic, uterine rupture, laminitis, uterine torsion, and premature udder development) displayed a mixed profile of progestagen concentrations (elevated, normal, or decreased); however, each type of pathology was represented by only a single mare. A similar finding of reduced progestagens following medical or surgical disease has previously been reported [132,133]. It was speculated [131] that placentitis causes fetal stress and precocious fetal adrenal maturation, which results in an overproduction of P_5, and that diseased uteroplacental tissues retain their

capacity to metabolize P_5 to 5α-DHP, βα-diol, and 2α-5P leading to increased circulating concentrations of these metabolites An alternative explanation suggested was that fetal production and metabolism are altered as well, not only leading to increased P_5 output, but increased fetal hepatic conversion of P_5 to reduced 5α-pregnanes. Placentally derived CRH has been shown to be increased in women with pre-term delivery [134,135], and is believed to be involved with fetal hypothalamic-pituitary-adrenal axis (HPAA) activation during pre-term delivery. Localization of CRH in the equine placenta has not yet been described, but if present would provide greater insight to the endocrinologic pathways leading to HPA activation and pre-term delivery due to placentitis.

An experimental model of induced placentitis in the mare measured maternal plasma progestin profiles (RIA-P4; cross reactivities were 5α-DHP: 10%, 3α-5P: 0.25%, 20α-5P: 0.25%) before and after transcervical inoculation of *S. zooepidemicus* [136]. The endpoint used for statistical analysis was defined as a >50% change in progestin from a baseline sample within a mare. Placentitis was confirmed by gross and histopathologic evaluation of the placenta. The following patterns were observed: a decrease in 7 of 15, an increase in 6 of 15, and no change in 2 of 15 inoculated mares. Mares that aborted < 7 d from inoculation exhibited a decrease in progestins, while those aborting > 7 d following inoculation exhibited an increase in progestins. As a diagnostic tool, a change in progestin concentration had a sensitivity and specificity of 87% and 100% respectively, and the positive and negative predictive value (defined as the proportion of mares testing positive that were truly affected or testing negative that were truly unaffected) were 100% and 78% respectively. The inconsistent pattern of change in inoculated mares was thought to result from instrumentation with myometrial electrodes and allantoic catheters in 11 of the 15 inoculated mares. Data that were not described were the days from inoculation to abortion for each mare and the days from inoculation to detected change in progestin profile.

1.4.2 Fetal Maturation

In most species, activation of the hypothalamic-pituitary-adrenal axis (HPAA) is a key event in the control of labor at term. In sheep the increase in fetal cortisol during the last 2 weeks of gestation causes a decline in the placental production of progesterone and an increase in estrogen production (Figure 1.4) [137,138]. Labor can either be delayed by hypophysectomy [139] or adrenalectomy [140], or accelerated by fetal ACTH administration [141]. The fetal pig also has a gradual increase in cortisol during the last 2 weeks, but the final surge is of a shorter duration than that of the fetal lamb [142,143]. In contrast, the cortisol in the fetal horse only rises just before birth (Figure 1.5) [142,144]. HPAA activation in primates is characterized by increased fetal cortisol, dehydroepiandrosterone sulfate, estrone, and progesterone only a few days prior to term vaginal delivery [116]. Similar to sheep, administration of glucocorticoids does promote fetal maturation, and is commonly given to affect pulmonary surfactant secretion [145,146] . Activation of the HPAA has also been shown to be a key event in pre-term delivery due to intrauterine infection in women. Patients with intrauterine infection and pre-term delivery, as defined by recovery of a microbial pathogen from amniotic fluid or an amniotic interleukin-6 (IL-6) concentration > 2 ng/mL, had significantly higher amniotic fluid concentrations of dehydroepiandrosterone and cortisol than patients with pre-term delivery without intrauterine

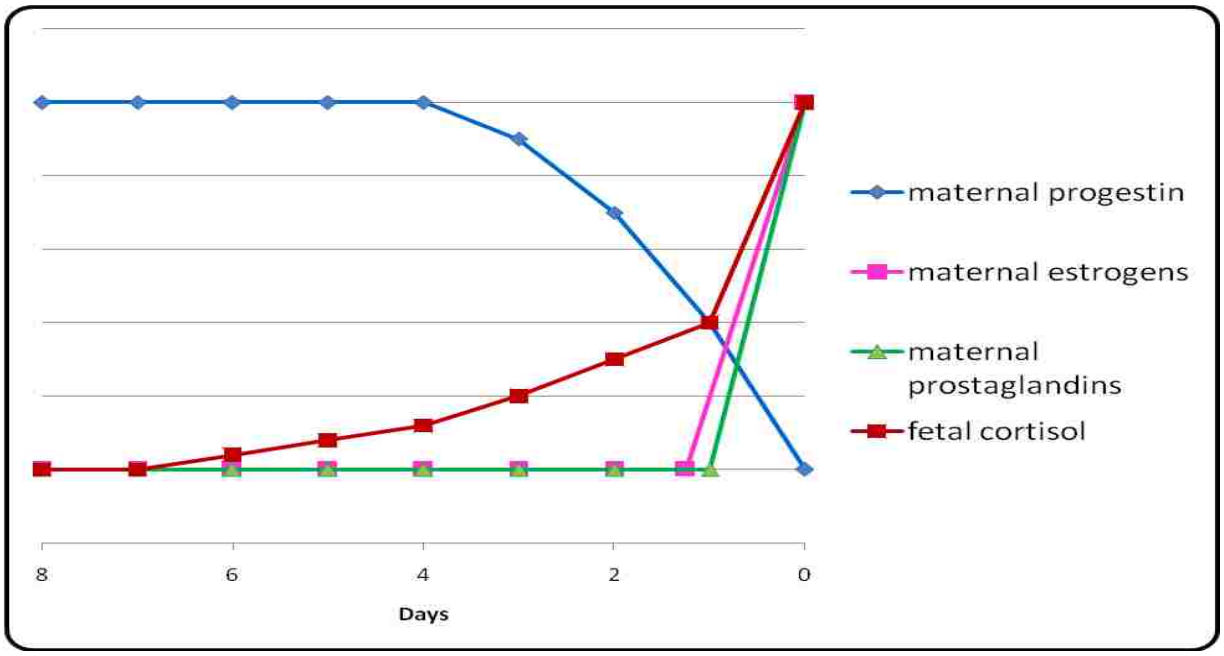

Figure 1.4 Relationship between fetal cortisol and maternal progesterone, estrogen, and prostaglandins in relation to parturition Day 0 in the ewe. Adapted from Challis et al. [138].

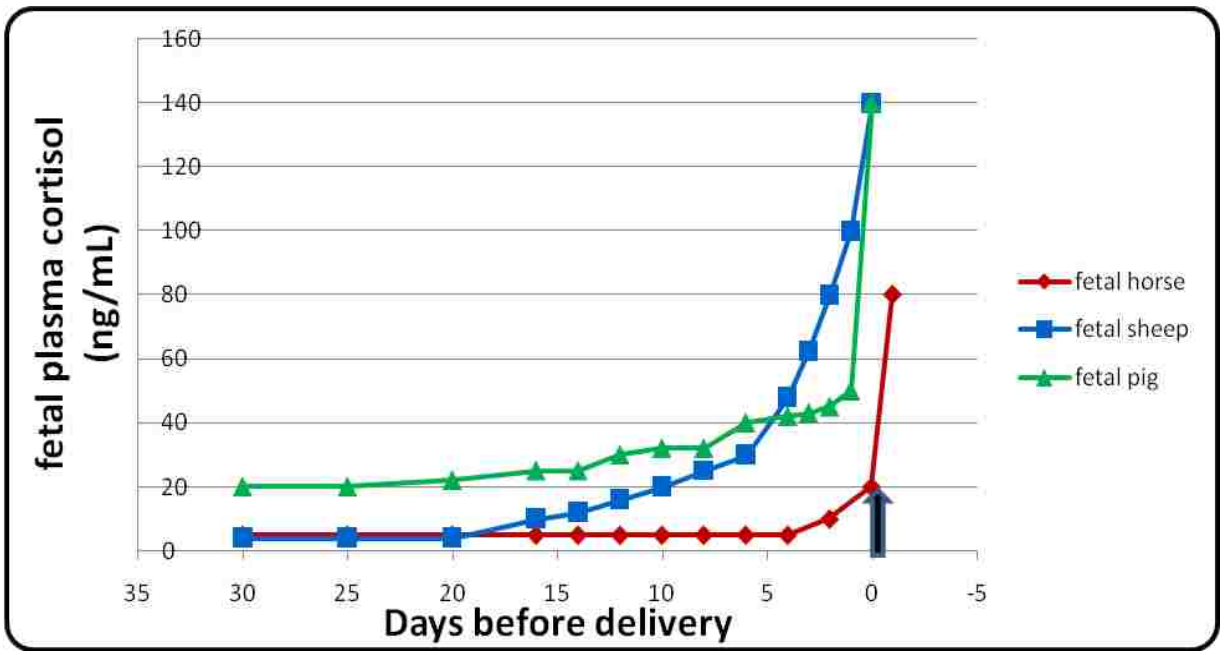

Figure 1.5 Prenatal changes in plasma cortisol in fetal sheep, pig, and horse in late gestation, Day 0 is day of delivery. Adapted from Silver et al. (1988) [142].

infection [147]. It was suggested that placental CRH is responsible for HPAA activation; however, elevated CRH has only been demonstrated in women in whom pre-term birth is not due to intrauterine infection [135].

Elevation of equine fetal cortisol was seen during the final 4 to 5 [148] or the final 9 d [144] of gestation. Levels for cortisol rose from 3.1 $\pm$ 1.0 to 13.4 $\pm$ 3.7 ng/mL, while significant increases in ACTH were not seen until the last 2 d of gestation (159 $\pm$ 21 to 246 $\pm$ 42 pg/mL) [144]. Fetuses less than 295 d of gestation failed to release cortisol in response to administration of intravenous $ACTH_{1-24}$ (the partially degraded product of ACTH having only amino acids 1-24 but maintaining all effects of the total molecule), those > 304 d showed small but significant releases in cortisol, while those > 313 d of gestation showed the greatest release of cortisol, demonstrating that the equine fetal adrenal responsiveness to exogenous ACTH does not develop until extremely late in gestation [148]. Based on the observed adrenal response in fetuses > 304 d, the investigators then compared the effects of intramuscular intrafetal $ACTH_{1-24}$ (n=16) administration daily for 3 d with saline administration (n=7), and whether induction of parturition 2 to 4 d after $ACTH_{1-24}$ administration was associated with a positive neonatal outcome (n=4) compared to spontaneous delivery. Five mares aborted immediately following the last $ACTH_{1-24}$ injection. Administration of $ACTH_{1-24}$ shortened gestation significantly in spontaneous delivery mares (312 to 314 vs. 327 to 333) and postnatal adaptive and endocrine patterns were normal; however, in pregnancies induced to deliver (306 to 308 d) 1 fetus died, and 5 of the remaining 6 foals required supportive care, and had lower plasma cortisol and elevated progestagens which failed to decline with the first 24 h of life [149] . Given the number of abortions and postnatal complications associated with intrafetal injections, the effect of maternally administered depot $ACTH_{1-24}$ (1 mg vs. 4 to 5 mg) at 300, 301, and 302 d on fetal

maturation was examined. Earlier parturition was significantly correlated with high dose ACTH given to mares mated late in the season (after July 1), compared to those mated early, although the strength of the correlation was not strong ($r^2 = 0.5$). Maternal plasma cortisol and progestagen concentrations were elevated following $ACTH_{1-24}$ administration, and this effect was greater and of a longer duration with high dose $ACTH_{1-24}$ [150]. It is apparent from these studies that not only does HPAA maturation occur only at the end of gestation, but that manipulations in an attempt to accelerate fetal maturation precociously are frequently complicated with a negative neonatal outcome. Effective interventional strategies to advance fetal maturation would be of great benefit in many instances of both reproductive and nonreproductive compromised pregnancies.

1.5 Immunologic Aspects of Infective Pre-term Delivery

1.5.1 Immunologic Aspects of the Non-pregnant Equine Uterus

The equine uterus is a mucous membrane; therefore, the predominant immunologic response to disease is characteristic of innate immunity. This is an obvious advantage when breeding on successive cycles to the same stallion, where a strong adaptive response would likely preclude establishment of pregnancy. Likewise, vigorous adaptive immunity would be disadvantageous during pregnancy, i.e., the fetus as an allograft [151]. From a historical perspective, much of the early work on defining immune parameters of the uterus and the cause of susceptibility to endometritis in mares focused primarily on the innate response to infection, namely opsonins in uterine secretions and the function of uterine-derived neutrophils. Once physical or mechanical clearance was determined to be an extremely important factor in the resolution of uterine infection [152-155], research emphasis shifted away from further investigation of the uterus as a mucosal immune system. In the meantime, the field of

immunology has expanded exponentially, and compared to other species, there are many basic aspects of equine uterine mucosal immunity that remain to be elucidated. Cells involved with adaptive immunity have been identified in endometria and the presence of mucociliary currents has been proposed; however, their role in diseases of the uterus is not yet clear.

1.5.1.1 Role of Immunoglobulins in Uterine defense Mechanisms

Immunoglobulin A [156-161], secretory IgA [159,160], IgG [157-159,161,162], IgG(T) [157-159], IgGa [156], IgGb [156], IgGc [156], and IgM [157,158] have been demonstrated in luminal secretions from mares resistant and susceptible to endometritis. Secretory IgA (11s peak) comprises a mean of 60% of the total IgA present in uterine secretions [161]. The former (and current) designations for the sub-isotypes of IgG are as follows: IgGa (IgG1, IgG2), IgG(T) (IgG3, IgG5), IgGb (IgG4), and IgGc (IgG6, IgG7) [163]. Demonstration of secretory IgA and higher Ig to albumin ratios in uterine secretions compared to those in serum supports the classification of the equine uterus as a local secretory immune system [161]. Transudation of Ig and active transport may also be a significant source of intraluminal Ig [160]. The relative contributions of active transport from serum versus local Ig production is largely unknown, but *in vitro* endometrial production of specific IgG, IgM, and IgA has been reported [164].

Results of studies on immunoglobulins in luminal secretions and immunohistology of the equine endometrium pose some interesting questions. First, that antigen processing in the equine uterus is variable. Intrauterine inoculation with *S. zooepidemicus* results in a local titer increase with no concurrent increase in systemic titer. Inoculation with *Taylorella equigenitalis* or dinitro-phenylated human serum albumin results in both local and systemic titer increases in antibody [165,166]. Despite no overall change in the peripheral titer, differences in the bactericidal activity of blood is seen following intrauterine inoculation with *S. zooepidemicus*; this activity is not only

strain specific, but also is comprised of heat-stabile and heat-labile components [167-169]. Antigen-processing and dendritic cell function in the equine uterus has not been studied. Second, since in general mares susceptible to endometritis have similar or higher levels of free Ig in the uterus, the common inference is that a dysfunction in the humoral response to intrauterine infection does not contribute significantly to increased susceptibility to endometritis. The suggestions that IgG_T [164] or IgA [170] may interfere with effective opsonization by specific antibody warrants further study. Investigation of bacterial proteins, such as immunoglobulin-binding proteins [171], which can reduce both antibody-mediated complement fixation and opsonization, would lead to a better understanding of the humoral response to intrauterine infection and its role in susceptibility to infection. Indeed, immunoglobulin-binding proteins have been identified in uterine secretions, and deplete hemolytic complement activity *in vitro* [172].

1.5.1.2 Chemotactic Properties of Uterine Secretions

The chemotactic activity of uterine fluid is influenced by ovarian hormones. Fluid collected during estrus possessed similar chemotactic activity to the activated serum control, but was significantly greater than that of diestrous uterine fluid [173]. Uterine washes from estrogen-supplemented ovariectomized mares prior to inoculation with *S. zooepidemicus* stimulated random migration of neutrophils under agarose, while those from control or progesterone-supplemented mares inhibited chemokinetic activity [174]. Infection [175], spermatozoa-induced complement activation [176], and susceptibility [177,178] appears to increase chemoattractants in uterine secretions, and susceptibility increases the chemotactic activity irrespective of stage of cycle or presence of uterine infection [177]. This pattern was thought to reflect the increased chemotactic activity present in uterine secretions from mares with active endometritis observed by Blue et al. [175]. Chemoattractants are composed of both heat-labile [176,179] and heat stabile [179] components, and are dose dependent [179]. This finding is specific to chemoattractants released by

the endometrium since *S. zooepidemicus* itself does not to possess chemotactic activity [180].

1.5.1.3 Opsonins in Uterine Secretions

Interest in characterization of opsonins in uterine luminal secretions began with the clinical response seen with the use of intrauterine plasma infusion in mares susceptible to endometritis [181]. The conclusion was that susceptibility to uterine infection may be due in part to deficiency either in quality or quantity of opsonins in uterine secretions [181]. *In vitro*, the addition of serum to uterine washings resulted in a significant increase in opsonization and phagocytosis by peripheral neutrophils, and this activity was reduced by complement depletion [182].

Non-infected estrous fluid promoted greater phagocytosis than diestrous fluid [183], and the opsonic activity of susceptible mares was either increased [177,184] or decreased [178] compared to that of resistant mares. The interaction between ovarian hormone status and susceptibility on opsonins is complex with opsonic activity being reported as greater in resistant mares during diestrus and greater in susceptible mares during estrus [184], while culture supernatant of endometria from mares treated with progesterone was less effective at bacterial opsonization than supernatant from control mares or those treated with estrogen [185]. The contribution of complement as an opsonin has also been controversial. In one study, opsonic activity was not reduced by heat inactivation, and the major opsonin was associated with IgG [184]. However, when standardized for protein content, both complement and specific IgG were found to be important opsonins in uterine washes from reproductively normal non-cycling mares [186]. An interesting observation of declining IgG concentration in susceptible mares by 36 h of infection [162] raises the question of whether decreased production or secretion, or increased consumption of IgG is involved with persistence of endometritis.

1.5.1.4 Complement in Uterine Secretions

Examination of complement components and hemolytic activity has yielded conflicting

results. Antigenic C3 and breakdown products of C3 were identified, yet no hemolytic complement (functional) activity was measured in uterine secretions [182], possibly due to the species of target red blood cells. Hemolytic complement activity using porcine red blood cells was significantly greater in uterine flushings from susceptible mares compared to resistant mares, although individual variability was high and stage of cycle was variable [177]. Lastly, immunoactive C3 was not significantly different between resistant and susceptible mares, although at 36 h after bacterial challenge immunoactive C3 was greater in resistant mares [162].

1.5.1.5 Antibacterial Activity of Uterine Secretions

Although it has been reported that cell-free uterine flushes from mares in mid-diestrus possesses antibacterial activity [173], other investigators failed to demonstrate inherent antibacterial activity of uterine fluid from noncycling mares [186] or from E_2- or P_4-supplemented ovariectomized mares [187]. The discrepancy in the results from these studies may lie in potential qualitative and quantitative differences between the uterine secretions from cycling and noncycling mares. Recently lactoferrin, an antimicrobial and immunomodulator member of the transferrin gene family which is expressed by epithelial cells and neutrophil granules, has been identified in the equine endometrium [188]. Lactoferrin's antibacterial property lies within its ability to sequester free iron, thereby inhibiting bacterial growth. Lactoferrin expression was upregulated during early estrus, protein staining was uninfluenced by cycle and was most intense in the glandular epithelium, and expression of lactoferrin was only increased in mares with delayed physical clearance during early estrus [188], which might represent a response to inflammation. While intriguing from a perspective of host-pathogen interactions, unless diminished response in lactoferrin expression and production were observed in susceptible mares, reduced lactoferrin activity is unlikely to be responsible for increased susceptibility to infection.

1.5.1.6 Modulators of Uterine Contractility

In cycling [189], and E_2- or P_4-supplemented ovariectomized mares [190,191], uterine luminal immunoreactive PGF, PGE_2 and leukotriene B_4 (LTB_4) dramatically increase following an inflammatory insult. Progesterone-supplemented, or P_4-supplemented followed by E_2-supplemented ovariectomized mares had higher concentrations of immunoreactive PGF and PGE_2, than those receiving E_2 alone [191]. Resistant mares have greater release of $PGF_{2\alpha}$ in response to antigenic stimulation (insemination) or oxytocin administration than do mares susceptible to persistent endometritis [192]. Since LTB_4 and PGE_2 cause dose-related chemokinesis of peripheral neutrophils, PGF_2 and LTB_4 increase bactericidal activity, and LTB_4 increases phagocytosis [193], their presence is unlikely to play a role in persistence of endometritis by adversely affecting neutrophil function. To the contrary, it has been suggested that increased eicosanoids in susceptible mares may contribute to enhanced phagocytic function [178].

Nitric oxide (NO), a bactericidal agent produced by macrophages and neutrophils following ingestion of microorganisms [194], can cause myometrial relaxation [195]. Nitric oxide concentration was higher in uterine secretions from mares susceptible to delayed clearance than in resistant mares [196]. Whether this is due to upregulation of NO-synthase or due to accumulation of inflammatory byproducts in susceptible mares was not determined, but it was speculated that NO accumulation may contribute to undesirable uterine relaxation following an antigenic challenge [196].

1.5.1.7 Functional Properties of Neutrophils

Uterine neutrophils from susceptible mares have been reported to have either reduced [197,198] or equal [178] chemokinetic function compared to resistant mares. Similarly, decreased [177,199] or increased [178,200] phagocytic function of uterine-derived neutrophils from susceptible mares has been reported. Clearly there is an interaction between stage of estrous cycle and category of mare and both may have a profound effect on the functional capacity of uterine

neutrophils. It is doubtful that some level of compromised uterine neutrophil function exists in the susceptible mare; the more likely explanation for findings to date is the effect of experimental design, specifically method of uterine neutrophil recruitment, stage of cycle, and differences in bacterial strain on in-vitro neutrophil function. Indeed, when a chemokine (recombinant human interleukin-8) was used to recruit neutrophils into the uterus not only was there no difference in phagocytic capacity of uterine neutrophils between normal mares and those with degenerative changes, but the capacity of uterine neutrophils from mares with degenerative uterine changes to generate reactive oxygen species was enhanced relative to peripheral neutrophils or uterine neutrophils from normal mares [201]. Diminished cellular clearance occurs most likely secondary to persistence of infection and continued bacterial growth, which exceeds the capability of normal phagocytic function.

1.5.1.8 Evidence of an Adaptive Immune Response in the Uterus

The endometrium of the mare is inhabited by T lymphocytes [202-206]. Genitally normal mares had greater numbers of CD4+ lymphocytes (T_H cell; "helper T cell") and CD8+ cells (T_C cell; "cytotoxic/suppressor cell") in the stratum compactum than in the stratum spongiosum [204]; the density of cells was either increased during the estrus [203] or found to be independent of cycle stage [204], was not influenced by age [206], and greater numbers of CD4+ and CD8+ cells were present in the uterine body than in the uterine horns [206]. Following insemination [205] or in the presence of endometritis [204] the number of CD4+ [204,205] and CD8+ cells [204] increased; the number of CD4+ cells doubled in lymphoid aggregates, and the number of CD8+ cells in the luminal and glandular epithelium increased [204] suggesting that an adaptive response is initiated following antigenic stimulation of the endometrium.

Exogenous antigens, such as those of *S. zooepidemicus*, are processed through an endocytic pathway and are presented on the surface of the membrane of cells which express class II Major

Histocompatability Molecules (MHC) [207]. Presentation of class II MHC to CD4+ T_H cells is restricted to "professional" antigen presenting cells (APCs), namely dendritic cells, macropages, and B cells. "Nonprofessional" APCs, such as certain epithelial cells, fibroblasts, and vascular endothelial cells, can present antigen in the context of class II MHC, but fail to deliver a costimulatory signal and present antigen for brief periods during a sustained inflammatory response. Cells in the equine endometrium expressing MHC class II staining include macrophages, monocytes, dendritic cells, lymphocytes, vascular endothelium, and uterine luminal epithelium [202]. Expression of MHC II was greater in genitally normal mares in estrus than in diestrus; the distribution was predominantly beneath the luminal epithelium, with occasional foci in the stratum compactum and rarely in the stratum spongiosum [204]. This pattern is consistent with antigen presentation. There was no difference in expression between infected and noninfected mares [204], which is similar to findings from murine models of intestinal Salmonellosis where little change from the steady state numbers of dendritic cells is seen with infection [208].

1.5.1.9 Cytokine Expression in Response to Infection and Insemination

It has been suggested that increased endometrial expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and IL-8 during estrus, increased IL-1β, TNF-α, and IL-8 during diestrus, and decreased expression of the anti-inflammatory cytokine IL-10 during estrus contributes to susceptibility to endometritis [209,210], although non-infected susceptible mares were not examined in these studies. This pattern would be predicted to occur with chronic-active inflammation; however, re-examination of mares susceptible to endometritis prior to initiation of an inflammatory insult would be useful to understand the potential roles of these cytokines during persistent endometritis. If elevated IL-8 expression and diminished IL-10 expression in susceptible mares in the absence of infection or inflammation exists, a potential factor in susceptibility to endometritis may be explained. These studies are a first step towards investigating the cytokine

signaling involved with endometritis, and clearly further study is needed in this area.

1.5.1.10 Mucociliary Clearance in the Mare

It has been recently suggested that mucociliary clearance may play an important role in the clearance of infections and that disruption of mucociliary currents in the susceptible mare leads to persistence of infection [211]. While mucus-producing cells and ciliated cells beneath a mucus blanket suggests a functional mucociliary apparatus in the equine uterus [212], *in vitro* documentation of ciliary movement is lacking [211]. Mucus production and secretion is increased during estrus [212] and inflammation [212,213], and the optical density of the mucus is increased in mares with delayed clearance [212]. The biological significance of alterations in the mucous blanket is unknown. The effects of fluid accumulations [214], intrauterine therapy [215], and uterine pathogens [214,216,217] on mucociliary clearance are deserving of future research, as is whether or not there is a primary defect in the mucociliary apparatus in mares susceptible to endometritis.

1.5.2 Animal Models for Infective Pre-term Delivery

The pre-term delivery (PTD) rate for human infants in 2005 was 12.7% [218], a rate that is on the rise, despite considerable efforts and progress towards defining the pathogenesis of pre-term births and advancing treatment strategies. Infection has been reported to be responsible for up to 10 to 50% of pre-term deliveries, with higher rates being seen in underdeveloped countries [219,220]. Intrauterine infection resulting from ascension of bacteria through the cervix with invasion of the chorioamnion and 41eciduas (maternal endometrium at the site of placental attachment) is a significant cause of pre-term delivery in women as it is in the mare [221,222]. A wide variety of animal models have been described to investigate the mechanisms of PTDs; but direct comparisons between models are hampered by differences in species used, the agents used to initiate PTD, and the route of inoculation or administration.

In most species studied, an increase in pro-inflammatory cytokines has been observed in fetal fluids in response to inflammation or infection [223-229]. Non-infectious models for pre-term delivery typically use lipopolysaccharide (LPS) administered into the maternal circulation [230], the maternal peritoneal space [231,232], or the uterus [233] to cause pregnancy loss. Intraamniotic, but not maternal, administration of LPS to sheep at approximately 80% gestation resulted in increases in amniotic and fetal IL-6 (immunoreactive) concentrations but no premature labor despite increased uterine activity, though there was a considerable degree of variability and the samples size were small [228]. Intraamniotic inoculation of group B streptococci in instrumented (fetal artery and vein, amniotic pressure catheter, myometrial EMG electrodes, maternal artery and vein) rhesus macaques at 77% gestation produced increased the bioactivity of TNF-α in amniotic fluid, and increased immunoreactive concentrations of IL-1β, and IL-6 as early as 9 h following inoculation. PGE_2 and $PGF_{2\alpha}$ concentrations paralleled the increases in cytokines, and uterine contractility occurred within 28 h. The peak concentrations of PGE_2, compared to control, were 16,046 pg/mL vs. 2,765 pg/mL ($p < 0.05$), and was 5,547 vs. 708 pg/mL for $PGF_{2\alpha}$ ($p < 0.05$) [224].

While intraamniotic inoculation may provide a greater uniformity of response due to precise deposition of the inflammatory agent, it does not reproduce what happens during the course of natural infection, namely ascension through the cervical canal. Other models have attempted to mimic natural infection by intracervical inoculation. Intracervical inoculation of rabbit does at 70% gestation with *E. coli* also produced increased bioactivity of TNF-α, and increased immunoreactive concentrations of IL-1β, and IL-6, and PGE_2 and $PGF_{2\alpha}$ in amniotic fluid as early as 4 h after inoculation [223]. Similarly, intracervical deposition of either LPS or *E. coli* in wild-type or IL-1β -/- mice produced increases in amniotic immunoreactive TNF-α, IL-β,

and IL-6 and premature delivery in 92% (*E. coli*) to 100% (LPS) of mice. Although decreased cytokine was observed in IL-1β -/- mice, pregnancy loss was the same [226].

One of the proposed mechanisms of infection-induced pre-term delivery is that pro-inflammatory cytokines (IL-1, IL-6, TNF-α) from the deciduas and cortisol from the mother cause activation of the fetal hypothalamic-pituitary-adrenal axis, causing elevation of fetal cortisol [48]. Increased fetal cortisol, either at term or in response to pro-inflammatory cytokines, decreases levels of PGDH, an enzyme responsible for inactivation of PGE_2 and $PGF_{2\alpha}$, leading to increased PGE_2 and $PGF_{2\alpha}$ and subsequent myometrial contractions. Pro-inflammatory cytokines also increase the activity of another key enzyme, prostaglandin H_2 synthase (PGHS-2), which increases prostaglandin synthesis. The decrease in PGDH and increase in PGHS-2 lead to increased PGE_2 and $PGF_{2\alpha}$ synthesis, myometrial contractions and premature labor (See Figure 1.6) [227,234-236].

1.5.3 A Model of Ascending Placentitis in the Mare

One model examining the relationship between inflammatory cytokines and ascending placentitis in pregnant mares has been reported [237]. Placentitis was induced by intracervical inoculation of 10^7 to 10^8 CFU of *S. zooepidemicus* in mares between 283 and 291 d of gestation. Groups consisted of mares instrumented with allantoic catheters 10 to 16 d prior to inoculation (n=4), non-instrumented inoculated mares (n=4), and non-inoculated control mares (n=4). Allantoic fluid samples were obtained from the catheter (if patent) or by allantocentesis from inoculated mares at pre-inoculation, 3 to 4 d post-inoculation, 6 to 10 d post-inoculation, and a sample within 48 h of delivery. Mares in the control group were sampled at 280, 288 to 290, 296 to 303 d, and within 48 h of delivery. Allanotic fluid was analyzed for TNF-α (cytolytic assay), IL-1 and IL-6 (lymphoproliferative assay), $PGF_{2\alpha}$ and PGE_2 (competitive binding RIA).

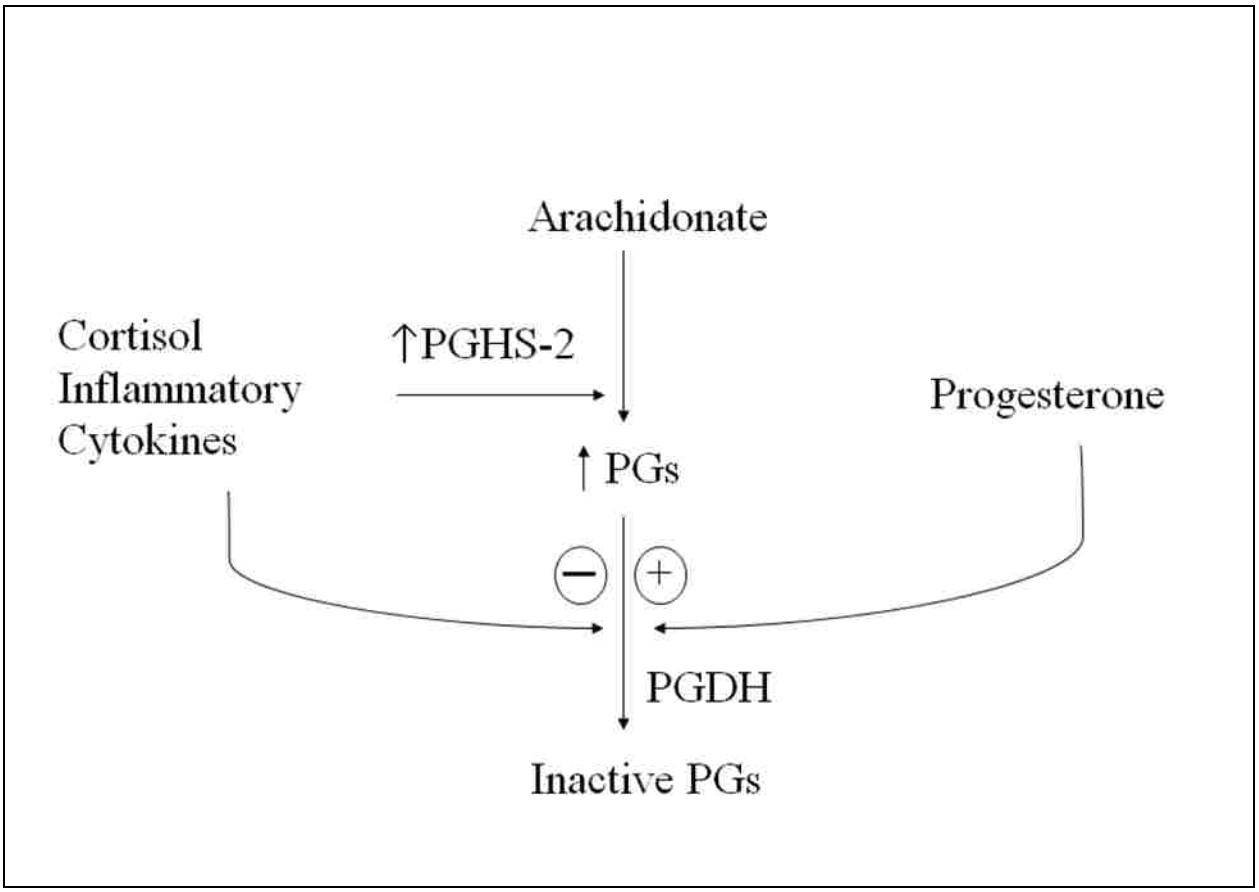

Figure 1.6 Role of progesterone and cytokines in regulating prostaglandin synthesis during pregnancy. From Challis et al., (2000) [48].

Placental tissue was collected for histopathology and mRNA expression of IL-1, IL-6, and IL-8. No differences were detected in the concentrations of soluble TNF-α, IL-1 and IL-6 between groups over time (means and standard deviations were not reported). Placental IL-6 and IL-8 mRNA was higher at the cervical star and IL-6 mRNA was higher in the placental body of inoculated mares compared to control mares (tissues not matched for gestational age). Based on the mRNA data it was hypothesized that bacterial infection causes increased expression of proinflammatory cytokines leading to the release of PGE_2 and $PGF_{2\alpha}$ into the allantoic fluid resulting in premature labor.

The failure to demonstrate an increase in the concentrations of pro-inflammatory cytokines in equine fetal fluids following bacterial infection differs from results from rabbits, monkeys, women, sheep, and mice [223-226,228,238]. There may be several explanations for this difference. In the study using mares [237], the number of days from inoculation to abortion ranged from 4 to 27, and the clinical and histologic characterization of the placentitis varied from acute to chronic, thus the experimental group was heterogeneous. This variation may have resulted from individual differences in response to the strain of *S. zooepidemicus* used [168], the variable dose of the inoculum, and difficulty in standardizing the site of inoculation within the cervical canal. Failure to detect soluble cytokines in the allantoic fluid may have been due to sampling intervals which were too infrequent to detect changes in cytokine concentrations. Amniotic levels of TNF-α, IL-1α, and IL-1β were elevated within 4 h of transcervical inoculation of pregnant rabbits with *E. coli* [223]. In pregnant ewes, the concentration of IL-6 in amniotic fluid peaked between 4 to 6 h following administration of LPS and had begun to decrease by 24 h [228], making it conceivable that changes in cytokine levels were missed in the equine study. Other potential sources of variability were the presence of allantoic catheters in some, but not all, of the mares; and repeated ultrasound-guided allantocentesis to obtain fluid from mares without catheters or those with non-patent catheters. Biochemical characterization [239,240] of fetal fluid was not performed, therefore the potential confounding effect of amniotic fluid contamination on analytes cannot be assessed.

1.6 Conclusions and Proposed Studies

The need still remains for a dependable method for collection of allantoic fluid that would permit reliable and frequent sampling within the first 24 h after transcervical inoculation. The temporal relationship between elevations in allantoic cytokines and prostaglandins is quite important. If the synthesis of $PGF_{2\alpha}$ and PGE_2 is driven by cytokines in concert with fetal cortisol, as has been suggested

in a model of ascending placentitis [48], then treatment strategies that fail to interrupt the inflammatory cascade and reduce fetal stress may be ineffective in delaying the onset of labor. In other species, activation of the fetal HPAA axis is likely a key event in placentitis-induced pre-term pregnancy loss [147]. However, this may not be the case for the horse, since the fetal HPA only matures near the end of gestation [126,241]. Therefore, it would be useful to delineate a noninfectious-inflammatory model from an infectious-inflammatory model. Inflammation of the chorioallantois will be induced by inoculation with live bacteria or heat-killed bacteria. Heat-killed *S. zooepidemicus* has been used both *in vivo* in an endometritis model in the mare [172] and *in vitro* in phagocytosis assays for equine neutrophils [184,200]. By comparing the inoculation with heat-killed *S. zooepidemicus* to the inoculation with live *S. zooepidemicus*, we may be able to separate the effects of a transient inflammatory response and resultant prostaglandin synthesis from the effects of placental, and possibly fetal, infection. We hypothesize that both types of inocula will induce an acute placentitis, that heat-killed *S. zooepidemicus* will produce a less variable pattern of inflammatory mediators than that produced by live *S. zooepidemicus*, and that live *S. zooepidemicus* will lead to pre-term delivery in all mares. Because the inflammatory insult initiated by heat-killed *S. zooepidemicus* would occur as a single event without continual replication and propagation of the inflammatory cascade as would occur with live *S. zooepidemicus*, it is unlikely to lead to pre-term delivery in all mares.

In the mare, ovarian progesterone is responsible for maintenance of myometrial quiescence for roughly the first third of gestation; metabolites of progesterone produced by the fetoplacental unit assume this role thereafter [241]. The fetus synthesizes pregnenolone, which is the precursor for a wide variety of progestagens. Two progestagens, 5α-pregnane-3,20-dione (5α-DHP) and 20α-hydroxy-5α-pregnan-3-one (20α-5P), are excreted into the umbilical and uterine circulation and are believed to be the most biologically important progestagens for preventing uterine contractions [126]. Changes observed in progestagen profiles during late pregnancy appear to depend on the nature of the stress and

the maturity of the fetal HPAA. Stress of the equine fetus due to infection-induced placentitis has been shown to result in two patterns of progestagen profiles: acute placentitis was associated with no change in total progestagens, whereas chronic placentitis was associated with elevations in maternal total progestagens [136]. In contrast, maternal progestagens tend to remain stable and then decline prior to abortion in cases of severe maternal stress without direct involvement of the fetus, such as colic or uterine torsion [133]. What is not clear from previous studies is why supplementation with synthetic progestins (altrenogest) in cases of placentitis is not always successful in preventing uterine contractions. Although maternal progestagens appear to provide some measure of placental health, what is needed is a measure of synthesis of pregnenolone from the fetus. Fetal output of pregnenolone and progestagens are typically measured in studies involving fetal umbilical vasculature, uterine vasculature, and maternal catheterization [126]. No attempts have been made to measure either cortisol or progestagens in allantoic fluid; therefore we propose to analyze allantoic samples serially to examine possible effects of placentitis on fetal synthesis of cortisol.

This is the first reported attempt to use laparoscopy rather than laparotomy for allantoic catheter placement in the mare [242]. Avoiding general anesthesia and laparotomy in a late gestation mare, with all the attendant problems of pre-operative fasting, intra-operative hypotension, post-operative ileus, and potentially increased concentrations of fetal cortisol due to maternal fasting, justify attempting the use of laparoscopy as a less invasive alternative to instrumentation of pregnant mares. We will test the hypothesis that TNF-α, IL-1 and IL-6, which increase in response to inflammation of the chorioallantois, are temporally related to increases in allantoic cortisol, $PGF_{2\alpha}$ and PGE_2, leading to myometrial contractions and premature foal delivery. Inflammation of the chorioallantois will be induced by inoculation with live or killed bacteria.

CHAPTER 2

LAPAROSCOPIC PLACEMENT OF AN INDWELLING ALLANTOIC CATHETER IN THE MARE: DEVELOPMENT OF THE TECHNIQUE

2.1 Introduction

Catheterization of fetal vasculature, maternal vasculature, and the allantoic and amniotic compartments has been used to study normal physiology and pathologic conditions of pregnancy in many species for several decades. Undoubtedly the knowledge gained has been invaluable; however, the invasiveness of the described surgical procedures are tremendous, leading to fetal demise, potential alteration of endocrine function of the dam and fetus, and limited ability to re-instrument the dam in future pregnancies. The mare has proven to be extremely problematic for these studies, especially with regards to allantoic catheterization. A recently reported model of placentitis in the mare showed increased expression of pro-inflammatory cytokines in the chorioallantois of mares transcervically inoculated with *Streptococcus equi* subsp. *zooepidemicus* compared to control mares, while no differences were detected in activities of soluble cytokines from both groups of mares [237]. In women, rhesus monkeys, rabbits, and mice increased concentrations or activity of soluble pro-inflammatory cytokines in amniotic or allantoic fluid are observed as a result of natural or experimental infection of the placenta [224-226,243] . The reason for this discrepancy between the mare and other species is not clear. It was felt that a non-invasive method of catheterizing the allantoic space of the mare was needed. The objective of this study was to develop a technique for laparoscopic placement of an allantoic catheter in the standing mare. Obstacles and concerns included side of placement, catheter design, bacterial contamination, catheter patency, locating the allantoic space, and future mare fertility.

2.2 Material and Methods

2.2.1 Experimental Animals

Twenty-nine adult pony mares and one horse mare of various ages were used in this study over a four-year period (2003 to 2007). Mares were maintained on pasture and supplemented with hay and a pelleted ration during the winter to maintain good body condition. All procedures were in accordance with Louisiana State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. The horse mare was artificially inseminated with a horse stallion, and the pony mares were pasture-bred with one of four different adult pony stallions. Two weeks after introduction of the stallions, mares were examined for pregnancy by transrectal ultrasonography every 7 to 14 d to determine gestational age of the early embryo. Ovulation dates were extrapolated from this information to provide ovulation dates that would be accurate within 2 to 3 d [11]. Laparoscopic procedures were performed between 234 and 285 d of gestation.

2.2.2 Allantoic Catheter Placement

Allantoic catheters were placed in sedated standing animals under local anesthesia using laparoscopic visualization. Mares were not fasted prior to surgery. Either immediately, or 24 h preceding surgery and again immediately prior to surgery, the hair of the left or right flank was clipped and the skin scrubbed with a povidone-iodine cleanser. Mares were sedated with 0.05 mg/kg detomidine iv and 0.1 mg/kg butorphanol iv, and then restrained in stocks. After regional anesthesia with 2% lidocaine (approximately 60 mL), the left or right flank was aseptically prepared prior to insertion of the laparoscopy cannulas (See Figure 2.1). From 2005 to 2007, following a stab incision through the skin, a 14 ga Veress needle (VS150000, VersaStep^{TM}, United States Surgical, Tyco Healthcare Group, Norwalk, CT) covered with a radially

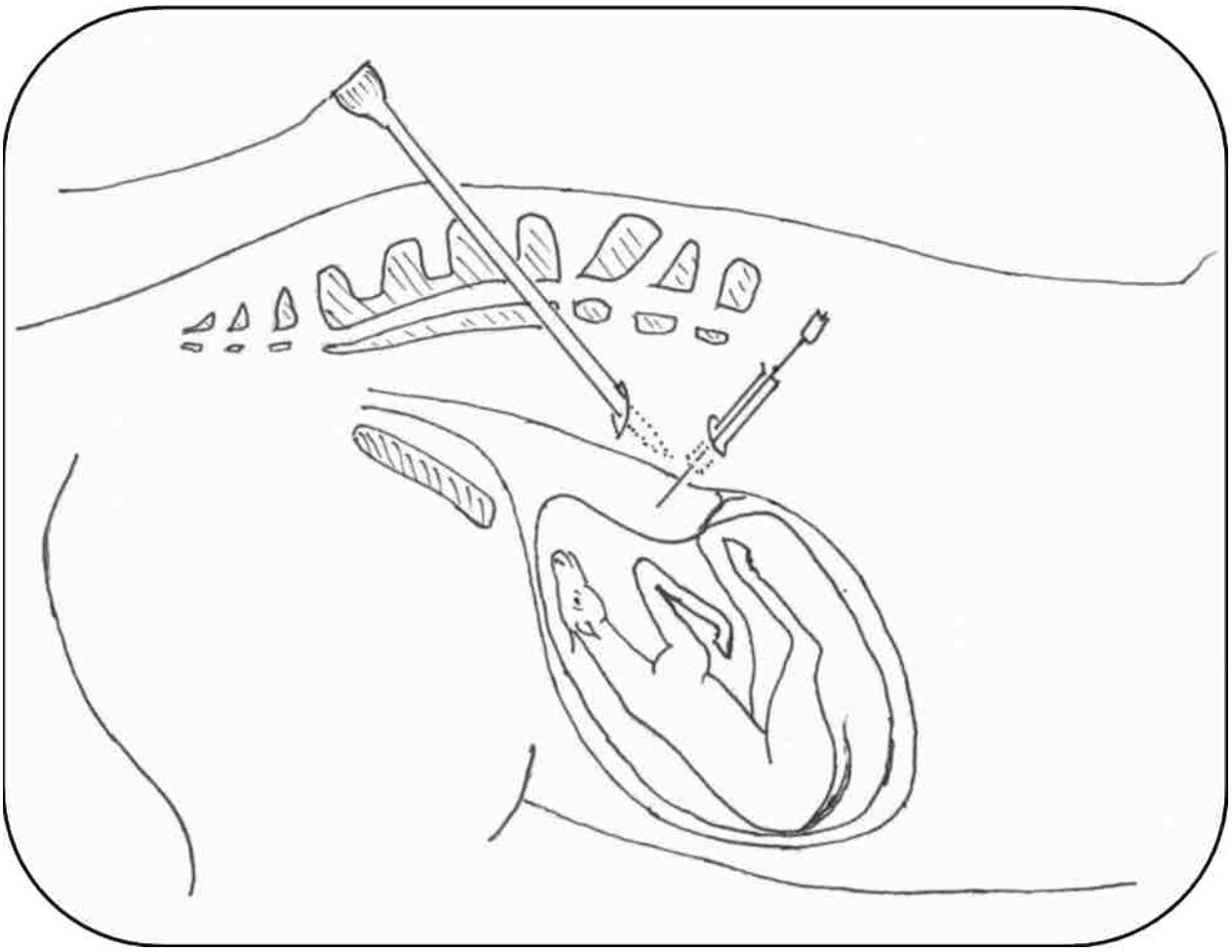

Figure 2.1 Laparoscopic portal placements for catheterization of the allantoic space in the standing mare.

expandable sleeve (VS101500, VeraStep™, United States Surgical, Tyco Healthcare Group, Norwalk, CT) was used to allow bladeless entry into the peritoneal space. A 12-mm cannula and dilator (VS101512P, VeraStep™, United States Surgical, Tyco Healthcare Group, Norwalk, CT) were inserted into the expandable sleeve, and pneumoperitoneum was created with CO_2 insufflation. Intraperitoneal pressure was maintained between 10 and 14 mm Hg. This step was repeated for each portal site. After identifying the gravid uterus, one of three types of catheter designs was introduced either through a cannula or along a rigid guide to the uterine wall. With a quick thrust, the catheter was introduced into the allantoic space. The dead space of the catheter

and attached access port system, if present, was measured for each individual. The skin incisions were sutured with 2-0 nylon suture in either a cruciate or a Ford-interlocking pattern (depending on incisional length) and a sterile adherent dressing with a triple-antibiotic ointment (Bacitracin-Neomycin-Polymyxin B, Clay-Park Labs, Inc., Bronx, NY) was applied.

Post-operatively, physical examinations were performed twice daily for one week to monitor body temperature, vaginal discharge, and pain, heat or swelling at the cannula entry sites. Fluid from the catheter was collected, centrifuged at 2,000 x g for 15 min and stored at 4°C until analyzed for creatinine, chloride, sodium, and calcium concentrations to confirm that it was of allantoic origin [239,240]. All samples were analyzed within 12 h of collection. In two mares, a multi-frequency rigid laparoscopic transducer (UST-5526L-7.5, Aloka, Inc. Wallingford, CT), a multi-frequency flexible laparoscopic transducer (UST-5536-7.5, Aloka, Inc. Wallingford, CT), or a 6.5-MHz transvaginal transducer (Medison, Universal Medical Systems, Inc., Bedford Hills, NY) was introduced through a separate portal site to evaluate the usefulness of laparoscopic ultrasonography in locating a suitable site for catheter insertion. Fluid samples were obtained at surgery, 4, 8, 12, 16, 20, 24, 28, 36, 42, and 50 h post-operatively, and then daily until fetal delivery. Biochemical and cytologic analysis of each sample verified the nature of the fluid as allantoic, amniotic, peritoneal, or an admixture of allantoic and amniotic fluid.

2.2.3 Catheter Design

Three custom-designed and one commercially available catheter were evaluated. The first catheter design was a 3-Fr, 75-cm, polypropylene, balloon-tipped catheter; placed by a 10-ga split-steel introducer (Figure 2.2A). The second catheter was a 3-Fr, 75-cm, polypropylene, through-the needle (12 ga) catheter with a collapsible disk at the terminal end and a moveable disk designed to anchor the catheter to the serosal surface of the uterus (Figure 2.2B). The third

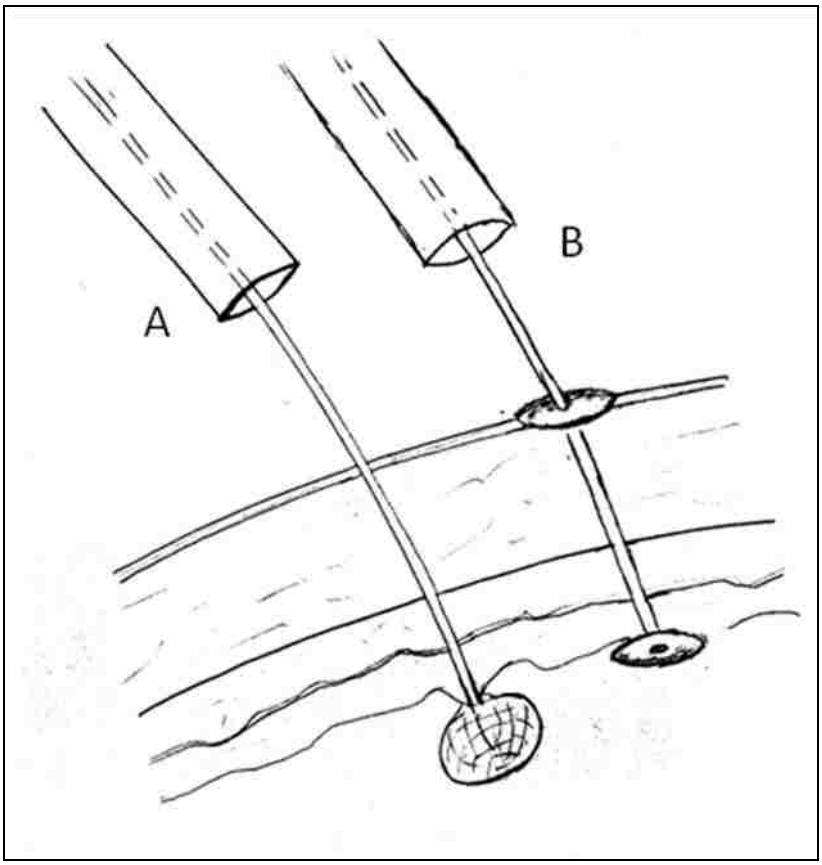

Figure 2.2 Schematic drawings for custom-designed catheters for catheterization of the allantoic space in the standing mare. A) A balloon-tipped catheter; placed by a 10-ga split-steel introducer. B) A through-the needle catheter with a collapsible disk at the terminal end and a moveable disk designed to anchor the catheter to the serosal surface of the uterus.

catheter design was a polypropylene, over-the needle, (10 ga) catheter with a bonded flange for anchoring the system to the serosal surface of the uterine wall. The fourth catheter design was a commercially available, over-the-needle, 7.5 Fr, 25-cm, polyurethane catheter, with a locking pigtail (Ultrathane® Suprapubic Catheter, Cook Urological, Spencer, IN) designed for percutaneous placement of a loop catheter in the renal pelvis for nephrostomy drainage (Figure 2.3A). Figure 2.3B depicts the placement of the locking pigtail within the allantoic space.

A) B)

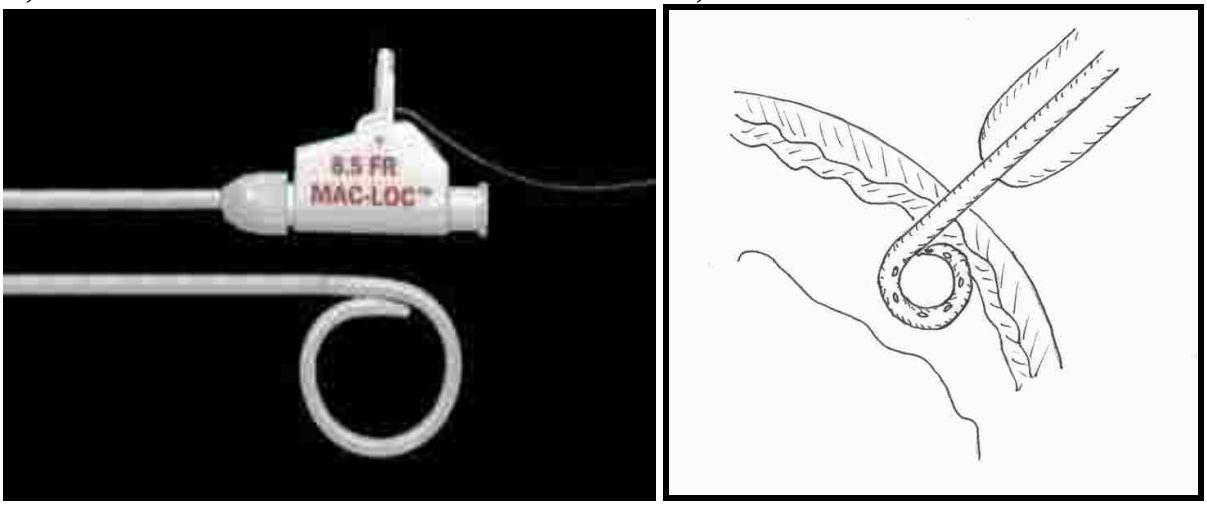

Figure 2.3 A) A commercially available over-the-needle, 7.5 Fr, 25-cm, polyurethane catheter, with a locking pigtail (Ultrathane® Suprapubic Catheter, Cook Urological, Spencer, IN) used for catheterization of the allantoic space in the standing mare. B) Schematic drawing of catheter placement within the allantoic space.

2.2.4 Modifications to Reduce Microbial Tracking along the Catheter System

2.2.4.1 Subcutaneous Access Port

For 13 of 17 of the catheterizations, a subcutaneous access port (PMIDA-SIL-C70 Custom, Instech Solomon, Plymouth Meeting, PA) was incorporated in the collection system. Attached to the port was a 12-inch section of silicone tubing ending in a locking male luer. After catheterization of the allantoic cavity, a 4-cm vertical incision was made 4 to 5 cm dorsal and 3 to 4 cm anterior to the exit wound of the allantoic catheter. A subcutaneous pouch under the anterior margin of the port incision was formed by blunt dissection. The access port was placed in this pouch. After bluntly dissecting a tunnel from the catheter incision to the port incision, the tubing of the port was connected to the catheter, and both incisions were closed with 2-0 nylon suture in a Ford-interlocking pattern (Figure 2.4).

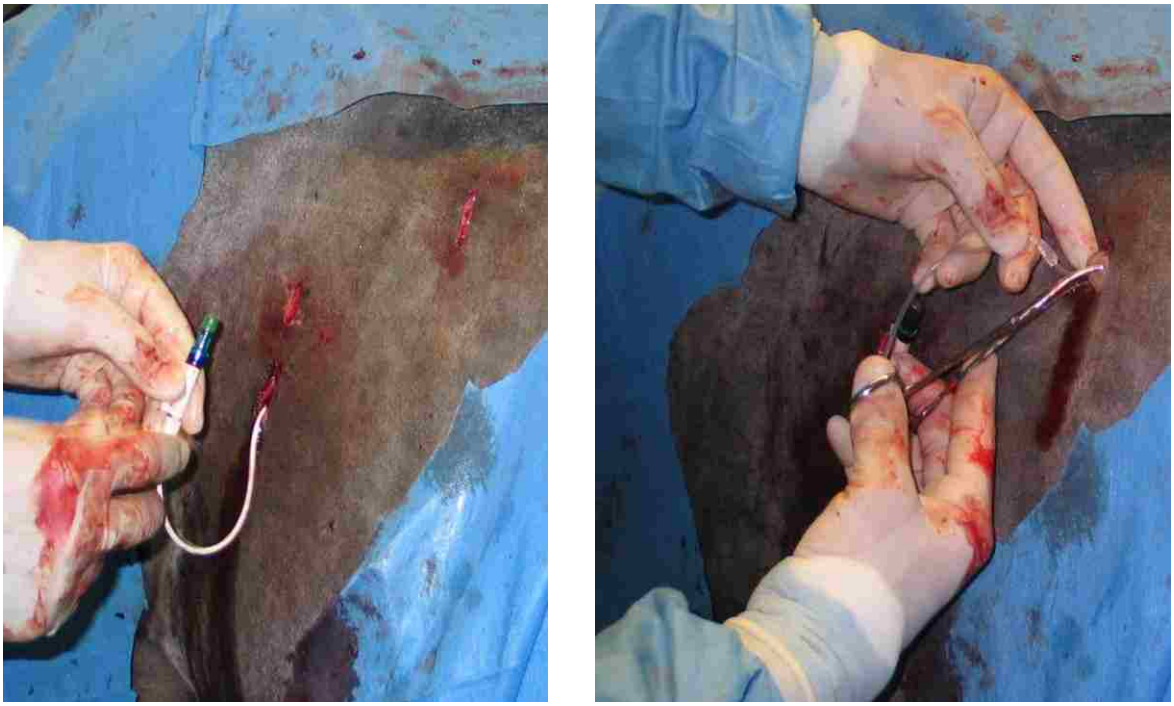

Figure 2.4 Placement of a subcutaneous access port in mares with an indwelling allantoic catheter.

2.2.4.2 Indwelling Huber Needle

Non-coring needles (Huber needles, Instech Solomon, Plymouth Meeting, PA) were used to obtain samples from the access ports. Straight single-use 22 ga x 3/4" Huber needles were used for sample acquisition in seven mares. For ten mares, a 22 ga x 5/8" right angle Huber needle with a 6-inch PVC tubing and female luer was inserted into the subcutaneous access port, and a sterile adherent bandage applied. Bandages and needles were changed every 72 h.

2.3 Results

2.3.1 Side of Approach

A total of 48 laparoscopic procedures were performed; eight during 2004, 30 during 2005, eight during 2006, and two during 2007. The first procedure was on a mare at 234 d of gestation and a left flank approach was utilized. The left uterine horn was adequately visualized; however, later in gestation (>260 d), the left uterine horn was totally obscured by small intestines

or small colon (n=2). For the remaining procedures, mares were between 260 and 285 d of gestation, and a right flank approach provided adequate visualization of the right uterine horn in all but two instances. In both of these cases fat, in addition to loops of small intestine, obscured visualization of the uterus.

2.3.2 Catheter Design

None of the custom-designed catheters could either be inserted into or retained within the allantoic space. The through-the-needle catheter with a terminal collapsible disk was too bulky to allow advancement of the catheter through the introducing trocar. Although the balloon-tipped catheter could be successfully inserted into the allantoic space, the balloon size was inadequate to retain the catheter within the allantoic cavity. The catheter material of the over-the-needle design created too much tissue drag, and prohibited advancement of the catheter through the uterine wall. The nephrostomy catheter proved to be an acceptable design, creating minimal tissue drag during insertion and having good retention of the locking pigtail. It was important however, that the tension on the pull tie was sufficient to create the maximal degree of loop fixation to retain the catheter. Failure to do so resulted in three catheters pulling out of the uterine wall. The peritoneal location of the catheter was determined by biochemical and cytologic evaluation of the sample. (See Table 2.1) Subsequent to identifying the appropriate catheter design, 17 catheters were successfully placed into the allantoic space (2005 to 2006). Two of the three catheters that pulled out were successfully replaced during a second laparoscopy; the third mare was not re-catheterized.

2.3.3 Catheter Patency

The number of days indwelling, patent, and number of potential and missing samples is summarized in Table 2.2. In 2004, four mares were successfully catheterized during the eight

Table 2.1 Biochemical Analysis of fluid samples collected from allantoic catheters

			All Fluid Categories				Allantoic Fluid		Admixed Fluid		Peritoneal Fluid	
Mare	**Treatment Group**	**Date**	**Creat.**	**Chloride**	**Calicum**	**Sodium**	**Creat.**	**Chloride**	**Creat**	**Chloride**	**Creat**	**Chloride**
25	sham	1/26/2005	39.8	36	26.5	112	39.8	36				
25		1/27/2005	27	84	18	130			27	84		
25		1/28/2005	27	93	21.7	127			27	93		
25		1/29/2005	25	102	18.5	129			25	102		
25		1/30/2005	25	102	17.6	122			25	102		
25		1/31/2005	23	109	16.4	120			23	109		
25		2/14/2005	20.8	99	25.4	105						
762	sham	5/30/2005	72	39	22.2	74.5	72	39				
762		5/31/2005	74	36	23.1	71	74	36				
762		6/1/2005	79	34	23.1	69	79	34				
762		6/2/2005	79	34	22.3	63	79	34				
762		6/3/2005	80	36	21.8	63	80	36				
762		6/4/2005	83	38	22.5	61	83	38				
762		6/5/2005	86	38	21.9	58	86	38				
762		6/6/2005	87	37	20.3	57	87	37				
762		6/7/2005	91	36	22.5	54	91	36				
847	sham	3/21/2005	97.9	22	39.8	50	97.9	22				
847		3/22/2005	101.8	19	41	47	101.8	19				
847		3/23/2005	87	35	37	65	87	35				
847		3/24/2005	83	46	34.7	73	83	46				
847		3/25/2005	56	24	20.8	108	56	24				
847		3/26/2005	90	48	34	74	90	48				
847		3/29/2005	141	13	50.1	51	141	13				

Table 2.1 continued

Mare	Treatment Group	Date	All Fluid Categories Creat.	Chloride	Calicum	Sodium	Allantoic Fluid Creat.	Chloride	Admixed Fluid Creat	Chloride	Peritoneal Fluid Creat	Chloride
790	PBS	2/22/2005	53	66	149	24	53	66				
790		2/24/2005	48	67	137.5	31	48	67				
790		2/25/2005	1	107	9.9	138					1	107
781	PBS	3/23/2005	27.4	57	13	116	27.4	57				
781		3/24/2005	53	21	24.1	107	53	21				
781		3/25/2005	88	48	33.6	75	88	48				
781		3/26/2005	57	22	22.2	113	57	22				
22	PBS	6/14/2005	49.9	23.7	9.6	116	49.9	23.7				
22		6/15/2005	48.2	36.3	10.8	118	48.2	36.3				
22		6/16/2005	49.2	41	10.8	122	49.2	41				
22		6/17/2005	50.3	45	10.9	122	50.3	45				
22		6/18/2005	53	50	12.1	123	53	50				
22		6/19/2005	51	52	11.2	118	51	52				
22		6/20/2005	53	54	13.3	116	53	54				
22		6/21/2005	55	57	15.6	115	55	57				
22		6/22/2005	54	59	19.3	110	54	59				

Table 2.1 continued

Mare	Treatment Group	Date	All Fluid Categories				Allantoic Fluid		Admixed Fluid		Peritoneal Fluid	
			Creat.	Chloride	Calicum	Sodium	Creat.	Chloride	Creat	Chloride	Creat	Chloride
682	killed	3/4/2005	58	13	37.1	92	58	13				
682		3/5/2005	1.5	110	9	142					1.5	110
682		3/6/2005	1.1	108	9.7	142					1.1	108
682		3/24/2005	82	64	12.4	114	82	64				
682		3/25/2005	82	61	11.7	109	82	61				
682		3/26/2005	84	59	12.1	107	84	59				
682		3/27/2005	77	64	12.2	111	77	64				
682		3/28/2005	88	62	12.8	109	88	62				
682		3/30/2005	86	65	13.3	109	86	65				
682		3/31/2005	89	63	13.3	107	89	63				
682		4/1/2005	101	57	13.1	105	101	57				
682		4/2/2005	82	71	12.5	112	82	71				
682		4/3/2005	53	82	13.8	127	53	82				
416	killed	3/30/2005	32	82	33	99			32	82		
416		3/31/2005	24	82	29.3	111			24	82		
416		4/1/2005	20	85	19.2	121			20	85		
416		4/2/2005	18	88	19.3	125			18	88		
416		4/3/2005	16	85	25	125			16	85		
416		4/4/2005	16	79	22.4	121			16	79		
12SG	killed	6/22/2005	32	80	13.5	118			32	80		
12SG		6/23/2005	27	95	12.9	126			27	95		
12SG		6/24/2005	27	97	13.2	131			27	97		
12SG		6/25/2005	29	104	11.5	128			29	104		
12SG		6/26/2005	31	107	10.7	129			31	107		
12SG		6/27/2005	31	109	10	126			31	109		
12SG		6/28/2005	30	112	10.5	124			30	112		
12SG		6/29/2005	26	113	10.2	124			26	113		
12SG		6/30/2005	25	113	10.2	123			25	113		

Table 2.1 continued

Mare	Treatment Group	Date	All Fluid Categories Creat.	Chloride	Calicum	Sodium	Allantoic Fluid Creat.	Chloride	Admixed Fluid Creat	Chloride	Peritoneal Fluid Creat	Chloride
20	live-low	2/28/2005	18.5	58	5.95	137			18.5	58		
20		3/1/2005	12	84	7.3	144			12	84		
20		3/2/2005	11	80	11.3	141			11	80		
20		3/3/2005	11	72	9.4	131			11	72		
20		3/4/2005	11.9	63	6.7	132			11.9	63		
20		3/5/2005	14.8	53	8	125			14.8	53		
12	live-low	3/28/2005	57	7	48.6	84	57	7				
12		3/30/2005	52	23	30.5	95	52	23				
12		3/31/2005	48	36	31.7	105	48	36				
12		4/1/2005	51	38	32.4	106	51	38				
12		4/2/2005	54	36	31.1	107	54	36				
12		4/3/2005	57	34	30.9	106	57	34				
12		4/4/2005	57	32	31.6	104	57	32				
12		4/5/2005	58	30	32.8	103	58	30				
12		4/6/2005	60	28	28.3	103	60	28				
12		4/7/2005	61	26	39.7	102	61	26				
12		4/8/2005	63	24	36.3	105	63	24				
12		4/14/2005	79	14	31.6	97	79	14				
24SG	live-low	7/5/2005	43	26	41.7	98			43	26		
24SG		7/6/2005	24	101	15.4	123			24	101		
24SG		7/7/2005	25	109	13.9	126			25	109		
24SG		7/8/2005	24	111	12.6	126			24	111		
24SG		7/9/2005	26	114	11.1	129			26	114		
24SG		7/10/2005	23	114	10	129			23	114		
24SG		7/11/2005	21	118	10.4	130			21	118		
24SG		7/12/2005	20	120	10.4	130			20	120		
24SG		7/13/2005	20	122	10.8	128			20	122		
24SG		7/14/2005	18	122	10.7	128			18	122		

Table 2.1 continued

Mare	Treatment Group	Date	All Fluid Categories Creat.	Chloride	Calicum	Sodium	Allantoic Fluid Creat.	Chloride	Admixed Fluid Creat	Chloride	Peritoneal Fluid Creat	Chloride
23	live - high	8/25/2006	54	19	30.7	130	54	19				
23		8/26/2006	58	20	32	129	58	20				
23		8/27/2006	60	17	33.7	129	60	17				
23		8/28/2006	65	14	36.3	130	65	14				
23		8/29/2006	67	15	36.8	129	67	15				
23		8/30/2006	66	17	37.3	127	66	17				
23		8/31/2006	65	20	38.1	128	65	20				
23		9/2/2006	66	30	34.3	127	66	30				
23		9/6/2006	61	31	32.2	126	61	31				
N=			104	104			64	64	36	36	3	3
AVE			50.2	60.1			68.1	38.2	23.2	94.1	1.2	108.3
STDEV			27.7	33.7			18.9	17.7	6.9	21.7	0.3	1.5
SEM			2.7	3.3			2.4	2.2	1.2	3.6	0.2	0.9
MEDIAN			53	57			62	36	24	99	1.1	108

Table 2.2 Summary results of allantoic catheterization for 2005 to 2006. Number of days indwelling, days patent, potential samples, missing samples, for 13 mares laparoscopically catheterized between 260 and 285 days of gestation.

Mare ID	Indwelling (d)	Patency (d)	Potential samples	Missing samples
Group A Single Huber sampling; spontaneous abortion				
25	19	19	13	0
790	7	2	13	4
20	10	5	18	5
Group B Indwelling Huber sampling; spontaneous abortion				
682	10	10	12	0
847	12	8	20	6
781	3	3	11	0
Group C Indwelling Huber sampling; spontaneous abortion; flank preparation 24 h pre-operatively				
12	17	16	24	1
416	5	5	13	0
23	11	8	19	3
Group D Indwelling Huber sampling; induced delivery day 8; flank preparation 24 h pre-operatively				
24SG	8	8	16	0
12SG	8	8	16	0
762	8	8	16	0
22SG	8	8	16	0
Mean	9.7	8.3	15.9	1.4
SEM	1.22	1.3	1.0	0.6

procedures; however, biochemical confirmation of the fluid was only performed in three mares due to limited funding resources. Catheters were patent for an average of 5.6 d, with a range of five to six d. In 2005, 12 mares were successfully catheterized during 30 procedures. In 2006, two mares were successfully catheterized during eight procedures. Fluid samples were obtained at surgery, 4, 8, 12, 16, 20, 24, 28, 36, 42, and 50 h post-operatively, and then daily until fetal delivery. Biochemical and cytological analysis of each sample verified the nature of the fluid as allantoic, amniotic, an admixture of allantoic and amniotic fluid, or peritoneal. (See Table 2.1) For all mares in 2005 and 2006, catheters were indwelling and patent for 9.7 $\pm$ 1.2 d and 8.3 $\pm$ 1.3 d, respectively (mean $\pm$ SEM). Of a potential 207 samples for all mares, there were only 19 sample times for which no fluid sample was obtained. For four of the last five mares instrumented, samples were acquired from all mares at each of the specified time points. (See Table 2.2) The majority of the samples were easily withdrawn using a 6- or 12-mL syringe. On occasion, fluid recovery was not immediate, presumably due to fetal position. In these instances, ballottement of the fetus resulted in sufficient fetal movement to allow sample retrieval.

2.3.4 Control of Bacterial Contamination

Tracking of skin microflora along the catheter system and into the allantoic cavity was a significant problem. During the pilot study of 2004, the catheter exited the body wall through the portal site created during laparoscopy. Because of the extreme caudal flank location of this portal site, maintaining a bandage over the exit wound was difficult. Contamination of the allantoic space usually occurred within 5 d of catheterization. The first day of bacterial contamination and cytologic inflammation, and the histologic description of the catheter site for mares catheterized in 2005 and 2006 are summarized in Table 2.3. During 2005, utilization of the subcutaneous access port and single-use Huber needles (three mares) for sampling delayed, but did not

Table 2.3 Summary results of allantoic catheterization for 2005 and 2006. Culture, cytology, and biochemical characterization of fetal fluids, and the histologic description of the catheter size for 13 mares laparoscopically catheterized between 260 and 285 days of gestation.

Mare ID	Day of bacterial contamination	Day of cytologic inflammation	Fluid Characterization	Histologic description of catheter site
Group A Single Huber sampling; spontaneous abortion				
25	none	Day 9	Admix	Cystic allantoic dysplasia; microcotyledonary atrophy; no inflammation
790	none	Day 2	Allantoic[1]	Vascular thrombi, transmural infarction; coccoid bacteria
20	>Day 5	NA	Admix	Severe diffuse placental necrosis; marked squamous metaplasia; diffuse allantoic fibrosis; allantoic vascular thrombosis
Group B Indwelling Huber sampling; spontaneous abortion				
682	Day 10	Day 10	Allantoic[2]	Focal subacute placentitis with bacterial cocci
847	Day 4	Day 8	Allantoic	Focal bacterial placentitis
781	Day 3	Day 3	Allantoic	Not identified at necropsy
Group C Indwelling Huber sampling; spontaneous abortion; flank preparation 24 h pre-operatively				
12	Day 17	Day 17	Allantoic	Chronic focal necrotizing placentitis
416	None	None	Admix	Not identified at necropsy
23	None[3]	Day 6	Allantoic	Placentitis at cervical star extended cranially to the catheter site
Group D Indwelling Huber sampling; induced delivery day 8; flank preparation 24 h pre-operatively				
24SG	None	None	Admix	Focal fibrosis; edema; no inflammation
12SG	None	None	Admix	Not identified at necropsy
762	None	None	Allantoic	Chronic focal reactive placental changes; no significant inflammation; edema
22SG	None	None	Allantoic	Not identified at necropsy

[1]Pulled out at 40 h, not re-catheterized
[2]Pulled out at 24 h, re-catheterized 10 d after first surgery
[3]Transcervical inoculation with *Streptococcus equi* subsp. *zooepidemicus*, single isolate from allantoic fluid.

eliminate, tracking along the catheter. This was evidenced by either focal placentitis at the catheter site or positive bacteriologic culture of the allantoic fluid. Bacterial tracking along the catheter system was associated with inflammatory changes of the chorioallantois at the catheter exit site (Figure 2.5A), while absence of bacterial tracking was typically evidenced by fibrotic changes (Figure 2.5B). Additionally, patient discomfort was considerable with single-use needles. Indwelling Huber needles (three mares), significantly improved patient comfort and ease of sample procurement, but did not significantly reduce tracking. Clipping and cleansing the flank with povidone-iodine scrub 24 h prior to laparoscopy resulted in no bacterial tracking in six of seven mares. The allantoic fluid of the seventh mare remained culture negative for 16 of the 17 d that the catheter was indwelling.

2.3.5 Location of Allantoic Space

In 2005, a sufficiently deep pocket of allantoic fluid in the allantoic space could not be located during 13 laparoscopies, due to either fetal positioning, or close proximity of the amniotic membrane to the allantoic membrane. Additionally, fluid from five mares successfully catheterized at surgery showed evidence of an admixture of the allantoic and amniotic compartments by 24 h post-laparoscopy (Tables 2.1 and 2.3). Three transducers for laparoscopic ultrasound were evaluated in two mares. All three types of probe configurations allowed imaging of the allantoic cavity, amnion, and fetus. The rigid side-fire laparoscopic transducer necessitated placing the probe on the ventrad portion of the uterine horn, making catheter insertion adjacent to the beam extremely difficult. Because the transvaginal transducer produced an end-fire sector beam, images and triangulation of the catheter direction were possible; however, the diameter of the probe necessitated a significantly larger incision. The side-fire flexible laparoscopic

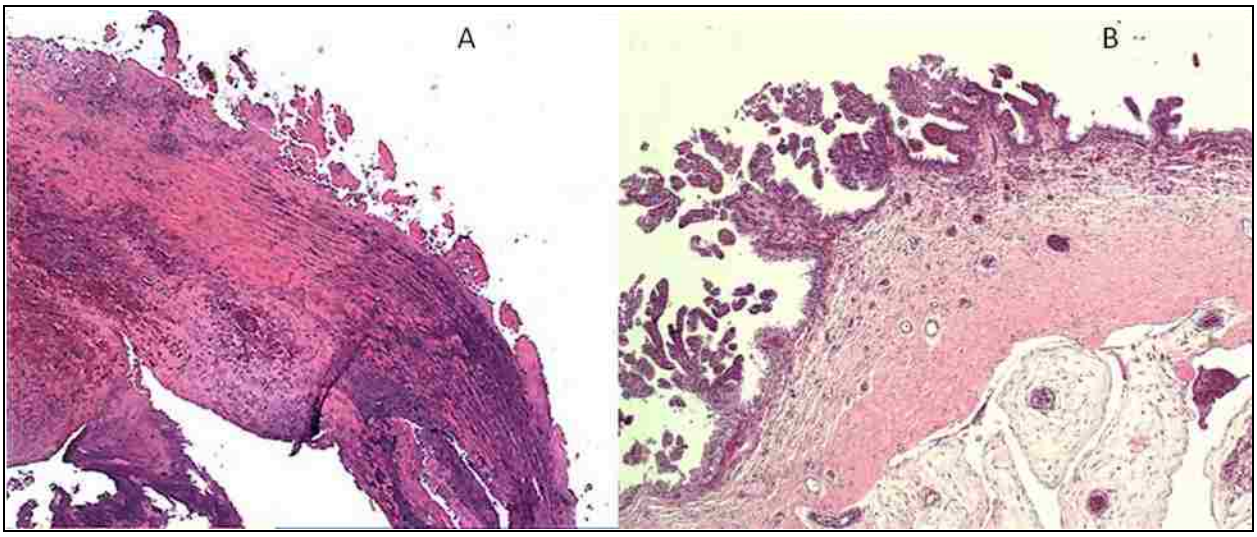

Figure 2.5 Histologic changes of the chorioallantoic membrane at the catheter site in response to bacterial tracking along the catheter system. Chronic necrotizing placentitis associated with bacterial tracking (A), or focal fibrosis in the absence of bacterial tracking (B).

transducer provided adequate visualization of allantoic fluid, amnion, and fetus, and could be introduced through a standard laparoscopic trocar or directly through the body wall (Figure 2.6).

2.3.6 Complications Encountered and Future Fertility

In 2006, the cecum of one mare was inadvertently punctured despite the use of the Veress-needle system for bladeless creation of a pneumoperitoneum. This mare was treated with flunixin meglumine, broad-spectrum antibiotics, intravenous fluids, and was withdrawn from the study. While peritonitis was successfully controlled, the mare subsequently developed irreversible hepatic lipidosis and was euthanized. All eight of the mares used in 2004, except for one, were successfully re-bred and were used again in 2005; this mare conceived in 2005 and foaled in 2006. Nine of the 12 mares catheterized in 2005 to 2006 were successfully rebred and foaled the following year; three mares were not rebred. Nine mares underwent unsuccessful attempts at catheterization in 2005; all foaled uneventfully at term, one was not rebred, and five were successfully rebred during 2005. One of the mares in this group that failed to become

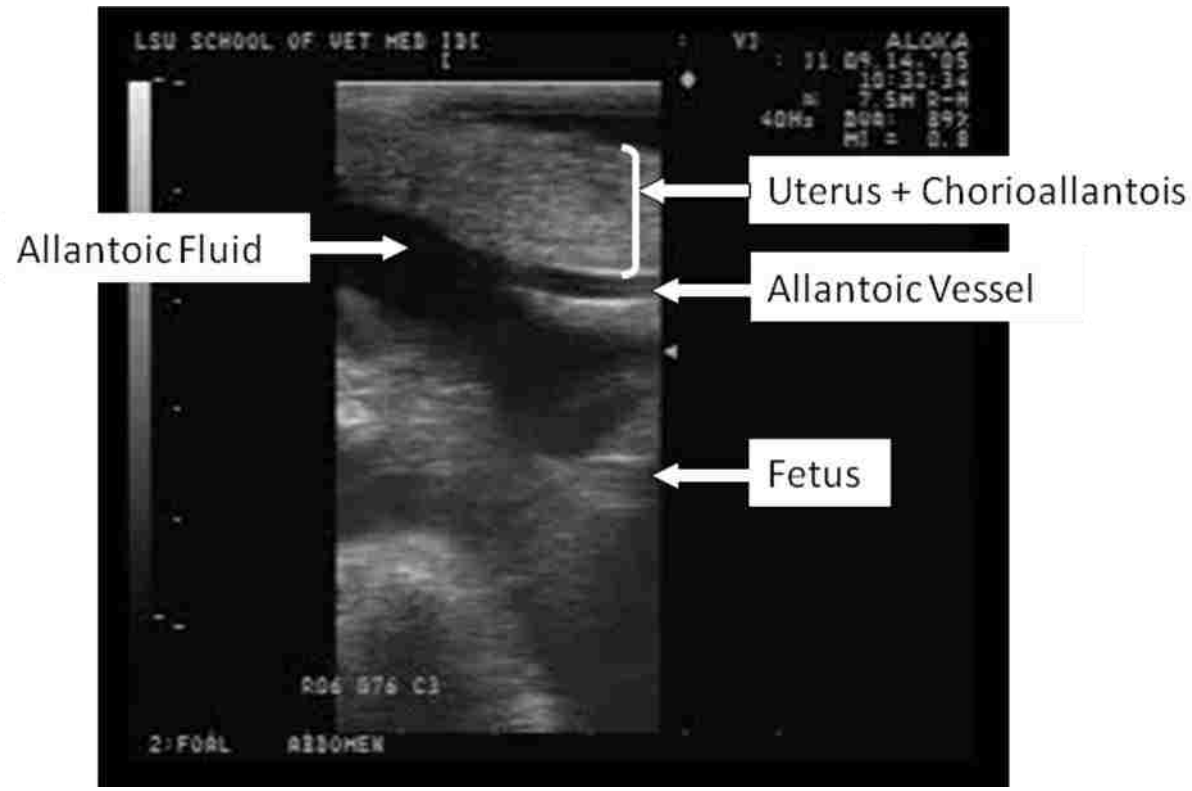

Figure 2.6 Laparoscopic ultrasonographic image of the uterus, allantoic fluid, and fetus prior to placement of an allantoic catheter.

pregnant was in low body condition score and suffered from summer-associated chronic obstructive pulmonary disease, the remaining two mares became pregnant in 2006. Four mares were used in 2006, three were successfully rebred and the fourth was euthanized as previously explained due to hepatic lipidosis.

2.4 Discussion

These findings describe using laparoscopy to catheterize the allantoic space with a nephrostomy catheter in the pregnant mare. Subcutaneous access ports, indwelling Huber needles and pre-operative skin preparation reduced bacterial tracking along the catheter system to a minimum. Although these modifications were applied sequentially, the latter may have had the greatest impact on tracking. Prophylactic antibiotics could provide a further reduction in the

incidence of contamination. Because the development of this instrumentation technique was done in conjunction with a placentitis model, antibiotic administration was not employed. In 2004 flunixin meglumine was administered perioperatively, but was not in 2005. Patient discomfort appeared to be minimal. Mares were alert upon return to the stall and resumed eating immediately. Only slight tenderness was noted along the incision sites, and mares tolerated the bandaging well. Despite no antibiotics or non-steroidal anti-inflammatory agents in 2005, no mares experienced a febrile episode post-operatively. The ability to repeatedly use this technique from year to year greatly improves the welfare and the economic aspects of gestational catheterization studies. Laparoscopic ultrasound produced a significant refinement in the procedure by localizing allantoic fluid. Retraction of the gastrointestinal tract by the laparoscopic transducer, as is done in human medicine, may allow for the use of a left-sided approach later in gestation and simplify the procedure from the right side especially when mares are obese. Ultrasound-guided laparoscopy can be successfully used to catheterize the allantoic space of the pregnant mare thereby providing a minimally invasive, reliable method of studying the pathophysiology of a variety of gestational diseases.

CHAPTER 3

AN EXPERIMENTAL MODEL OF INFECTIVE PRE-TERM DELIVERY IN THE MARE: RESPONSE TO INOCULATION

3.1 Introduction

Placentitis is responsible for approximately one-third of abortions and perinatal loss in horses [3]. The ascension of aerobic bacteria through the vagina and cervix is the most common cause of placentitis; *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is the species most frequently isolated [5]. Clinically, mares may have a vaginal discharge, show udder development, prenatally lactate, and deliver a premature or dead foal. Mares suspected or diagnosed as having placentitis are treated empirically with progestins to maintain uterine quiescence, broad-spectrum antibiotics to eliminate bacterial infection, and anti-inflammatory agents to prevent prostaglandin synthesis. Although these agents should address the basic problems of infection-induced prostaglandin synthesis which leads to uterine contractions and fetal expulsion, the efficacy of these regimes is frequently poor [6]. In a controlled trial the best results in neonatal outcome were achieved when pentoxifylline was combined with trimethopim-sulfamethoxazole and therapy was initiated very early in the course of infection [6,244] . There are two major areas where the basic knowledge of the pathophysiology of equine placentitis is lacking: (1) the temporal relationship between the inflammatory cascade and increases in prostaglandin synthesis and (2) the endocrinological events associated with loss of myometrial quiescence and activation of the fetal hypothalamic-pituitary-adrenal axis.

To the investigators' knowledge, only one study has examined the relationship between inflammatory cytokines and ascending placentitis in pregnant mares [237]. Induction of placentitis by intracervical inoculation of *S. zooepidemicus* increased mRNA expression of IL-6

and IL-8 at the cervical star and IL-6 at the placental body and resulted in greater concentrations of $PGF_{2\alpha}$ and PGE_2 in allantoic fluid, whereas no differences were detected in the activities of TNF-α, IL-1 and IL-6 in allantoic fluid between groups over time. The failure to demonstrate an increase in the activities of pro-inflammatory cytokines in equine fetal fluids following bacterial infection differs from the results from monkeys, rabbits, women and mice [223-226]. The reasons for this discrepancy between the mare and other species is not clear, but may have resulted from sampling intervals and the heterogenic response of experimental animals. This latter variation may have been due to individual differences in response to the strain of *S. zooepidemicus* used [168], and the dose and site of inoculation chosen for this study.

The temporal relationship between elevations in allantoic cytokines and prostaglandins is quite important. If the synthesis of $PGF_{2\alpha}$ and PGE_2 is driven by cytokines in concert with fetal cortisol, as has been suggested in a model of ascending placentitis [48], then treatment strategies that fail to interrupt the inflammatory cascade and fetal stress may be ineffective in preventing pre-term labor. Therefore, it would be useful to delineate a noninfectious-inflammatory response from an infectious-inflammatory response. Inflammation of the chorioallantois was induced by inoculation with live bacteria or heat-killed bacteria. Heat-killed *S. zooepidemicus* has been used both *in vivo* in an endometritis model in the mare [172] and *in vitro* in phagocytosis assays for equine neutrophils [184,200]. By comparing the inoculation with heat-killed *S. zooepidemicus* to the inoculation with live *S. zooepidemicus*, the effects of the inflammatory cascade and resultant prostaglandin synthesis can be separated from the effects of placental, and possibly fetal, infection. We hypothesized that both types of inocula would induce an acute placentitis, that heat-killed *S. zooepidemicus* would produce a less variable pattern of inflammatory mediators than that produced by live *S. zooepidemicus*, and that live *S. zooepidemicus* would lead to pre-term delivery in all mares. Because the inflammatory insult initiated by heat-killed *S.*

zooepidemicus would occur as a single event without continual replication (and thus propagation of the inflammatory cascade) as would occur with live *S. zooepidemicus*, it would be unlikely to lead to pre-term delivery in all mares.

3.2 Material and methods

3.2.1 Experimental Animals

Fourteen adult pony mares of various ages were used in this study over a 2-year period (2005-2006). Mares were maintained on pasture and supplemented with hay and a pelleted ration during the winter to maintain good body condition. All procedures were in accordance with Louisiana State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. Only mares with normal perineal conformation and no previous history of spontaneous abortion due to placentitis were included. The mares have been in the herd for several years and accurate reproductive records were available for all individuals. Mares were pasture bred with one of four different adult pony stallions. Beginning two weeks after introduction of the stallions, mares were examined by transrectal ultrasonography for pregnancy every 7 to 14 d to determine gestational age of the early embryo. Ovulation dates were extrapolated from this information. This method provided ovulation dates that are accurate within 2 to 3 d [11]. Mares were grouped by age and by body condition score and randomly assigned to one of four treatment groups. Prior to the placement of allantoic catheters, fetal heart rate (FHR) was measured by transabdominal ultrasonography, and combined thickness of the uterus and placenta (CTUP) ventrad to the internal cervical os was measured by transrectal ultrasonography to avoid inclusion of any mares with pre-existing placentitis. To avoid inclusion of a mare with preexisting placentitis, any pregnancy with a sustained FHR less than 60 bpm or greater than 120 bpm [245], or a CTUP greater than 7 mm [246] was excluded from the study. Pony mares were assigned to one of four treatment groups: low STREP, high STREP, KILLED

STREP, PBS CONTROL, and SHAM CONTROL groups. Procedures were performed between 260 and 285 d of gestation.

3.2.2 Inoculates

3.2.2.1 Live Bacterial Inoculates

One colony of a *S. zooepidemicus* isolate from a clinical case of endometritis (provided by Dr. Robert Causey, University of Maine, Orono, ME) was used to inoculate 200 mL of Todd Hewitt broth and incubated at 37°C for 24 h. Following centrifugation, the pellet was resuspended in sterile PBS supplemented with 10% sterile glycerol to a concentration of 10×10^7 CFU/mL, determined spectrophotometrically [200] and confirmed by serial dilution. Two-mL aliquots were frozen at -70°C until needed for inoculation. Upon thawing at 37°C for 2 min, 100 µL were added to 900 µL of sterile PBS to provide an inoculum of 1×10^7 CFU (low STREP). A 100-µL aliquot was streaked on blood agar and incubated at 37°C for 48 h to document purity and viability of the inoculum. This technique ensured consistency among inocula. Preparation of the high STREP was similar except the concentration of the aliquots was adjusted to 5.1×10^8 CFU/mL, and confirmed by serial dilution. Upon thawing at 37°C for 2 min, 1 mL provided an inoculum of 5.1×10^8 CFU (high STREP).

3.2.2.2 Heat-Killed Bacterial Inoculate

One colony of the same *S. zooepidemicus* isolate used for the live inoculate was used to inoculate 200 mL of Todd Hewitt broth and incubated at 37°C for 24 h. Following autoclaving and centrifugation, the pellet was resuspended in sterile PBS supplemented with 10% sterile glycerol to a concentration of 10×10^7 CFU/mL, determined spectrophotometrically [200]. Two-mL aliquots were frozen at -70°C until needed for inoculation. Upon thawing at 37°C for 2 min, 100 µL was added to 900 µL of sterile PBS and used for inoculation (KILLED STREP). A 100-

µl aliquot was streaked on blood agar and incubated at 37°C to document that no viable organisms were present.

3.2.2.3 PBS Inoculate

The PBS CONTROL inoculate consisted of 990 µL of PBS and 10 µL of sterile glycerol. Two-mL aliquots were frozen at -70°C until needed for inoculation. Upon thawing at 37°C for 2 min, one mL was used for inoculation. A 100-µl aliquot was streaked on blood agar and incubated at 37°C to document that no viable organisms were present.

3.2.3 Procedures

3.2.3.1 Allantoic Catheter Placement

Allantoic catheters were placed in 13 of the 14 sedated standing animals under local anesthesia using laparoscopic visualization as previously described (Chapter 2). The type of pre-operative and post-operative approach to control bacterial tracking is listed in Table 3.1. The dead space of the catheter and attached port system was measured for each individual. Body wall portal sites were closed routinely and a sterile adherent dressing with an antibiotic ointment applied. Post-operatively, physical examinations were performed twice daily for one week to monitor body temperature, vaginal discharge, and pain, heat or swelling at the cannula entry sites. Each sample of fluid obtained via the catheter was analyzed for creatinine, chloride, and sodium concentrations to define the character of the fetal fluid [239,240]. (Table 2.1)

3.2.3.2 Transcervical Inoculation

Transcervical inoculation was performed 4 to 6 h after placement of the allantoic catheters and was designated Time 0. In two instances inoculation was delayed one or four days due to cytologic evidence of intra-allantoic hemorrhage following the catheterization procedure. Mares were restrained in stocks and sedated with 0.05 mg/kg detomidine, iv. Following aseptic preparation of the perineum, a guarded culture instrument was used to obtain a culture of the

vaginal fornix and external os of the cervix. Obtaining a culture of the microflora of the vaginal fornix and external os at the time of inoculation was useful in determining the source of contaminants (from the vagina at the time of inoculation or from the skin due to bacterial tracking along the catheter system) that might be isolated from the placenta, fetal fluids, or fetus. A sheathed sterile artificial insemination pipette (IUI Pipette with Inner Catheter, Minitube of America, Verona, WI; or Equi-Flex-EzeTM, Reproduction Resources, Walworth, WI) was introduced manually through the vagina. Once the pipette was positioned at the external os, the outer sheath was penetrated as the pipette entered the cervical canal. The examiner's arm was withdrawn, and transrectal ultrasonography was used to monitor advancement of the pipette to the internal os, positioning it adjacent to the surface of the chorioallantois, where the inoculum was deposited. Inoculated mares received either 1×10^7 live *S. zooepidemicus* in 1 mL sterile phosphate-buffered saline (low STREP; n=3), 5.1×10^8 live *S. zooepidemicus* in 1 mL (high STREP; n=2), 1×10^7 heat-killed *S. zooepidemicus* in 1 mL of PBS (KILLED STREP; n=3), or 1 mL of PBS (PBS CONTROL; n=3). SHAM CONTROL mares (n=3) were sedated, restrained in the stocks, had the vaginal fornix and external os sampled for culture, but did not receive a transcervical inoculation. We were unable to instrument one of the high STREP mares with an allantoic catheter, but elected to inoculate the mare in order to provide tissue for expression analysis and histologic evaluation.

3.2.3.3 Sample Collection and Patient Monitoring

Ultrasonography was performed daily following catheter placement to monitor the health of the fetoplacental unit. Fetal heart rate was evaluated daily with B/M-mode transabdominal ultrasonography using a 3.5 to 5.0-MHz sector-array transducer (SonoAce PICO, Medison Co., Ltd., Seoul, Korea). Transrectal evaluation of the combined uteroplacental thickness (CTUP) at

Table 3.1 Pre- and post-operative methods, type of delivery, and presence of inflammation at the catheter site from mares (n=14) in an experimental model of ascending placentitis. The allantoic space was catheterized laparoscopically between 265-280 days gestation: SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (n=3) received 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (n=3) received 10^7 *S. zooepidemicus* transcervically; and high STREP mares (n=1) received 5.1 x 10^8 *S. zooepidemicus* transcervically.

Mare ID	Prep method	Type of delivery	Inflammation at catheter site
SHAM treatment group			
25	A[1]	induced	0
847	B[2]	abortion	2+
762	D[4]	induced	0
PBS treatment group			
790	A	abortion	1+
781	B	abortion	NA[5]
22SG	D	induced	NA
KILLED STREP			
682	B	abortion	2+
416	C[3]	abortion	NA
12SG	D	induced	NA
low STREP			
20	A	abortion	2+
12	C	abortion	2+
24SG	D	induced	0
high STREP			
23	D	abortion	3+[6]
42	no catheter	abortion	NA

[1]Group A: Single Huber sampling; spontaneous abortion
[2]Group B: Indwelling Huber sampling; spontaneous abortion
[3]Group C: Indwelling Huber sampling; spontaneous abortion; flank preparation 24 h pre-operatively
[4]Group D Indwelling Huber sampling; induced delivery day 8; flank preparation 24 h pre-operatively
[5]Not identified at necropsy
[6]Placentitis extended from the cervical star region cranially to encompass the catheter site

the internal cervical os was examined daily using a 5.0 to 7.5-MHz linear-array scanner. Six mL of fetal fluid for soluble cytokine and prostaglandin concentration determination, were collected at 0, 4, 8, 12, 16, 20, 24, 32, 40, and 48 h, and then daily until delivery or induction of parturition. An additional 5 mL was collected at 24 h intervals for progestagen and cortisol analysis. Catheters were removed either immediately prior to induced delivery, or immediately after spontaneous delivery.

Fetal fluid samples were collected and immediately placed on ice for transport to the laboratory. Prior to centrifugation, 1 mL was removed for culture, biochemical profile, and cytologic evaluation. The presence of neutrophils, with or without intracellular and extracellular bacteria, was defined as cytologic inflammation. The remainder of the sample was centrifuged at 2,000 x g for 15 min at 4ºC, and the supernatant aliquoted into one-mL units. Samples for future analysis were stored at -70ºC. All samples were evaluated for the presence of bacteria by incubating 100 µL on a blood agar plate at 37ºC for 48 h. If bacteria were recovered, the Louisiana Veterinary Medical Diagnostic Laboratory identified all isolates using standard microbiological techniques. Any isolate determined to be *S. zooepidemicus* was characterized by pulsed-field gel electrophoresis. Maternal blood was collected into heparin once daily and plasma stored at -70ºC until assayed for future cortisol and progestagens determination.

At delivery of the fetus, chorioallantoic tissue was collected from the region of the cervical star, any grossly abnormal areas, the catheter site (if identifiable), and from one normal area located at least 10 cm from any grossly abnormal areas. Tissue was also collected from the amnion, umbilical cord, fetal lung, and fetal liver. The gender of the fetus, crown rump length, and placental and fetal weights were recorded. Contents from the fetal lung and stomach were submitted for aerobic bacterial culture. All tissues were fixed in formalin, paraffin embedded,

stained using hematoxylin and eosin, and evaluated by a pathologist. From each site of the chorioallantois sampled for histopathology, three 0.2-g pieces of tissue were placed in 2 mL of RNA stabilization reagent (RNAlater, Qiagen Inc., Valencia, CA) and stored at -20°C for later analysis of cytokine expression.

3.2.4 Induction of Parturition

Two mares from the SHAM CONTROL group, one mare from the PBS CONTROL group, one mare from the KILLED STREP group and one mare from the low STREP group were induced to deliver fetuses to provide gestational age-matched placental and fetal tissue, and control for the variability from inoculation to delivery. Parturition was induced by insertion of a 1000-µg alprostadil suppository (MUSE, Vivus, Inc., Mountain View, CA) to achieve cervical dilation [247]. Two h after alprostadil administration, mares were treated with 5 mg of dinoprost tromethamine, im (Lutalyse, Pfizer, New York, NY) followed one hour later by oxytocin (10 IU, iv), repeated as needed until delivery. Any fetuses alive at delivery were humanely euthanized with an overdose of barbiturate (100 mg/kg, iv).

3.2.5 Differentiation of Isolates by Pulsed-Field Gel Electrophoresis

Any streptococcal isolate determined to be *S. zooepidemicus* from chorioallantois, allantoic fluid, fetal stomach and lung contents was differentiated by pulsed-field gel electrophoresis to determine if it originated from the inoculum or from a contaminant source. Each isolate was grown on brain heart infusion agar overnight at 37°C. Cells were harvested, resuspended in a cell suspension buffer and the cell suspension added to a 1% agarose solution (SeaKem Gold Agarose, BioWhittaker Molecular Applications, Rockland ME) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 55°C. The cell/agarose suspension was pipetted into disposable agarose gel plug molds, and allowed to solidify. Each plug was incubated in two

different cell wall lysis solutions: one containing lysozyme for 2 h, the second containing proteinase overnight. Following removal of the cell lysis solution each plug was washed twice in sterile reagent grade water, and then washed 4 times in TE buffer. Plugs were stored in fresh TE buffer at 4°C. For restriction endonuclease digestion, a 2-mm wide slice of each plug was placed in commercial buffer containing 40 units of *Sma1* restriction endonuclease (New England Biolabs, Beverly, MA). Digestion took place over a 16-hour incubation period at 25°C and plugs were stored in TE buffer for up to 14 d at 4°C.

In situ digested genomic DNA-containing plugs was adhered to gel comb teeth, using a drop of warm 1% agarose solution in 0.5 X TBE buffer (45 mM Tris-HCl, 45 mM borate, 1.0 mM EDTA, pH 8.0). A 1 % agarose gel in 0.5 X TBE was poured around the comb and allowed to solidify. The comb was removed and the gel placed in a pulsed-field gel electrophoresis cell (Chef Dr-II, Bio-Rad Laboratories, Hercules, CA) with 0.5 X TBE as the running buffer. The running buffer was maintained at 14°C using an external cooling module (Chef Dr-II, Colling Module, Bio-Rad Laboratories, Hercules, CA). Following electrophoresis, the gel was stained with aqueous ethidium bromide solution and destained twice in distilled water. The gel was transilluminated with ultraviolet radiation (302 nm) and the image recorded digitally (ChemiImager 4400, Alpha Innotech Corp., San Leandro, CA).

3.2.6 Statistical Analysis

Because of the small samples sizes within treatment group, effect of treatment group on the response to inoculation was not possible. The effect of infection (positive fetal fluid culture), type of delivery (spontaneous abortion or induced delivery), and fluid characterization (allantoic or admixture) on various parameters was compared by a Student’s t test, using a statistical

analysis software program (SAS 9.1, Cary, NC) with significance set at $p<0.05$, including Satterthwaite's and Cochran's methods for sample populations with unequal variances.

3.3 Results

3.3.1 Response of the Fetoplacental Unit to Inoculation

The individual daily CTUP measurements and FHRs are shown in Table 3.2, and Figures 3.1 and 3.2. Small sample size precluded analysis of effect of treatment group on CTUP. Mares with spontaneous abortion had significantly increased ($p=0.038$) CTUP compared to mares in which delivery was induced (Figure 3.4A). There was not a significant effect of infection within the allantoic space on CTUP ($p=0.11$). Two mares (one SHAM CONTROL and one KILLED STREP mare) had significant edema of the chorioallantois, but no infection of fetal fluids and no histologic inflammation of the chorioallantois. One mare in the PBS CONTROL group had infected fetal fluids, minimal inflammatory changes of the chorioallantois, and aborted within four d of instrumentation; contamination of the allantoic fluid likely occurred at the time of laparoscopy. Removal of these three mares from analysis resulted in mares with allantoic infection having increased CTUP approaching significance ($p=0.051$; Figure 3.5B). There was no significant effect of fluid characterization (allantoic or admixture) on CTUP ($p=0.23$; Figure 3.6). There was no difference in FHR with infection or type of delivery. In only one instance (a mare in the high STREP group) was *in utero* fetal death documented during a daily exam. This mare aborted two hours following examination. Measurement of FHR was not possible in one mare in the KILLED STREP group (#416) due to excessive fetal motion.

3.3.2 Microbiology and Cytology

The results of microbiological and cytological evaluation of allantoic fluid samples from thirteen mares are presented in Table 3.3. Bacterial tracking along the catheter system was a

Table 3.2 Daily individual combined thickness of uterus and placenta (CTUP) and fetal heart rates (FHR) for mares (n=13) in an experimental model of placentitis.

Mare	Date	Time (d)	FHR	CTUP ventrad	CTUP @ cervix	Type of delivery
Sham Mares						
25	1/26/2005	0	120	4		
25	1/27/2005	1	88	3.9		
25	1/28/2005	2	124	4.3		
25	1/29/2005	3	132	4		
25	1/30/2005	4	132	4.2		
25	1/31/2005	5	136	4.8		
25	2/4/2005	9	113	4.4		
25	2/14/2005	19				induction
847	3/21/2005	0	88	4	4	
847	3/22/2005	1	112	4.4	4,4	
847	3/23/2005	2	120	4.2	4.2	
847	3/24/2005	3	122	4	4	
847	3/25/2005	4	110	5.8	5.8	
847	3/26/2005	5	80	4.8	11	
847	3/27/2005	6		5.6	12	
847	3/28/2005	7	100	5.68	8.5	
847	3/29/2005	8	99	5.6	8.1	
847	3/30/2005	9	128	5.5	14	
847	3/31/2005	10	122	5.1	14	
847	4/1/2005	11	130	7.1	25	
847	4/2/2005	12	111	14.7	22.6	
847	4/3/2005	13				abortion
762	5/26/2005	0	118	5.5		
762	5/26/2005	-D4				
762	5/27/2005	-D3				
762	5/30/2005	0	115	6.5		
762	5/31/2005	1	99	7		
762	6/1/2005	2	120	7		
762	6/2/2005	3	106	10		
762	6/3/2005	4	93	9		
762	6/4/2005	5	106	9		
762	6/5/2005	6	107	9.5		
762	6/6/2005	7	111	9.5		
762	6/7/2005	8	109	10		induction

Table 3.2 continued

Mare	Date	Time (d)	FHR	CTUP ventrad	CTUP @ cervix	Type of delivery
PBS Control Mares						
790	2/23/2005	0	102	5		
790	2/24/2005	1	114	4.9		
790	2/25/2005	2	113	4.3		
790	2/26/2005	3	89	4		
790	2/27/2005	4		6		
790	2/28/2005	5	?	?		
790	3/2/2005	7				abortion
781	3/23/2005	0	107	4		
781	3/24/2005	1	99	5.4		
781	3/25/2005	2	92	5		
781	3/26/2005	3	73	5.9		
781	3/27/2005	4				abortion
22SG	6/14/2005	0	102	4.9		
22SG	6/15/2005	1		4.8		
22SG	6/16/2005	2	71	4		
22SG	6/17/2005	3	88	4.4		
22SG	6/18/2005	4	96	4.5		
22SG	6/19/2005	5	95	4.9		
22SG	6/20/2005	6		5.1		
22SG	6/21/2005	7	85	5.9		
22SG	6/22/2005	8	96	5.1		induction

Table 3.2 continued

Mare	Date	Time (d)	FHR	CTUP ventrad	CTUP @ cervix	Type of delivery
Killed STREP mares						
682	3/24/2005	0	118	4	6.5	
682	3/25/2005	1	98	6.6	6.6	
682	3/26/2005	2	89	6	6	
682	3/27/2005	3	95	6.7	6.7	
682	3/28/2005	4	97	6.4	6.4	
682	3/29/2005	5	99	9.30	15.6	
682	3/30/2005	6	102	6.1	14	
682	3/31/2005	7	96	6	17	
682	4/1/2005	8	98	6.8	15	
682	4/2/2005	9	90	7.3	15.5	
682	4/3/2005	10	96	7.2	12	
682	4/4/2005	11				abortion
416	3/30/2005	0	(active)*	?	4.5	
416	3/31/2005	1	(active)*	4.5	10.2	
416	4/1/2005	2	(active)*	4.4	10.4	
416	4/2/2005	3	(active)*	6.3	15.4	
416	4/3/2005	4	(active)*	6.1	15.8	
416	4/4/2005	5	(no activity)	5.9		
416	4/5/2005	6				abortion
12SG	6/22/2005	0		?		
12SG	6/23/2005	1	126	5.6		
12SG	6/24/2005	2	109	6.6		
12SG	6/25/2005	3	119	5.7		
12SG	6/26/2005	4	104	6		
12SG	6/27/2005	5	110	6		
12SG	6/28/2005	6	113	6		
12SG	6/29/2005	7	99	6.1		
12SG	6/30/2005	8	100	6.5		induction

Table 3.2 continued

Mare	Date	Time (d)	FHR	CTUP ventrad	CTUP @ cervix	Type of delivery
low STREP mares						
20	2/28/2005	0	106	4.1		
20	3/1/2005	1	97	4.9		
20	3/2/2005	2	107	6.5		
20	3/3/2005	3	82	6.6		
20	3/4/2005	4	115	6.9		
20	3/5/2005	5	123	5.5		
20	3/6/2005	6	115	6.1		
20	3/7/2008	7				
20	3/8/2005	8	87	5.1		
20	3/9/2005	9	62	11.1		
20	3/10/2005	10	73	10.1		
20	3/11/2005	11				abortion
12	3/28/2005	0	91	5	5	
12	3/29/2005	1	91	6.5	8.1	
12	3/30/2005	2				
12	3/31/2005	3	95	6.3	15	
12	4/1/2005	4	97	6.2	18.1	
12	4/2/2005	5	107	5.7	16.3	
12	4/3/2005	6	107	5.5	9.7	
12	4/4/2005	7	88	5.8	14.6	
12	4/5/2005	8		6.5	15.5	
12	4/6/2005	9	91	4.5	11.1	
12	4/7/2005	10	95	6.4	15.9	
12	4/8/2005	11				
12	4/9/2005	12	77	6	21.3	
12	4/10/2005	13	95	5.6	12	
12	4/11/2005	14	91	6.8	12	
12	4/13/2005	16	88	6.3	16	
12	4/14/2005	17	87	7.4	16	
12	4/15/2005	18				abortion

Table 3.2 continued

Mare	Date	Time (d)	FHR	CTUP ventrad	CTUP @ cervix	Type of delivery
low STREP Mares						
24SG	7/6/2005	0	106	5.3		
24SG	7/7/2005	1	106	5.5		
24SG	7/8/2005	2	111	5.7		
24SG	7/9/2005	3	91	5		
24SG	7/10/2005	4	104	5.9		
24SG	7/11/2005	5	98	5.9		
24SG	7/12/2005	6	87	6		
24SG	7/13/2005	7	92	5.9		
24SG	7/14/2005	8	84	?		induction
high STREP Mares						
23	8/25/2006	0	78	4.5		
23	8/26/2006	1	98	14.5		
23	8/27/2006	2	78	17.5		
23	8/28/2006	3	101	20.8		
23	8/29/2006	4	76	24.7		
23	8/30/2006	5	79	30.5		
23	8/31/2006	6	79	26.9		
23	9/1/2006	7	78	36.4		
23	9/2/2006	8	72	48.9		
23	9/3/2006	9	64	48.2		
23	9/4/2006	10	91	54.8		
23	9/5/2006	11	0	39.4		abortion

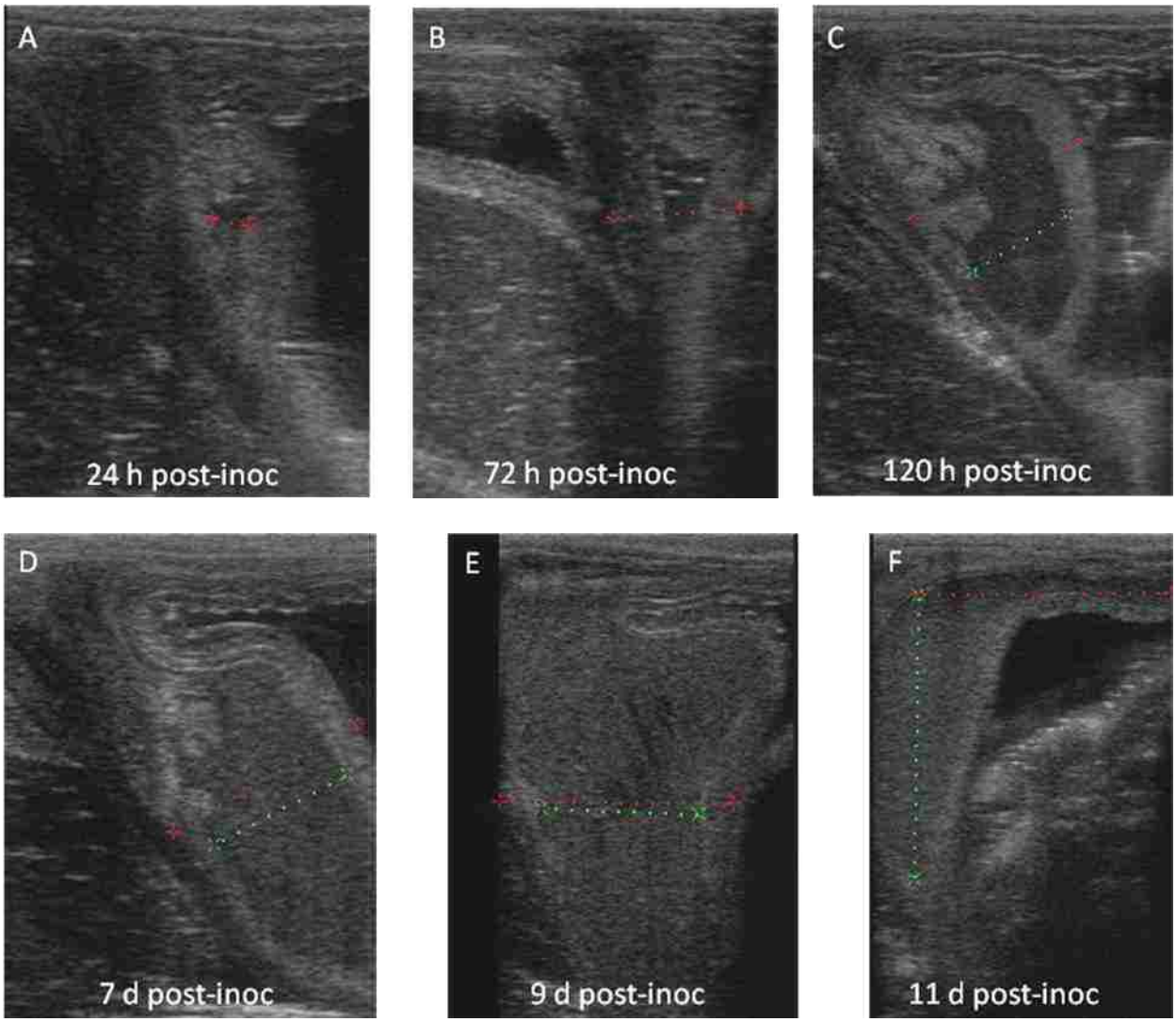

Figure 3.1 Ultrasonographic images from a mare following transcervical inoculation with 5.1 x 10^8 CFU/mL *S. zooepidemicus.* Inoculation is Day 0. Note the progressive widening of the combined thickness of the uterus and placenta (CTUP), separation of the chorioallantois from the endometrium, and accumulation of exudate.

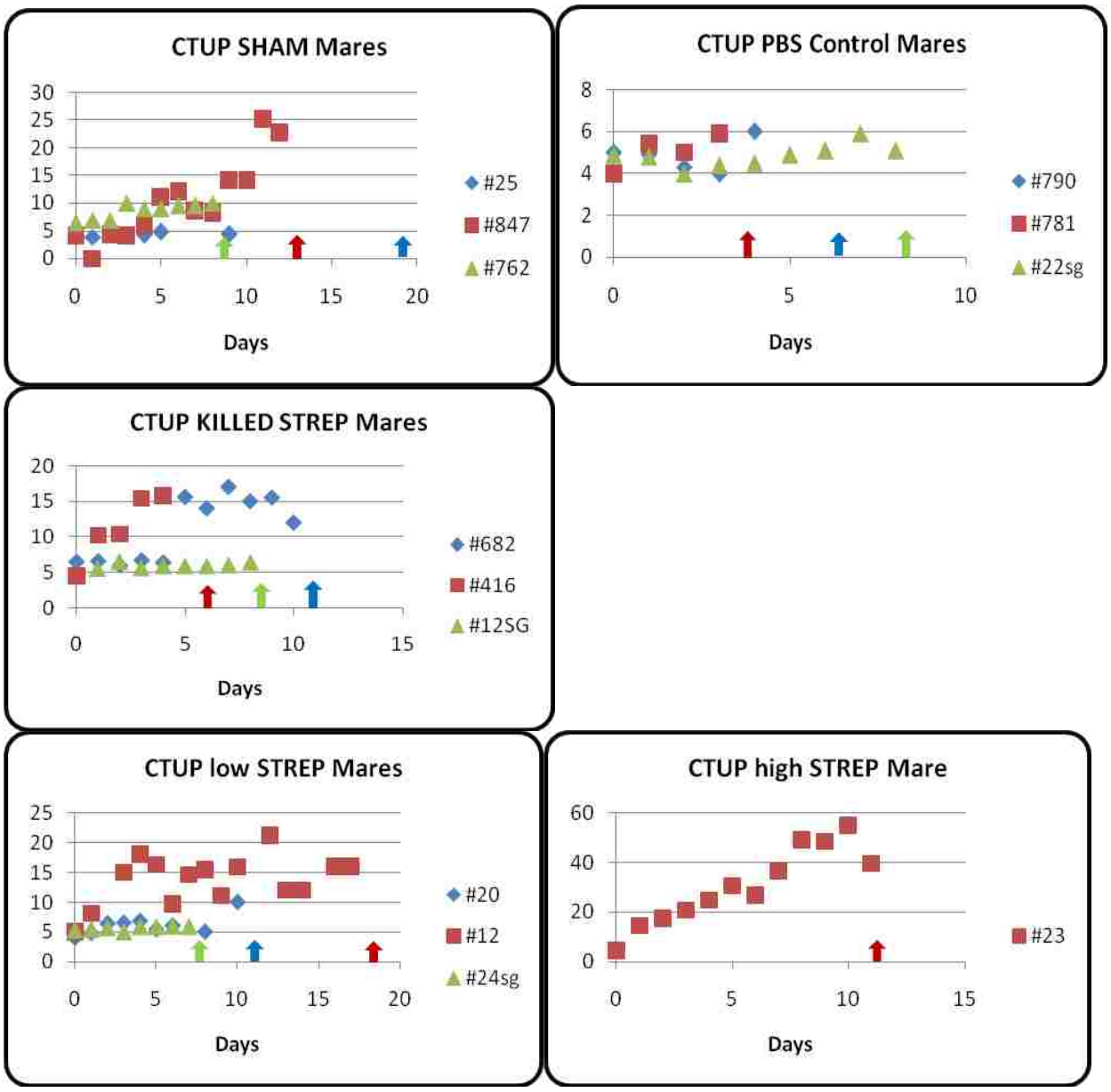

Figure 3.2 Combined thickness of uterus and placenta (CTUP) from mares (n=13) in an experimental model of placentitis. The allantoic space was catheterized laparoscopically between 265-280 days gestation. Day 0 was day of inoculation. SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (n=3) received 1 x 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (n=3) received 1 x 10^7 *S. zooepidemicus* transcervically; and high STREP mares (n=1) received 5.1 x 10^8 *S. zooepidemicus* transcervically. Measurements were performed with a 5 to 7.5-MHz linear transducer, and were obtained from the caudal uterine segment, ventrad to the cervix. Arrows along the x axis denote the day of delivery.

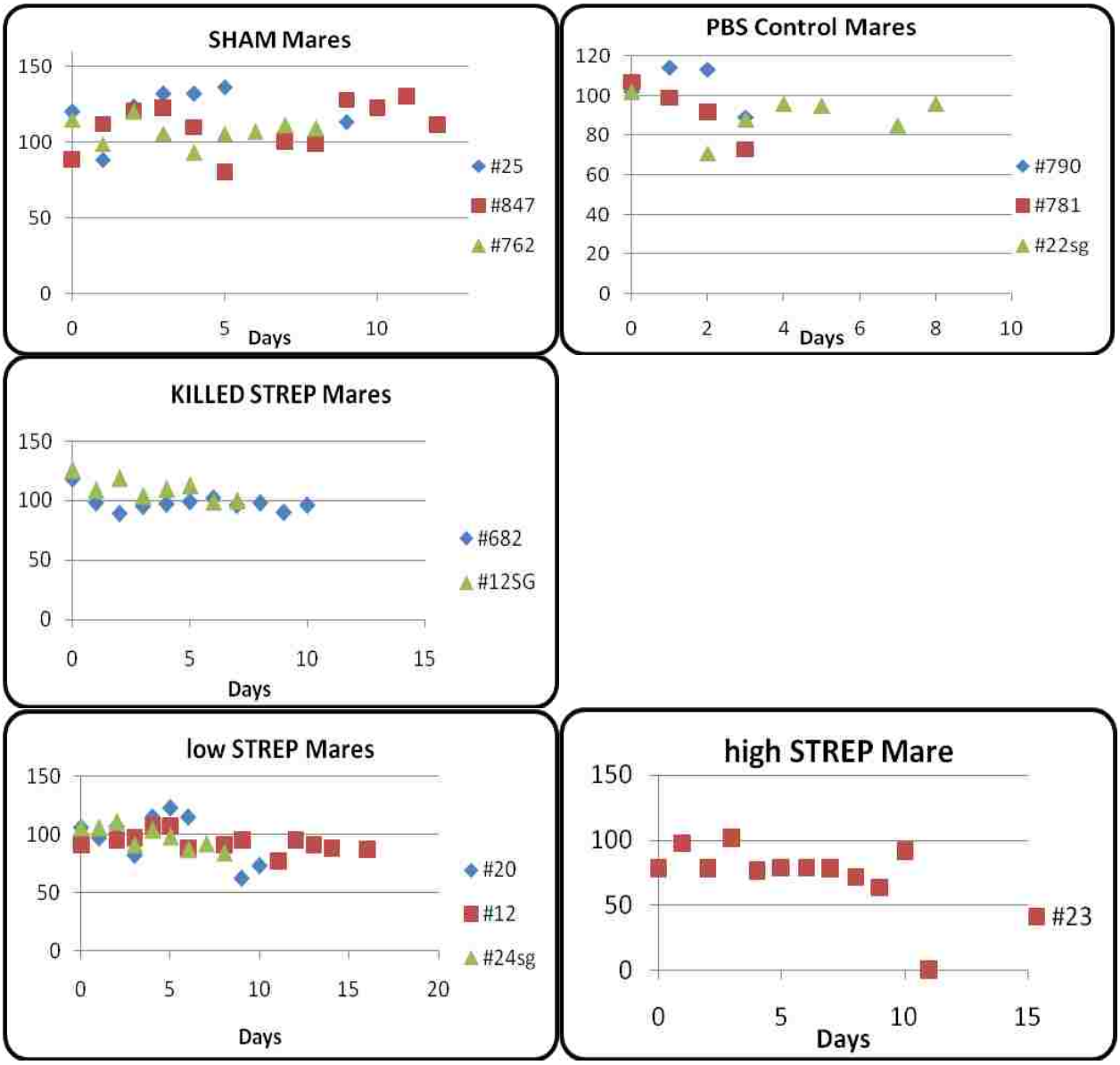

Figure 3.3 Fetal heart rates (FHR) from mares (n=12) in an experimental model of placentitis. The allantoic space was catheterized laparoscopically between 265-280 days gestation. Day 0 was day of inoculation. SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (n=3) received 1 x 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (n=3) received 1 x 10^7 *S. zooepidemicus* transcervically; and high STREP mares (n=1) received 5.1 x 10^8 *S. zooepidemicus* transcervically. Measurements were performed with a 5 to 7.5-MHz linear transducer, and were obtained from the caudal uterine segment, ventrad to the cervix.

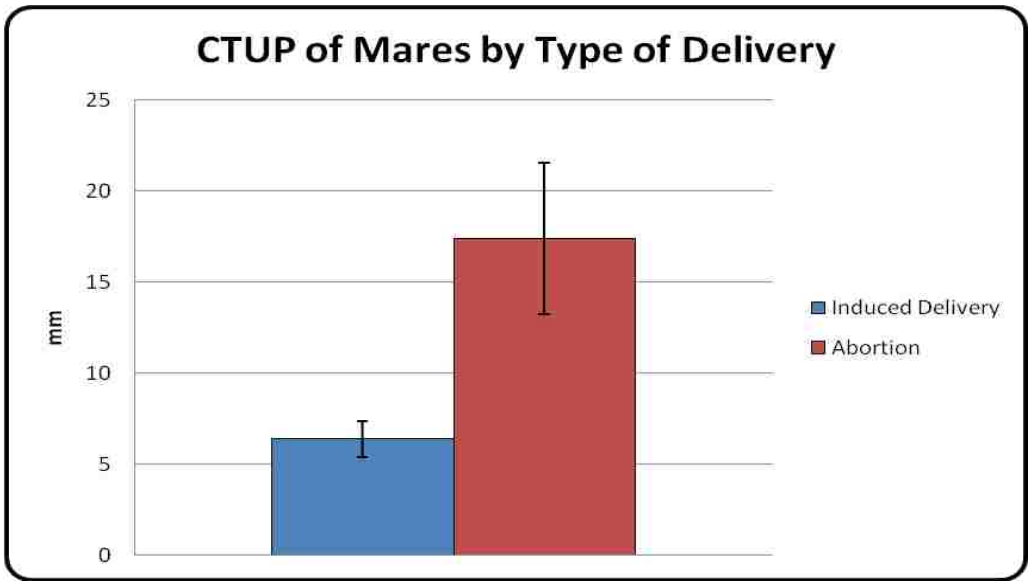

Figure 3.4 Relationship between type of delivery and the combined thickness of the uterus and placenta (CTUP) within 24 h of delivery in an experimental model of placentitis for mares with induced delivery (n=5) and mares with spontaneous abortion (n=7). CTUP was significantly greater (p=0.038) in mares with spontaneous abortion.

considerable problem. Use of an indwelling Huber needle for sample collection and clipping and sterile preparation of the skin 24 h prior to laparoscopy were associated with a reduction in bacterial tracking. Comparing the bacterial isolates with the histologic inflammatory changes at both the cervical star and catheter site made it possible to determine the source of infection. *Streptococcus zooepidemicus* was not isolated from any fetal fluid or fetal samples from SHAM CONTROL, PBS CONTROL, or KILLED STREP mares; however, five of nine mares in these groups became infected with bacteria via the catheter system. *Streptococcus zooepidemicus* was recovered from only one of three mares in the low STREP; one mare had *Staphylococcus* spp, and the other mare had no growth. Evidence of inflammation preceded the recovery of bacteria in one instance, was on the same day of bacterial recovery in three instances, and in one instance bacterial recovery preceded the presence of inflammation in the fetal fluids.

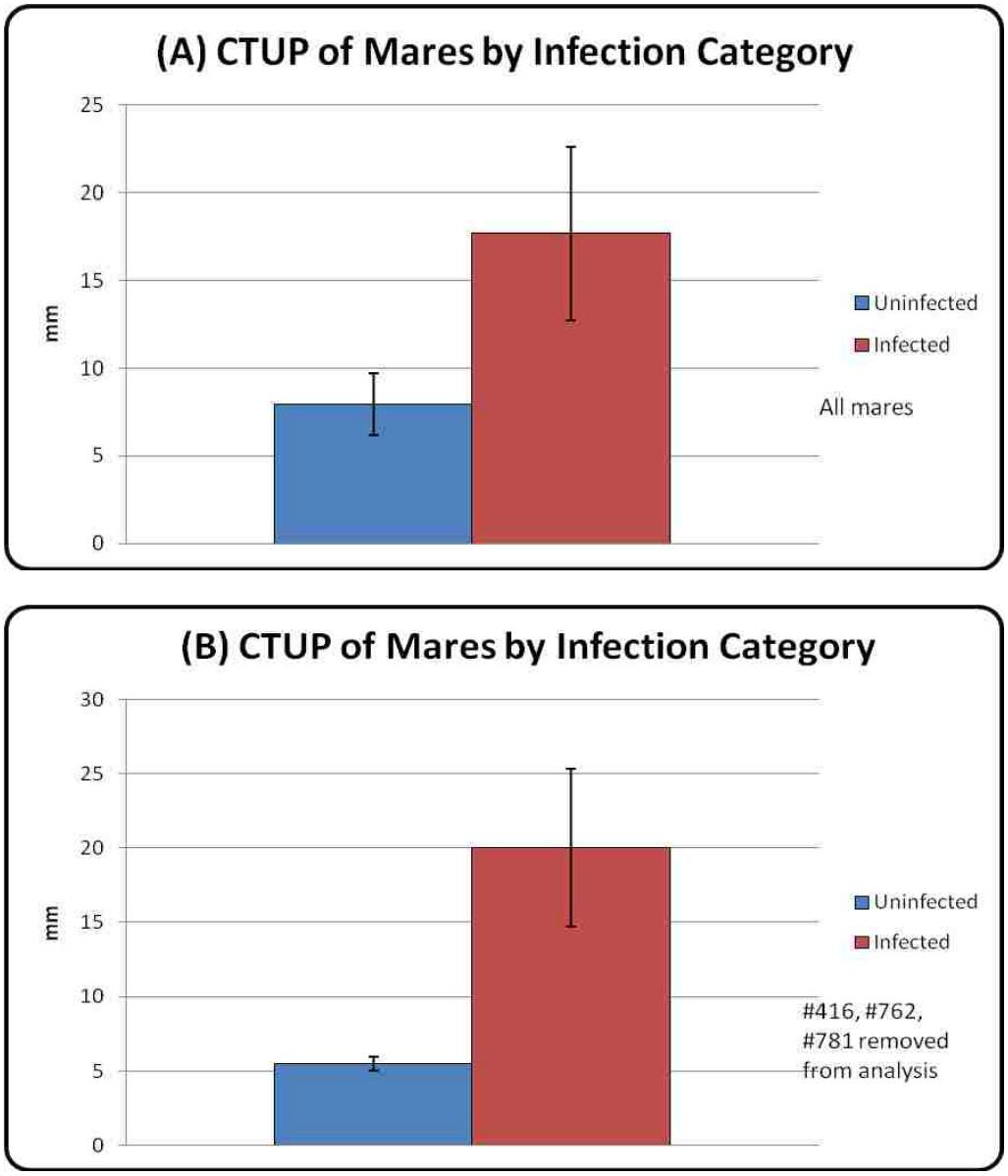

Figure 3.5 Relationship between infection of the fetal fluids and the combined thickness of the uterus and placenta (CTUP) within 24 h of delivery in an experimental model of placentitis. (A) No significant effect between infected mares (n=6) and uninfected mares (n=6) on CTUP including all mares (p=0.11); (B) removal of mares #416, #762, #781 results in infection (n=5) having increased CTUP compared to uninfected (n=4) approaching significance (p=0.051).

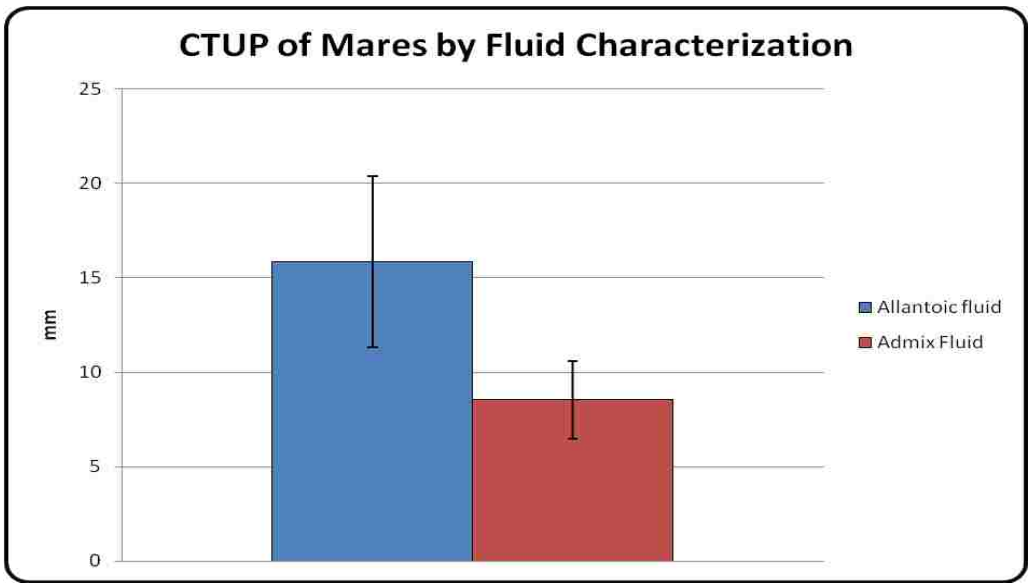

Figure 3.6 Relationship between characterization of the fetal fluid and the combined thickness of the uterus and placenta in an experimental model of placentitis. No significant effect of allantoic fluid (n=7) and admixed fluid (n=5) was observed (p=0.23).

3.3.3 Histopathology

The results of histologic evaluation of the individual fetal membranes and fetal tissue from fourteen mares are presented in Appendix: Histopathology of placental and fetal tissue.

3.3.3.1 Changes at the Cervical Star

The predominant histologic change at the cervical star in mares in the SHAM CONTORL, PBS CONTROL, and KILLED STREP groups was of edema (four of nine mares); two of these mares spontaneously aborted, two had induced deliveries. Inflammatory changes at the cervical star in mares not receiving a live inoculum were classified as mild in one of the SHAM mares (#25) and two of the PBS mares (#790, #781), and none of the KILLED STREP mares. In mares receiving a live inoculum, chronic necrotizing placentitis of the cervical star was present in three of five mares (#20, #23, #42), with extensive cranial extension from the cervical star in the high STREP mares (#23, #42), to encompass the catheter site in one of these mares

Table 3.3 Cytologic findings and culture results of fetal fluid samples from mares (n=13) in an experimental model of placentitis. The allantoic space was catheterized laparoscopically between 265-280 days gestation. Day 0 was the day of inoculation. SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (n=3) received 1 x 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (n=3) received 1 x 10^7 *S. zooepidemicus* transcervically; and high STREP mares (n=1) received 5.1 x 10^8 *S. zooepidemicus* transcervically.

Mare ID	Fluid type	Day of cytological inflammation	Day of positive culture	Delivery day	Delivery type	Inflammatory changes Cervical star	Inflammatory changes Catheter site	Bacterial isolate
SHAM Control Mares								
25	Admix	9	None	19	induced	1+	0	No growth
847	Allantoic	5	4	13	abortion	0 (necrosis)	2+	*Staphylococcus aureus*
762	Allantoic	None	None	8	induced	0 (edema)	0 (edema)	No growth
PBS Control Mares								
790	Allantoic[1]	Unknown[1]	Unknown[1]	7	abortion	0 (edema)	1+	*Corynebacterium spp.* (fetus)
781	Allantoic	3	3	4	abortion	1+	Not identified	*Staphylococcus* spp. (coagulase +)
22SG	Allantoic	None	none	8	induced	0 (edema)	Not identified	No growth[3]
KILLED STREP Mares								
682	Allantoic	10	10	11	abortion	0	2+	*Staphylococcus aureus*
416	Admix	None	None	6	abortion	0 (edema)	Not identified	No growth
12SG	Admix	None	None	8	induced	0	Not identified	*S. equisimilis*[4], *E. coli*[4]
Low STREP Mares								
20	Admix	>5[2]	>5[2]	11	abortion	2+	2+	*S. zooepidemicus*[6], *R. equi*
12	Allantoic	17	17	18	abortion	0 (edema)	2+	*Staphylococcus* spp.
24SG	Admix	None	None	8	induced	0 (edema)	0 (edema)	No growth
High STREP Mares								
23		6	11	11	abortion	3+	3+[5]	*S. zooepidemicus*[6]

[1]Pulled out at 40 h, not re-catheterized; positive cytology of peritoneal fluid.
[2]Pulled out at 24 h, re-catheterized 10 d after first surgery.
[3]*S. zooepidemicus* isolated from cervix prior to induction (not inoculum strain), allantoic fluid no growth.
[4]*S. equisimilis* isolated from cervix pre-induction, fetal lung, fetal stomach; *E. coli* isolated from fetal lung, fetal stomach.
[5]Inflammatory lesions from cervical star extended cranially to encompass the catheter site.
[6]*S. zooepidemicus* isolate identical to inoculum (confirmed by pulsed-field gel electrophoresis)

(#23). Because only *S. zooepidemicus* was recovered from the fetus and from fetal fluids in this mare, the change at the catheter site was presumed to be extension from an ascending placentitis. Edematous changes only were present in the remaining two low STREP mares (#12, #24SG).

3.3.3.2 Changes at the Catheter Site

The catheter site was identified at necropsy in 9 of the 13 catheterized mares. Inflammatory changes at the catheter site were present in five of these mares and extended from the cervical star to encompass the catheter site one of the high STREP mares (#23). Excluding the mare with cranial extension of ascending placentitis, the affected area of the chorioallantois ranged from 7 cm^2 to 75 cm^2. The pattern varied from subacute placentitis to chronic necrotizing placentitis. Two mares had chronic reactive placental changes with no inflammation, three mares had edema, one mare had infarction with transmural necrosis, and one mare had focal fibrotic changes. The non-inflammatory changes were seen either singly or in combination with other non-inflammatory changes.

3.3.3.3 Changes in the Umbilical Cord

Inflammation of the umbilical cord (funicitis) was present in 7 of the 14 mares (two SHAM CONTROL mares, three PBS CONTROL mares, one low STREP mare, and one high STREP mare), six of which had positive cultures of the fetus and fetal fluids, and three of which received a live inoculum (two low STREP and one high STREP). One of the PBS CONTROL mares had mild cord hemorrhage, and the remainder of the mares had no inflammatory changes.

3.3.3.4 Fetal Pneumonia

In mares not receiving a live inoculum (SHAM CONTROL, PBS CONTROL, and KILLED STREP groups), fetal pneumonia was present in three of the nine fetuses, no changes were present in three fetuses, and one fetus had bacteria with minimal inflammation (PBS CONTROL group). In the five mares receiving a live inoculum, pneumonia was present in two

fetuses, autolysis with bacteria was present in two fetuses, and no changes were present in the remaining fetus. Four of the five fetuses with pneumonia had a positive culture of the fetal lungs and fetal fluids, one fetus (KILLED STREP group) had mild fibrinous pneumonia with negative cultures. Bacteria were present with either minimal inflammation once (PBS CONTROL) or with autolysis on two occasions (high STREP group); in all three instances positive cultures were obtained from both fetal lung and fetal fluids.

3.4 Discussion

Initiation of placentitis was attempted by inoculation with either heat-killed or live *S. zooepidemicus* in pony mares instrumented with allantoic catheters between 260 and 285 d of gestation. Despite visualization of the pipette tip at the surface of the chorioallantois during inoculation, an ascending placentitis subsequent to a single inoculum with live *S. zooepidemicus* was only established in two of four animals. No animals receiving heat-killed bacteria or sterile PBS developed an ascending placentitis due to the introduction of vaginal microflora. Two possible reasons for a failure to establish infection of the chorioallantois are incomplete deposition of inocula and the dose of inoculum. In 2005, inoculations were performed with a double-lumen low-dose insemination pipette, in which seating of the inner catheter within the inner lumen is essential for complete delivery of the desired fluid. It is likely that failure to seat the two catheters tightly together allowed all or part of the inoculum to escape within the inner lumen of the outer catheter. In 2006, a single-lumen low-dose insemination pipette was used and an ascending placentitis was successfully induced in all mares receiving a live inoculum, with a consistent interval from inoculation to abortion. We considered the possibility that 1×10^7 CFU of *S. zooepidemicus* was not sufficient to establish an infection. During the winter of 2005 to 2006, three un-instrumented mares were inoculated with 1×10^{10} live *S. zooepidemicus* in a 2-mL volume, using the single lumen catheter. All three mares aborted, one less than 48 h, one less

than 72 h, and one 7 d post-inoculation. Based on these findings, the dose of 5.1 x 10^8 live *S. zooepidemicus* was chosen for further investigations.

We were unable to initiate an inflammatory response with heat-killed *S. zooepidemicus.* Whether this was due to the type of pipette used (all mares were inoculated in 2005) or that heat-killed *S. zooepidemicus* produces negligible inflammation of the chorioallantois cannot be determined from this study. During the winter of 2005 to 2006 inoculation of a single un-instrumented mare with 1 x 10^{10} heat-killed *S. zooepidemicus* produced a transient mild edema of the chorioallantois of less than 48 h of duration, but abortion failed to occur. This pregnancy continued without complication until delivery at term. Further development of a non-infectious inflammatory model may be useful in evaluating potential anti-inflammatory therapeutics for ascending placentitis.

The presence of neutrophils in fetal fluids was associated with a positive bacterial culture, and was usually present on the first day of a positive culture. In instances where fetal fluid was available for evaluation within 24 h of abortion, abortion was preceded by a positive culture of fetal fluid by 24 h or less except for one of the SHAM CONTROL mares. Small sample sizes and bacterial tracking along the catheter system (Chapter 2) produced considerable confounding effects, making comparison among treatment groups not possible. Therefore the effects of infection of the fetal fluid, characterization of fetal fluid, and abortion on the CTUP were compared. Mares spontaneously aborting had significantly increased CTUP compared to mares in which delivery was induced; however, there was not a significant effect of the presence of infection on the CTUP. This later finding was somewhat surprising; however, three mares had measurements considerably different from the other mares within their respective category. One of the KILLED STREP mares and one of the SHAM CONTROL mares had significantly increased CTUPs and placental edema but did not have intrauterine infection or histologic

inflammation at the cervical star; both mares spontaneously aborted. Edema is associated with impending term delivery [248]. It is unknown what initiated labor in these mares. One of the PBS CONTROL mares had intrauterine infection, but no histologic changes at the cervical star; based on the interval from catheter placement to abortion in this mare (less than 96 h), bacterial introduction presumably occurred at the time of surgery. When these mares were excluded from analysis, mares with infected fetal fluid tended to have an increased CTUP. These findings are in agreement with experimentally induced streptococcal placentitis [249] and clinical observations of mares with naturally occurring ascending placentitis [244]. Our findings also emphasize that increases in CTUP of the chorioallantois may signal impending abortion, but that the abortion is not necessarily due to *in utero* infection. Increased CTUP due to edema, but without concurrent separation of the chorioallantois from the endometrium and accumulation of exudate warrants careful monitoring, but antimicrobial therapy may not be indicated.

Chronic necrotizing placentitis was the most common histologic description in mares with an ascending infection of the chorioallantois; and, similar to a previous report [250], funicitis was associated with infection of the fetal fluids. A surprising finding was the inconsistent presence of fetal pneumonia in response to bacteria within the lung with *in utero* infection. While the interpretation by the pathologist was bacterial overgrowth presumed to be post-mortem contamination; this was not the case. In all of these instances, fetal fluids were culture positive prior to abortion; identical isolates were recovered from fetal fluid and fetal lung. Whether this finding represents a peracute fetal infection and death prior to the initiation of an inflammatory response, or the failure of the fetus to mount an immunologic response to infection is unknown. In two of the fetuses with bacteria and no pneumonia, there was no increase in fetal fluid cortisol (Chapter 6), so an incompetent immunologic response is possible.

Clearly this study was hampered by small samples sizes and bacterial contamination due to bacterial tracking along the catheter system. Our original study design included sample sizes of six in each treatment group; however, we were forced to conclude the study with the animals presented herein. Although the intent was to produce a model of ascending placentitis in mares inoculated with *S. zooepidemicus,* comparison of variables from groups with *in utero* infection to those without infection may provide valuable information. Despite these limitations, useful descriptive data was obtained, and the acquisition of fetal fluids for measurement of soluble TNF-α, prostaglandins, and cortisol should contribute to the body of knowledge on equine placentitis.

CHAPTER 4

THE POTENTIAL ROLE OF CYTOKINES IN AN EXPERIMENTAL MODEL OF INFECTIVE PRE-TERM DELIVERY IN THE MARE

4.1 Introduction

Intrauterine infection resulting from ascension of bacteria through the cervix with invasion of the chorioamnion and deciduas (maternal endometrium at the site of placental attachment) is a significant cause of pre-term delivery (PTD) in women [221] as it is in the mare. A wide variety of animal models have been described to investigate the mechanisms of PTDs [251]; but direct comparisons between models are hampered by differences in species used, agent used to initiate PTD, and route of inoculation/administration. In most species studied, an increase in pro-inflammatory cytokines has been detected in fetal fluids in response to inflammation or infection [223-229]. Non-infectious models for preterm delivery typically use lipopolysaccharide (LPS) administered into the maternal circulation [230], the maternal peritoneal space [231,232], or the uterus [233] to cause pregnancy loss. Intraamniotic, but not maternal, administration of LPS to sheep at approximately 80% gestation resulted in increases in amniotic and fetal IL-6 concentrations but no premature labor despite increased uterine activity, though there was a considerable degree of variability and the sample sizes were small [228]. Intraamniotic inoculation of group B streptococci in instrumented (fetal artery and vein, amniotic pressure catheter, myometrial EMG electrodes, maternal artery and vein) rhesus macques at 77% gestation produced increases in the amniotic concentrations of TNF-α, IL-1β, and IL-6 as early as 9 h following inoculation. PGE_2 and $PGF_{2\alpha}$ concentrations paralleled the increases in cytokines, and the hourly contraction area (mm Hg x sec / h) increased significantly within 28 h [224].

While intraamniotic inoculation may provide a greater uniformity of response due to precise deposition of the inflammatory agent, it does not reproduce what happens during the course of natural infection, namely ascension through the cervical canal. Other models have attempted to mimic natural infection by intracervical inoculation. Intracervical inoculation of rabbit does at 70% gestation with *E. coli* also produced increased concentrations of TNF-α, IL-β, and IL-6, and PGE_2 and $PGF_{2\alpha}$ in amniotic fluid as early as four h after inoculation [223]. Similarly, intracervical deposition of either LPS or *E. coli* in wild-type or IL-1β -/- mice produced increases in amniotic TNF-α, IL-β, and IL-6 and premature delivery in 92% (*E. coli*) to 100% (LPS) of mice. Although decreased cytokine was observed in IL-1β -/- mice, pregnancy loss was the same [226].

One of the proposed mechanisms of infection-induced PTD is that pro-inflammatory cytokines (IL-1, IL-6, TNF-α) from the deciduas and cortisol from the mother cause activation of the fetal hypothalamic-pituitary-adrenal axis, causing an increase of fetal cortisol [48]. Increased fetal cortisol, either at term or in response to pro-inflammatory cytokines, decreases concentrations of PGDH, an enzyme responsible for inactivation of PGE_2 and $PGF_{2\alpha}$, leading to increased PGE_2 and $PGF_{2\alpha}$ and subsequent myometrial contractions. Pro-inflammatory cytokines also increase the activity of another key enzyme, prostaglandin H_2 synthase (PGHS-2 or COX-2), which increases prostaglandin synthesis. The decrease in PGDH and increase in PGHS-2 leads to increased PGE_2 and $PGF_{2\alpha}$ synthesis, myometrial contractions and premature labor [227,234,235,252].

One model examining the relationship between inflammatory cytokines and ascending placentitis in pregnant mares has been reported [237]. Placentitis was induced by intracervical inoculation of 10^7 to 10^8 CFU of *S. zooepidemicus* in mares between 283 and 291 d of gestation.

No differences were detected in the activities of TNF-α, IL-1, and IL-6 between groups over time. Placental IL-6 and IL-8 mRNA were higher at the cervical star and IL-6 mRNA was higher in the placental body of inoculated mares compared to term-delivery control mares. Based on the expression data it was hypothesized that bacterial infection causes increased expression of pro-inflammatory cytokines leading to the release of PGE_2 and $PGF_{2\alpha}$ into the allantoic fluid resulting in premature labor.

The failure to demonstrate an increase in the concentrations of pro-inflammatory cytokines in equine fetal fluids following bacterial infection differs from results from rabbits, monkeys, women, sheep, and mice [223-226,228]. Variability in response to inoculation [168,227], histologic characterization of placentitis, dose of inoculum, and site of inoculation may have contributed to the failure to detect increased concentrations of pro-inflammatory cytokines in fetal fluids. Failure to detect soluble cytokines in the allantoic fluid may have also been due to sampling intervals that were too infrequent to detect changes in cytokine concentrations. Amniotic concentrations of TNF-α, IL-1α, and IL-1β were elevated within four h of transcervical inoculation of pregnant rabbits with *E. coli* [223]. In pregnant ewes, the concentration of IL-6 in amniotic fluid peaked between four to six h following administration of LPS and had begun to decrease by 24 h [228], making it conceivable that changes in cytokine levels were missed in the equine study [237]. Other potential sources of variability were the presence of allantoic catheters in some, but not all, of the mares; and repeated ultrasound-guided allantocentesis to obtain fluid from mares without catheters or those with non-patent catheters. Biochemical characterization for creatinine, chloride, sodium, and calcium of fetal fluids to determine its origin [239,240] was not performed; therefore, the nature of fetal fluid may have affected differences between groups.

The temporal relationship between increases in allantoic cytokines and prostaglandins is quite important. If the synthesis of $PGF_{2\alpha}$ and PGE_2 is driven by cytokines in concert with fetal cortisol, as has been suggested in a model of ascending placentitis [48], then a therapeutic regime that is unsuccessful in interrupting the signaling of cytokines and prostaglandins will fail to prevent the onset of labor. The hypothesis was that the concentration of soluble TNF-α in fetal fluids and the expression of pro-inflammatory cytokines in chorioallantoic tissue would be increased in mares with *in utero* infection or spontaneous abortion.

4.2 Material and Methods

4.2.1 Experimental Animals**,** Instrumentation, and Inoculation

Animal care, instrumentation, and inoculation were as previously described (Chapter 3). Briefly, indwelling allantoic catheters were placed laparoscopically in 13 adult pony mares between 260 and 285 d of gestation. All procedures were in accordance with Louisiana State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. Mares received either 1×10^7 CFU live *S. zooepidemicus* (n=3), 5.1×10^8 CFU live *S. zooepidemicus* (n=1), 1×10^7 heat-killed *S. zooepidemicus* (n=3), 1 mL sterile PBS (n=3). Sham conrol mares did not receive a transcervical inoculation (n=3). One mare received 5.1×10^8 CFU live *S. zooepidemicus* but was not instrumented with an allantoic catheter. Two sham control mares, one PBS control mare, one killed-Strep mare and one low-dose Strep mare were induced to deliver fetuses as previously described (Chapter 3) to provide gestational age-matched placental and fetal tissue, and control for the variability from inoculation to delivery. Preparation of the inocula, transcervical inoculations, patient monitoring, and sample collection were performed as previously described (Chapter 3). Five mL of fetal fluid was collected at 0, 4, 8, 12, 16, 20, 24, 32, 40, 48, h and then daily until spontaneous delivery or induction of parturition. Fetal fluid samples were collected and immediately placed

on ice for transport to the laboratory, centrifuged at 2,000 x g for 15 minutes at 4°C, and the supernatant separated into 1-mL aliquots and stored at -70°C for future analysis. At delivery of the fetus, the chorioallantois was cleaned of debris with sterile saline, placed within a sterilized bag, and transported on ice to the laboratory for processing. Three 0.2-g pieces of tissue were collected from the region of the cervical star, any grossly abnormal area, the catheter site if identifiable, and from one normal area at least 10 cm from any grossly abnormal site, placed in 2 mL (wt/vol ratio of 10%) of RNA stabilization reagent (RNAlater, Qiagen Inc., Valencia, CA), and stored at -20°C for later analysis of cytokine expression.

4.2.2 TNF-α Enzyme-Linked ImmunoSorbant Assay

Concentrations of soluble TNF-α protein (TNFp) were determined using a commercially available equine-specific screening kit (Equine TNF-α Screening Set, Endogen®, Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions except for the following modifications. This assay has been validated for equine serum samples [253]. Preliminary data showed a reduction in the optical density by the presence of allantoic fluid, so a standard diluent with allantoic fluid from a sample collected at surgery (T-4SD) was prepared at a 1:100 dilution (vol/vol). The high standard (10,000 pg/mL) was diluted in T-4SD at a 1:40 dilution to provide the top standard in the assay at 250 pg/mL. Serial dilutions (1:2; vol/vol) were prepared so that the lowest value was 1.95 pg/mL. The same allantoic sample (T-4) was used to prepare T-4SD for all assays. Samples were diluted 1:100 in reagent diluents, and 100 µL of each sample was added to individual wells. Blocking and coating of 96-well plates was as directed by the manufacturer. Plates were incubated for 1 hr at room temperature, and all remaining steps were as directed by the manufacturer. Absorbance was read at A_{450} on a microplate spectrophotometer (Benchmark Plus, Bio-Rad Laboratories, Hercules, CA) and associated software (Microplate Manager 5.2, Bio-Rad Laboratories, Hercules, CA). For any samples with detectable values,

samples were re-evaluated at either a 1:2 or 1:10 dilution and serially diluted within the plate, with the upper and lower standards of the assay at 1000 and 7.8125 pg/mL, respectively. In order to efficiently delineate a potential relationship between time of infection and detection of TNFp, samples from two mares with, and two mares without, *in utero* infection were analyzed at the following time points: 0, 4, 8, 12, 16, 20, 48, 72, 96, and 120h, and 6, 7, and 8 d relative to inoculation. Based on these findings, samples from the remainder of mares were analyzed at the last four samples available prior to spontaneous abortion or induced delivery.

4.2.3 Expression of Cytokines

4.2.3.1 RNA Extraction

RNeasy® System – Approximately one-half of the tissue from a site was placed in 600 µL of RLT with β-mercaptoethanol (Qiagen, Inc., Valencia, CA), minced with a sterile blade, and placed in a snap-cap vial. The tissue was homogenized with a rotor-stator homogenizer (Power Gen 125, fisher Scientific, Hampton, NH) and a sterile generator probe (Power Gen disposable 7 x 110mm, Fisher Scientific, Hampton, NH) while remaining in RLT buffer. The lysate was pipetted into a microcentrifuge shredder spin-column (QIAshredder, Qiagen, Valencia, CA), placed inside a 2-mL collection tube and centrifuged for 2 minutes at 8,000 x g. The spin-column was removed, the lysate centrifuged a second time for 3 minutes, and the resultant supernatant transferred to a new microcentrifuge tube. An equal volume of 70% ethanol was added to the lysate, mixed, and transferred to a spin-column (RNeasy spin-column, Qiagen Inc., Valencia, CA) in a 2-mL collection tube. Following centrifugation at 8,000 x g, the spin-column was removed and the flow-through discarded. The spin-column was washed three times by the addition of 500 µL of Buffer RPE (Qiagen, Valencia, CA) and centrifugation at 8000 x g for 2 minutes removed any residual ethanol. The spin-column was placed in a new 2-mL collection tube and centrifuged at 8,000 x g for one minute. Finally, the spin-column was transferred to a

new 1.5-mL collection tube, and RNA eluted twice by the addition of 30 to 50 µL of RNase-free H_2O, centrifuged at 8,000 x g, and stored at -80°C until needed for further assay.

RNA-STAT Method – Approximately one-half of the tissue from a site was placed in 1000 µL of RNA stabilization reagent (RNA-STAT, Fisher Scientific, Hampton, NH), minced with a sterile blade, and placed in a 5-mL snap-cap vial. The tissue was homogenized with a rotor-stator homogenizer (Power Gen 125, Fisher Scientific, Hampton, NH) and a sterile generator probe (Power Gen disposable 7 x 110mm, Fisher Scientific, Hampton, NH) while remaining in stabilization reagent. After vortexing to further homogenize the sample, 200 µL of chloroform was added and mixed vigorously for 15-20 seconds. Samples were allowed to stand for 3 minutes. Following centrifugation at 12,000 x g for 15 minutes at 4°C, the upper 400 µL of the aqueous layer was transferred to a new 1.5-mL centrifuge tube and 320 µL of isopropyl alcohol was added and pulse-vortexed. Samples were incubated at 20°C for a minimum of 30 minutes. Following centrifugation at 20,000 x g for 10 minutes at 4°C, the supernatant was decanted, the rim of the tube blotted, and 800 µL of 75% ethanol added to the pellet. Following vortexing, samples were centrifuged at 14,000 x g for 5 minutes at 4°C. The supernatant was carefully removed and discarded, and the tubes placed on their side to allow the pellets to dry while under the path of an air-flow hood for four to five minutes. A 60-µL volume of RNase-free water was added and the samples placed in a 60°C water bath for 10 minutes, pulse-vortexed, and stored at -80°C until needed for further assay.

4.2.3.2 Reverse Transcription Reaction

The concentration of RNA was determined by measuring the absorbance at 260 nm, and the volume of sample containing a maximum of 1 µg of RNA was calculated. RNase-free water was added to each PCR sample to give a final volume of 41.5 µL. The Master Mix is provided in Table 4.1. To each PCR tube, 35.8 µL of Master Mix was added, pipetted repeatedly

to completely mix, and placed in a themocycler (Bio-Rad Laboratories, Hercules, CA) with reaction settings at 42°C for 15 minutes, 95°C for 5 minutes, and a 4°C holding time. Samples were then either immediately assayed or stored at -20°C.

Table 4.1 Master Mix reaction components for reverse transcription reaction.

Master Mix	Each reaction	Promega, Madison, WI
AMV buffer (5X)	16 µL	Supplied with PR-M9004
MgCl2 (25 mM)	16 µL	PR-A3511
dNTP	4 µL	PR-U1515
RNAsin (40 U/µL)	1 µL	PR-N2615
AMV Rtase	0.5 µL	PR-M9004
Oligo dT15 primer (500 µg/mL	1 µL	PR-C1101
Total volume Master Mix per reaction	38.5 µl	

4.2.3.3 Quantitative Real-Time PCR (qRT-PCR)

Quantitative RT-PCR was performed using a fast real-time detection system (7900HT Fast Real-Time PCR System, Applied Biosystems, Foster City, CA). Primer probe sets (Table 4.2) for the detection of equine-specific IL-1β, IL-6, IL-8, IL-10, IL-15, IL-18, iNOS, MCP-1, TNF-α, TGF-β, and β-GUS were designed using a commercially available service (Assays-By-Design, Applied Biosystems, Foster City, CA). The reaction in each well consisted of 5 µL of Taq (TaqNab Gene Expression Master Mix, Applied Biosystems, Foster City, CA), 0.5 µL of primer probe (20X), and 4.5µL of cDNA template. PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds (45 cycles), and 60°C for 1 minute. Equine β-glucuronidase (β-BUS) was chosen for the housekeeping gene because of its low variability and lack of pseudogenes [254,255]. The individual amplification efficiency for each PCR in each

Table 4.2 Primer probe set sequences for equine-specific cytokines

β-glucuronidase	
fwd	GCTCATCTGGAACTTTGCTGATTTT
rev	CTGACGAGTGAAGATCCCCTTTT
probe	FAM CTCTCTGCGGTGACTGG NFQ
IL-1β	
fwd	CCGACACCAGTGACATGATGA
rev	ATCCTCCTCAAAGAACAGGTCATTC
probe	FAM ATTGCCGCTGCAGTAAG NFQ
IL-6	
fwd	GGATGCTTCCAATCTGGGTTCAAT
rev	TCCGAAAGACCAGTGGTGATTTT
probe	FAM ATCAGGCAGGTCTCCTG NFQ
IL-8	
fwd	GCCGTCTTCCTGCTTTCTG
rev	CCGAAGCTCTGCAGTAATTCTTGAT
probe	FAM CAACCGCAGCTTCAC NFQ
IL-10	
fwd	AGGACCAGCTGGACAACATG
rev	GGTAAAACTGGATCATCTCCGACAA
probe	FAM CCAGGTAACCCTTAAAGTC NFQ
IL-15	
fwd	TGAGGCTGGCATTCATGTCTT
rev	CCAGTTTGCCTCTGTTTTAGGAAGA
probe	FAM CTGATACAGCCCAAAATG NFQ
IL-18	
fwd	CCTGTGTTTGAGGATATGCCTGATT
rev	GCTAGACCTCTAGTGAGGCTATCTT
probe	FAM ATTGTACAGACAACGCACCC NFQ
iNOS	
fwd	GCGTTACTCCACCAACAATGG
rev	CCAGATCCGGAAGTCATGCTTTC
probe	FAM ATGGCCGACCTGATGTT NFQ
IFN-γ	
fwd	AGCAGCACCAGCAAGCT
rev	TTTGCGCTGGACCTTCAGA
probe	FAM ATTCAGATTCCGGTAAATGA NFQ
TGF-β	
fwd	CCCTGCCCCTACATTTGGA
rev	TGTACAGGGCCAGGACCTT
probe	FAM CCTGGACACGCAGTACAG NFQ
TNF-α	
fwd	TTACCGAATGCCTTCCAGTCAAT
rev	GGGCTACAGGCTTGTCACTT
probe	FAM CCAGACACTCAGATCAT NFQ
MCP-1	
fwd	GCGGCCGCCTTCAG
rev	CAGCAGGTGACTGGAGAATTAATTG
probe	FAM CAGGTGCTGGCTCAGC NFQ

well was subjected to a linear regression analysis [256,257] prior to calculation of the $\Delta\Delta C_T$ [258], where

$\Delta\Delta C_T$ = [gene of interest C_T – β-GUS C_T]$_{sample}$ – [gene of interest C_T – β-GUS C_T]$_{calibrator}$

and

C_T = the amplification cycle at which the gene reaches a threshold level of fluorescence

and the relative quantification, or fold change in gene expression is

$RQ = 2^{-\Delta\Delta CT}$.

Three different calibrators were used:

- No inflammation = average of $\Delta\Delta C_T$ for all normal sites for all mares
- No inflammation/No infection = average of $\Delta\Delta C_T$ for normal sites of uninfected mares
- Individual = normal site for that mare

4.2.4 Statistical Analysis

Because of the small samples sizes within treatment groups, analysis of effect of treatment group on cytokine expression was not possible. Mares were categorized according to infection and type of delivery. Using the three different calibrators, the difference in relative quantification within each site between infected and uninfected mares, or aborting and induced-delivery mares was compared by a student's t test, using a statistical analysis software program (SAS 9.1, Cary, NC) with significance set at $p \leq 0.10$, including Satterthwaite's and Cochran's methods for sample populations with unequal variances.

4.3 Results

4.3.1 Soluble TNF-α in Fetal Fluid

In only three samples of all mares at all time-points assayed was TNFp detectable (Table 4.3). One PBS control mare, one SHAM control mare, and one mare receiving 1 x 10^7 CFU/mL of *S. zooepidemicus* had detectable concentrations of TNFp; all three mares experienced *in utero*

Table 4.3 Soluble TNF-α protein (TNFp) in fetal fluid from mares with experimental in utero infection. The allantoic space was catheterized laparoscopically between 265-280 d gestation: SHAM mares (n=3) received no transcervical procedure; PBS Control mares (PBS; n=3) received 1 mL sterile PBS transcervically; KILLED STREP (KS; n=3) received 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (LS; n=3) received 10^7 *S. zooepidemicus* transcervically; and high STREP mares (HS; n=1) received 5.1 x 10^8 *S. zooepidemicus* transcervically.

Mare	Group	Day of infection	Day of delivery	Sample	TNFp (pg/mL)
Infected mares					
20	LS	>120h	11d	0h	BD[1]
				4h	BD
				8h	BD
				12h	BD
				16h	BD
				20h	BD
				48h	BD
				72h	BD
				96h	BD
				120h	24.5
				>120 h	no sample available[2]
23	HS	11d	11d	0h	BD
				4h	BD
				8h	BD
				12h	BD
				16h	BD
				20h	BD
				40h	BD
				48h	BD
				72h	BD
				96h	BD
				120h	BD
				6d	BD
				7d	BD
682	KS	10d	11d	6d	BD
				7d	BD
				8d	BD
				9d	BD

[1] Value below detection limit of assay.
[2] Sampling after 120h was not possible due to catheter system failure.

Table 4.2 continued

Mare	Group	Day of infection	Day of delivery	Sample	TNFp (pg/mL)
781	PBS	72h	4d	32h	BD
				40h	BD
				48h	BD
				72h	10,304
12	LS	17d	18d	11d	BD
				12d	BD
				13d	BD
				17d	BD
847	SHAM	96h	13d	32h	12,129
				48h	BD
				72h	BD
				8d	BD
790	PBS	??	7d	32h	BD
				40h	BD
				48h	BD
				72h	BD
Uninfected mares					
762	SHAM	—	8d	0h	BD
				4h	BD
				8h	BD
				12h	BD
				16h	BD
				20h	BD
				48h	BD
				72h	BD
				96h	BD
				120h	BD
				6d	BD
				7d	BD
				8d	BD
22SG	PBS	—	8d	0h	BD
				4h	BD
				8h	BD
				12h	BD
				16h	BD
				20h	BD
				48h	BD

Table 4.2 continued

Uninfected mares					
22SG	PBS	—		72h	BD
				96h	BD
				120h	BD
				6d	BD
				7d	BD
				8d	BD
416	KS	—	6d	48h	BD
				72h	BD
				96h	BD
				120h	BD
24SG	LS	—	8d	120h	BD
				6d	BD
				7d	BD
				8d	BD
12SG	KS	—	8d	120h	BD
				6d	BD
				7d	BD
				8d	BD
25	SHAM	—	19d	96h	BD
				120h	BD
				8d	BD
				19d	BD

infection. Tumor necrosis factor-α protein was detectable prior to a positive culture of the fetal fluids twice (64 h once and interval unknown once), and in one instance the detection of TNFp and bacterial isolation from fetal fluids were at the same time point. In all other time points sampled, TNFp was below the detection limit of the assay. The mares and time points with detectable TNFp were: one mare from the PBS control group (#781; 10,304 pg/mL), one mare from the SHAM control group (#847; 12,129 pg/mL), and one mare in the low-dose STREP group (#20; 24.5 pg/mL).

4.3.2 Expression of Cytokines in Chorioallantoic Tissues

Of the eleven equine-specific cytokines evaluated at four sites (normal, abnormal, catheter site, cervical star) from the chorioallantois of thirteen mares, significant differences in relative quantitative expression (RQ) for IL-1β, IL-18, IL-15, iNOS, MCP-1, and IFN-γ, were found, while that of IL-8 approached significance.

4.3.2.1 Change in Expression of Cytokines with Intrauterine Infection

At normal sites of the chorioallantois, the mean RQ of IL-1β **(**Table 4.4, p=0.069) and IL-18 (Table 4.9, p=0.052) were greater in infected mares than in uninfected mares. At abnormal sites of the chorioallantois, the mean RQ of IL-15 (Table 4.8, p=0.067) was greater in infected mares than uninfected mares; and the mean RQ of IL-1β (Table 4.4, p=0.033), IL-6 (Table 4.5, p=0.13), IL-8 (Table 4.6, p=0.11), and TNF-α (Table 4.14, p=0.14) tended to be greater in infected mares than in uninfected mares. At the cervical star, the mean RQ of IFN-γ (Table 4.10, p=0.13) tended to be greater in infected mares than in uninfected mares, while the RQ of MCP-1 (Table 4.12, p=0.04) was greater in uninfected than in infected mares.

4.3.2.2 Change in Expression of Cytokines with Spontaneous Abortion

At normal sites of the chorioallantois, IL-18 (Table 4.9, p=0.052) and IFN-γ (Table 4.10, p=0.092) were greater in aborting mares than in induced-delivery mares. At abnormal sites of the chorioallantois**,** IL-1β (Table 4.4, p=0.045) and IL-18 (Table 4.9, p=0.052) were greater in aborting mares than in induced-delivery mares, while IL-6 (Table 4.5, p=0.11), IL-8 (Table 4.6, p=0.11), and TNF-α (Table 4.14, p=0.13) tended to be greater in aborting mares than in induced-delivery mares. At the cervical star, iNOS (Table 4.11, p=0.049) was greater in aborting mares than in induced-delivery mares, while MCP-1 (Table 4.12, p=0.049) was greater in induced-delivery mares than in aborting mares.

Table 4.4 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interleukin-1 (IL-1) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	no inflamm	2.4. ± 0.82 (8)	0.61 ± 0.20 (4)	0.069	Satterthwaite
	no infect/no inflamm	5.40 ± 1.85 (8)	1.376 ± 0.45 (4)	0.069	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	74.45 ± 37.87 (5)	0.41 ± 0.021 (3)	0.12	Satterthwaite
	no infect/no inflamm	167.54 ± 85.22 (5)	0.9182 ± 0.05 (3)	0.12	Satterthwaite
	Individual	22.93 ± 6.83 (5)	1.114 ± 0.28 (3)	0.033	Satterthwaite
Cath site	No inflamm	6.418 ± 2.48 (4)	0.30 (1)	.	.
	No infect/no inflamm	14.44 ± 5.57 (4)	0.67 (1)	.	.
	Individual	16.44 ± 11.65 (4)	0.9406 (1)	.	.
Cervical star	No inflamm	70.44 ± 57.3 (8)	2.065 ± 0.87 (4)	0.27	Satterthwaite
	No infect/no inflamm	158.50 ± 128.90 (8)	4.65 ± 2.0 (4)	0.27	Satterthwaite
	Individual	18.58 ± 10.84 (8)	4.32 ± 2.26 (4)	0.24	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	2.218 ± 0.7d (9)	0.56 ± 0.27 (3)	0.25	Pooled
	No infect/no inflamm	4.99 ± 1.68 (9)	1.254 ± 0.62 (3)	0.25	Pooled
	Individual	1.00 ± 0 (9)	1.00 ± 0 (3)	.	.
Abnormal	no inflamm	62.12 ± 33.29 (6)	0.39 ± 0.01 (2)	0.12	Satterthwaite
	no infect/no inflamm	139.80 ± 74.91 (6)	0.871 ± 0.01 (2)	0.12	Satterthwaite
	Individual	19.21 ± 6.71 (6)	1.38 ± 0.14 (2)	0.045	Satterthwaite
Cath site	No inflamm	6.42 ± 2.48 (4)	0.30 (1)	.	.
	No infect/no inflamm	14.44 ± 5.57 (4)	0.67 (1)	.	.
	Individual	16.44 ± 11.65 (4)	0.94 (1)	.	.
Cervical star	no inflamm	62.70 ± 51.12 (9)	2.47 ± 1.088 (3)	0.27	Satterthwaite
	No infect/no inflamm	141.10 ± 115.00 (9)	5.55 ± 2.24 (3)	0.27	Satterthwaite
	individual	16.64 ± 9.76 (9)	5.39 ± 2.82 (3)	0.53	Pooled

Table 4.5 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interleukin-6 (IL-6) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	3.12 ± 1.61 (8)	1.61 ± 0.91 (4)	0.56	Pooled
	No infect/no inflamm	8.73 ± 4.64 (8)	4.50 ± 2.53 (4)	0.56	Pooled
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	11.20 ± 5.61 (5)	0.60 ± 0.31 (3)	0.13	Satterthwaite
	No infect/no inflamm	31.32 ± 15.69 (5)	1.69 ± 0.88 (3)	0.13	Satterthwaite
	Individual	3.38 ± 1.15 (5)	1.65 ± 1.03 (3)	0.35	Pooled
Cath site	No inflamm	2.28 ± 0.86 (4)	0.31 (1)	.	.
	No infect/no inflamm	6.36 ± 2.40 (4)	0.86 (1)	.	.
	Individual	5.81 ± 2.69 (4)	0.85 (1)	.	.
Cervical star	No inflamm	13.97 ± 10.32 (8)	4.59 ± 1.86 (4)	0.40	Satterthwaite
	No infect/no inflamm	39.03 ± 28.85 (8)	12.82 ± 5.20 (4)	0.40	Satterthwaite
	Individual	2.87 ± 0.96 (8)	13.90 ± 9.05 (4)	0.31	Satterthwaite
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	3.22 ± 1.47 (9)	0.81 ± 0.61 (3)	0.38	Pooled
	No infect/no inflamm	9.00 ± 4.10 (9)	2.27 ± 1.69 (3)	0.38	Pooled
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	9.54 ± 4.88 (6)	0.291 ± 0.06 (2)	0.12	Satterthwaite
	No infect/no inflamm	26.67 ± 13.63 (6)	0.87 ± 0.02 (2)	0.11	Satterthwaite
	Individual	2.87 ± 1.07 (6)	2.326 ± 1.36 (2)	0.80	Pooled
Cath site	No inflamm	2.28 ± 0.86 (4)	0.31 (1)	.	.
	No infect/no inflamm	6.361 ± 2.402 (4)	0.87 (1)	.	.
	Individual	5.81 ± 2.69 (4)	0.85 (1)	.	.
Cervical star	No inflamm	12.66 ± 9.20 (9)	5.37 ± 2.39 (3)	0.46	Satterthwaite
	No infect/no inflamm	35.39 ± 25.70 (9)	15.02 ± 6.67 (3)	0.46	Satterthwaite
	Individual	2.61 ± 0.889 (9)	18.35 ± 11.15 (3)	0.29	Pooled

Table 4.6 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interleukin-8 (IL-8) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	10.64 ± 8.68 (8)	0.146 ± 0.06 (3)	0.27	Satterthwaite
	No infect/no inflamm	97.81 ± 79.84 (8)	1.34 ± 0.53 (3)	0.27	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	121.50 ± 59.59 (5)	1.73 ± 1.68 (3)	0.11	Satterthwaite
	No infect/no inflamm	1117.00 ± 547.90 (5)	15.96 ± 15.48 (3)	0.11	Satterthwaite
	Individual	82.80 ± 43.72 (5)	0.56 + 0.17 (2)	0.13	Satterthwaite
Cath site	No inflamm	9.71 ± 3.87 (4)	0.023(1)	.	.
	No infect/no inflamm	89.25 ± 35.60 (4)	0.24 (1)	.	.
	Individual	15.59 + 12.50 (4)	0.12 (1)	.	.
Cervical star	No inflamm	101.20 ± 76.87 (8)	3.74 ± 2.62 (4)	0.25	Satterthwaite
	No infect/no inflamm	930.50 ± 706.80 (8)	34.42 ± 24.10 (4)	0.25	Satterthwaite
	Individual	45.67 + 28.46 (8)	7.90 + 3.20 (3)	0.23	Satterthwaite
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	10.64 ± 8.68 (8)	0.146 ± 0.06 (3)	0.27	Satterthwaite
	No infect/no inflamm	97.81 ± 79.84 (8)	1.34 ± 0.53 (3)	0.27	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	102.10 ± 52.38 (6)	0.05 ± 0.03 (2)	0.11	Satterthwaite
	No infect/no inflamm	939.00 ± 481.6 0(6)	0.48 ± 0.27 (2)	0.11	Satterthwaite
	Individual	82.80 ± 43.72 (5)	0.56 ± 0.175(2)	0.13	Satterthwaite
Cath site	No inflamm	9.71 ± 3.87 (4)	0.03 (1)	.	.
	No infect/no inflamm	89.25 ± 35.60 (4)	0.24 (1)	.	.
	Individual	15.59 + 12.50 (4)	0.12 (1)	.	.
Cervical star	No inflamm	91.23 ± 68.52 (9)	1.18 ± 0.76 (3)	0.23	Satterthwaite
	No infect/no inflamm	838.80 ± 630.00 (9)	10.83 ± 6.99 (3)	0.23	Satterthwaite
	Individual	45.67 + 28.46 (8)	7.90 + 3.20 (3)	0.23	Satterthwaite

Table 4.7 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interleukin-10 (IL-10) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	1.27 ± 0.46 (8)	5.39 ± 3.22 (3)	0.33	Satterthwaite
	No infect/no inflamm	0.34 ± 0.12 (8)	1.45 ± 0.87 (3)	0.33	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	22.12 ± 17.77 (5)	0.73 ± 0.36 (3)	0.30	Satterthwaite
	No infect/no inflamm	5.95 ± 4.78 (5)	0.20 ± 0.10 (3)	0.30	Satterthwaite
	Individual	51.81 ± 38.93 (5)	0.46 ± 0.03 (2)	0.26	Satterthwaite
Cath site	No inflamm	6.85 ± 3.64 (4)	0.65 (1)	.	.
	No infect/no inflamm	1.84 ± 0.98 (4)	0.18 (1)	.	.
	Individual	8.14 ± 1.54 (4)	0.23 (1)	.	.
Cervical star	No inflamm	26.53 ± 22.19 (8)	3.89 ± 1.32 (3)	0.34	Satterthwaite
	No infect/no inflamm	7.13 ± 5.97 (8)	1.05 ± 0.36 (3)	0.34	Satterthwaite
	Individual	55.70 ± 49.48 (8)	2.16 ± 1.67 (2)	0.32	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	1.27 ± 0.46 (8)	5.39 ± 3.22 (3)	0.33	Satterthwaite
	No infect/no inflamm	0.34 ± 0.12 (8)	1.45 ± 0.87 (3)	0.33	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	18.45 ± 14.96 (6)	1.02 ± 0.369 (2)	0.3	Satterthwaite
	No infect/no inflamm	4.96 ± 4.02 (6)	0.27 ± 0.10 (2)	0.3	Satterthwaite
	Individual	51.81 ± 38.93 (5)	0.46 ± 0.03 (2)	0.26	Satterthwaite
Cath site	No inflamm	6.85 ± 3.64 (4)	0.65 (1)	.	.
	No infect/no inflamm	1.84 ± 0.978 (4)	0.18 (1)	.	.
	Individual	8.14 ± 1.54 (4)	0.23 (1)	.	.
Cervical star	No inflamm	24.07 ± 19.72 (9)	3.62 ± 2.24 (2)	0.65	Pooled
	No infect/no inflamm	6.47 ± 5.30 (9)	0.97 ± 0.60 (2)	0.65	Pooled
	Individual	55.70 ± 49.48 (8)	2.16 ± 1.67 (2)	0.32	Satterthwaite

Table 4.8 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interleukin-15 (IL-15) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	1.33 ± 0.34 (8)	1.01 ± 0.19 (4)	0.54	Pooled
	No infect/no inflamm	1.14 ± 0.29 (8)	0.87 ± 0.16 (4)	0.54	Pooled
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	3.12 ± 0.99 (5)	0.67 ± 0.20 (3)	0.067	Satterthwaite
	No infect/no inflamm	2.67 ± 0.84 (5)	0.5704 ± 0.17 (3)	0.067	Satterthwaite
	Individual	5.71 ± 2.90 (5)	0.71 ± 0.16 (3)	0.16	Satterthwaite
Cath site	No inflamm	1.49 ± 0.63 (4)	0.36 (1)	.	.
	No infect/no inflamm	1.28 ± 0.54 (4)	0.30 (1)	.	.
	Individual	1.11 ± 0.20 (4)	0.34 (1)	.	.
Cervical star	No inflamm	1.66 ± 0.42 (8)	0.99 ± 0.28 (4)	0.32	Pooled
	No infect/no inflamm	1.42 ± 0.36 (8)	0.85 ± 0.24 (4)	0.32	Pooled
	Individual	1.47 ± 0.25 (8)	1.14 ± 0.42 (4)	0.50	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	1.24 ± 0.31 (9)	1.18 ± 0.12 (3)	0.91	Pooled
	No infect/no inflamm	1.06 ± 0.27 (9)	1.01 ± 0.10 (3)	0.91	Pooled
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	2.68 ± 0.92 (6)	0.75 ± 0.32 (2)	0.29	Pooled
	No infect/no inflamm	2.30 ± 0.79 (6)	0.64 ± 0.27 (2)	0.30	Pooled
	Individual	4.91 ± 2.50 (6)	0.58 ± 0.17 (2)	0.38	Pooled
Cath site	No inflamm	1.49 ± 0.63 (4)	0.36 (1)	.	.
	No infect/no inflamm	1.28 ± 0.54 (4)	0.30 (1)	.	.
	Individual	1.11 ± 0.20 (4)	0.34 (1)	.	.
Cervical star	No inflamm	1.60 ± 0.37 (9)	0.95 ± 0.39 (3)	0.38	Pooled
	No infect/no inflamm	1.37 ± 0.32 (9)	0.82 ± 0.33 (3)	0.38	Pooled
	Individual	1.54 ± 0.24 (9)	0.82 ± 0.37 (3)	0.15	Pooled

Table 4.9 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interleukin-18 (IL-18) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM)	Uninfected (mean ± SEM)	Pr > |t|	Method
Normal	No inflamm	1.50 ± 0.23 (8)	0.56 ± 0.29 (3)	0.052	Satterthwaite
	No infect/no inflamm	3.45 ± 0.53 (8)	1.29 ± 0.68 (3)	0.052	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	5.92 ± 2.86 (5)	1.46 ± 0.89 (3)	0.29	Pooled
	No infect/no inflamm	13.64 ± 6.59 (5)	3.38 ± 2.06 (3)	0.29	Pooled
	Individual	3.61 ± 1.68 (5)	1.24 ± 0.65 (2)	0.44	Pooled
Cath site	No inflamm	1.52 ± 0.20 (4)	0.58 (1)	.	.
	No infect/no inflamm	3.51 ± 0.45 (4)	1.35 (1)	.	.
	Individual	1.46 ± 0.52 (4)	0.00 (1)	.	.
Cervical star	No inflamm	11.12 ± 9.26 (8)	1.01 ± 0.56 (4)	0.31	Satterthwaite
	No infect/no inflamm	25.63 ± 21.35 (8)	2.32 ± 1.30 (4)	0.31	Satterthwaite
	Individual	6.40 ± 4.98 (8)	1.18 ± 0.41 (3)	0.33	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM)	Induction (mean ± SEM)	Pr > |t|	Method
Normal	No inflamm	1.50 ± 0.23 (8)	0.56 ± 0.29 (3)	0.052	Pooled
	No infect/no inflamm	3.45 ± 0.53 (8)	1.29 ± 0.68 (3)	0.052	Pooled
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	5.47 ± 2.37 (6)	0.57 ± 0.11 (2)	0.094	Satterthwaite
	No infect/no inflamm	12.62 ± 5.48 (6)	1.32 ± 0.25 (2)	0.094	Satterthwaite
	Individual	3.61 ± 1.68 (5)	1.24 ± 0.65 (2)	0.44	Pooled
Cath site	No inflamm	1.52 ± 0.20 (4)	0.58 (1)	.	.
	No infect/no inflamm	3.51 ± 0.45 (4)	1.35 (1)	.	.
	Individual	1.46 ± 0.52 (4)	0.00 (1)	.	.
Cervical star	No inflamm	10.18 ± 8.22 (9)	0.46 ± 0.07 (3)	0.27	Satterthwaite
	No infect/no inflamm	23.47 ± 18.95 (9)	1.03 ± 0.16 (3)	0.27	Satterthwaite
	Individual	6.40 ± 4.981 (8)	1.18 ± 0.41 (3)	0.33	Satterthwaite

Table 4.10 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for interferon-γ (IFN-γ) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	3.11 ± 1.15 (8)	0.64 ± 0.15 (4)	0.069	Satterthwaite
	No infect/no inflamm	4.23 ± 1.56 (8)	0.87 ± 0.20 (4)	0.069	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	15.31 ± 8.22 (5)	0.71 ± 0.54 (2)	0.34	Pooled
	No infect/no inflamm	20.79 ± 11.17 (5)	0.96 ± 0.73 (2)	0.34	Pooled
	Individual	5.96 ± 2.07 (5)	1.07 ± 0.79 (2)	0.22	Pooled
Cath site	No inflamm	0.78 ± 0.423 (3)	1.36 (1)	.	.
	No infect/no inflamm	1.06 ± 0.58 (3)	1.85 (1)	.	.
	Individual	1.42 ± 0.81 (3)	2.04 (1)	.	.
Cervical star	No inflamm	5.66 ± 2.73 (7)	0.65 ± 0.31 (3)	0.12	Satterthwaite
	No infect/no inflamm	7.69 ± 3.72 (7)	0.89 ± 0.43 (3)	0.12	Satterthwaite
	Individual	2.98 ± 1.06 (7)	1.20 ± 0.38 (3)	0.32	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	2.80 ± 1.06 (9)	0.75 ± 0.12 (3)	0.092	Satterthwaite
	No infect/no inflamm	3.80 ± 1.44 (9)	1.02 ± 0.17 (3)	0.09	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	15.31 ± 8.22 (5)	0.71 ± 0.54 (2)	0.34	Pooled
	No infect/no inflamm	20.79 ± 11.17 (5)	0.96 + 0.73 (2)	0.34	Pooled
	Individual	5.96 ± 2.07 (5)	1.07 ± 0.79 (2)	0.22	Pooled
Cath site	No inflamm	0.78 ± 0.43 (3)	1.36 (1)	.	.
	No infect/no inflamm	1.06 ± 0.58 (3)	1.85 (1)	.	.
	Individual	1.42 ± 0.81 (3)	2.04 (1)	.	.
Cervical star	No inflamm	4.99 ± 2.46 (8)	0.83 ± 0.45 (2)	0.44	Pooled
	No infect/no inflamm	6.78 ± 3.34 (8)	1.12 ± 0.61 (2)	0.44	Pooled
	Individual	2.74 ± 0.95 (8)	1.27 ± 0.64 (2)	0.48	Pooled

Table 4.11 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for inducible nitric oxide synthase (iNOS) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	2.30 ± 0.70 (8)	1.74 ± 0.90 (4)	0.65	Pooled
	No infect/no inflamm	4.94 ± 1.5 (8)	3.74 ± 1.93 (4)	0.64	Pooled
	Individual	1.00	1.00	.	.
Abnormal	No inflamm	5.08 ± 2.06 (5)	2.29 ± 0.60 (3)	0.35	Pooled
	No infect/no inflamm	10.91 ± 4.42 (5)	4.91 ± 1.29 (3)	0.35	Pooled
	Individual	1.28 ± 0.37 (5)	3.56 ± 2.96 (3)	0.52	Satterthwaite
Cath site	No inflamm	3.63 ± 3.38 (4)	1.67 (1)	.	.
	No infect/no inflamm	7.79 ± 7.26 (4)	3.59 (1)	.	.
	Individual	1.38 ± 0.68 (4)	5.08 (1)	.	.
Cervical star	No inflamm	1.24 ± 0.60 (8)	0.52 ± 0.48 (3)	0.51	Pooled
	No infect/no inflamm	2.66 ± 1.29 (8)	1.11 ± 1.04 (3)	0.51	Pooled
	Individual	0.73 ± 0.25 (8)	0.35 ± 0.13 (3)	0.39	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	2.37 ± 0.62 (9)	1.35 ± 1.14 (3)	0.44	Pooled
	No infect/no inflamm	5.09 ± 1.34 (9)	2.89 ± 2.45 (3)	0.44	Pooled
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	4.67 ± 1.73 (6)	2.12 ± 1.00 (2)	0.45	Pooled
	no infect/no inflamm	10.03 ± 3.72 (6)	4.54 ± 2.15 (2)	0.45	Pooled
	Individual	1.22 ± 0.31 (6)	4.89 ± 4.59 (2)	0.57	Pooled
Cath site	No inflamm	3.63 ± 3.38 (4)	1.67 (1)	.	.
	No infect/no inflamm	7.79 ± 7.26 (4)	3.59 (1)	.	.
	Individual	1.38 ± 0.68 (4)	5.08 (1)	.	.
Cervical star	no inflamm	1.26 ± 0.53 (9)	0.04 ± 0.00 (2)	0.049	Satterthwaite
	No infect/no inflamm	2.72 ± 1.14 (9)	0.08 ± 0.01 (2)	0.049	Satterthwaite
	individual	0.71 ± 0.22 (9)	0.277 ± 0.18 (2)	0.40	Pooled

Table 4.12 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for monocyte chemoattractant protein-1 (MCP-1) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	no inflamm	1.47 ± 0.48 (8)	1.84 ± 0.75 (4)	0.68	Pooled
	no infect/no inflamm	1.75 ± 0.56 (8)	2.18 ± 0.89 (4)	0.68	Pooled
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	1.79 ± 0.623 (5)	1.48 ± 0.70 (3)	0.76	Pooled
	no infect/no inflamm	2.12 ± 0.74 (5)	1.75 ± 0.83 (3)	0.76	Pooled
	Individual	1.35 ± 0.537 (5)	1.85 ± 1.01 (3)	0.64	Pooled
Cath site	No inflamm	2.03 ± 0.73 (4)	0.63 (1)	.	.
	No infect/no inflamm	2.40 ± 0.86 (4)	0.75 (1)	.	.
	Individual	1.53 ± 0.27 (4)	0.68 (1)	.	.
Cervical star	No inflamm	1.85 ± 0.64 (8)	5.03 ± 1.50 (3)	0.044	Pooled
	No infect/no inflamm	2.19 ± 0.76 (8)	5.96 ± 1.78 (3)	0.044	Pooled
	Individual	1.88 ± 0.50 (8)	2.46 ± 0.70 (3)	0.54	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	1.67 ± 0.46 (9)	1.38 ± 0.83 (3)	0.77	Pooled
	No infect/no inflamm	1.98 ± 0.55 (9)	1.64 ± 0.99 (3)	0.77	Pooled
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	1.97 ± 0.54 (6)	0.78 ± 0.05 (2)	0.27	Pooled
	no infect/no inflamm	2.33 ± 0.64 (6)	0.93 ± 0.05 (2)	0.27	Pooled
	Individual	1.27 ± 0.44 (6)	2.33 ± 1.54 (2)	0.37	Pooled
Cath site	No inflamm	2.03 ± 0.73 (4)	0.64 (1)	.	.
	No infect/no inflamm	2.40 ± 0.86 (4)	0.75 (1)	.	.
	Individual	1.53 ± 0.27 (4)	0.68 (1)	.	.
Cervical star	no inflamm	2.06 ± 0.60 (9)	5.67 ± 2.35 (2)	0.049	Pooled
	No infect/no inflamm	2.44 ± 0.71 (9)	6.72 ± 2.79 (2)	0.049	Pooled
	individual	1.80 ± 0.45 (9)	3.11 ± 0.44 (2)	0.23	Pooled

Table 4.13 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for transforming growth factor-β (TGF-β) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection or inflammation ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	no inflamm	1.58 ± 0.75 (8)	1.06 ± 0.19 (3)	0.52	Satterthwaite
	no infect/no inflamm	1.54 ± 0.73 (8)	1.03 ± 0.19 (3)	0.52	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	4.03 ± 1.28 (5)	2.52 ± 1.10 (3)	0.45	Pooled
	no infect/no inflamm	3.91 ± 1.24 (5)	2.44 ± 1.07 (3)	0.45	Pooled
	Individual	5.81 ± 1.54 (5)	1.97 ± 1.19 (2)	0.21	Pooled
Cath site	No inflamm	0.82 ± 0.31 (4)	2.93 (1)	.	.
	No infect/no inflamm	0.80 ± 0.31 (4)	2.84 (1)	.	.
	Individual	1.59 ± 1.04 (4)	2.04 (1)	.	.
Cervical star	No inflamm	2.91 ± 0.73 (8)	2.59 ± 0.76 (4)	0.80	Pooled
	No infect/no inflamm	2.82 ± 0.71 (8)	2.52 ± 0.74 (4)	0.80	Pooled
	Individual	2.51 ± 0.49 (8)	1.93 ± 0.47 (3)	0.53	Pooled
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	1.58 ± 0.75 (8)	1.06 ± 0.19 (3)	0.52	Satterthwaite
	No infect/no inflamm	1.54 ± 0.73 (8)	1.03 ± 0.19 (3)	0.52	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	3.74 ± 1.09 (6)	2.64 ± 1.89 (2)	0.63	Pooled
	no infect/no inflamm	3.63 ± 1.06 (6)	2.57 ± 1.83 (2)	0.63	Pooled
	Individual	5.81 ± 1.54 (5)	1.97 ± 1.19 (2)	0.21	Pooled
Cath site	No inflamm	0.82 ± 0.31 (4)	2.93 (1)	.	.
	No infect/no inflamm	0.80 ± 0.31 (4)	2.84 (1)	.	.
	Individual	1.59 ± 1.04 (4)	2.04 (1)	.	.
Cervical star	no inflamm	3.00 ± 0.65 (9)	2.20 ± 0.92 (3)	0.54	Pooled
	No infect/no inflamm	2.91 ± 0.64 (9)	2.14 ± 0.89 (3)	0.54	Pooled
	individual	2.51 ± 0.49 (8)	1.93 ± 0.47 (3)	0.53	Pooled

Table 4.14 Mean relative quantitative expression, standard error of the mean, and the probabilities that means are different for tumor necrosis factor-α (TNF-α) with infection or type of delivery at different sites of the chorioallantoic membrane in a model of infective preterm delivery in the mare (n=13). Calibrator was either a pool of normal sites from all mares ("no inflamm"), a pool of normal sites from mares without infection or inflammation ("no infect/no inflamm"), or that individual's normal site ("individual").

Infection					
Site	Calibrator	Infected (mean ± SEM) (n)	Uninfected (mean ± SEM) (n)	Pr > |t|	Method
Normal	no inflamm	2.30 ± 1.10 (8)	0.98 ± 0.34 (4)	0.29	Satterthwaite
	no infect/no inflamm	2.64 ± 1.27 (8)	1.13 ± 0.39 (4)	0.29	Satterthwaite
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	36.70 ± 19.37 (5)	0.78± 0.37 (3)	0.14	Satterthwaite
	no infect/no inflamm	42.19 ± 22.28 (5)	0.90 ± 0.43 (3)	0.14	Satterthwaite
	Individual	33.17 ± 21.76 (5)	1.12 ± 0.422(3)	0.21	Satterthwaite
Cath site	No inflamm	2.64 ± 0.94 (4)	0.33 (1)	.	.
	No infect/no inflamm	3.03 ± 1.08 (4)	0.38 (1)	.	.
	Individual	5.22 ± 2.10 (4)	0.49 (1)	.	.
Cervical star	No inflamm	20.87 ± 14.59 (8)	1.83 ± 0.452 (4)	0.23	Satterthwaite
	No infect/no inflamm	23.99 ± 16.78 (8)	2.11 ± 0.52 (4)	0.23	Satterthwaite
	Individual	6.71 ± 4.85 (8)	2.65 ± 1.10 (4)	0.44	Satterthwaite
Type of Delivery					
Site	Calibrator	Abortion (mean ± SEM) (n)	Induction (mean ± SEM) (n)	Pr > |t|	Method
Normal	No inflamm	2.13 ± 0.99 (9)	1.05 ± 0.47 (3)	0.56	Pooled
	No infect/no inflamm	2.45 ± 1.14 (9)	1.21 ± 0.54 (3)	0.56	Pooled
	Individual	1.00	1.00	.	.
Abnormal	no inflamm	30.83 ± 16.87 (6)	0.41 ± 0.074 (2)	0.13	Satterthwaite
	no infect/no inflamm	35.45 ± 19.40 (6)	0.4682 ± 0.08 (2)	0.13	Satterthwaite
	Individual	27.97 ± 18.51 (6)	0.70 ± 0.00 (2)	0.20	Satterthwaite
Cath site	No inflamm	2.64 ± 0.94 (4)	0.33 (1)	.	.
	No infect/no inflamm	3.04 ± 1.08 (4)	0.38 (1)	.	.
	Individual	5.22 ± 2.10 (4)	0.48 (1)	.	.
Cervical star	no inflamm	18.83 ± 13.03 (9)	1.60 ± 0.55 (3)	0.22	Satterthwaite
	No infect/no inflamm	21.65 ± 14.98 (9)	1.84 ± 0.63 (3)	0.22	Satterthwaite
	individual	6.33 ± 4.30 (9)	2.44 ± 1.53 (3)	0.63	Pooled

4.4 Discussion

This data describes the change in expression pattern of equine-specific cytokines in chorioallantoic tissue from mares with experimental *in utero* infection and spontaneous abortion. Experimentally-induced infection increased the expression of IL-1β, IL-18, IL-15, IFN-γ, and possibly IL-8, in a site-dependant manner. Mares spontaneously aborting also had increased expression of IL-1β, IL-18, IFN-γ, and iNOS in a site dependant manner. Expression of MCP-1 at the cervical star was reduced with spontaneous abortion. This expression pattern of pro-inflammatory cytokines in response to infection of equine placental tissue is slightly different from a previous report [237], where IL-6 and IL-8 were increased at the cervical star and IL-6 was increased at the uterine body. Two possible reasons for this difference are the housekeeping genes used, and lack of gestational age-matched tissue. The previous study [237] used GAPDH as the housekeeping gene, which has since been shown to have a high degree of variability [254,255]. For this reason, β-GUS was chosen for this study. In this study presented here, induced delivery provided gestational age-matched tissue for comparison. The expression of various enzymes and cytokines can vary greatly with gestation [48]; therefore, the appropriate controls for experimentally infected fetal membranes are those of the same gestational age. The transient presence of soluble TNF-α was detected in a limited number of fetal fluids. Although this was somewhat surprising, TNF-α may only be present for a brief period of time in response to infection of fetal fluids [228]; therefore, sampling time in relation to an inflammatory insult is crucial. The experimental design attempted to account for this by frequent sampling within the first 24 h; however, the lag phase from inoculation to inflammation of the chorioallantois was much longer than expected.

IL-1β is a pro-inflammatory cytokine belonging to the IL-1 superfamily, which includes IL-1α, IL-18, and IL-1 receptor antagonist (IL-1Ra), among others (review, [259]). It is secreted by a variety of cell types, such as monocytes, macrophages, endothelial cells, and epithelial cells to produce pro-inflammatory responses such as increased vascular permeability, increased adhesion molecules on endothelial cells, chemokine induction (IL-8), induction of IL-6, activation of T and B cells, synthesis of acute phase proteins in the liver, proliferation of platelets and fibroblasts, and fever. The production of fever is linked to its induction of COX-2 (PGHS-2) and PGE_2 synthesis [260-262]. Il-1β has been shown in a variety of species and models to play a pivotal role in infective pre-term delivery [76,223,224,263,264]. Following binding to the type 1 IL-1 receptor (IL-1R1), signaling pathways that lead to target cell effector functions involve adaptor molecules (myeloid differentiation primary response gene (88); MyD88) and IL-1R associated kinases (IRAK), and ultimately activate nuclear factor (NF)-κB through the degradation of inhibitor of NF-κB (IκB) [265].

IL-18, also known as IFN-γ inducing factor, signals through a receptor in the IL-1 receptor family that is distinct from IL-R1[266]. Following binding of IL-18 to IL-18R, signaling also involves MyD88 and IRAK; however, further signaling through NF-κB does not occur with IL-18, but rather is primarily through the p38-mitogen-activated protein kinase (MAPK) / activator protein 1 (AP-1) pathway and does not lead to COX-2 upregulation in epithelial cells [267]. IL-2, IL-12 and IL-15 are considered co-stimulants for IL-18, and are thought to be required for IL-18-induced IFN-γ production [268]. Both IL-1β and IL-18 can induce IL-1α, IL-6, and IL-8 secretion from epithelial cells; the ability of IL-18 to do so is independent of IL-1β [267]. Increased IL-18 in amniotic fluid [269,270] and cervical mucus [270] is seen with intra-amniotic infection in women. Interestingly, either IL-18 gene disruption or IL-18 neutralization

led to an increased susceptibility to fetal loss secondary to LPS-induced intrauterine inflammation [271], highlighting the complex interaction of cytokines and pre-term delivery due to inflammation.

Following stimulation with IL-12 or IL-18, IFN-γ is secreted by T_H1 cells (CD4+), CD8+ cells, natural killer (NK) cells, B cells, NKT cells and professional antigen-presenting cells to cause macrophage activation and increased expression of class I and II MHC molecules, thereby increasing antigen presentation [272]. The role of INF-γ in pre-term delivery has been infrequently described, but increased expression of INF-γ was found in the placentas of women in pre-term labor compared to normal delivery at term [273]. Increased expression of INF-γ was only observed at sites were IL-18 was increased. An increase in IL-15 (co-stimulant for IL-18) was observed in abnormal sites from infected mares where IL-18 and INF-γ were also increased.

Nitric oxide (NO) is an important mediator of inflammation [274], as well as a wide variety of reproductive processes [275]. Inducible nitric oxide synthase (iNOS) is one of three synthases that produces NO [276], and is upregulated in human cervical tissue at term [277,278], human fetal trophoblast cells at term [279], and murine placenta subsequent to LPS-induced pre-term delivery [280]. IFN-γ stimulation of macrophages causes upregulation of iNOS [281]. Similarly, increased expression of iNOS was observed in tissue from the cervical star region of the chorioallantois in spontaneously aborting mares.

Monocyte chemoattractant protein-1 (MCP-1; CCL2) is a chemokine secreted by endothelial cells and cells of the monocyte-macrophage cell linage in response to inflammatory stimuli, such as IL-1, TNF-α, and LPS. Its action is to attract monocytes, basophils, T cells, and NK cells to the site of inflammation [282,283]. Monocyte chemoattractant protein-1 was increased in amniotic fluid of women with pre-term labor with or without intra-amniotic

infection [284] and expression of MCP-1 was increased in cervical biopsies from women in term labor without infection [285]. Both MCP-1 expression and protein production were increased in a murine model of intrauterine LPS-induced pre-term delivery [286]. Increased expression of MCP-1 was observed at the cervical star in mares without infection or in those induced to deliver. The reason for this discrepancy is unknown. Collection of term-in-labor samples from mares without infection would be useful to defining the role of MCP-1 in term delivery and that complicated by *in utero* infection.

In summary, infection or spontaneous abortion is associated with increased expression of IL-1β, IL-18, IL-15, IFN-γ, and iNOS in both abnormal and normal equine placenta. Although extremely transient, there is evidence to suggest that soluble TNF-α is secreted into the allantoic fluid. These findings are in agreement with those of previously published experimental models and clinical findings from women with intra-amniotic infection and pre-term delivery [76,223-228,263,270,273,280]. The potential consequence of increased IL-1β in infected chorioallantois of the mare is the induction of COX-2 and PGE_2 production leading to labor.

CHAPTER 5

THE ROLE OF THE PROSTAGLANDINS PGE_2 AND $PGF_{2\alpha}$ IN AN EXPERIMENTAL MODEL OF INFECTIVE PRE-TERM DELIVERY IN THE MARE

5.1 Introduction

One of the proposed mechanisms of infection-induced pre-term delivery is that pro-inflammatory cytokines (IL-1, IL-6, TNF-α) from the deciduas and cortisol from the mother cause activation of the fetal hypothalamic-pituitary-adrenal axis, causing elevation of fetal cortisol [48]. Increased fetal cortisol, either at term or in response to pro-inflammatory cytokines, decreases concentrations of PGDH, an enzyme responsible for inactivation of PGE_2 and $PGF_{2\alpha}$, leading to increased PGE_2 and $PGF_{2\alpha}$ and subsequent myometrial contractions. Pro-inflammatory cytokines also increase the activity of another key enzyme, prostaglandin H_2 synthase (PGHS-2), which increases prostaglandin synthesis. The decrease in PGDH and increase in PGHS-2 lead to increased PGE_2 and $PGF_{2\alpha}$ synthesis, myometrial contractions and premature labor [227,234,235,252].

One model examining the relationship between inflammatory cytokines and prostaglandin concentration in fetal fluids during ascending placentitis in pregnant mares has been reported [237]. Placentitis was induced by intracervical inoculation of *S. zooepidemicus* in mares between 283 and 291 d of gestation. Allantoic fluid samples were obtained from an allantoic catheter or by allantocentesis from inoculated and control mares at several-day intervals until abortion or term delivery. Concentrations of PGE_2 (competitive binding RIA) in allantoic fluid rose from pre-inoculation values of 108 $\pm$ 29 pg/mL to 13,972 $\pm$ 6255 pg/mL within 48 h of delivery, while those in control mares ranged from 25 to 432 pg/mL. Concentrations of $PGF_{2\alpha}$

rose from pre-inoculation values of 295.7 ± 50 pg/mL to 3241 ± 1487 pg/mL within 48 h of delivery, while control mares ranged from 105 to 432 pg/mL. Allantoic fluid samples were obtained from control mares on a 7 to 10 d interval until spontaneous parturition, therefore a sample within 48 h of delivery was likely not available from the majority of mares within this group. Based on the increased expression of placental IL-6 and IL-8 at the cervical star and IL-6 at the uterine body, it was hypothesized that bacterial infection causes increased expression of pro-inflammatory cytokines leading to the release of PGE_2 and $PGF_{2\alpha}$ into the allantoic fluid resulting in premature labor [237].

The temporal relationship between increases in allantoic cytokines and prostaglandins is quite important. If the synthesis of $PGF_{2\alpha}$ and PGE_2 is driven by cytokines in concert with fetal cortisol, as has been suggested in a model of ascending placentitis [48], then a therapeutic regime that is unsuccessful in interrupting the signaling of cytokines and abrogating the production of prostaglandins will fail to prevent the onset of labor. We hypothesized that the concentrations of PGE_2 and $PGF_{2\alpha}$ would be increased in mares with spontaneous abortion or intrauterine infection.

5.2 Material and Methods

5.2.1 Experimental Animals, Instrumentation, and Inoculation

Thirteen adult pony mares mare of various ages were used in this study over a 2-year period (2005 to 2006). Mares were maintained on pasture and supplemented with hay and a pelleted ration during the winter to maintain good body condition. All procedures were in accordance with Louisiana State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. Allantoic catheters were placed in sedated standing animals under local anesthesia using laparoscopic visualization as previously

described (Chapter 2). Pony mares were assigned to receive one of four transcervical inoculations: low STREP (1 x 10^7 CFU *S. zooepidemicus*; n=3), high STREP (5.1 x 10^8 CFU *S. zooepidemicus*; n=1), KILLED STREP (1 x 10^7 heat-killed *S. zooepidemicus*' n=3), PBS CONTROL (1 mL sterile PBS; n=3), and SHAM CONTROL groups (n=3). Procedures were performed between 260 and 285 d of gestation. Two SHAM CONTROL mares, one PBS CONTROL mare, one KILLED STREP mare, and one low STREP mare were induced to deliver fetuses as previously described (Chapter 3), to provide gestational age-matched placental and fetal tissue, and control for the variability from inoculation to delivery. Preparation of the inocula, transcervical inoculations, patient monitoring, and sample collection were performed as previously described (Chapter 3). Briefly, 12 mL of fetal fluid was collected at 24 h intervals, and immediately placed on ice for transport to the laboratory. Samples were centrifuged at 2,000 x g for 15 min at 4ºC, and the supernatant separated into one 5-mL aliquot and four 1-mL aliquots and stored at -70ºC for future analysis.

5.2.2 Determination of PGE_2 Concentration in Fetal Fluids

The concentration of PGE_2 in fetal fluid was assessed using a commercially available competitive-binding immunoassay (Prostaglandin E_2 Parameter™ Assay, R&D Systems, Minneapolis, MN) according to manufacturer's directions. The assay was validated for use with fetal fluid by demonstrating parallelism of the serially diluted allantoic fluid curve and the kit curve (slopes 4.148 and 3.258 respectively), and linearity of serially diluted allantoic fluid (R^2 = 0.9923). The mean percent recovery was 90.3%. The sample volume per well was 2.5 µL; samples were assayed in duplicate. Plates were read at 450 and 540 nm on a microplate spectrophotometer (Synergy™ HT, Bio-Tek, Winooski, VT) with associated software (KC4, Bio-Tek, Winooski, VT). The standard curve ranged from 0 to 2500 pg/mL, and the intra- and interassay coefficients of variation were 12% and 20% µg/dL respectively.

5.2.3 Determination of $PGF_{2\alpha}$ Concentration in Fetal Fluids

The concentration of $PGF_{2\alpha}$ in fetal fluid was assessed using a commercially available competitive-binding enzyme immunoassay (Prostaglandin $F_{2\alpha}$ EIA kit, Cayman Chemical, Ann Arbor, MI) according to manufacturer's directions. The assay was validated for use with fetal fluid by demonstrating parallelism of the serially diluted allantoic fluid curve and the kit curve (slopes 3.0728 and 3.3457 respectively), and linearity of the serially diluted allantoic fluid (R^2 = 0.9973). The mean percent recovery was 90.8%. The sample volume per well was 10 µL, and samples were assayed in duplicate. Plates were read at 405 nm on a microplate spectrophotometer (Synergy™ HT, Bio-Tek, Winooski, VT) with associated software (KC4, Bio-Tek, Winooski, VT). The standard curve ranged from 3.9 to 500 pg/mL, and the intra- and inter-assay coefficients of variation were 7.3% and 21%, respectively.

5.2.4 Statistical Analysis

Because of the small samples sizes within treatment group, effect of treatment group on the concentration of PGE_2 or $PGF_{2\alpha}$ in fetal fluid was not possible. The effect of type of delivery (spontaneous abortion or induced delivery) on the difference in PGE_2 or $PGF_{2\alpha}$ concentration between the sample immediately prior to delivery (T_{END}) and that at the time of inoculation (T_0) was compared by a Student's t test, using a statistical analysis software program (SAS 9.1, Cary, NC) with significance set at $p<0.05$, including Satterthwaite's method for sample populations with unequal variances. Examination of the daily pattern of PGE_2 concentration data revealed that PGE_2 concentrations rose transiently from T_0 for the following 1 to 2 days following laparoscopy, and then declined. Consequently, a separate calculation of $T_{END} - T_{LOW}$ was made, where T_{LOW} represents the lowest daily PGE_2 concentration for that individual mare.

5.3 Results

Data from two mares were removed; one because the fluid was determined to be of peritoneal origin (PBS control mare), and one because sample viscosity interfered with assay performance (SHAM control mare). The individual fetal fluid concentrations of PGE_2 for the remaining eleven mares are shown in Table 5.1 and Figure 5.1. The difference in $T_{END} - T_0$ was not statistically significant (p=0.1021) between spontaneously aborting mares and induced-delivery mares; even after removal of mares in which a sample within 24 h of delivery was unavailable for analysis (p=0.0807; Table 5.3). The difference in $T_{END} - T_{LOW}$ for all mares was not statistically significant (p=0.0950) between spontaneously aborting mares and induced-delivery mares; however, the difference in $T_{END} - T_{LOW}$ for all spontaneously aborting mares in which a sample within 24 h of delivery was available for analysis was statistically significant from induced-delivery mares (p=0.0455; Table 5.3) The individual fetal fluid concentrations of $PGF_{2\alpha}$ for eleven mares are shown in Table 5.2 and Figure 5.2. There was a significant difference in $T_{END} - T_0$ between all spontaneously aborting mares and induced-delivery mares (p=0.0312); there was also a significant difference in $T_{END} - T_0$ between all spontaneously aborting mares and induced-delivery mares in which a sample within 24 h of delivery was available for analysis (p<0.0001; Table 5.3).

There was no significant effect of the presence of infection on the difference ($T_{END} - T_0$) in PGE_2 concentration for mares (p=0.3166); however, after removal of mares in which a sample with 24 h of delivery was not available for analysis $T_{END} - T_0$ was statistically different (p=0.0306). There was no significant effect of the presence of infection on the difference ($T_{END} - T_0$) in $PGF_{2\alpha}$ concentration for all mares (p=0.1518; Table 5.3); however, after removal of mares in which a sample with 24 h of delivery was unavailable for analysis $T_{END} - T_0$ was statistically different (p=0.0324; Table 5.3).

Table 5.1 Daily PGE_2 concentrations and differences in concentrations in fetal fluids from mares in an experimental model of ascending placentitis. The allantoic space was catheterized laparoscopically between 265-280 d gestation: SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (n=3) received 1 x 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (n=3) received 1 x 10^7 *Streptococcus zooepidemicus* transcervically; and high STREP mares (n=1) received 5.1 x 10^8 *Streptococcus zooepidemicus* transcervically.

Pony	Treatment group	Day	pg/ml	T_{END}-T_0 [1]	T_{END}-T_{LOW} [2]	Type of delivery	Infection	Day of delivery
781	PBS	0	5598.8					
781		1	4806.6					
781		3	17107.6	11508.8	12301.0	abortion	infected	4
416	KILLED	0	6561.8					
416		1	5213.2					
416		2	1752.8					
416		3	2640.2					
416		4	8123					
416		6	14204.6	7642.8	12451.8	abort	uninfected	6
762	SHAM	0	4405.8					
762		1	3767.3					
762		2	3063.1					
762		3	3658.5					
762		4	4104.7					
762		5	4156.9					
762		6	4475.8					
762		7	4639.4					
762		8	5058.2	652.4	1995.1	induct	uninfected	8
20	low STREP	0	4881.5					
20		1	3063.1					
20		2	3658.5					
20		3	4104.7					
20		4	4156.9					
20		5	4475.8	-405.7	1412.7	abort	infected	11
12	low STREP	1	1335.7					
12		3	2112.8					
12		4	1958.1					
12		6	1295.9					
12		7	1475.2					
12		8	714.32					
12		9	986.8					
12		11	1230.6					
12		12	2042.1	706.4	1327.8	abort	infected	18

Table 5.1 continued

Pony	Treatment group	Day	pg/ml	TEND-T0[1]	TEND-TLOW[2]	Type of delivery	Infection	Day of delivery
682	KILLED	0	1327.6					
682		1	474.9					
682		2	843.3					
682		3	1165.9					
682		4	3383.7					
682		5	815.9					
682		6	1656.8					
682		7	1624.8					
682		8	23314.8	21987.2	22471.5	abort	infected	11
22	PBS	0	1913.1					
22		1	2669.9					
22		2	2085.9					
22		3	2345.3					
22		4	1208.3					
22		5	1105.3					
22		6	1539.2					
22		8	2318.8	405.7	1110.5	induct	uninfected	8
12SG	KILLED	0	2207.0					
12SG		1	1980.6					
12SG		2	2145.2					
12SG		3	2674.7					
12SG		4	1372.7					
12SG		6	1155.8					
12SG		7	875.2					
12SG		8	737.9	-1469.1	-137.4	induct	uninfected	8
24	low STREP	0	2492.1					
24		1	3001.8					
24		2	3096.6					
24		3	3533.7					
24		4	2080.7					
24		5	1734.3					
24		6	1169.5					
24		7	1259.1					
24		8	1871.4	-620.7	612.3	induct	uninfected	8

Table 5.1 continued

Pony	Treatment group	Day	pg/ml	TEND-T0[1]	TEND-TLOW[2]	Type of delivery	Infection	Day of delivery
23	high STREP	0	1635.8					
23		1	1542.7					
23		2	1639.9					
23		3	573.3					
23		4	90.9					
23		5	22.8					
23		6	619.8	-1016.0	529.0	abort	infected	11
25	SHAM	0	3676.52					
25		1	563.5					
25		2	0					
25		3	281.5					
25		4	1853.7					
25		5	1680.3	-1996.2	1398.8	induct	uninfected	19

[1] T_{END}-T_0 is the difference in concentration from the last sample obtained and T0.

[2] T_{END}-T_{LOW} is the difference in concentration from the last sample obtained and the lowest value from that individual mare.

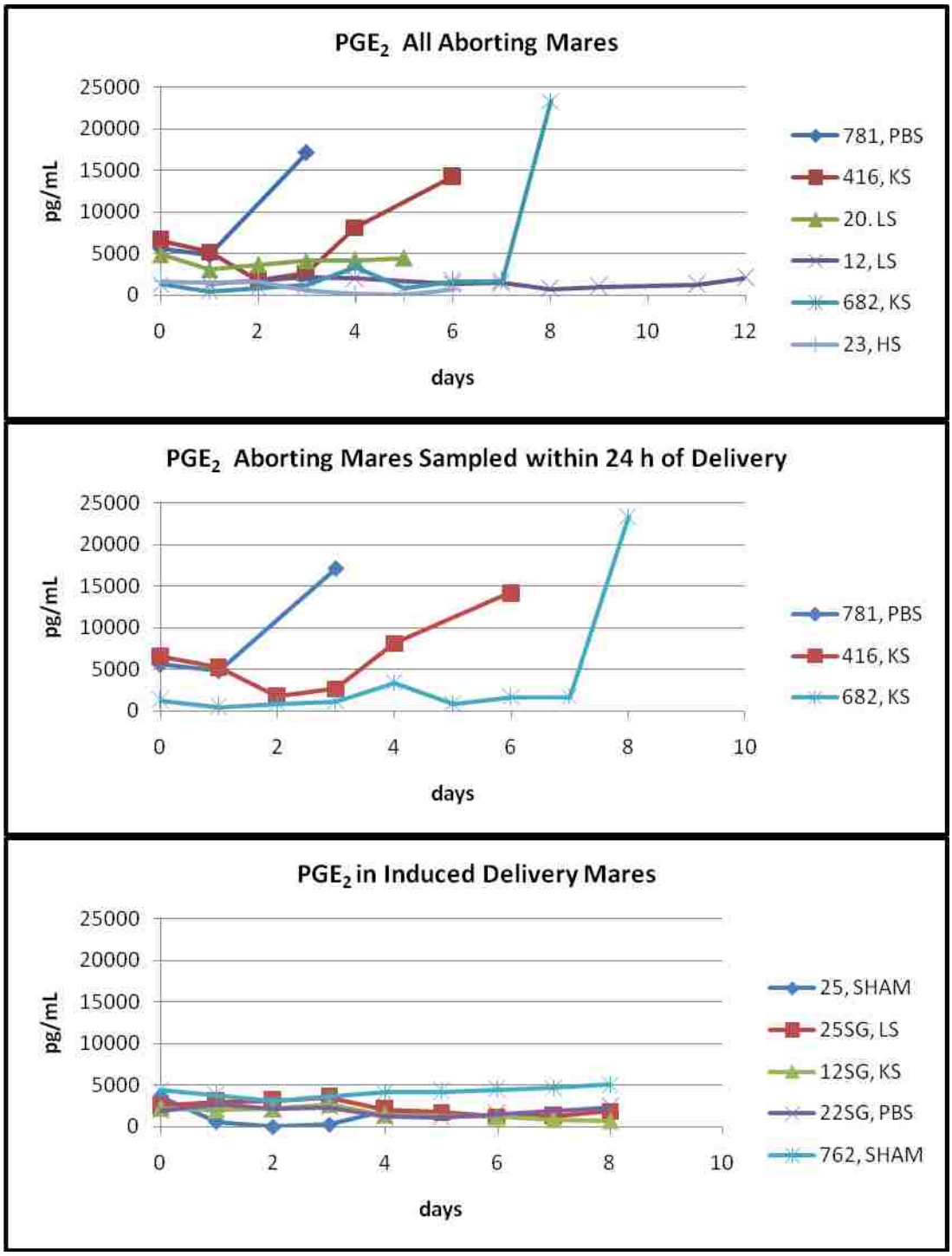

Figure 5.1 Daily PGE_2 concentrations in fetal fluids from mares in an experimental model of ascending placentitis with type of delivery. The allantoic space was catheterized laparoscopically between 265-280 d gestation: SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (KS, n=3) received 1 x 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (LS, n=3) received 1 x 10^7 *Streptococcus zooepidemicus* transcervically; and high STREP mares (HS, n=1) received 5.1 x 10^8 *Streptococcus zooepidemicus* transcervically.

Table 5.2 Daily $PGF_{2\alpha}$ concentrations and differences in concentrations in fetal fluids from mares in an experimental model of ascending placentitis. The allantoic space was catheterized laparoscopically between 265-280 d gestation: SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (n=3) received 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (n=3) received 10^7 *Streptococcus zooepidemicus* transcervically; and high STREP mares (n=1) received 5.1 x 10^8 *Streptococcus zooepidemicus* transcervically.

Pony	Treatment group	Day	pg/mL	T_{END}-T_0[1]	Type of delivery	Infection	Day of delivery
781	PBS	0	122.0				
781		2	106.9				
781		2	176.4				
781		3	2624.0	2502.0	abort	infected	4
416	KILLED	0	518.3				
416		1	964.2				
416		2	207.6				
416		3	759.0				
416		4	2624.0				
416		5	2624.0	2105.8	abort	unifected	6
762	SHAM	0	149.0				
762		1	152.0				
762		2	161.3				
762		3	185.2				
762		4	161.7				
762		5	165.6				
762		6	166.1				
762		7	185.1				
762		8	253.1	104.1	induct	uninfected	8
20	low STREP	0	370.2				
20		1	268.2				
20		2	2624.0				
20		3	990.4				
20		4	2624.0				
20		5	2624.0	2253.8	abort	infected	11
12	low STREP	0	102.1				
12		3	75.7				
12		4	70.1				
12		6	73.5				
12		7	77.2				
12		8	69.7				
12		9	68.8				
12		11	74.3				
12		12	82.2	-20.0	abort	infected	18

Table 5.2 continued

Pony	Treatment group	Day	pg/mL	T_{END}-T_0 [1]	Type of delivery	Infection	Day of delivery
682	KILLED	0	100.2				
682		24	96.9				
682		48	64.5				
682		72	48.9				
682		0	61.0				
682		1	62.0				
682		2	65.6				
682		3	76.6				
682		4	68.4				
682		5	65.6				
682		6	68.0				
682		7	67.8				
682		8	2624.0	2563.1	abort	infected	11
22	PBS	0	244.9				
22		1	194.6				
22		2	168.2				
22		3	143.9				
22		4	136.6				
22		5	135.9				
22		6	116.2				
22		8	113.3	-131.6	induct	uninfected	8
12SG	KILLED	0	322.4				
12SG		1	186.0				
12SG		2	93.9				
12SG		3	72.0				
12SG		4	64.8				
12SG		6	49.8				
12SG		7	68.6				
12SG		8	59.3	-263.1	induct	uninfected	8
24	low STREP	0	254.2				
24		1	125.3				
24		2	72.4				
24		3	68.0				
24		4	65.2				
24		5	63.7				
24		6	59.2				
24		7	114.5				
24		8	65.5	-188.7	induct	uninfected	8

Table 5.2 continued

Pony	Treatment group	Day	pg/mL	T_{END}-T_0[1]	Type of delivery	Infection	Day of delivery
23	high STREP	0	484.1				
23		1	107.4				
23		2	59.4				
23		3	50.8				
23		4	47.6				
23		5	42.4				
23		6	117.8	-366.3	abort	infected	11
25	SHAM	0	132.0				
25		1	127.5				
25		2	154.3				
25		3	111.1				
25		4	100.1				
25		5	99.7	-32.3	induct	uninfected	19

[1] T_{END}-T_0 is the difference in concentration from the last sample obtained and T0.

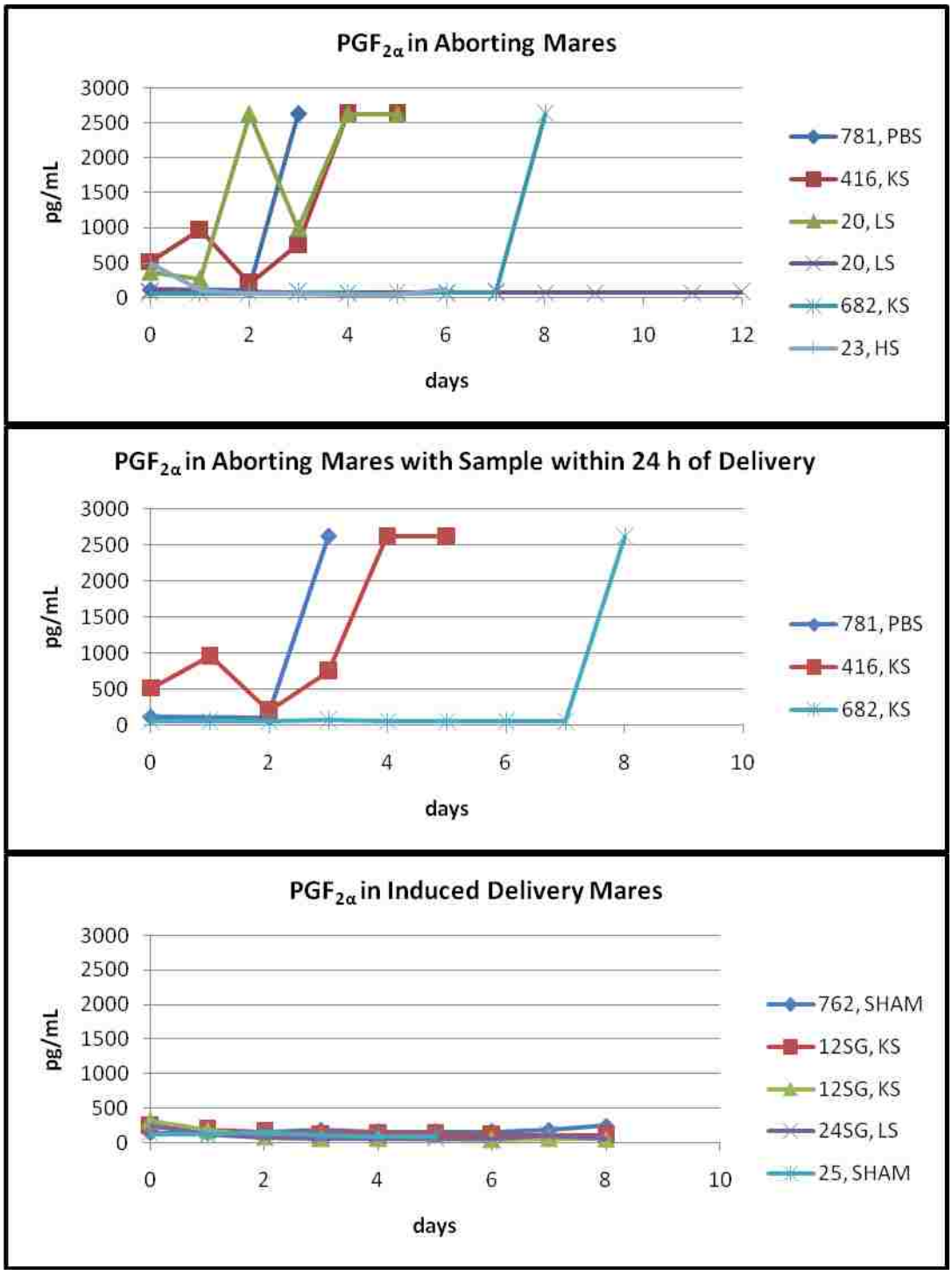

Figure 5.1 Daily $PGF_{2\alpha}$ concentrations in fetal fluids from mares in an experimental model of ascending placentitis with type of delivery. The allantoic space was catheterized laparoscopically between 265-280 d gestation: SHAM mares (n=3) received no transcervical procedure; PBS Control mares (n=3) received 1 mL sterile PBS transcervically; KILLED STREP (KS, n=3) received 1 x 10^7 heat-killed *Streptococcus zooepidemicus* transcervically; low STREP mares (LS, n=3) received 1 x 10^7 *Streptococcus zooepidemicus* transcervically; and high STREP mares (HS, n=1) received 5.1 x 10^8 *Streptococcus zooepidemicus* transcervically.

Table 5.3 Analysis of the difference in PGE_2 or $PGF_{2\alpha}$ concentration between pre-inoculation and prior to delivery in a model of placentitis (n=11).

Parameter	Category	Mean (pg/mL)	SEM (pg/mL)	P value	Method[1]
PGE_2 T_{END}-T_0	Abortion	6737.3	3668.1	0.10	Satterthwaite
	Induced	-605.6	514.1		
PGE_2 T_{END}-T_0^2	Abortion	13713	4285	0.081	Satterthwaite
	Induced	-257.9	488.8		
PGE_2 T_{END}-T_{LOW}	Abortion	8415.6	3607.6	0.095	Satterthwaite
	Induced	995.9	360.9		
PGE_2 T_{END}-T_{LOW}^2	Abortion	15741.0	3365.3	0.045	Satterthwaite
	Induced	895.1	447.4		

Parameter	Category	Mean (pg/mL)	SEM (pg/mL)	P value	Method[1]
$PGF_{2\alpha}$ T_{END}-T_0	Abortion	1506.4	543.5	0.031	Satterthwaite
	Induced	-102.3	63.9		
$PGF_{2\alpha}$ T_{END}-T_0^2	Abortion	2390.3	143.3	<0.0001	Pooled
	Induced	-119.8	79.4		

Parameter	Category	Mean (pg/mL)	SEM (pg/mL)	P value	Method[1]
PGE_2 T_{END}-T_{LOW}	infected	7608.4	4306.3	0.3166	Pooled
	uninfected	2905.2	1931.9		
PGE_2 T_{END}-T_{LOW}^2	infected	17386.0	5085.3	0.0306	Pooled
	uninfected	3206.5	2337.2		

Parameter	Category	Mean (pg/mL)	SEM (pg/mL)	P value	Method[1]
$PGF_{2\alpha}$ T_{END}-T_0	infected	1386.5	649.3	0.1518	Pooled
	uninfected	-102.3	63.9		
$PGF_{2\alpha}$ T_{END}-T_0^2	infected	2532.5	30.5	0.0324	Pooled
	uninfected	325.3	449.3		

[1]Where variances were found to be unequal (*F′* statistic; folded), Satterthwaite's method for calculation of degrees of freedom associated with the approximate *t* is used, and the approximate *t′* statistic is computed Where variances were found to not be significantly different, the t statistic uses a pooled variance.

[2]Mares without a sample within 24 h of delivery removed from analysis.

5.4 Discussion

Significant increases in the concentrations of PGE_2 and $PGF_{2\alpha}$ in fetal fluids were observed when a sample was obtained within 24 h of delivery in mares with spontaneous abortion or intrauterine infection in an experimental model of ascending placentitis. Previously published mean concentrations of PGE_2 and $PGF_{2\alpha}$ in allantoic fluid from chronically catheterized mares during a similar stage of gestation were 3000 pg/mL and 2000 ng/mL, respectively [287], which are comparable to the values observed in this study. The daily pattern from a single mare from catheterization to abortion (17 d post-operatively) was also similar to the daily pattern of most mares from our study. A mild increase in both PGE_2 and $PGF_{2\alpha}$ concentration was observed for the first several days following surgery (an increase of 1500 pg/mL and 1000 pg/mL for PGE_2 and $PGF_{2\alpha,}$ respectively), followed by either an eight-day plateau ($PGF_{2\alpha}$) or a decline (PGE_2), followed by a large increase (10,000 pg/mL and 5,000 pg/mL for PGE_2 and $PGF_{2\alpha}$, respectively) just prior to abortion [287]. Similarly a more consistent increase in PGE_2 concentration than in $PGF_{2\alpha}$ concentration was observed following surgery; therefore, the calculated rise in PGE_2 from the peak prior to delivery in relationship to the lowest value for that individual mare ($T_{END} - T_{LOW}$) was used for statistical analysis. The absolute values of PGE_2 concentration were lower than those in a previously published model of placentitis [237], although in both study populations, variances were high and the instrumentation of the sample populations were different. All mares in the study described herein were instrumented with allantoic catheters, while in the previous study [237] some mares were catheterized and some were sampled by repeated allantocentesis. Despite these differences, both studies documented significant increases in concentrations of PGE_2 and $PGF_{2\alpha}$ in fetal fluids in response to an ascending placentitis.

Prostaglandins are produced from both fetal membranes and from maternal uterine tissues. PGHS-1 and PGHS-2 expression was localized to human amniotic epithelium and mesoderm [66,67,288] , while the chorion laeve was found to either express both PGHS-1 and PGHS-2 [67,68], PGHS-1 [66], or PGSH2 [288]. In most studies, the expression of PGHS-2 has been shown to increase with labor [66,69,288] and the activity of PGHS-2 (measured as the ability of the microsomal fraction of chorionic tissue to convert arachidonate to PGE_2) was significantly greater in chorion from term spontaneous labor patients compared to chorion from term prior to labor patients [69]. Similar to human fetal membranes, PGHS-1 and PGHS-2 mRNA were expressed in cotyledonary tissues from ewes at 40 d to term; and while no change in the relative abundance of PGHS-1 was observed during this period, the relative abundance of PGHS-2 mRNA increased sevenfold between 40 to 85 d and 140 to 145 d. No significant change in PGHS-2 was observed between ewes not in labor at 140 to 145 d and ewes in labor [70]. No change in PGE_2 or $PGF_{2\alpha}$ receptor subtype with respect to onset of spontaneous labor has been identified [71].With experimental Group B streptococcal infection in rhesus monkeys, extravillous trophoblast cell necrosis was associated with loss of immunoreactive PGDH staining and relative sparing of ir-PGHS-2 in amniotic epithelial cells, thereby leading to a net increased production of prostaglandins. Comparable studies have not been carried out with tissues from equine pregnancies.

The source of PGE_2 and $PGF_{2\alpha}$ in fetal fluids, although not specifically defined in the literature, is presumably through a paracrine route (i.e., secretion from fetal membranes into the allantoic or amniotic compartment), with potential contribution through endocrine pathways. The major consequence of fetal membrane and intrauterine production of PGE_2 and $PGF_{2\alpha}$ is myometrial contraction, by either paracrine or endocrine signaling. Secretion of PGE_2 in the fetal fluids could have a secondary end result of paracrine action on fetal membranes with further

upregulation and release of interleukin-8 (IL-8) contributing to cervical relaxation (ripening) [289].

The effect of cytokines on prostaglandin production is well documented in a variety of tissues. Interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) have been shown to increase prostaglandin production by cultured human amnion cells [92,93], human chorion laeve cells [290] and myometrial cells [94], through increased expression of PGHS-2 [291], and while the administration of an IL-1β receptor antagonist prevented IL-β-induced labor in mice [95] it was unable to prevent delivery in endotoxin-induced pre-term labor [96]. The observed response to direct stimulation with IL-6 has been less consistent, varying from causing an increase in $cPLA_2$ expression by myometrial cells [75], to having no significant effect on PGE_2 production by human myocytes [94].

Several studies would advance the knowledge of signaling pathways involved in the pro-inflammatory cytokine-induced production of PGE_2 and $PGF_{2\alpha}$ in equine uterine and placental tissues. Measurement of a simultaneous increase in the expression of PGHS-2 (COX-2), along with IL-β, and IL-18 (as was seen in Chapter 4), would confirm the relationship between upregulation of pro-inflammatory cytokines and PGHS-2 in response to infection. Immunohistochemical staining for PGHS-2 in both the chorioallantois and endometrium of mares following experimental induction of ascending placentitis would provide further corroborating evidence. Both techniques have been previously validated for equine tissues [292,293]. Additional recommended studies would include *in vitro* production of PGE_2 and $PGF_{2\alpha}$ from chorioallantoic and endometrial cell lines in response to IL-1β and IL-6.

One area of research in therapeutic regimes for equine placentitis that has not been adequately addressed is the use of selective, rather than non-selective, cyclooxygenase inhibitors

to reduce the production of PGE_2 and $PGF_{2\alpha}$. Conventional therapy currently involves non-selective agents such as flunixin meglumine or phenylbutazone [244,294]. Results from a sheep model of preterm delivery documenting the effectiveness of meloxicam, a selective PGHS-2 (COX-2) inhibitor, decreased PGHS-2 expression in endometrium, myometrium, and amnion [295], and attenuated amplitude and frequency of contractions, increased contraction duration, and delayed labor, without altering either fetal renal or cotyledonary blood flow [296]. These findings warrant investigation of its use as a tocolytic in the mare.

In summary, increased concentrations of PGE_2 and $PGF_{2\alpha}$ in fetal fluids were observed within 24 h of spontaneous abortion or *in utero* infection in an experimental model of infective pre-term delivery in the mare. Further research is needed to define more the localization and regulation of PGHS-2 in equine placental and maternal tissues, and to assess the effectiveness of selective PGHS-2 inhibitors as a tocolytic.

CHAPTER 6

EVIDENCE OF FETAL HYPOTHALAMIC-PITUITARY-ADRENAL AXIS ACTIVATION IN AN EXPERIMENTAL MODEL OF INFECTIVE PRE-TERM DELIVERY IN THE MARE

6.1 Introduction

In most species, activation of the hypothalamic-pituitary-adrenal axis (HPAA) is a key event in the control of labor at term. In sheep the increase in fetal cortisol concentration during the last 2 weeks of gestation causes a decline in the placental production of progesterone and an increase in estrogen production [137,138]. Parturition can either be delayed by hypophysectomy [139] or adrenalectomy [140], or accelerated by fetal ACTH administration [141]. The fetal pig also has a gradual increase in cortisol concentrations during the last 2 weeks, but the final surge is of a shorter duration than that of the fetal lamb [142,143]. In contrast, cortisol concentration in the fetal horse only rises just before birth [142,144]. Activation of the HPAA in primates is characterized by increased fetal cortisol, dehydroepiandrosterone sulfate, estrone, and progesterone concentrations only a few days prior to term vaginal delivery [116]. Similar to sheep, administration of glucocorticoids promotes fetal maturation, and is commonly given to affect pulmonary surfactant secretion [145,146] . The HPAA activation has also been shown to be a key event in preterm delivery due to intrauterine infection in women. Patients with intrauterine infection and preterm delivery, as defined by recovery of a microbial pathogen from amniotic fluid or an amniotic IL-6 concentration > 2 ng/ml, had significantly higher amniotic fluid concentrations of cortisol (5.28 $\pm$ 1.0 µg/dL) and dehydroepiandrosterone (539 $\pm$ 79 pg/mL) than patients with preterm delivery without intrauterine infection (1.61 $\pm$ 1.05 µg/dL and 273 $\pm$ 82 pg/mL respectively) [147].

Increased concentrations of equine fetal plasma cortisol were seen during the final 4 to 5 [148] or the final 9 d [144] of gestation. Cortisol concentrations rose from 3.1 $\pm$ 1.0 to 13.4 $\pm$ 3.7 ng/mL, while significant increases in ACTH concentrations were not seen until the last 2 d of gestation (159 $\pm$ 21 to 246 $\pm$ 42 pg/mL) [144]. Fetuses less than 295 d of gestation failed to release cortisol in response to administration of intravenous $ACTH_{1-24}$, those greater than 304 d showed small but significant releases in cortisol, while those greater than 313 d of gestation showed the greatest release of cortisol, demonstrating that the equine fetal adrenal responsiveness to exogenous ACTH does not develop until extremely late in gestation [148]. Based on the observed adrenal response in fetuses greater than 304 d, the investigators then compared the effects of intramuscular intrafetal $ACTH_{1-24}$ (n=16) administration daily for three days with saline administration (n=7), and whether induction of parturition 2 to 4 days after $ACTH_{1-24}$ administration was associated with a positive neonatal outcome (n=4) compared to spontaneous delivery. Five mares aborted immediately following the last $ACTH_{1-24}$ injection. Administration of $ACTH_{1-24}$ did shorten gestation significantly in mares delivering spontaneously (312 to 314 vs. 327 to 333) and postnatal adaptive and endocrine patterns were normal; however, in pregnancies induced to deliver (306 to 308 days) one fetus died, and five of the remaining six foals required supportive care, and had lower plasma cortisol and increased, progestagen concentrations which failed to decline within the first 24 h of life [149] . Given the number of abortions and postnatal complications associated with intrafetal injections, the effect of maternally administered depot $ACTH_{1-24}$ (1 mg vs. 4 to 5 mg) on days 300, 301, and 302 on fetal maturation was examined. Earlier parturition was significantly correlated with high dose ACTH given to mares mated late in the season (after July 1), compared to those mated early, although the strength of the correlation, as measured by the coefficient of determination, was not strong (r^2=0.5). Maternal plasma cortisol and progestagen concentrations were increased

following $ACTH_{1-24}$ administration, and this effect was greater and of a longer duration with high dose $ACTH_{1-24}$ [297]. Investigations on maternally-administered dexamethazone on delivery and precocious fetal development have yielded conflicting results. Administration of 100 mg/d, for three days beginning at either 321 or 315 d of gestation has been shown to induce parturition with no dystocia and good eventual neonatal outcome [298,299] while administration beginning on 331-347 d resulted in dystocia and fetal death in two of three mares [300]. Betamethazone administration on 305, 306 , and 307 d advanced spontaneous delivery by 7 to 14 d, but did not improve outcome if delivery was induced on day 320 [301]. One study evaluating the effect of maternally administered dexamethazone in combination with antibiotics to mares with experimentally induced streptococcal placentitis, failed to demonstrate that dexamethazone improved neonatal viability or postnatal outcome [302].

It is apparent from these studies that not only does maturation of the equine HPAA occur only at the end of gestation in normal pregnancies, but that manipulations in an attempt to accelerate fetal maturation precociously are frequently complicated with a negative neonatal outcome. Effective interventional strategies to advance fetal maturation would be of great benefit in many instances of both reproductive and non-reproductive high risk pregnancy. Changes in fetal plasma cortisol concentration in response to *in utero* infection have not previously been reported, nor have concentrations of cortisol in fetal fluid. The objective of this study was to measure cortisol concentrations in fetal fluid from mares in an experimental model of infective pre-term delivery. We hypothesized that cortisol concentrations in equine fetal fluids would increase subsequent to infection of the chorioallantois, fetal fluids, or the fetus.

6.2 Material and Methods

6.2.1 Experimental Animals, Instrumentation, and Inoculation

Thirteen adult pony mares of various ages were used in this study over a 2 year period (2005-2006). Mares were maintained on pasture and supplemented with hay and a pelleted ration during the winter to maintain good body condition. All procedures were in accordance with Louisiana State University's Institutional Animal Care and Use Committee's guidelines for the humane treatment of research animals. Allantoic catheters were placed in sedated standing animals under local anesthesia using laparoscopic visualization as previously described (Chapter 2). Pony mares were assigned to receive one of five transcervical inoculations: low STREP (1 x 10^7 CFU *S. zooepidemicus*; n=3), high STREP (5.1 x 10^8 CFU *S. zooepidemicus*; n=1), KILLED STREP (1 x 10^7 heat-killed *S. zooepidemicus*' n=3), PBS CONTROL (1 mL sterile PBS; n=3), and SHAM CONTROL groups (n=3). Procedures were performed between 260 and 285 d of gestation. Two SHAM CONTROL mares, one PBS CONTROL mare, one KILLED STREP mare and one low STREP mare were induced to deliver fetuses as previously described (Chapter 3), to provide gestational age-matched placental and fetal tissue, and control for the variability from inoculation to delivery. Preparation of the inocula, transcervical inoculations, patient monitoring, and sample collection were performed as previously described (Chapter 3). Briefly, 12 mL of fetal fluid was collected at twenty-four hour intervals, and immediately placed on ice for transport to the laboratory. Samples were centrifuged at 2,000 x g for 15 min at 4ºC, and the supernatant separated into one 5-mL aliquot and four 1-mL aliquots and stored at -70ºC for future analysis.

6.2.2 Determination of Cortisol Concentration in Fetal Fluid

The concentration of cortisol in fetal fluid was assessed using commercially available radioimmunoassay reagents (Cortisol RIA, Diagnostic Systems Laboratories, Inc., Webster, TX)

and methods as previously described [303], and validated for equine samples [304]. Samples were assayed in duplicates. The intra- and interassay coefficients of variation and assay sensitivity were 6%, 8%, and 0.11 µg/dL, respectively.

6.2.3 Statistical Analysis

Because of the small sample sizes within treatment group, effect of treatment group on the cortisol concentration in fetal fluid was not possible. The presence of infection (positive allantoic fluid culture), type of fetal fluid (allantoic or admixture), type of delivery (spontaneous abortion or induced delivery), and presence of histologic inflammation on cortisol concentrations was compared by a Student's t test, using a statistical analysis software program (SAS 9.1, Cary, NC) with significance set at $p<0.05$, including Satterthwaite's method for sample populations with unequal variances. Cortisol to creatinine ratios (CCR) were calculated and also compared by the Student's t test to assess the potential influence of the relative dilutional effect of amniotic fluid on cortisol concentrations.

6.3 Results

Two mares were excluded from the analysis – one PBS CONTROL mare due to peracute abortion (less than 96 h following laparoscopy), and one PBS CONTROL mare because the catheter pulled out of the uterus within 24 h following laparoscopy. The individual daily fetal fluid cortisol concentrations, creatinine concentrations, and CCRs for the eleven mares are found in Table 6.1. Significant increases in cortisol concentration were found in fetal fluids from three mares (one KILLED STREP, one SHAM CONTROL, and one low STREP mare), and were observed prior to spontaneous abortion. Because there were different number of days from laparoscopy to delivery for each mare, the difference between the final (T_{END}) and initial (T_0) cortisol concentration and CCR ($Cort_{diff}$, CCR_{diff}; Table 6.2) were used for statistical analysis. A summary of the means, standard error of the mean, and probabilities that $Cort_{diff}$ and CCR_{diff} are

Table 6.1 Individual daily fetal fluid cortisol and creatine concentration and cortisol to creatinine ratios (CCR) in an experimental model of placentitis.

ID	Group	day	cortisol µg/dL	cortisol T_{end}-T_0	Creat	CCR	CCR T_{end}-T_0	infection	Type of fetal fluid	Type of delivery	Inflammation
25	SHAM	0	0.36		39.8	0.0090		inf	admix	induced	inflamm
25		1	0.29		27	0.0107		inf	admix	induced	inflamm
25		2	0.29		27	0.0107		inf	admix	induced	inflamm
25		3	0.18		25	0.0072		inf	admix	induced	inflamm
25		4	0.18		25	0.0072		inf	admix	induced	inflamm
25		5	0.11		23	0.0048		inf	admix	induced	inflamm
25		8	0.74	0.38	20.8	0.0356	0.02653	inf	admix	induced	inflamm
847	SHAM	0	0.4		97.9	0.0041		inf	allantoic	abortion	inflamm
847		1	0.36		101.8	0.0035		inf	allantoic	abortion	inflamm
847		2	0.35		87	0.0040		inf	allantoic	abortion	inflamm
847		3	0.38		83	0.0046		inf	allantoic	abortion	inflamm
847		4	0.34		56	0.0061		inf	allantoic	abortion	inflamm
847		5	0.34		90	0.0038		inf	allantoic	abortion	inflamm
847		8	1.7	1.3	141	0.0121	0.00797	inf	allantoic	abortion	inflamm
762	SHAM	0	0.32		70	0.0046		uninf	allantoic	induced	none
762		1	0.37		74	0.0050		uninf	allantoic	induced	none
762		2	0.35		79	0.0044		uninf	allantoic	induced	none
762		3	0.12		79	0.0015		uninf	allantoic	induced	none
762		4	0.33		80	0.0041		uninf	allantoic	induced	none
762		5	0.36		83	0.0043		uninf	allantoic	induced	none
762		6	0.33		86	0.0038		uninf	allantoic	induced	none
762		7	0.32		87	0.0037		uninf	allantoic	induced	none
762		8	0.38	0.06	91	0.0042	-0.00040	uninf	allantoic	induced	none

Table 6.1 continued

ID	Group	day	cortisol µg/dL	cortisol Tend-T0	Creat	CCR	CCR Tend-T0	infection	Type of fetal fluid	Type of delivery	Inflammation
22sg	PBS	0	0.48		50.8	0.0094		uninf	allantoic	induced	none
22sg		1	0.32		49	0.0065		uninf	allantoic	induced	none
22sg		2	0.39		49.2	0.0079		uninf	allantoic	induced	none
22sg		3	0.44		50.3	0.0087		uninf	allantoic	induced	none
22sg		4	0.46		53	0.0087		uninf	allantoic	induced	none
22sg		5	0.4		51	0.0078		uninf	allantoic	induced	none
22sg		6	0.4		53	0.0075		uninf	allantoic	induced	none
22sg		7	0.27		55	0.0049		uninf	allantoic	induced	none
22sg		8	0.35	-0.13	54	0.0065	-0.00297	uninf	allantoic	induced	none
682	KILLED	1	0.29		84	0.0035		inf	allantoic	abortion	inflamm
682		2	0.31		77	0.0040		inf	allantoic	abortion	inflamm
682		3	0.32		88	0.0036		inf	allantoic	abortion	inflamm
682		4	0.33			#DIV/0!		inf	allantoic	abortion	inflamm
682		5	0.28		86	0.0033		inf	allantoic	abortion	inflamm
682		6	0.34		89	0.0038		inf	allantoic	abortion	inflamm
682		7	0.33		101	0.0033		inf	allantoic	abortion	inflamm
682		8	0.28	-0.04	82	0.0034	-0.00049	inf	allantoic	abortion	inflamm
416	KILLED	0	0.46		32	0.0144		uninf	admix	abortion	inflamm
416		1	0.71		24	0.0296		uninf	admix	abortion	inflamm
416		2	0.65		20	0.0325		uninf	admix	abortion	inflamm
416		3	0.67		18	0.0372		uninf	admix	abortion	inflamm
416		4	2.08		16	0.1300		uninf	admix	abortion	inflamm
416		5	3.12	2.66	16	0.1950	0.18063	uninf	admix	abortion	inflamm
12 SG	KILLED	0	0.5		37	0.0135		uninf	admix	induced	none
12 SG		1	0.3		28	0.0107		uninf	admix	induced	none
12 SG		2	0.36		27	0.0133		uninf	admix	induced	none
12 SG		3	0.28		29	0.0097		uninf	admix	induced	none

Table 6.1 continued

ID	Group	day	cortisol µg/dL	cortisol Tend-T0	Creat	CCR	CCR Tend-T0	infection	Type of fetal fluid	Type of delivery	Inflammation
12 SG	KILLED	4	0.26		31	0.0084		uninf	admix	induced	none
12 SG		5	0.18		31	0.0058		uninf	admix	induced	none
12 SG		6	0.31		30	0.0103		uninf	admix	induced	none
12 SG		7	0.21		26	0.0081		uninf	admix	induced	none
12 SG		8	0.27	-0.23	25	0.0108	-0.0027	uninf	admix	induced	none
12	low STREP	0	0.38		57	0.0067		inf	allantoic	abortion	inflamm
12		3	0.38		48	0.0079		inf	allantoic	abortion	inflamm
12		4	0.33		51	0.0065		inf	allantoic	abortion	inflamm
12		5	0.28		54	0.0052		inf	allantoic	abortion	inflamm
12		6	0.23		57	0.0040		inf	allantoic	abortion	inflamm
12		8	0.25		58	0.0043		inf	allantoic	abortion	inflamm
12		9	0.2		60	0.0033		inf	allantoic	abortion	inflamm
12		10	0.17		61	0.0028		inf	allantoic	abortion	inflamm
12		11	0.3		63	0.0048		inf	allantoic	abortion	inflamm
12		12	0.27					inf	allantoic	abortion	inflamm
12		13	0.24					inf	allantoic	abortion	inflamm
12		14	0.58					inf	allantoic	abortion	inflamm
12		17	0.49	0.11	79	0.0062	-0.00046	inf	allantoic	abortion	inflamm
24SG	low STREP	0	0.29		28	0.0104		uninf	admix	induced	none
24SG		1	0.23		26	0.0088		uninf	admix	induced	none
24SG		3	0.27		26	0.0104		uninf	admix	induced	none
24SG		4	0.22		23	0.0096		uninf	admix	induced	none
24SG		5	0.24		21	0.0114		uninf	admix	induced	none
24SG		6	0.15		20	0.0075		uninf	admix	induced	none
24SG		7	0.2		20	0.0100		uninf	admix	induced	none
24SG		8	0.2	-0.09	18	0.0111	0.00075	uninf	admix	induced	none

Table 6.1 continued

ID	Group	day	cortisol µg/dL	cortisol Tend-T0	Creat	CCR	CCR Tend-T0	infection	Type of fetal fluid	Type of delivery	Inflammation
20	low STREP	0	0.23		21	0.0110		inf	admix	abortion	inflamm
20		1	0.3		12	0.0250		inf	admix	abortion	inflamm
20		2	0.62		11	0.0564		inf	admix	abortion	inflamm
20		4	1.49	1.26	11.9	0.1252	0.11426	inf	admix	abortion	inflamm
23	high STREP	0	0.37		53	0.0070		inf	allantoic	abortion	inflamm
23		1	0.37		57	0.0065		inf	allantoic	abortion	inflamm
23		2	0.37		60	0.0062		inf	allantoic	abortion	inflamm
23		3	0.4		65	0.0062		inf	allantoic	abortion	inflamm
23		4	0.41		67	0.0061		inf	allantoic	abortion	inflamm
23		5	0.39		66	0.0059		inf	allantoic	abortion	inflamm
23		6	0.34		65	0.0052		inf	allantoic	abortion	inflamm
23		7	0.28	-0.09	66	0.0042	-0.00274	inf	allantoic	abortion	inflamm

Table 6.2 Differences in resting and final cortisol concentrations and cortisol to creatinine ratios (CCR) in an experimental model of placentitis (n=11).

ID		day of final sample	day of delivery	Cort diff µg/dL	CCR diff µg/dL	infection	category	delivery	histopath
25	SHAM	8	19	0.38	0.02653	inf	admix	induced	inflamm
847	SHAM	8	8	1.3	0.00797	inf	allantoic	abortion	inflamm
762	SHAM	8	8	0.06	-0.00040	uninf	allantoic	induced	none
781	PBS	2	4	-0.05	-0.00987	inf	allantoic	abortion	inflamm
22SG	PBS	8	8	-0.13	-0.00297	uninf	allantoic	induced	none
682	KILLED	10	10	-0.04	-0.00049	inf	allantoic	abortion	inflamm
416	KILLED	5	6	2.66	0.18063	uninf	admix	abortion	inflamm
12 SG	KILLED low	8	8	-0.23	-0.00271	uninf	admix	induced	none
20	STREP low	4	10	1.26	0.11426	inf	admix	abortion	inflamm
12	STREP low	17	18	0.11	-0.00046	inf	allantoic	abortion	inflamm
24SG	STREP high	8	8	-0.09	0.00075	uninf	admix	induced	none
23	STREP	7	11	-0.09	-0.00274	inf	allantoic	abortion	inflamm

Table 6.3 Means, standard error of the means, and the probabilities that means are different for cortisol concentrations (Cort diff) and cortisol to creatinine ratios (CCR diff) between pre-inoculation and prior to delivery in a model of placentitis.

Parameter	Category	Mean	Std Err	P value	Method[1]
Cort diff	Admixed (n=5)	0.796	0.534	0.30	Pooled
	Allantoic (n=6)	0.202	0.223		
CCR diff	Admixed (n=5)	63.9×10^{-3}	36×10^{-3}	0.15	Satterthwaite
	Allantoic (n=6)	0.153×10^{-3}	1.6×10^{-3}		
Cort diff	Infection (n=6)	0.487	0.260	0.96	Pooled
	No infection (n=5)	0.454	0.553		
Cort diff[2]	Infection (n=6)	0.487	0.26	0.075	Satterthwaite
	No infection (n=4)	-0.098	0.060		
CCR diff	Infection (n=6)	24.2×10^{-3}	18.5×10^{-3}	0.79	Pooled
	No infection (n=5)	35.1×10^{-3}	36.4×10^{-3}		
CCR diff[2]	Infection (n=6)	24.2×10^{-3}	18.5×10^{-3}	0.23	Satterthwaite
	No infection (n=4)	1×10^{-3}	0.9×10^{-3}		
Cort diff	Abortion (n=6)	0.867	0.442	0.11	Satterthwaite
	Induced (n=5)	-0.00200	0.106		
CCR diff	Abortion (n=6)	49.9×10^{-3}	32.1×10^{-3}	0.22	Satterthwaite
	Induced (n=5)	4.24×10^{-3}	5.6×10^{-3}		
Cort diff	Inflammation (n=6)	0.487	0.260	0.96	Pooled
	No inflammation (n=5)	0.454	0.554		
Cort diff[2]	Inflammation (n=6)	0.462	0.246	0.072	Satterthwaite
	No inflammation (n=4)	-0.0975	00.0602		
CCR diff	Inflammation (n=6)	24.2×10^{-3}	18.5×10^{-3}	0.79	Satterthwaite
	No inflammation (n=5)	35.1×10^{-3}	36.4×10^{-3}		
CCR diff[2]	Inflammation (n=6)	24.2×10^{-3}	18.5×10^{-3}	0.22	Satterthwaite
	No inflammation (n=4)	1.33×10^{-3}	9×10^{-3}		

[1]Where variances were found to be unequal (*F′* statistic; folded), Satterthwaite's method for calculation of degrees of freedom associated with the approximate *t* is used, and the approximate *t′* statistic is computed Where variances were found to not be significantly different, the t statistic uses a pooled variance.

[2]Mare #416 removed from analysis

significantly different are provided in Table 6.3. There was no significant difference in $Cort_{diff}$ (p=0.30) or CCR_{diff} (p=0.15) between admixed fetal fluid and allantoic fluid. There was no significant difference in $Cort_{diff}$ (p=0.96) between mares with infected fetal fluids (Figure 6.1) compared to those with uninfected fetal fluids. However, only a single mare in the uninfected group (KILLED STREP mare; #416) had a substantial increase in cortisol concentration. This mare did not have histologic evidence of inflammation of the chorioallantois, and removal of her data from the analysis led to the effect of infection on cortisol concentration approaching significance (p=0.075). There was no difference in CCR_{diff} (p=0.79) between mares with infected fetal fluids compared to those with uninfected fetal fluids; no change in significance (p=0.23) was seen when #416 was removed from analysis. All three mares with elevated cortisol spontaneously aborted (Fig. 6.2); however, the $Cort_{diff}$ (p=0.11) and CCR_{diff} (p=0.22) were not significant between spontaneously aborting mares and those induced to deliver. There was no difference in $Cort_{diff}$ between mares with histologic inflammation of the chorioallantois and those without inflammation (p=0.96); removal of mare #416 from the analysis led to the effect of infection on cortisol concentration approaching significance (p=0.072). There was no significant difference in CCR_{diff} between mares with or without histologic inflammation (p=0.79); no change in significance was seen with removal of mare #416 from the analysis (p=0.22)

6.4 Discussion

These results suggest that increased cortisol concentration in fetal fluid may be seen with infection or histologic inflammation of the chorioallantois at 80% gestation. None of the fetal fluids from mares induced to deliver, or without inflammation of the chorioallantois, had increased cortisol concentrations. Failure of intra-fetal administration of ACTH at a similar stage of gestation to cause fetal cortisol release has led to the conclusion that the HPAA was immature and incapable of response [149]. The data presented here suggest that this may not be the case,

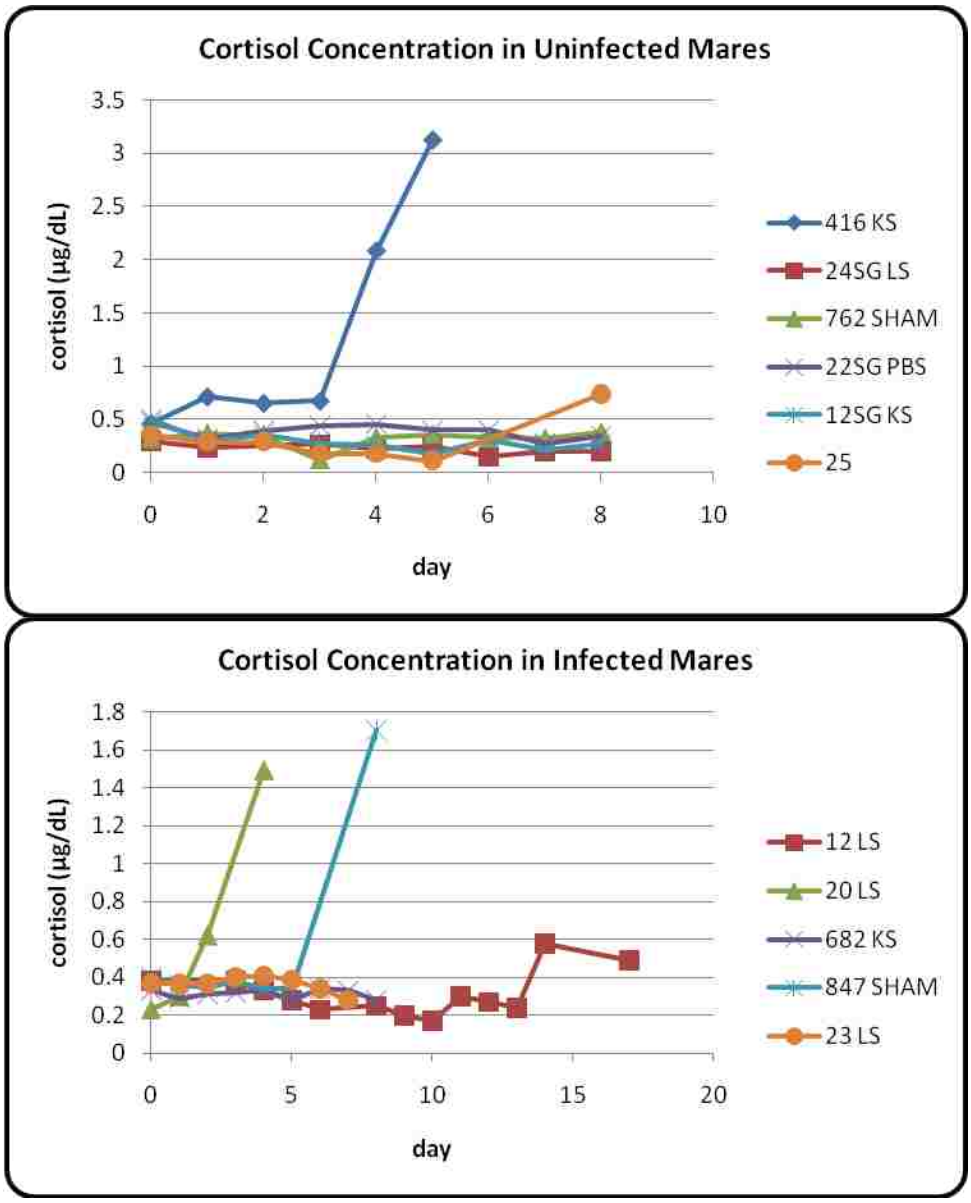

Figure 6.1 Cortisol concentrations (µg/dL) in fetal fluids from mares uninfected (n=6) and uninfected (n=5) in an experimental model of placentitis.

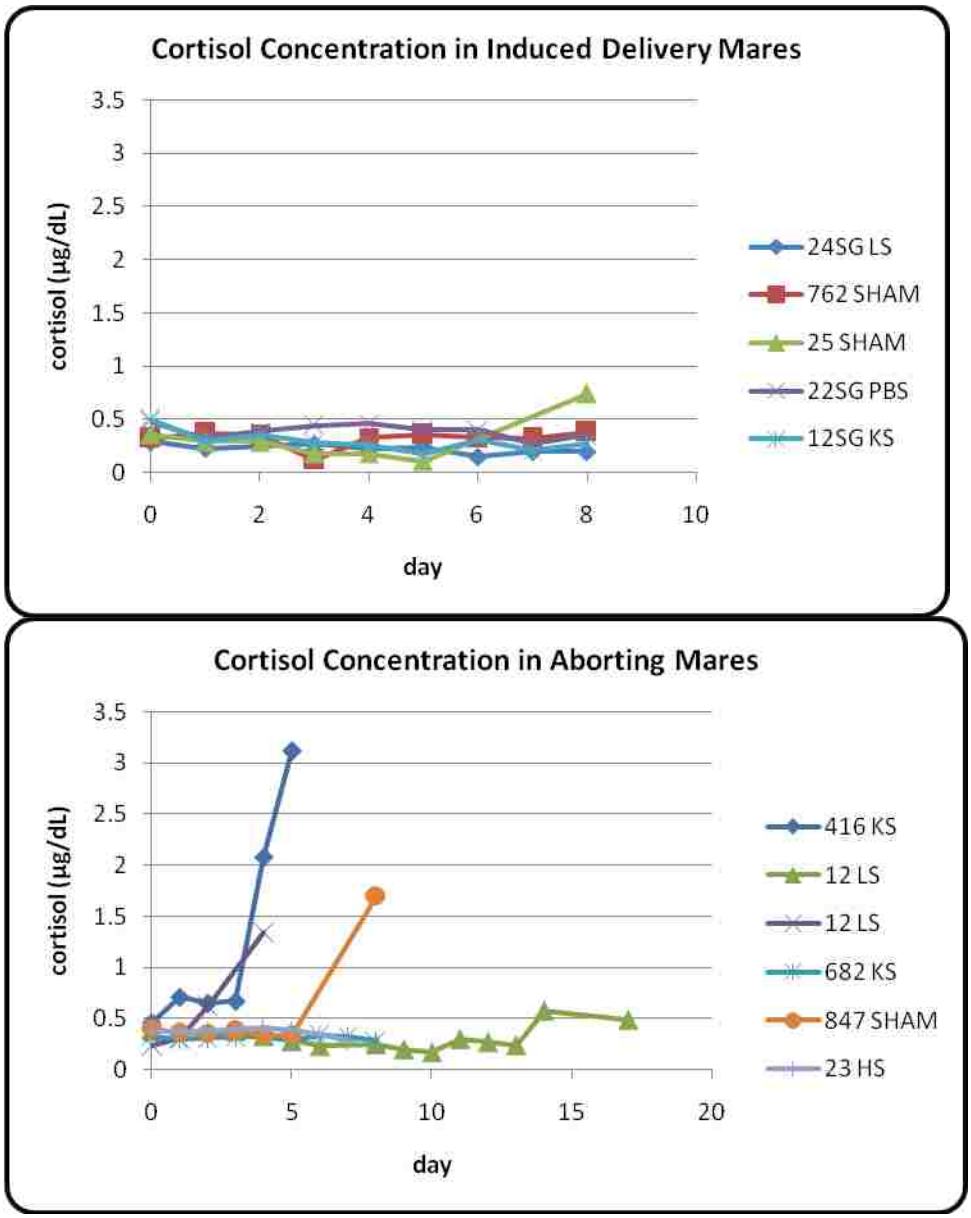

Figure 6.2 Cortisol concentrations (µg/dL) in fetal fluids from mares where fetal delivery was induced (n=5) or spontaneous (n=6) in an experimental model of placentitis.

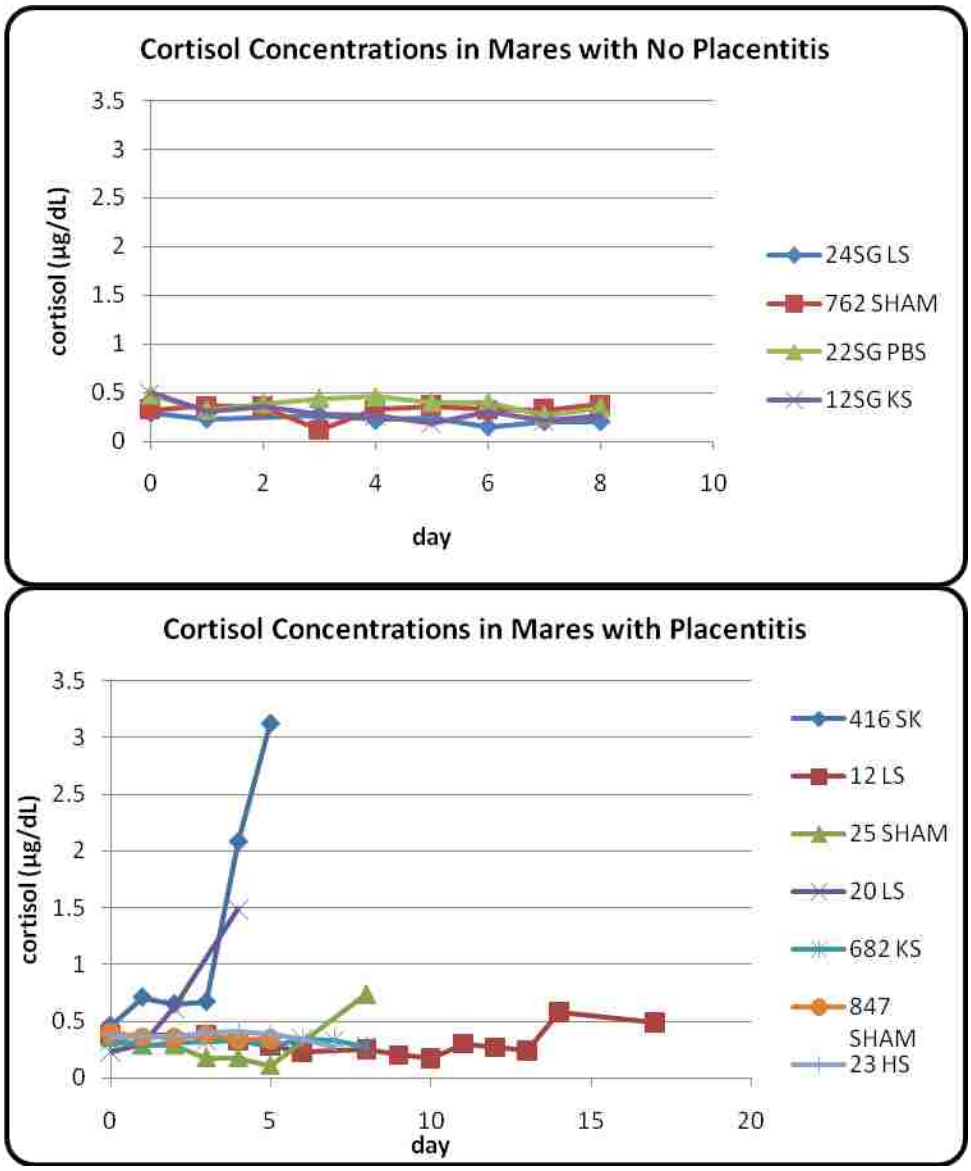

Figure 6.3 Cortisol concentrations (µg/dL) in fetal fluids from mares with (n=5) or without (n=4) histologic evidence of inflammation in an experimental model of placentitis.

and that the equine fetal adrenal less than 295 d may be capable of response to stimuli. Unfortunately the sample sizes are small, and evaluation of additional animals would be useful. The highest cortisol concentrations were seen in one of the KILLED STREP mares (#416) which is difficult to explain. From instrumentation until abortion, this fetus was extremely active, precluding measurement of fetal heart rate. No infection was detected in either fetal fluids or fetal samples at delivery, and minimal inflammation was found in the chorioallantois. This mare spontaneously aborted and had a significant increase in CTUP (Table 3.1) and edema at the cervical star (Table 3.3), both of which are characteristic of changes at term in normal pregnancies [248]. At necropsy the fetus had a fibrinous pneumonia but no bacteria were present, either from culture or histologic specimens. It would appear that this fetus was experiencing *in utero* stress, although the source of the trigger is unknown. While it is tempting to speculate that this was secondary to the effects of a non-infectious inflammation produced by inoculation with heat-killed *S. zooepidemicus*, this is unlikely. This mare was inoculated in 2005, when difficulties were encountered with the type of pipette used for inoculation (Chapter 3). It was suspected that either all or part of the inoculum was not deposited at the surface of the chorioallantois, leading to a failure to produce inflammation or infection.

The signaling pathways responsible for release of cortisol from the equine fetal adrenal gland subsequent to intrauterine infection are unknown, but data from other species would suggest that pro-inflammatory cytokines is one likely mechanism resulting in fetal HPAA activation. Administration of full length IL-1 stimulates preterm delivery in mice [264] and guinea pigs [305], and increased fetal cortisol concentration [305] and pulmonary lung function [305,306] in guinea pigs. We have previously shown (Chapter 4) that expression of IL-1, IL-18 and IFN-γ in the equine chorioallantois increased with infection and spontaneous abortion. All

three of the mares with increased fetal fluid cortisol concentrations had greater than a 25-fold expression change in IL-1β at the cervical star, and normal and abnormal areas of the chorioallantois. Further investigations, such as *in vitro* fetal adrenal cell responsiveness to IL-1 and ACTH at various stages of gestation may provide insights into the signaling pathways leading to cortisol secretion subsequent to IL-1 treatment exposure.

Several studies suggest another potential signaling pathway leading to increased fetal cortisol output is by prostaglandin E_2 (PGE_2) stimulation. Administration of PGE_2 to chronically catheterized fetal sheep resulted in premature labor [307], and produced significant increases of fetal immunoreactive ACTH and cortisol within 30 min of infusion compared to control fetuses from gestational age 119 d to term, with the greatest response see with fetuses between 119 to 125 d [308]. Meloxicam (a specific PGHS-2 inhibitor) significantly decreased fetal plasma concentrations of PGE_2, ACTH, and cortisol in a competitive progesterone receptor antagonist (RU486, mifepristone)-induced model of pre-term delivery in the ewe [309]. Prostaglandin E_2 enhanced the ability of arginine vasopressin to stimulate ACTH secretion by cultured pituitary cells [310]. These results have led to the conclusion that PGE_2 plays a role in activation of the HPAA at term; however, this hypothesis is questioned by the failure of nimesulide, a selective PGHS-2 inhibitor, to reduce fetal ACTH or cortisol concentration in the late gestation ewe despite significantly decreasing fetal PGE_2 concentrations [311]. There is insufficient data available on fetal fluid PGE_2 concentrations from the three mares with increased fetal fluid cortisol concentrations in the study presented here to draw conclusions on PGE_2 signaling equine fetal cortisol secretion.

It is unlikely that the elevated cortisol concentrations in fetal fluid described here are due to elevated maternal cortisol. Placental 11β-hydroxysteroid dehydrogenase type 2 enzyme (11β-

HSD2) converts cortisol to cortisone, protecting the fetus from excessive exposure to maternal cortisol [312-314]. The equine endometrium has been shown to express 11β-HSD2 [315], and presumably the equine chorioallantois would as well. It would be useful to confirm the presence and activity of 11β-HSD2 in the equine chorion during gestation, and whether its activity is altered with infection, inflammation, or stage of gestation. In the future, documenting no increase in maternal cortisol concentration in samples contemporaneous with fetal fluid samples would provide evidence that an increase in fetal fluid cortisol concentration is solely of fetal origin.

In summary, these data provide preliminary evidence that the equine adrenal gland may be capable of cortisol secretion in response to stimuli prior to 295 d. Precocious fetal maturation is observed with some cases of naturally occurring equine placentitis, and neonatal survivability is most probably due to accelerated activation of the HPAA. If IL-1β is indeed the signal for HPAA activation, then perhaps intra-allantoic or intra-amniotic treatment with either full length IL-1β [305] or a non-inflammatory IL-1β-fragment [306] might provide a means of accelerating HPAA activation and enhancing neonatal outcome in high risk pregnancies.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Ascending placentitis is a significant cause of abortions, stillbirths, and perinatal loss in horses. Empirical treatment for placentitis includes progestins to maintain uterine quiescence, broad-spectrum antibiotics to eliminate bacterial infection, and anti-inflammatory agents to prevent prostaglandin synthesis. Although these agents should address the basic problems of infection-induced prostaglandin synthesis that leads to uterine contractions and fetal expulsion, the efficacy of these regimes is often poor [316]. Recent investigations suggest that if therapy is instituted early in the course of infection, a favorable outcome is more likely [244,294]. Unfortunately, the diagnosis in clinical cases often lags the initiation of infection. There are two major areas where the basic knowledge of the pathophysiology of equine placentitis is lacking: (1) the endocrinological events associated with loss of myometrial quiescence and (2) the temporal relationship between the inflammatory cascade, increases in prostaglandin synthesis and release, and activation of the fetal hypothalamic-pituitary-adrenal axis.

In the first study (Chapter 2), we developed a technique for laparoscopic-guided catheterization of the allantoic space. The goal was to define a minimally invasive technique that would allow reliable allantoic fluid sample acquisition, and would allow for repeated animal use during subsequent pregnancies. Many technical hurdles, such as portal placement, catheter design, repeated use in subsequent years, and bacterial tracking were resolved. Laparoscopic ultrasound proved extremely useful in identifying a suitable pocket of allantoic fluid for catheter placement; however, a high percentage of attempts where fetal positioning precluded catheter insertion were still encountered. Attempts to rectify this problem by laparoscopic retractors and

pharmacologic intervention (propantheline as a myometrial relaxant) were unsuccessful in creating a deeper pocket of allantoic fluid. Despite these limitations, this technique holds promise in future investigations. It is likely that instrumentation earlier in gestation, when the relative ratio of allantoic to amniotic fluid volume is greater [317], would simplify placement of a catheter.

In the second study (Chapter 3), we described an experimental model of streptococcal infective pre-term delivery using mares instrumented with allantoic fluid catheters. Bacterial tracking confounded the data, and in only one-half of mares inoculated with live *S. zooepidemicus* was placentitis established. Clearly this study was hampered by small samples sizes and bacterial contamination due to tracking along the catheter system. Our original study design included sample sizes of six in each treatment group; however, we were forced to conclude the study with only three animals per treatment group. Despite these limitations, useful descriptive data was obtained, and fetal fluids were acquired for future measurement of soluble TNF-α, prostaglandin, cortisol, and progestagen concentrations, and chorioallantoic tissue was obtained for analysis of gene expression patterns. Of particular interest were the histologic changes in the fetal lung in response to infection. Fetal pneumonia in response to the presence of bacteria within the lung was inconsistent. The interpretation of the post-mortem bacterial contamination; however, this was not the case. In all of these instances, fetal fluids were culture positive prior to abortion, and identical isolates were recovered from fetal fluid and fetal lung. Whether this finding represents a peracute fetal infection and death prior to the initiation of an inflammatory response, or the failure of the fetus to mount an immunologic response to infection is unknown. In two of the fetuses with bacteria and no pneumonia, there was no increase in fetal fluid cortisol (Chapter 6), so an incompetent immunologic response is possible.

In the third study (Chapter 4), we described the change in expression pattern of equine-specific cytokines in chorioallantoic tissue from mares with experimental *in utero* infection and spontaneous abortion. Experimentally-induced infection increased the expression of IL-1β, IL-18, IL-15, and IFN-γ in a site-dependant manner. Spontaneous abortion also increased the expression of IL-1β, IL-18, IFN-γ, and iNOS in a site dependant manner. This expression pattern of pro-inflammatory cytokines in response to infection of equine placental tissue is different from a previous report [237], where IL-6 and IL-8 were increased at the cervical star and IL-6 was increased at the uterine body. The findings presented here are in agreement with those of previously published experimental animal models and clinical findings from women with intra-amniotic infection and pre-term delivery [76,224-228,243,263,270,273,280]. The consequence of increased IL-1β is the induction of COX-2 and PGE_2 production leading to labor. We were also able to detect the transient presence of soluble TNF-α in a limited number of fetal fluids. Although this was somewhat surprising, TNF-α may only be present for a brief period of time in fetal fluids [228], therefore sampling time in relation to an inflammatory insult is crucial.

In the fourth study (Chapter 5), we described the changes in concentrations of PGE_2 and $PGF_{2\alpha}$ in fetal fluids from mares in an experimental model of ascending placentitis. The concentrations of PGE_2 and $PGF_{2\alpha}$ in fetal fluids were increased within 24 h of delivery in mares with spontaneous abortion or intrauterine infection. Similar to a previously reported catheterized model [287] , we observed a fairly consistent modest increase in PGE_2 concentrations following surgery, followed by a decline. The absolute values of PGE_2 concentration immediately prior to delivery we observed were lower than those in a previously published model of placentitis [237], although in both study populations, variances were high and the instrumentation of the sample populations were different. IL-1β has been shown to upregulate PGHS-2 [267]; similar studies

with equine cell lines are recommended. Conventional therapy for equine placentitis currently involves non-selective agents such as flunixin meglumine or phenylbutazone [244,294]; however, meloxicam, a selective cyclooxygenase inhibitor, has been shown to effectively prevent pre-term labor in sheep [295,296], warranting investigation of its use as a tocolytic in the mare.

In the final study (Chapter 6), we observed increased cortisol concentrations in fetal fluid in some mares with infection or histologic inflammation of the chorioallantois. None of the fetal fluids from mares induced to deliver or without inflammation of the chorioallantois had increased cortisol concentrations. Previous research has led to the conclusion that the hypothalamic-pituitary-adrenal axis (HPAA) in fetuses less than 295 d are immature [149]; however, the data presented here suggest that this may not be the case, and that the equine fetal adrenal gland less than 295 d is capable of responding to stimuli. The signaling pathways responsible for release of cortisol from the equine fetal adrenal gland subsequent to intrauterine infection are unknown, but data from other species suggest that IL-1 stimulation is the likely mechanism producing fetal HPAA activation [264,305,306]. The *in vitro* response of equine adrenal cells throughout gestation to IL-1β would significantly add to the knowledge of what occurs during the course of *in utero* infection, and possibly delineate potential novel therapeutics in cases of high risk pregnancy. Precocious fetal maturation is observed with some cases of naturally occurring equine placentitis, and neonatal survivability is most probably due to accelerated activation of the HPAA. If IL-1β is indeed the signal for HPAA activation in equids, then perhaps intra-allantoic or intra-amniotic treatment with either full length IL-1β [305] or a non-inflammatory IL-1β-fragment [306] might provide a means of accelerating HPAA activation and enhancing neonatal outcome in high risk pregnancies. Other conditions associated with high

risk pregnancy in the mare, such as endotoxemia, are areas where further knowledge is needed of the signaling events leading to pre-term delivery. Although amniocentesis is commonly used by physicians to diagnose chorioamnionitis in women and assess fetal maturity, allantocentesis is rarely used in cases of suspected placentitis in the mare to either document infection of the fetal fluid compartments or to ascertain readiness for birth. The risk of allantocentesis causing fetal infection is considered by some high [318]. Increased cortisol concentration in fetal fluid appears to provide evidence of fetal HPAA activation. Continued research is needed to determine whether cortisol concentration determination in allantoic fluid can be used as an indicator of equine fetal maturity. Until reliable markers for fetal maturity and for infection are identified, the use of allantocentesis in the diagnosis and management of placentitis should be undertaken with caution.

In summary, we have developed a novel method of catheterization of the allantoic space in the mare, and have applied this method of instrumentation to a model of streptococcal placentitis. Based on our observations, we propose the following sequence of events leading from intrauterine infection to infective pre-term delivery. Following infection of the chorioallantois, IL-1β, IL-18, IL-15, and IFN-γ are upregulated in a site-dependant manner. IL-1β causes increased PGHS-2 (COX-2) expression, resulting in increased PGE_2 and $PGF_{2\alpha}$ production, and ultimately labor. IL-1β also accelerates fetal HPAA activation, thereby promoting precocious *in utero* fetal maturation. The eventual outcome of pre-term labor, i.e., neonatal survivability, will depend on the degree of HPAA activation at the onset of labor.

REFERENCES

1. Smith KC, Blunden AS, Whitwell KE, Dunn KA, Wales AD. A survey of equine abortion, stillbirth and neonatal death in the UK from 1988 to 1997. Equine Vet J 2003;35: 496-501.

2. Hong CB, Donahue JM, Giles RC, Jr., Petrites-Murphy MB, Poonacha KB, Roberts AW, Smith BJ, Tramontin RR, Tuttle PA, Swerczek TW. Etiology and pathology of equine placentitis. J Vet Diagn Invest 1993;5: 56-63.

3. Giles RC, Donahue JM, Hong CB, Tuttle PA, Petrites-Murphy MB, Poonacha KB, Roberts AW, Tramontin RR, Smith B, Swerczek TW. Causes of abortion, stillbirth, and perinatal death in horses: 3,527 cases (1986-1991). J Am Vet Med Assoc 1993;203: 1170-1175.

4. Hong CB, Donahue JM, Giles RC, Jr., Petrites-Murphy MB, Poonacha KB, Roberts AW, Smith BJ, Tramontin RR, Tuttle PA, Swerczek TW. Equine abortion and stillbirth in central Kentucky during 1988 and 1989 foaling seasons. J Vet Diagn Invest 1993;5: 560-566.

5. Whitwell KE. Infective placentitis in the mare. In: Powell DG (ed), Equine Infectious Dieseases. Lexington, KY, University Press of Kentucky, 1988;172-180.

6. Renaudin CD, Liu IKM, Troedsson MHT, Schrenzel MD. Transrectal ultrasonographic diagnosis of ascending placentitis in the mare: A report of two cases. Equine Vet Educ 1999;11: 69-74.

7. Mossman HW. Vertebrate Fetal Membranes. New Brunswick, NT, Rutgers University Press, 1987,271-275.

8. Oguri N, Tsutsumi Y. Non-surgical recovery of equine eggs, and an attempt at non-surgical egg transfer in horses. J Reprod Fertil 1972;31: 187-195.

9. Betteridge KJ, Eaglesome MD, Mitchell D, Flood PF, Beriault R. Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. J Anat 1982;135: 191-209.

10. Betteridge KJ. Comparative aspects of equine embryonic development. Anim Reprod Sci 2000;60-61: 691-702.

11. Ginther OJ. Reproductive Biology of the Mare, 2nd edition. Cross Plains, WI, Equisciences, 1992,345-418.

12. Clegg MT, Boda JM, Cole HH. The endometrial cups and allantochorionic pouches in the mare with emphasis on the source of equine gonadotrophin. Endocrinology 1954;54: 448-463.

13. Allen WR. Fetomaternal interactions and influences during equine pregnancy. Reproduction 2001;121: 513-527.

14. Allen WR, Moor RM. The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. J Reprod Fertil 1972;29: 313-316.

15. Flood PF, Marrable AW. A histochemical study of steroid metabolism in the equine fetus and placenta. J Reprod Fertil Suppl 1975: 569-573.

16. Samuel CA, Allen WR, Steven DH. Studies on the equine placenta. I. Development of the microcotyledons. J Reprod Fertil 1974;41: 441-445.

17. Cottrill CM, Jeffers-Lo J, Ousey JC, McGladdery AJ, Ricketts SW, Silver M, Rossdale PD. The placenta as a determinant of fetal well-being in normal and abnormal equine pregnancies. J Reprod Fertil Suppl 1991;44: 591-601.

18. Cassar TIY, Fallon LH, Martinez EH, Schlafer DH. Segmental ossification of involuted yolk sacs in equine umbilical cords. Anim Reprod Sci 2006;94: 439-442.

19. Lambert FL, Pelletier G, Dufour M, Fortier MA. Specific properties of smooth muscle cells from different layers of rabbit myometrium. Am J Physiol - Cell Physiol 1990;258: C794-802.

20. Challis JRG, Lye SJ. Parturition. In: Knobil E,Neil JD (eds), The Physiology of Reproduction. New York, Raven Press, 1994;985-1031.

21. Chow EH, Marshall JM. Effects of catecholamines on circular and longitudinal uterine muscle of the rat. Eur J Pharmacol 1981;76: 157-165.

22. Hirsbrunner G, Reist M, Couto SS, Steiner A, Snyder J, Vanleeuwen E, Liu I. An in vitro study on spontaneous myometrial contractility in the mare during estrus and diestrus. Theriogenology 2006;65: 517-527.

23. Ousey JC, Freestone N, Fowden AL, Mason WT, Rossdale PD. The effects of oxytocin and progestagens on myometrial contractility in vitro during equine pregnancy. J Reprod Fertil Suppl 2000;56: 681-691.

24. Rigby SL, Barhoumi R, Burghardt RC, Colleran P, Thompson JA, Varner DD, Blanchard TL, Brinsko SP, Taylor T, Wilkerson MK, Delp MD. Mares with delayed uterine clearance have an intrinsic defect in myometrial function. Biol Reprod 2001;65: 740-747.

25. Nickel R, Schummer A, Seiferle E, W.O. S. The Viscera of the Domestic Animals. New York, Springer-Verlag, 1973,351-392.

26. Morizaki N, Morizaki J, Hayashi RH, Garfield RE. A functional and structural study of the innervation of the human uterus. Am J Obstet Gynecol 1989;160: 218-228.

27. Bae SE, Corcoran BM, Watson ED. Immunohistochemical study of the distribution of adrenergic and peptidergic innervation in the equine uterus and the cervix. Reproduction 2001;122: 275-282.

28. Riley M, Wu X, Baker PN, Taggart MJ. Gestational-dependent changes in the expression of signal transduction and contractile filament-associated proteins in mouse myometrium. J Soc Gynecol Investig 2005;12: e33-43.

29. Shynlova O, Tsui P, Dorogin A, Chow M, Lye SJ. Expression and localization of alpha-smooth muscle and gamma-actins in the pregnant rat myometrium. Biol Reprod 2005;73: 773-780.

30. Taggart MJ, Morgan KG. Regulation of the uterine contractile apparatus and cytoskeleton. Semin Cell Dev Biol 2007;18: 296-304.

31. Sjuve R, Arner A, Li Z, Mies B, Paulin D, Schmittner M, Small JV. Mechanical alterations in smooth muscle from mice lacking desmin. J Muscle Res Cell Motil 1998;19: 415-429.

32. Salomonis N, Cotte N, Zambon AC, Pollard KS, Vranizan K, Doniger SW, Dolganov G, Conklin BR. Identifying genetic networks underlying myometrial transition to labor. Genome Biol 2005;6: R12.

33. Mason CW, Swaan PW, Weiner CP. Identification of interactive gene networks: a novel approach in gene array profiling of myometrial events during guinea pig pregnancy. Am J Obstet Gyn 2006;194: 1513-1523.

34. Small JV, Gimona M. The cytoskeleton of the vertebrate smooth muscle cell. Acta Physiol Scand 1998;164: 341-348.

35. Williams SJ, White BG, MacPhee DJ. Expression of alpha5 integrin (Itga5) is elevated in the rat myometrium during late pregnancy and labor: implications for development of a mechanical syncytium. Biol Reprod 2005;72: 1114-1124.

36. Macphee DJ, Lye SJ. Focal adhesion signaling in the rat myometrium is abruptly terminated with the onset of labor. Endocrinology 2000;141: 274-283.

37. White BG, Williams SJ, Highmore K, Macphee DJ. Small heat shock protein 27 (Hsp27) expression is highly induced in rat myometrium during late pregnancy and labour. Reproduction 2005;129: 115-126.

38. Li Y, Je HD, Malek S, Morgan KG. ERK1/2-mediated phosphorylation of myometrial caldesmon during pregnancy and labor. Am J Physiol Regul Integr Comp Physiol 2003;284: R192-199.

39. Gabella G. Morphology of smooth muscle. In: Kao CY,Carsten ME (eds), Cellular Aspects of Smooth Muscle Function. Cambridge, Cambridge University Press, 1997;1-47.

40. Meiss RA. Mechanics of smooth muscle contraction. In: Kao CY,Carsten ME (eds), Cellular Aspects of Smooth Muscle Contraction. Cambridge, Cambridge University Press, 1997;169-208.

41. Hai CM, Murphy RA. Cross-bridge phosphorylation and regulation of latch state in smooth muscle. Amer J of Physiol - Cell Physiol 1988;254.

42. Inoue Y, Sperelakis N. Gestational change in Na+ and Ca2+ channel current densities in rat myometrial smooth muscle cells. Am J Physiol 1991;260: C658-663.

43. Savineau JP, Mironneau J, Mironneau C. Influence of the sodium gradient on contractile activity in pregnant rat myometrium. Gen Physiol Biophys 1987;6: 535-559.

44. Maigaard S, Forman A, Brogaard-Hansen KP, Andersson KE. Inhibitory effects of nitrendipine on myometrial and vascular smooth muscle in human pregnant uterus and placenta. Acta Pharmacol Toxicol (Copenh) 1986;59: 1-10.

45. Arnaudeau S, Lepretre N, Mironneau J. Oxytocin mobilizes calcium from a unique heparin-sensitive and thapsigargin-sensitive store in single myometrial cells from pregnant rats. Pflugers Arch 1994;428: 51-59.

46. Young RC, Zhang P. Inhibition of in vitro contractions of human myometrium by mibefradil, a T-type calcium channel blocker: support for a model using excitation-contraction coupling, and autocrine and paracrine signaling mechanisms. J Soc Gynecol Investig 2005;12: e7-12.

47. Word RA, Kamm KE. Regulation of smooth muscle contraction by myosin phosphorylation. In: Kao CY,Carsten ME (eds), Cellular Aspects of Smooth Muscle Function. Cambridge, Cambridge University Press, 1997;209-252.

48. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev 2000;21: 514-550.

49. Williams MT, Davis HN, McCrea AE, Hennessy MB. The distribution of radiolabeled corticotropin-releasing factor in pregnant rats: an investigation of placental transfer to the fetuses. Int J Dev Neurosci 1998;16: 229-234.

50. Haeberle JR, Hathaway DR, DePaoli-Roach AA. Dephosphorylation of myosin by the catalytic subunit of a type-2 phosphatase produces relaxation of chemically skinned uterine smooth muscle. J Biol Chem 1985;260: 9965-9968.

51. Mackenzie LW, Word RA, Casey ML, Stull JT. Myosin light chain phosphorylation in human myometrial smooth muscle cells. Am J Physiol 1990;258: C92-98.

52. Fuchs AR, Fuchs F, Husslein P, Soloff MS, Fernstrom MJ. Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Science 1982;215: 1396-1398.

53. Riemer RK, Goldfien AC, Goldfien A, Roberts JM. Rabbit uterine oxytocin receptors and in vitro contractile response: abrupt changes at term and the role of eicosanoids. Endocrinology 1986;119: 699-709.

54. Sanborn BM. Hormones and calcium: mechanisms controlling uterine smooth muscle contractile activity. The Litchfield Lecture. Exp Physiol 2001;86: 223-237.

55. Chan WY, Berezin I, Daniel EE, Russell KC, Hruby VJ. Effects of inactivation of oxytocin receptor and inhibition of prostaglandin synthesis on uterine oxytocin receptor and gap junction formation and labor in the rat. Can J Physiol Pharmacol 1991;69: 1262-1267.

56. Gross GA, Imamura T, Luedke C, Vogt SK, Olson LM, Nelson DM, Sadovsky Y, Muglia LJ. Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. Proc Natl Acad Sci U S A 1998;95: 11875-11879.

57. Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N, Knopf JL. A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. Cell 1991;65: 1043-1051.

58. Rajabi MR, Cybulsky AV. Phospholipase A2 activity is increased in guinea pig uterine cervix in late pregnancy and at parturition. Am J Physiol 1995;269: E940-947.

59. Skannal DG, Brockman DE, Eis AL, Xue S, Siddiqi TA, Myatt L. Changes in activity of cytosolic phospholipase A2 in human amnion at parturition. Am J Obstet Gyn 1997;177: 179-184.

60. Skannal DG, Eis AL, Brockman D, Siddiqi TA, Myatt L. Immunohistochemical localization of phospholipase A2 isoforms in human myometrium during pregnancy and parturition. Am J Obstet Gyn 1997;176: 878-882.

61. Zhang Q, Wu WX, Brenna JT, Nathanielsz PW. The expression of cytosolic phospholipase A2 and prostaglandin endoperoxide synthase in ovine maternal uterine and fetal tissues during late gestation and labor. Endocrinology 1996;137: 4010-4017.

62. Slater DM, Astle S, Bennett PR, Thornton S. Labour is associated with increased expression of type-IIA secretory phospholipase A2 but not type-IV cytosolic phospholipase A2 in human myometrium. Mol Hum Reprod 2004;10: 799-805.

63. Xu X-M, Hajibeige A, Tazawa R, Loose-Mitchell D, Want L-H, Wu KK. Characterization of human prostaglandin H synthase genes. In: Samuelsson B,Paoletti R (eds), Advances in Prostaglandin, Thromboxane, and Leukotriene Research. New York, Raven Press, 1995;105-107.

64. Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 1996;271: 33157-33160.

65. Kniss DA. Cyclooxygenases in reproductive medicine and biology. J Soc Gynecol Investig 1999;6: 285-292.

66. Slater DM, Berger LC, Newton R, Moore GE, Bennett PR. Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. Am J Obstet Gyn 1995;172: 77-82.

67. Gibb W, Matthews SG, Challis JR. Localization and developmental changes in prostaglandin H synthase (PGHS) and PGHS messenger ribonucleic acid in ovine placenta throughout gestation. Biol Reprod 1996;54: 654-659.

68. Mijovic JE, Zakar T, Nairn TK, Olson DM. Prostaglandin endoperoxide H synthase (PGHS) activity and PGHS-1 and -2 messenger ribonucleic acid abundance in human chorion throughout gestation and with preterm labor. J Clin Endocrinol Metab 1998;83: 1358-1367.

69. Mijovic JE, Zakar T, Nairn TK, Olson DM. Prostaglandin-endoperoxide H synthase-2 expression and activity increases with term labor in human chorion. Am J Physiol 1997;272: E832-840.

70. Rice GE, Freed KA, Aitken MA, Jacobs RA. Gestational- and labour-associated changes in the relative abundance of prostaglandin G/H synthase-1 and -2 mRNA in ovine placenta. J Mol Endocrinol 1995;14: 237-245.

71. Gyomorey S, Lye SJ, Gibb W, Challis JR. Fetal-to-maternal progression of prostaglandin H(2) synthase-2 expression in ovine intrauterine tissues during the course of labor. Biol Reprod 2000;62: 797-805.

72. Tanioka T, Nakatani Y, Semmyo N, Murakami M, Kudo I. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. J Biol Chem 2000;275: 32775-32782.

73. Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh S, Kudo I. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000;275: 32783-32792.

74. Forsberg L, Leeb L, Thoren S, Morgenstern R, Jakobsson P. Human glutathione dependent prostaglandin E synthase: gene structure and regulation. FEBS Lett 2000;471: 78-82.

75. Sooranna SR, Grigsby PL, Engineer N, Liang Z, Sun K, Myatt L, Johnson MR. Myometrial prostaglandin E2 synthetic enzyme mRNA expression: spatial and temporal variations with pregnancy and labour. Mol Hum Reprod 2006;12: 625-631.

76. Astle S, Newton R, Thornton S, Vatish M, Slater DM. Expression and regulation of prostaglandin E synthase isoforms in human myometrium with labour. Mol Hum Reprod 2007;13: 69-75.

77. Kubota K, Kubota T, Kamei D, Murakami M, Kudo I, Aso T, Morita I. Change in prostaglandin E synthases (PGESs) in microsomal PGES-1 knockout mice in a preterm delivery model. J Endocrinol 2005;187: 339-345.

78. Palliser HK, Ooi GT, Hirst JJ, Rice G, Dellios NL, Escalona RM, Young IR. Changes in the expression of prostaglandin E and F synthases at induced and spontaneous labour onset in the sheep. J Endocrinol 2004;180: 469-477.

79. Palliser HK, Hirst JJ, Rice GE, Ooi GT, Dellios NL, Escalona RM, Young IR. Labor-associated regulation of prostaglandin E and F synthesis and action in the ovine amnion and cervix. J Soc Gynecol Investig 2006;13: 19-24.

80. Filion F, Bouchard N, Goff AK, Lussier JG, Sirois J. Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. J Biol Chem 2001;276: 34323-34330.

81. Premyslova M, Chisaka H, Okamura K, Challis JR. IL-1beta treatment does not co-ordinately up-regulate mPGES-1 and COX-2 mRNA expression, but results in higher degree of cellular and intracellular co-localization of their immunoreactive proteins in human placenta trophoblast cells. Placenta 2006;27: 576-586.

82. Arosh JA, Banu SK, Kimmins S, Chapdelaine P, Maclaren LA, Fortier MA. Effect of interferon-tau on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2. Endocrinology 2004;145: 5280-5293.

83. Watanabe K. Prostaglandin F synthase. Prostaglandins Other Lipid Mediat 2002;68-69: 401-407.

84. Westbrook C, Jarabak J. Purification and partial characterization of an NADH-linked delta13-15-ketoprostaglandin reductase from human placenta. Biochem Biophys Res Commun 1975;66: 541-546.

85. Kankofer M, Wiercinski J. Prostaglandin E2 9-keto reductase from bovine term placenta. Prostaglandins Leukot Essent Fatty Acids 1999;61: 29-32.

86. Asselin E, Fortier MA. Detection and regulation of the messenger for a putative bovine endometrial 9-keto-prostaglandin E(2) reductase: effect of oxytocin and interferon-tau. Biol Reprod 2000;62: 125-131.

87. Kankofer M, Wiercinski J, Zerbe H. Prostaglandin E(2) 9-keto reductase activity in bovine retained and not retained placenta. Prostaglandins Leukot Essent Fatty Acids 2002;66: 413-417.

88. Farina MG, Billi S, Sordelli MS, Ribeiro ML, Di Girolamo G, Lombardi E, Franchi AM. Nitric oxide (NO) inhibits prostaglandin E2 9-ketoreductase (9-KPR) activity in human fetal membranes. Prostaglandins Other Lipid Mediat 2006;79: 260-270.

89. Franczak A, Woclawek-Potocka I, Oponowicz A, Kurowicka B, Kotwica G. Oxytocin stimulates prostaglandin F2alpha secretion and prostaglandin F synthase protein expression in porcine myometrial tissue. Reprod Biol 2004;4: 177-184.

90. Burgess JR, Reddy CC. Isolation and characterization of an enzyme from sheep seminal vesicles that catalyzes the glutathione-dependent reduction of prostaglandin H2 to prostaglandin F2 alpha. Biochem Mol Biol Int 1997;41: 217-226.

91. Kubata BK, Duszenko M, Kabututu Z, Rawer M, Szallies A, Fujimori K, Inui T, Nozaki T, Yamashita K, Horii T, Urade Y, Hayaishi O. Identification of a novel prostaglandin f(2alpha) synthase in Trypanosoma brucei. J Exp Med 2000;192: 1327-1338.

92. Albert TJ, Su HC, Zimmerman PD, Iams JD, Kniss DA. Interleukin-1 beta regulates the inducible cyclooxygenase in amnion-derived WISH cells. Prostaglandins 1994;48: 401-416.

93. Mitchell MD, Romero RJ, Edwin SS, Trautman MS. Prostaglandins and parturition. Reprod Fertil Dev 1995;7: 623-632.

94. Todd HM, Dundoo VL, Gerber WR, Cwiak CA, Baldassare JJ, Hertelendy F. Effect of cytokines on prostaglandin E2 and prostacyclin production in primary cultures of human myometrial cells. J Matern Fetal Med 1996;5: 161-167.

95. Romero R, Tartakovsky B. The natural interleukin-1 receptor antagonist prevents interleukin-1-induced preterm delivery in mice. Am J Obstet Gyn 1992;167: 1041-1045.

96. Fidel PL, Jr., Romero R, Cutright J, Wolf N, Gomez R, Araneda H, Ramirez M, Yoon BH. Treatment with the interleukin-I receptor antagonist and soluble tumor necrosis factor receptor Fc fusion protein does not prevent endotoxin-induced preterm parturition in mice. J Soc Gynecol Investig 1997;4: 22-26.

97. Schlegel W, Demers LM, Hildebrandt-Stark HE, Behrman HR, Greep RO. Partial purification of human placental 15-hydroxy-prostaglandin dehydrogenase: kinetic properties. Prostaglandins 1974;5: 417-433.

98. Sangha RK, Walton JC, Ensor CM, Tai HH, Challis JR. Immunohistochemical localization, messenger ribonucleic acid abundance, and activity of 15-hydroxyprostaglandin dehydrogenase in placenta and fetal membranes during term and preterm labor. J Clin Endocrinol Metab 1994;78: 982-989.

99. Han X, Rossdale PD, Ousey J, Holdstock N, Allen WR, Silver M, Fowden AL, McGladdery AJ, Labrie F, Belanger A, et al. Localisation of 15-hydroxy prostaglandin dehydrogenase (PGDH) and steroidogenic enzymes in the equine placenta. Equine Vet J 1995;27: 334-339.

100. Harding R, Poore ER, Bailey A, Thorburn GD, Jansen CA, Nathanielsz PW. Electromyographic activity of the nonpregnant and pregnant sheep uterus. Am J Obstet Gyn 1982;142: 448-457.

101. Taverne MA, Scheerboom JE. Myometrial electrical activity during pregnancy and parturition in the pygmy goat. Res Vet Sci 1985;38: 120-123.

102. Taverne MA, Naaktgeboren C, Elsaesser F, Forsling ML, van der Weyden GC, Ellendorff F, Smidt D. Myometrial electrical activity and plasma concentrations of progesterone, estrogens and oxytocin during late pregnancy and parturition in the miniature pig. Biol Reprod 1979;21: 1125-1134.

103. Zerobin K, Sporri H. Motility of the bovine and porcine uterus and fallopian tube. Adv Vet Sci Comp Med 1972;16: 303-354.

104. van Engelen E, Taverne MA, Everts ME, van der Weijden GC, Doornenbal A, Breeveld-Dwarkasing VN. EMG activity of the muscular and stromal layer of the cervix in relation to EMG activity of the myometrium and cervical dilatation in PGF2alpha induced parturition in the cow. Theriogenology 2007;67: 1158-1167.

105. Germain G, Cabrol D, Visser A, Sureau C. Electrical activity of the pregnant uterus in the cynomolgus monkey. Am J Obstet Gyn 1982;142: 513-519.

106. Taylor NF, Martin MC, Nathanielsz PW, Seron-Ferre M. The fetus determines circadian oscillation of myometrial electromyographic activity in the pregnant rhesus monkey. Am J Obstet Gynecol 1983;146: 557-567.

107. Farber DM, Giussani DA, Jenkins SL, Mecenas CA, Winter JA, Wentworth RA, Nathanielsz PW. Timing of the switch from myometrial contractures to contractions in late-gestation pregnant rhesus monkeys as recorded by myometrial electromyogram during spontaneous term and androstenedione-induced labor. Biol Reprod 1997;56: 557-562.

108. Haluska GJ, Lowe JE, Currie WB. Electromyographic properties of the myometrium correlated with the endocrinology of the pre-partum and post-partum periods and parturition in pony mares. J Reprod Fertil Suppl 1987;35: 553-564.

109. McGlothlin JA, Lester GD, Hansen PJ, Thomas M, Pablo L, Hawkins DL, LeBlanc MM. Alteration in uterine contractility in mares with experimentally induced placentitis. Reproduction 2004;127: 57-66.

110. Thorburn GD. A speculative review of parturition in the mare. Equine Vet J Suppl 1993;(14): 41-49.

111. Thorburn GD, Challis JR, Currie WB. Control of parturition in domestic animals. Biol Reprod 1977;16: 18-27.

112. Thorburn GD, Challis JR. Endocrine control of parturition. Physiol Rev 1979;59: 863-918.

113. Linzell JL, Heap RB. A comparison of progesterone metabolism in the pregnant sheep and goat: sources of production and an estimation of uptake by some target organs. J Endocrinol 1968;41: 433-438.

114. Chavatte P, Rossdale PD, Tait AD. Cortiosteroid synthesis by the equine fetal adrenal. Biol Reprod Mono 1995;1: 13-20.

115. Albrecht ED, Pepe GJ. Effect of the antiestrogen ethamoxytriphetol (MER-25) and luteectomy on serum progesterone concentrations in pregnant baboons. Endocrinology 1984;115: 1717-1721.

116. Walsh SW, Stanczyk FZ, Novy MJ. Daily hormonal changes in the maternal, fetal, and amniotic fluid compartments before parturition in a primate species. J Clin Endocrinol Metab 1984;58: 629-639.

117. Haluska GJ, Cook MJ, Novy MJ. Inhibition and augmentation of progesterone production during pregnancy: effects on parturition in rhesus monkeys. Am J Obstet Gyn 1997;176: 682-691.

118. Holtan DW, Squires EL, Lapin DR, Ginther OJ. Effect of ovariectomy on pregnancy in mares. J Reprod Fertil Suppl 1979: 457-463.

119. Pashen RL, Allen WR. The role of the fetal gonads and placenta in steroid production, maintenance of pregnancy and parturition in the mare. J Reprod Fertil Suppl 1979: 499-509.

120. Short RV. Progesterone in blood. IV. Progesterone in the blood of mares. J Endocrinol 1959;19: 207-210.

121. Barnes RJ, Nathanielsz PW, Rossdale PD, Comline RS, Silver M. Plasma progestagens and oestrogens in fetus and mother in late pregnancy. J Reprod Fertil Suppl 1975: 617-623.

122. Ganjam VK, Kenney RM, Flickinger G. Plasma progestagens in cyclic, pregnant and post-partum mares. J Reprod Fertil Suppl 1975: 441-447.

123. Lovell JD, Stabenfeldt GH, Hughes JP, Evans JW. Endocrine patterns of the mare at term. J Reprod Fertil Suppl 1975: 449-456.

124. Holtan DW, Houghton E, Silver M, Fowden AL, Ousey J, Rossdale PD. Plasma progestagens in the mare, fetus and newborn foal. J Reprod Fertil Suppl 1991;44: 517-528.

125. Hamon M, Clarke SW, Houghton E, Fowden AL, Silver M, Rossdale PD, Ousey JC, Heap RB. Production of 5 alpha-dihydroprogesterone during late pregnancy in the mare. J Reprod Fertil Suppl 1991;44: 529-535.

126. Ousey JC, Forhead AJ, Rossdale PD, Grainger L, Houghton E, Fowden AL. Ontogeny of Uteroplacental Progestagen Production in Pregnant Mares During the Second Half of Gestation. Biol Reprod 2003;69: 540-548.

127. Rossdale PD, Ousey JC, Cottrill CM, Chavatte P, Allen WR, McGladdery AJ. Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. J Reprod Fertil Suppl 1991;44: 579-590.

128. Han X, Fowden AL, Silver M, Holdstock N, McGladdery AJ, Ousey JC, Allen WR, Rossdale PD, Challis JR. Immunohistochemical localisation of steroidogenic enzymes and phenylethanolamine-N-methyl-transferase (PNMT) in the adrenal gland of the fetal and newborn foal. Equine Vet J 1995;27: 140-146.

129. Chavatte PM, Rossdale PD, Tait AD. Modulation of 3 beta-hydroxysteroid dehydrogenase (3 beta-HSD) activity in the equine placenta by pregnenolone and progesterone metabolites. Equine Vet J 1995;27: 342-347.

130. Clapp JF, 3rd, Auletta FJ, Farnham J, Larrow RW, Mann LI. The ovine fetoplacental endocrine response to placental damage. Am J Obstet Gyn 1982;144: 47-54.

131. Ousey JC, Houghton E, Grainger L, Rossdale PD, Fowden AL. Progestagen profiles during the last trimester of gestation in Thoroughbred mares with normal or compromised pregnancies. Theriogenology 2005;63: 1844-1856.

132. Santschi EM, Slone DE, Gronwall R, Juzwiak JS, Moll HD. Types of colic and frequency of postcolic abortion in pregnant mares: 105 cases (1984-1988). J Am Vet Med Assoc 1991;199: 374-377.

133. Santschi EM, LeBlanc MM, Weston PG. Progestagen, oestrone sulphate and cortisol concentrations in pregnant mares during medical and surgical disease. J Reprod Fertil Suppl 1991;44: 627-634.

134. Frim DM, Emanuel RL, Robinson BG, Smas CM, Adler GK, Majzoub JA. Characterization and gestational regulation of corticotropin-releasing hormone messenger RNA in human placenta. J Clin Invest 1988;82: 287-292.

135. Korebrits C, Ramirez MM, Watson L, Brinkman E, Bocking AD, Challis JR. Maternal corticotropin-releasing hormone is increased with impending preterm birth. J Clin Endocrinol Metab 1998;83: 1585-1591.

136. Morris S, Kelleman AA, Stawicki RJ, Hansen PJ, Sheerin PC, Sheerin BR, Paccamonti DL, LeBlanc MM. Transrectal ultrasonography and plasma progestin profiles identifies feto-placental compromise in mares with experimentally induced placentitis. Theriogenology 2007;67: 681-691.

137. Bassett JM, Thorburn GD. Foetal plasma corticosteroids and the initiation of parturition in sheep. J Endocrinol 1969;44: 285-286.

138. Challis JR, Jones CT, Robinson JS, Thorburn GD. Development of fetal pituitary-adrenal function. J Steroid Biochem 1977;8: 471-478.

139. Liggins GC, Kennedy PC, Holm LW. Failure of initiation of parturition after electrocoagulation of the pituitary of the fetal lamb. Am J Obstet Gyn 1967;98: 1080-1086.

140. Drost M, Holm LW. Prolonged gestation in ewes after foetal adrenalectomy. J Endocrinol 1968;40: 293-296.

141. Liggins GC. Premature parturition after infusion of corticotrophin or cortisol into foetal lambs. J Endocrinol 1968;42: 323-329.

142. Silver M, Fowden A. Induction of labour in domestic animals: endocrine changes and neonatal viability. In: Jensen KW (ed), The Endocrine Control of the Fetus. Berlin, Springer-Verlag, 1988;401-411.

143. Fowden AL, Silver M. Adrenocortical activity in the fetal pig. J Physiol 1988;403: 124P.

144. Cudd TA, LeBlanc M, Silver M, Norman W, Madison J, Keller-Wood M, Wood CE. Ontogeny and ultradian rhythms of adrenocorticotropin and cortisol in the late-gestation fetal horse. J Endocrinol 1995;144: 271-283.

145. Liggins GC, Howie RN. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics 1972;50: 515-525.

146. Ballard PL, Ballard RA. Scientific basis and therapeutic regimens for use of antenatal glucocorticoids. Am J Obstet Gyn 1995;173: 254-262.

147. Gravett M, Hitti J, Hess D, Eschenbach D. Intrauterine infection and preterm delivery: Evidence for activation of the fetal hypothalamic-pituitary-adrenal axis. Am J Obstet Gyn 2000;182: 1404-1413.

148. Silver M, Fowden AL. Prepartum adrenocortical maturation in the fetal foal: responses to ACTH. J Endocrinol 1994;142: 417-425.

149. Ousey JC, Rossdale PD, Dudan FE, Fowden AL. The effects of intrafetal ACTH administration on the outcome of pregnancy in the mare. Reprod Fertil Dev 1998;10: 359-367.

150. Ousey JC, Rossdale PD, Palmer L, Grainger L, Houghton E. Effects of maternally administered depot $ACTH_{1-24}$ on fetal maturation and the timing of parturition in the mare. Equine Vet J 2000;32: 489-496.

151. Blois SM, Kammerer U, Alba Soto C, Tometten MC, Shaikly V, Barrientos G, Jurd R, Rukavina D, Thomson AW, Klapp BF, Fernandez N, Arck PC. Dendritic cells: key to fetal tolerance? Biol Reprod 2007;77: 590-598.

152. Evans MJ, Hamer JM, Gason LM, Graham CS, Asbury AC, Irvine CH. Clearance of bacteria and non-antigenic markers following intra-uterine inoculation into maiden mares: Effect of steroid hormone environment. Theriogenology 1986;26: 37-50.

153. LeBlanc MM, Asbury AC, Lyle SK. Uterine clearance mechanisms during the early postovulatory period in mares. Am J Vet Res 1989;50: 864-867.

154. Troedsson MH, Liu IK. Uterine clearance of non-antigenic markers (^{51}Cr) in response to a bacterial challenge in mares potentially susceptible and resistant to chronic uterine infections. J Reprod Fertil Suppl 1991;44: 283-288.

155. Troedsson MH, Liu IK, Ing M, Pascoe J, Thurmond M. Multiple site electromyography recordings of uterine activity following an intrauterine bacterial challenge in mares susceptible and resistant to chronic uterine infection. J Reprod Fertil 1993;99: 307-313.

156. Kenney RM, Kahleel SA. Bacteriostatic activity of the mare uterus: a progress report on immunoglobulins. J Reprod Fertil Suppl 1975: 357-358.

157. Asbury AC, Halliwell RE, Foster GW, Longino SJ. Immunoglobulins in uterine secretions of mares with differing resistance to endometritis. Theriogenology 1980;14: 299-308.

158. Mitchell G, Liu IK, Perryman LE, Stabenfeldt GH, Hughes JP. Preferential production and secretion of immunoglobulins by the equine endometrium - a mucosal immune system. J Reprod Fertil Suppl 1982;32: 161-168.

159. Williamson P, Dunning A, O'Connor J, Penhale WJ. Immunoglobulin levels, protein concentrations and alkaline phosphatase activity in uterine flushings from mares with endometritis. Theriogenology 1983;19: 441-448.

160. Tunón AM, Rodriguez-Martinez H, Hultén C, Nummijarvi A, Magnusson U. Concentrations of total protein, albumin and immunoglobulins in undiluted uterine fluid of gynecologically healthy mares. Theriogenology 1998;50: 821-831.

161. Widders PR, Stokes CR, David JS, Bourne FJ. Quantitation of the immunoglobulins in reproductive tract secretions of the mare. Res Vet Sci 1984;37: 324-330.

162. Troedsson MH, Liu IK, Thurmond M. Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of Streptococcus zooepidemicus in mares susceptible to versus resistant to chronic uterine infection. Biol Reprod 1993;49: 502-506.

163. Wagner B. Immunoglobulins and immunoglobulin genes of the horse. Dev Comp Immunol 2006;30: 155-164.

164. Watson ED, Stokes CR. Effect of susceptibility to endometritis on specific antibody in the endometria of mares. Theriogenology 1990;34: 39-45.

165. Widders PR, Stokes CR, David JSE, Bourne FJ. Effect of cyclic stage on immunoglobulin concentrations in reproductive tract secretions of the mare. J Reprod Immunol 1985;7: 233-242.

166. Widders PR, Stokes CR, David JS, Bourne FJ. Specific antibody in the equine genital tract following local immunisation and challenge infection with contagious equine metritis organism (Taylorella equigenitalis). Res Vet Sci 1986;40: 54-58.

167. Causey R, Weber J, Emmans E, Small P, Knapp K, Pelletier D. Immunoblotting of Streptococcus zooepidemicus antigens following intrauterine inoculation in mares. Theriogenology 2002;58: 487-489.

168. Causey RC, Paccamonti DL, Todd WJ. Antiphagocytic properties of uterine isolates of Streptococcus zooepidemicus and mechanisms of killing in freshly obtained blood of horses. Am J Vet Res 1995;56: 321-328.

169. Causey RC, Weber JA, Emmans EE, Stephenson LA, Homola AD, Knapp KR, Crowley IF, Pelletier DC, Wooley NA. The equine immune response to Streptococcus equi subspecies zooepidemicus during uterine infection. Vet J 2006;172: 248-257.

170. LeBlanc M, Ward L, Tran T, Widders P. Identification and opsonic activity of immunoglobulins recognizing Streptococcus zooepidemicus antigens in uterine fluids of mares. J Reprod Fertil Suppl 1991;44: 289-296.

171. Boyle MDP. Complement activation and bacterial immunoglobulin-binding proteins. In: Boyle MDP (ed), Bacterial Immunoglobulin-Binding Proteins. Volume 1. Microbiology, Chemistry, and Biology, Academic Press, 1990;295-304.

172. Lyle SK. Investigations on the uterine environment of the mare and its relationship to defense mechanisms. Masters Thesis,Veterinary Medicine, University of Florida, Gainesville, FL, 1991.

173. Strzemienski PJ, Do D, Kenney RM. Antibacterial activity of mare uterine fluid. Biol Reprod 1984;31: 303-311.

174. Watson ED. Effect of ovarian steroids on migration of uterine lumenal neutrophils and on chemokinetic factors in uterine secretions from mares. Equine Vet J 1988;20: 368-370.

175. Blue HB, Blue MG, Kenney RM, Merritt TL. Chemotactic properties and protein of equine uterine fluid. Am J Vet Res 1984;45: 1205-1208.

176. Troedsson MHT, Steiger BN, Ibrahim NM, King VL, Foster DN, Crabo BG. Mechanism of sperm-induced endometritis in the mare. Biol Reprod, Suppl 1995;52: 307.

177. Watson ED, Stokes CR, Bourne FJ. Cellular and humoral defence mechanisms in mares susceptible and resistant to persistent endometritis. Veterinary Immunology and Immunopathology 1987;16: 107-121.

178. Troedsson MH, Liu IK, Thurmond M. Function of uterine and blood-derived polymorphonuclear neutrophils in mares susceptible and resistant to chronic uterine infection: phagocytosis and chemotaxis. Biol Reprod 1993;49: 507-514.

179. Pycock JF, Allen WE. Pre-chemotactic and chemotactic properties of uterine fluid from mares with experimentally induced endometritis. Vet Rec 1988;123: 193-195.

180. Pycock JF, Allen WE. Equine neutrophil locomotion in response to Streptococcus zooepidemicus. Equine Vet J 1988;20: 448-450.

181. Asbury AC. Uterine defense mechanisms in the mare: The use of intrauterine plasma in the management of endometritis. Theriogenology 1984;21: 387-393.

182. Asbury AC, Gorman NT, Foster GW. Uterine defense mechanisms in the mare: Serum opsonins affecting phagocytosis of Streptococcus zooepidemicus by equine neutrophils. Theriogenology 1984;21: 375-385.

183. Blue MG, Brady AA, Davidson JN, Kenney RM. Studies on the composition and antibacterial activity of uterine fluid from mares. J Reprod Fertil Suppl 1982;32: 143-149.

184. Hansen PJ, Asbury AC. Opsonins of Streptococcus in uterine flushings of mares susceptible and resistant to endometritis: control of secretion and partial characterization. Am J Vet Res 1987;48: 646-650.

185. Watson ED. The influence of estrogen and progesterone on antibody synthesis by the endometrium of the mare, Equine Infectious Diseases V. Lexington, KY, The University Press of Kentucky, 1987;181-185.

186. Watson ED. Opsonins in uterine washings influencing in vitro activity of equine neutrophils. Equine veterinary journal 1988;20: 435-437.

187. Johnson JU, Oxender WD, Berkhoff HA. Influence of estrogen on antibacterial and immunoglobulin secretory activities of uterine fluids from ovariectomized mares. Am J Vet Res 1994;55: 643-649.

188. Kolm G, Klein D, Knapp E, Watanabe K, Walter I. Lactoferrin expression in the horse endometrium: relevance in persisting mating-induced endometritis. Vet Immunol Immunopathol 2006;114: 159-167.

189. Watson ED, Stokes CR, David JS, Bourne FJ, Ricketts SW. Concentrations of uterine luminal prostaglandins in mares with acute and persistent endometritis. Equine Vet J 1987;19: 31-37.

190. Watson ED, Stokes CR, Bourne FJ. Concentrations of immunoreactive leukotriene B4 in uterine lavage fluid from mares with experimentally induced and naturally occurring endometritis. J Vet Pharmacol Ther 1988;11: 130-134.

191. Watson ED, Stokes CR, Bourne FJ. Effect of exogenous ovarian steroids on the uterine luminal prostaglandins in ovariectomised mares with experimental endometritis. Res Vet Sci 1988;44: 361-365.

192. Nikolakopoulos E, Kindahl H, Watson ED. Oxytocin and $PGF_{2\alpha}$ in mares resistant and susceptible to persistent mating-induced endometritis. J Reprod Fertil Suppl 2000;56: 363-372.

193. Watson ED, Stokes CR, Bourne FJ. Influence of arachidonic acid metabolites in vitro and in uterine washings on migration of equine neutrophils under agarose. Res Vet Sci 1987;43: 203-207.

194. Janeway CA, Travers P, Walport M, Shlomchik MJ. Immunobiology: the immune system in health and disease, 6th edition. New York: Garland Science Publishing, 2005;43-44.

195. Demirkoprulu N, Cetin M, Bagcivan I, Kaya T, Soydan AS, Karadas B, Cetin A. Comparative relaxant effects of YC-1 and DETA/NO on spontaneous contractions and the levels of cGMP of isolated pregnant rat myometrium. Eur J Pharmacol 2005;517: 240-245.

196. Alghamdi AS, Troedsson MHT. Concentration of nitric oxide in uterine secretion from mares susceptible and resistant to chronic post-breeding endometritis. Theriogenology 2002;58: 445-448.

197. Liu IK, Cheung AT, Walsh EM, Miller ME, Lindenberg PM. Comparison of peripheral blood and uterine-derived polymorphonuclear leukocytes from mares resistant and susceptible to chronic endometritis: chemotactic and cell elastimetry analysis. Am J Vet Res 1985;46: 917-920.

198. Liu IK, Cheung AT, Walsh EM, Ayin S. The functional competence of uterine-derived polymorphonuclear neutrophils (PMN) from mares resistant and susceptible to chronic uterine infection: a sequential migration analysis. Biol Reprod 1986;35: 1168-1174.

199. Cheung AT, Liu IK, Walsh EM, Miller ME. Phagocytic and killing capacities of uterine-derived polymorphonuclear leukocytes from mares resistant and susceptible to chronic endometritis. Am J Vet Res 1985;46: 1938-1940.

200. Asbury AC, Hansen PJ. Effects of susceptibility of mares to endometritis and stage of cycle on phagocytic activity of uterine-derived neutrophils. J Reprod Fertil Suppl 1987;35: 311-316.

201. Zerbe H, Engelke F, Klug E, Schoon HA, Leibold W. Degenerative endometrial changes do not change the functional capacity of immigrating uterine neutrophils in mares. Reprod Domest Anim 2004;39: 94-98.

202. Watson ED, Dixon CE. An immunohistological study of MHC class II expression and T lymphocytes in the endometrium of the mare. Equine Vet J 1993;25: 120-124.

203. Frayne J, Stokes CR. MHC Class II positive cells and T cells in the equine endometrium throughout the oestrous cycle. Vet Immunol Immunopathol 1994;41: 55-72.

204. Watson ED, Thomson SR. Lymphocyte subsets in the endometrium of genitally normal mares and mares susceptible to endometritis. Equine Vet J 1996;28: 106-110.

205. Tunón AM, Katila T, Magnusson U, Nummijärvi A, Rodriguez-Martinez H. T-cell distribution in two different segments of the equine endometrium 6 and 48 hours after insemination. Theriogenology 2000;54: 835-841.

206. Tunon AM, Rodriguez-Martinez H, Nummijarvi A, Magnusson U. Influence of age and parity on the distribution of cells expressing major histocompatibility complex class II, CD4, or CD8 molecules in the endometrium of mares during estrus. Am J Vet Res 1999;60: 1531-1535.

207. Kindt TJ, Goldsby RA, Osborne BA. Kuby Immunology, 6th edition edition. New York: W.H. Freeman & Co., 2007;189-222.

208. Wick MJ. Monocyte and dendritic cell recruitment and activation during oral Salmonella infection. Immunol Lett 2007;112: 68-74.

209. Fumuso E, Giguere S, Wade J, Rogan D, Videla-Dorna I, Bowden RA. Endometrial IL-1beta, IL-6 and TNF-alpha, mRNA expression in mares resistant or susceptible to post-breeding endometritis. Effects of estrous cycle, artificial insemination and immunomodulation. Vet Immunol Immunopathol 2003;96: 31-41.

210. Fumuso EA, Aguilar J, Giguere S, Rivulgo M, Wade J, Rogan D. Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: effects of immunomodulation. Vet Immunol Immunopathol 2007;118: 30-39.

211. Causey RC. Mucus and the mare: how little we know. Theriogenology 2007;68: 386-394.

212. Causey RC, Ginn PS, Katz BP, Hall BJ, Anderson KJ, LeBlanc MM. Mucus production by endometrium of reproductively healthy mares and mares with delayed clearance. J Reprod Fert Suppl 2000;56: 333-229.

213. Freeman KP, Roszel JF, Slusher SH, Castro M. Variation in glycogen and mucins in the equine uterus related to physiologic and pathologic conditions. Theriogenology 1990;33: 799-808.

214. Troedsson MH, Liu IK. Measurement of total volume and protein concentration of intrauterine secretion after intrauterine inoculation of bacteria in mares that were either resistant or susceptible to chronic uterine infection. Am J Vet Res 1992;53: 1641-1644.

215. Al-Bagdadi FK, Eilts BE, Richardson GF. Scanning electron microscopy of the endometrium of mares infused with gentamicin. Microsc Microanal 2004;10: 280-285.

216. Pycock JF, Allen WE. Inflammatory components in uterine fluid from mares with experimentally induced bacterial endometritis. Equine Vet J 1990;22: 422-425.

217. Dimock WW, Edwards PR. Genital infection in mares by an organism of the encapsulatus group. J Am Vet Med Assoc 1927;70: 469-480.

218. Hamilton BE, Minino AM, Martin JA, Kochanek KD, Strobino DM, Guyer B. Annual summary of vital statistics: 2005. Pediatrics 2007;119: 345-360.

219. Goldenberg RL, Thompson C. The infectious origins of stillbirth. Am J Obstet Gynecol 2003;189: 861-873.

220. Klein LL, Freitag BC, Gibbs RS, Reddy AP, Nagalla SR, Gravett MG. Detection of intra-amniotic infection in a rabbit model by proteomics-based amniotic fluid analysis. Am J Obstet Gyn 2005;193: 1302-1306.

221. Norwitz ER, Robinson JN, Challis JR. The control of labor. N Engl J Med 1999;341: 660-666.

222. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and Paracrine Regulation of Birth at Term and Preterm. Endocr Rev 2000;21: 514-550.

223. McDuffie RS, Jr., Sherman MP, Gibbs RS. Amniotic fluid tumor necrosis factor-alpha and interleukin-1 in a rabbit model of bacterially induced preterm pregnancy loss. Am J Obstet Gyn 1992;167: 1583-1588.

224. Gravett MG, Witkin SS, Haluska GJ, Edwards JL, Cook MJ, Novy MJ. An experimental model for intraamniotic infection and preterm labor in rhesus monkeys. Am J Obstet Gynecol 1994;171: 1660-1667.

225. Romero R, Mazor M, Brandt F, Sepulveda W, Avila C, Cotton DB, Dinarello CA. Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. Am J Reprod Immunol 1992;27: 117-123.

226. Reznikov LL, Fantuzzi G, Selzman CH, Shames BD, Barton HA, Bell H, McGregor JA, Dinarello CA. Utilization of endoscopic inoculation in a mouse model of intrauterine infection-induced preterm birth: role of interleukin 1beta. Biol Reprod 1999;60: 1231-1238.

227. Romero R, Gomez R, Ghezzi F, Yoon B, Mazor M, Edwin S, Berry S. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. Am J Obstet Gynecol 1998;179: 186-193.

228. Grigsby PL, Hirst JJ, Scheerlinck JP, Phillips DJ, Jenkin G. Fetal responses to maternal and intra-amniotic lipopolysaccharide administration in sheep. Biol Reprod 2003;68: 1695-1702.

229. Gendron RL, Nestel FP, Lapp WS, Baines MG. Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor-alpha. J Reprod Fertil 1990;90: 395-402.

230. Schlafer DH, Yuh B, Foley GL, Elssaser TH, Sadowsky D, Nathanielsz PW. Effect of Salmonella endotoxin administered to the pregnant sheep at 133-142 days gestation on fetal oxygenation, maternal and fetal adrenocorticotropic hormone and cortisol, and maternal plasma tumor necrosis factor alpha concentrations. Biol Reprod 1994;50: 1297-1302.

231. Gross G, Imamura T, Vogt SK, Wozniak DF, Nelson DM, Sadovsky Y, Muglia LJ. Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. Am J Physiol Regul Integr Comp Physiol 2000;278: R1415-1423.

232. Buhimschi IA, Buhimschi CS, Weiner CP. Protective effect of N-acetylcysteine against fetal death and preterm labor induced by maternal inflammation. Am J Obstet Gyn 2003;188: 203-208.

233. Elovitz MA, Wang Z, Chien EK, Rychlik DF, Phillippe M. A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. Am J Pathol 2003;163: 2103-2111.

234. Gibb W, Challis JR. Mechanisms of term and preterm birth. J Obstet Gynaecol Can 2002;24: 874-883.

235. Keelan JA, Sato T, Mitchell MD. Interleukin (IL)-6 and IL-8 production by human amnion: regulation by cytokines, growth factors, glucocorticoids, phorbol esters, and bacterial lipopolysaccharide. Biol Reprod 1997;57: 1438-1444.

236. Gravett M, Hitti J, Hess D, Eschenbach D. Intrauterine infection and preterm delivery: Evidence for activation of the fetal hypothalamic-pituitary-adrenal axis. Am J Obstet Gynecol 2000;182: 1404-1413.

237. LeBlanc MM, Giguere S, Brauer K, Paccamonti DL, Horohov DW, Lester GD, O'Donnell LJ, Sheerin BR, Pablo L, Rodgerson DH. Premature delivery in ascending placentitis is associated with increased expression of placental cytokines and allantoic fluid prostaglandins E_2 and $F_{2\alpha}$. Theriogenology 2002;58: 841-844.

238. Romero R, Gomez R, Ghezzi F, Yoon B, Mazor M, Edwin S, Berry S. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. American Journal of Obstetrics and Gynecology 1998;179: 186-193.

239. Holdstock NB, McGladdery AJ, Ousey J, Rossdale PD. Assessing methods of collection and changes of selected biochemical constituents in amniotic and allantoic fluid throughout equine pregnancy. Biol Reprod 1995: 21-38.

240. Paccamonti D, Swiderski CE, Marx B, Gaunt S, Blouin D. Electrolytes and biochemical enzymes in amniotic and allantoic fluid of the equine fetus during late gestation. Biol Reprod 1995;Mono Ser I: 39-48.

241. Ousey JC. Peripartal endocrinology in the mare and foetus. Reprod Domest Anim 2004;39: 222-231.

242. Lyle SK, Paccamonti DL, Hubert JD, Schlafer DH, Causey RC, Eilts BE, Johnson JR. Laparoscopic placement of an indwelling catheter in the mare: Biochemical, cytologic, histologic, and microbiologic findings. Anim Reprod Sci 2006;94: 428-431.

243. McDuffie RS, Jr., Sherman MP, Gibbs RS. Amniotic fluid tumor necrosis factor-alpha and interleukin-1 in a rabbit model of bacterially induced preterm pregnancy loss. Amer J Obstet Gynecol 1992;167: 1583-1588.

244. Macpherson ML. Diagnosis and treatment of equine placentitis. Vet Clin North Am Equine Pract 2006;22: 763-776.

245. Adams-Brendemuehl C, Pipers FS. Antepartum evaluations of the equine fetus. J Reprod Fertil Suppl 1987;35: 565-573.

246. Renaudin CD, Troedsson M, Gillis CL. Transrectal ultrasonographic evaluation of the normal equine placenta. Equine Vet Educ 1999;11: 75-76.

247. Niemantsverdriet-Murton AS, Paccamonti DL, Eilts BE, Pinto CF. Prostaglandin E1 as an adjunct to prostaglandin F2? induced abortion in mares. Proc Soc Theriogenol 2001: 28.

248. Bucca S, Fogarty U, Collins A, Small V. Assessment of feto-placental well-being in the mare from mid- gestation to term: Transrectal and transabdominal ultrasonographic features. Theriogenology 2005;64: 542-557.

249. Kelleman AA, Luznar SL, Lester GD, Paccamonti DL, LeBlanc MM. Evaluation of transrectal ultrasonographic combined thickness of the uterus and placenta (CTUP) in a model of induced ascending placentitis in late gestation in the pony mare. Theriogenology 2002;58: 845-848.

250. Calderwood Mays MB, LeBlanc MM, Paccamonti D. Route of fetal infection in a model of ascending placentitis. Theriogenology 2002;58: 791-792.

251. Elovitz MA, Mrinalini C. Animal models of preterm birth. Trends Endocrinol Metab 2004;15: 479-487.

252. Gravett M, Hitti J, Hess D, Eschenbach D. Intrauterine infection and preterm delivery: Evidence for activation of the fetal hypothalamic-pituitary-adrenal axis. Am J Obstet Gynecol 2000;182: 1404-1413.

253. Vick MM, Adams AA, Murphy BA, Sessions DR, Horohov DW, Cook RF, Shelton BJ, Fitzgerald BP. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J Anim Sci 2007;85: 1144-1155.

254. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques 2004;37: 112-119.

255. Aerts JL, Gonzales MI, Topalian SL. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. BioTechniques 2004;36: 84-91.

256. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 2003;339: 62-66.

257. Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. J Mol Med 2006;84: 901-910.

258. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25: 402-408.

259. Barksby HE, Lea SR, Preshaw PM, Taylor JJ. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 2007;149: 217-225.

260. Dinarello CA, Thompson RC. Blocking IL-1: interleukin 1 receptor antagonist in vivo and in vitro. Immunol Today 1991;12: 404-410.

261. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 1996;87: 2095-2147.

262. Reznikov LL, Kim SH, Westcott JY, Frishman J, Fantuzzi G, Novick D, Rubinstein M, Dinarello CA. IL-18 binding protein increases spontaneous and IL-1-induced prostaglandin production via inhibition of IFN-gamma. Proc Natl Acad Sci U S A 2000;97: 2174-2179.

263. Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ. Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. Am J Obstet Gyn 2006;195: 1578-1589.

264. Romero R, Mazor M, Tartakovsky B. Systemic administration of interleukin-1 induces preterm parturition in mice. Am J Obstet Gyn 1991;165: 969-971.

265. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124: 783-801.

266. Parnet P, Garka KE, Bonnert TP, Dower SK, Sims JE. IL-1Rrp is a novel receptor-like molecule similar to the type I interleukin-1 receptor and its homologues T1/ST2 and IL-1R AcP. J Biol Chem 1996;271: 3967-3970.

267. Lee JK, Kim SH, Lewis EC, Azam T, Reznikov LL, Dinarello CA. Differences in signaling pathways by IL-1beta and IL-18. Proc Natl Acad Sci U S A 2004;101: 8815-8820.

268. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. Annu Rev Immunol 2001;19: 423-474.

269. Pacora P, Romero R, Maymon E, Gervasi MT, Gomez R, Edwin SS, Yoon BH. Participation of the novel cytokine interleukin 18 in the host response to intra-amniotic infection. Am J Obstet Gyn 2000;183: 1138-1143.

270. Jacobsson B, Holst RM, Mattsby-Baltzer I, Nikolaitchouk N, Wennerholm UB, Hagberg H. Interleukin-18 in cervical mucus and amniotic fluid: relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation and preterm delivery. Bjog 2003;110: 598-603.

271. Wang X, Hagberg H, Mallard C, Zhu C, Hedtjarn M, Tiger CF, Eriksson K, Rosen A, Jacobsson B. Disruption of interleukin-18, but not interleukin-1, increases vulnerability to preterm delivery and fetal mortality after intrauterine inflammation. Am J Pathol 2006;169: 967-976.

272. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 2004;75: 163-189.

273. El-Shazly S, Makhseed M, Azizieh F, Raghupathy R. Increased expression of pro-inflammatory cytokines in placentas of women undergoing spontaneous preterm delivery or premature rupture of membranes. Am J Reprod Immunol 2004;52: 45-52.

274. Mollace V, Muscoli C, Masini E, Cuzzocrea S, Salvemini D. Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. Pharmacol Rev 2005;57: 217-252.

275. Maul H, Longo M, Saade GR, Garfield RE. Nitric oxide and its role during pregnancy: from ovulation to delivery. Curr Pharm Des 2003;9: 359-380.

276. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 1994;298 (Pt 2): 249-258.

277. Tschugguel W, Schneeberger C, Lass H, Stonek F, Zaghlula MB, Czerwenka K, Schatten C, Kaider A, Husslein P, Huber JC. Human cervical ripening is associated with an increase in cervical inducible nitric oxide synthase expression. Biol Reprod 1999;60: 1367-1372.

278. Ledingham MA, Thomson AJ, Young A, Macara LM, Greer IA, Norman JE. Changes in the expression of nitric oxide synthase in the human uterine cervix during pregnancy and parturition. Mol Hum Reprod 2000;6: 1041-1048.

279. Marinoni E, Di Iorio R, Villaccio B, Alberini A, Rota F, Cosmi EV. Amniotic fluid nitric oxide metabolite levels and nitric oxide synthase localization in feto-placental tissues are modified in association with human labor. Eur J Obstet Gynecol Reprod Biol 2000;89: 47-54.

280. Paintlia MK, Paintlia AS, Singh AK, Singh I. Attenuation of Lipopolysaccharide Induced Inflammatory Response and Phospholipids Metabolism at the Feto-Maternal Interface by N-Acetyl-Cysteine. Pediatr Res 2008.

281. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997;15: 323-350.

282. Baggiolini M, Moser B, Clark-Lewis I. Interleukin-8 and related chemotactic cytokines. The Giles Filley Lecture. Chest 1994;105: 95S-98S.

283. Sozzani S, Locati M, Zhou D, Rieppi M, Luini W, Lamorte G, Bianchi G, Polentarutti N, Allavena P, Mantovani A. Receptors, signal transduction, and spectrum of action of monocyte chemotactic protein-1 and related chemokines. J Leukoc Biol 1995;57: 788-794.

284. Esplin MS, Romero R, Chaiworapongsa T, Kim YM, Edwin S, Gomez R, Mazor M, Adashi EY. Monocyte chemotactic protein-1 is increased in the amniotic fluid of women who deliver preterm in the presence or absence of intra-amniotic infection. J Matern Fetal Neonatal Med 2005;17: 365-373.

285. Tornblom SA, Klimaviciute A, Bystrom B, Chromek M, Brauner A, Ekman-Ordeberg G. Non-infected preterm parturition is related to increased concentrations of IL-6, IL-8 and MCP-1 in human cervix. Reprod Biol Endocrinol 2005;3: 39.

286. Diamond AK, Sweet LM, Oppenheimer KH, Bradley DF, Phillippe M. Modulation of monocyte chemotactic protein-1 expression during lipopolysaccharide-induced preterm delivery in the pregnant mouse. Reprod Sci 2007;14: 548-559.

287. Silver M, Barnes RJ, Comline RS, Fowden AL, Clover L, Mitchell MD. Prostaglandins in maternal and fetal plasma and in allantoic fluid during the second half of gestation in the mare. J Reprod Fertil Suppl 1979: 531-539.

288. Giannoulias D, Haluska GJ, Gravett MG, Sadowsky DW, Challis JR, Novy MJ. Localization of prostaglandin H synthase, prostaglandin dehydrogenase, corticotropin releasing hormone and glucocorticoid receptor in rhesus monkey fetal membranes with labor and in the presence of infection. Placenta 2005;26: 289-297.

289. Denison FC, Calder AA, Kelly RW. The action of prostaglandin E2 on the human cervix: stimulation of interleukin 8 and inhibition of secretory leukocyte protease inhibitor. Am J Obstet Gynecol 1999;180: 614-620.

290. Lundin-Schiller S, Mitchell MD. Prostaglandin production by human chorion laeve cells in response to inflammatory mediators. Placenta 1991;12: 353-363.

291. Rauk PN, Chiao JP. Interleukin-1 stimulates human uterine prostaglandin production through induction of cyclooxygenase-2 expression. Am J Reprod Immunol 2000;43: 152-159.

292. Boerboom D, Brown KA, Vaillancourt D, Poitras P, Goff AK, Watanabe K, Dore M, Sirois J. Expression of Key Prostaglandin Synthases in Equine Endometrium during Late Diestrus and Early Pregnancy. Biol Reprod 2004;70: 391-399.

293. Blikslager AT, Yin C, Cochran AM, Wooten JG, Pettigrew A, Belknap JK. Cyclooxygenase expression in the early stages of equine laminitis: a cytologic study. J Vet Intern Med 2006;20: 1191-1196.

294. Macpherson ML. Treatment strategies for mares with placentitis. Theriogenology 2005;64: 528-534.

295. Rac VE, Scott CA, Small C, Adamson SL, Rurak D, Challis JR, Lye SJ. Dose-dependent effects of meloxicam administration on cyclooxygenase-1 and cyclooxygenase-2 protein expression in intrauterine tissues and fetal tissues of a sheep model of preterm labor. Reprod Sci 2007;14: 750-764.

296. Rac VE, Small C, Scott CA, Adamson SL, Rurak D, Challis JR, Lye SJ. Meloxicam effectively inhibits preterm labor uterine contractions in a chronically catheterized pregnant sheep model: impact on fetal blood flow and fetal-maternal physiologic parameters. Am J Obstet Gynecol 2006;195: 528-534.

297. Ousey JC, Rossdalet PD, Palmer L, Grainger L, Houghton E. Effects of maternally administered depot $ACTH_{1-24}$ on fetal maturation and the timing of parturition in the mare. Equine Vet J 2000;32: 489-496.

298. Alm CC, Sullivan JJ, First NL. The effect of a corticosteroid (dexamethasone), progesterone, oestrogen and prostaglandin F2alpha on gestation length in normal and ovariectomized mares. J Reprod Fertil Suppl 1975: 637-640.

299. Ousey JC. Hormone profiles and treatments in the late pregnant mare. Vet Clin North Am Equine Pract 2006;22: 727-747.

300. Jeffcott LB, Rossdale PD. A critical review of current methods for induction of parturition in the mare. Equine Vet J 1977;9: 208-215.

301. Christiansen D, Olsen G, Smith J, Hopper R, LeBlanc MM, Ryan P. The use of betamethasone to advance fetal maturation in the equine. Havemeyer Foundation Monograph Series 2008: 19-20.

302. Ryan P, Crouch J, Sykes D, Moulton K, Christiansen D, Hopper R, Read R, Bennett W, LeBlanc MM. Experimentally induced placentitis in late gestation mares with Streptococcus equi zooepidemicus: prevention of pre-term birth, 2005;35-36.

303. Kitchalong L, Fernandez JM, Bunting LD, Southern LL, Bidner TD. Influence of chromium tripicolinate on glucose metabolism and nutrient partitioning in growing lambs. J Anim Sci 1995;73: 2694-2705.

304. Cartmill JA, Thompson DL, Jr., Storer WA, Gentry LR, Huff NK. Endocrine responses in mares and geldings with high body condition scores grouped by high vs. low resting leptin concentrations. J Anim Sci 2003;81: 2311-2321.

305. Ye X, Acharya R, Herbert JB, Hamilton SE, Folkesson HG. IL-1beta stimulates alveolar fluid absorption in fetal guinea pig lungs via the hypothalamus-pituitary-adrenal gland axis. Am J Physiol Lung Cell Mol Physiol 2004;286: L756-766.

306. Li T, Varadarajulu S, Beard LL, Yun J, Folkesson HG. A noninflammatory interleukin-1beta fragment stimulates fetal lung fluid absorption in guinea pigs. J Pharmacol Exp Ther 2007;320: 877-884.

307. Young IR, Deayton JM, Hollingworth SA, Thorburn GD. Continuous intrafetal infusion of prostaglandin E2 prematurely activates the hypothalamo-pituitary-adrenal axis and induces parturition in sheep. Endocrinology 1996;137: 2424-2431.

308. Hollingworth SA, Deayton JM, Young IR, Thorburn GD. Prostaglandin E2 administered to fetal sheep increases the plasma concentration of adrenocorticotropin (ACTH) and the proportion of ACTH in low molecular weight forms. Endocrinology 1995;136: 1233-1240.

309. McKeown KJ, Challis JR, Small C, Adamson L, Bocking AD, Fraser M, Rurak D, Riggs KW, Lye SJ. Altered fetal pituitary-adrenal function in the ovine fetus treated with RU486 and meloxicam, an inhibitor of prostaglandin synthase-II. Biol Reprod 2000;63: 1899-1904.

310. Brooks AN, Gibson F. Prostaglandin E2 enhances AVP-stimulated but not CRF-stimulated ACTH secretion from cultured fetal sheep pituitary cells. J Endocrinol 1992;132: 33-38.

311. Reimsnider SK, Wood CE. Does reduction of circulating prostaglandin E2 reduce fetal hypothalamic-pituitary-adrenal axis activity? J Soc Gynecol Investig 2005;12: e13-19.

312. Yang K. Placental 11 beta-hydroxysteroid dehydrogenase: barrier to maternal glucocorticoids. Rev Reprod 1997;2: 129-132.

313. Burton PJ, Waddell BJ. Dual function of 11beta-hydroxysteroid dehydrogenase in placenta: modulating placental glucocorticoid passage and local steroid action. Biol Reprod 1999;60: 234-240.

314. Seckl JR, Meaney MJ. Glucocorticoid programming. Ann N Y Acad Sci 2004;1032: 63-84.

315. Ganjam VK, Evans TJ. Equine endometrial fibrosis correlates with 11beta-HSD2, TGF-beta1 and ACE activities. Mol Cell Endocrinol 2006;248: 104-108.

316. Renaudin CD, Liu-IKM, Troedsson-MHT, Schrenzel-MD, (ed.) R-P, (ed.) M-T, Green-RE. Transrectal ultrasonographic diagnosis of ascending placentitis in the mare: a report of two cases. Equine Vet Educ 1999;11: 69-74.

317. Ginther OJ. Reproductive Biology of the Mare. Cross Plains, WI, Equisciences, 1992,377-378.

318. Bain FT, Wolfsdorf KE. Placental Hydrops. In: Robinson NE (ed), Current Therapy in Equine Medicine. St. Louis, Missouri, Saunders, 2003;301-302.

APPENDIX
HISTOPATHOLOGY OF PLACENTAL AND FETAL TISSUE

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
25 / SHAM						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	Chorio-all: norm	Diffuse villous atrophy with stromal mineralization	1+										Squamous metaplasia of trophoblast, some synctia	Mild diffuse placental degeneration with loss of villous tips (though labeled normal, there are notable microscopic changes in these tissues..
B	Cervical Star; chorio-all	Mild vasculitis with squamous metaplasia of trophoblast			1+			1+		1+		2+	Mild squamous metaplasia	Mild subacute vasculitis; mild placentitis
C	Chorio-all at cather site	Marked nodular cystic allantoic dysplasia								1+	1+			Marked cystic allantoic dysplasia, microcotyledonary atrophy
D	lung	Acute fetal pneumonia		None seen on H+E	1+	2+		1+			2+			Diffuse neutrophilic pneumonia
E	Chorio-all:abn	Marked nodular cystic allantoic dysplasia	2+ (focal)		2+			2+			1+		Mild squamous metaplasia	Marked cystic allantoic dysplasia, ulceration of allantois with intense focal placentitis - mixed inflammatory infiltrate admixed with keratin (apparent breechment of amnion - these are fetal cutaneous squames; microcotyledonary atrophy
F*	amniotic UC	Severe chronic ulcerative funicitis; granulomatous amnion nodosum	3+		3+	3+				2+	3+			Severe necrotizing funicitis, limited to superficial 1/3 of tissue below ulcerated amnion; embedded kerating within a granulomatous inflammatory cirumferential band.
G	Amnion	Severe locally extensive suppurative amnionitis	3+		3+	3+					1+			Severe chronic locally extensive suppurative amnionitis; vasculitis

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
						PMNs	Lymphs	Mixed	Edema					
847 / SHAM														
A														
	lung	Severe widespread non-suppurative pneumonia	1+	1+ (intracellular)	3+			3+ (With scattered giant cells)						Severe bronchopneumonia. There is marked mononuclear cell exudate accumulated within the larger airways and alveolar lumen. This is an unusual inflammatory cell infiltrate as there are relatively few neutrophils.
B*	Chorio-all at cather site	Chronic bacterial placentitis	1+	2+	2+	1+					1+		Trophoblast cell phagocytosis of large cocci (Staph?)	Marked focal bacterial placentitis
C	Cervical Star	Necrosis of cotyledonary villi with no significant inflammation	2+										Trophoblast necrosis	Locally extensive placental degeneration and villous necrosis
D	Chorio-all: norm	Mild alllantoic cystic dysplasia											Autolytic sloughing from tips of villi; mild squamous metaplasia	
E	amnion	Fibrin adhere to surface												
	amniotic UC	Severe necrotizing funicitis	2+	1+ (large cocci)	2+			2+						Subacute necrotizing funicitis with vascular thrombosis, scattered cocci on surface of cord
F	allantoic UC	Fibrin deposits within coelomic spaces												

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
762 / SHAM						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	Liv - Lung	Aerated lung!												The foal breathed
B	Chorio-all: abn	Placental edema, Squamous metaplasia of trophoblast cells, villous atrophy							3+					Moderate diffuse placenta edema and degeneration
C	Chorio-all: norm	Moderate placental edema							2+					
D	UC - amn									1+				Mild edema of amnion, no UC changes
E*	Cervical Star	Vacuolar degeneration of trophoblastic cells; marked edema							3+	1+				The dramatic vacuolization of trophoblast cytoplasm is unusal:interpreted as a degenerative change (perhaps in response to experimental exposure to ??)
F	UC - all	Cord hematoma								3+				
G	Chorio-all at cather site	Extensive allantoic edema, moderate acute hemorhage, allantoic cystic dysplasia												Chronic focal reactive placental changes. No significant inflammation other than the edema.

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
790 / PBS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	Chorio-all: norm	Placentitis	Necrosis and mineralization of individual trophoblast cells		2+			2+	1+				Reactive; hyperplastic trophoblast cells	Subacute placentits with mild to moderate allantoic stromal infiltration by macrophages and neutrophils; focal stromal mineralization
B	Cervical Star	edematous with mild placentitis						1+	2+					
C	Chorio-all at cather site	Infarction-transmural necrosis; intralesional bacteria	3+	3+	1+									Vascular thrombi, transmural infarction; coccoid bacteria in great abundance
D	liver	Congestion and hemorrhae								2+				
	lung	Pneumonia		1+	1+			1+		1+ with marked congestion				Diffuse bronchopneumonia, many alveolar macrophages contain bacteria
E	Chorio-all:abn	Chorionic villous atrophy and necrosis; non-suppurative placentitis	1+ fibrinoid necrosis of walls of some vessels		1+			1+				1+		Non-suppurative placentitis with locally extensive chorionic villous atrophy and necrosis.
F	UC	Funicitis; Amnionic segment		2+	2+			2+		3+				Moderate hemorrhagic necrotizing funicitis
G	allantoic UC													Fibrin on surface of umbilical vessel (fibrinous funicitis)

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation				Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
781/PBS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	Chorio-all: norm	Mild placental degeneration; vascular mineralization	1+	1+						1+			Villous stromal mineralization with scattered troph necrosis	
B	Cervical Star	Mild placentitis; villous necrosis	1+	1+				1+			1+			Subacute placentitis with coccal bacteria on the chorionic surface
C	UC - all	Mininal inflammation, but cocci on the surface		2+						2+				
D	UC - amn	Fibrin deposits in coelomic space												Mild fibrino-hemorrhagic funicitis
		Colonies of cocci on amnionic surface		2+										
		Mild cord hemorrhage								2+				
E	liver	Hepatic congestion and hemorrhage								2+				
	lung	Intra-airway cocci and keratinized squamous debris		3+										Despite the presence of large numbers of bacteria within the lung, there is only mild inflammation (post mortem proliferation, presumptive)

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
22SG/ PBS						PMNs	Lymphs	Mixed	Edema					
A	Amn													
B	Cervical Star	Edema + Hemorrhage							2+	1+				
C	liver													
	lung	Partially airated; few squames							1+					
D	Chorio-all: norm								1+					
682 / KILLED														
A	Chorio-all: norm													
B	Liver	Mild hepatic congestion							1+					
C	allantoic membrane		1+			1+								
	allantoic UC	Mild fibrino-suppurative funicitis	1+		1+									Mild fibrino-suppurative funicitis; fibrin within coelomic space
D	Cervical Star				1+									
E	amnion		1+							1+				
	amniotic UC	Fibrino-suppurative funicitis	1+		2+	1+				1+				Fibrino-suppurative funicitis
F	Chorio-allat cather site	Subacute placentitis, villous atrophy; placental separation		3+	2+			2+	2+	2+	2+	1+		Focal subacute placentitis with bacterial cocci

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
416/KS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	UC - all	Reactive hyperplastic allantoic epithelium												
	allantoic UD													
B	Chorio-all: norm	Allantoic epithelial hyperplasia												
C	Chorio-all:abn	Villous necrosis and mineralization, mild non-suppurative placentitis						2+			2+			Placental mineralization and villous necrosis
D	Cervical Star	Villous mineralization; allantoic stromal edema							2+					Minimal placentitis
E	UC - amn	minimal funicitis												
F	liver	Diffuse hepatic congestion												
	lung	Pulmonary congestion and mild fibrinous pneumonia												

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
12SG/ KS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	amniotic UC													
B	Cervical Star	Autolysis (slight) - mucoid exudate with bacteria (cocci)		Colonies of cocci										Although there are cocci on the chorionic surface and a mixed bacterial population within the allantois stroma, there is no significant placentitis present- this raised the possibility that the bacteria are postmortem overgrowth
C*	liver													
	lung	Fetal distress - inhaled squames							1+	1+				Fetal distress - inhaled squames
D	UC - all													
E	Chorio-all: norm								1+					
F*	Chorio-all:abn	Atrophy and necrosis of chorionic villi, fibrosis, reactive trophoblast syncytial formation											Many reactive trophoblast syncytia	Locallly extensive placental degeneration; (but no significant placentitis)

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
20/LS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	Chorio-all: norm	Mild segmental subacute placentitis with allantoic hyperplasia			**1+**			**1+**			**1+**	**1+**	Diffuse squamous metaplasia	Chronic placentits
B	Cervical Star	Chronic necrotizing placentitis	**3+**	**3+**	**2+**			**2+**			**2+**			Chronic necrotizing placentitis with vascular thrombosis and intralesional colonies of coccal bacteria
C	Chorio-all at cather site	Chronic necrotizing placentitis	**3+**		**2+**			**2+**			**2+**		Diffuse squamous metaplasia	Severe diffuse placental necrosis with marked squamous metaplasia of trophoblast cells; Diffuse allantoic fibrosis; ulceration of allantoic surface; allantoic vascular thrombosis
D*	liver													
	lung	Severe diffuse suppurative pneumonia with many giant cells			**3+**	**3+**								Severe neutrophilic and giant cell pneumonia; many sqames and some meconium are present in the deep airways.
E	allantois	Allantoic epithelial hyperplasia												Mild hemorrhagic funicitis
	allantoic UC	Mild hemorrhagic funicitis						**1+**		**2+**				
F*	amnoin	Necrotizing amnionitis	**2+**		**2+**			**2+**						Severe necrotizing amnionitis with vascular thrombosis
	amniotic UC	Marked funicitis with external pavementing with colonies of cocci		**3+**		**3+**								Severe necrotozing funicitis

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
12/LS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A*	Chorio-allat cather site	Chronic focal necrotizing placentitis	2+ (focal)		2+			2+	2+	1+	3+	2+	Villous atrophy, some villous necrosis	Marked focal necrosis, villous atroph, cystic allantoic dysplasia, allantoic fibrosis, vascular infarction, mild stromal mineralization
B	allantoic													No change in allantoic epithelium
	allantoic UC	Small amount of fibrin in coelomic space												
C*	amnion													
	amniotic UC	Mild focal funicitis - reaction to embedded keratin						1+	1+					The focal granulomatous inflammation in the cord may have been secondary to mechanical trauma as keratin flakes were embedded a short distance into the stroma
D*	liver	Moderate hepatic congestion with mild focal areas of hemorrhage								1+				Moderate hepatic congestion and sub-capsular hemorrage
	lung	Pneumonia		3+	2+			2+		1+ with Pulmonary congestion				Subacute fetal pneumonia with intralesional phagocytized bacteria, fetal distress as indicated by inhaled keratin squames embedded deep in the airways
E	Cervical Star								1+	1+	1+			
F	Chorio-all:abn	locally extensive allantoic hemorrhage								3+	1+			No inflammatory infiltrate
G	Chorio-all: norm													

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
24SG/LS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	Chorio-all: norm													
	Chorio-all:abn	Allantoic hematoma-small;acute												
B	amnon													
	amniotic UC													
C	Chorio-allat cather site	Focal fibrosis;edema; adhesion of amorphic debris - allantois calcular material									2+	2+		The CA has a focal area of fibrosis and granulation tissue; no significant inflammation.There is mild cystic allantoic dysplasia and adhesion of hippomane type debris on the allantoic surface.
D	liver													
	lung	Partial aeration; some squames in alveioli												
E	Cervical Star	Congestion and edema							2+	1+	1+			
G	Chorio-all:abn								1+		1+			Mild myxomatous change in stroma surrounding intermediate sized arteries, No placentitis is present.
H	Chorio-all: norm	Moderate edema and slight vilous atrophy							2+					

Appendix continued

Case / Slide #	Tissue	Abnormal findings	Necrosis	*Bacteria ?	Inflammation					Hemorrhage	Fibrosis	Allantoic cystic hyperplasia	Trophoblast	Summary
23 /HS						**PMNs**	**Lymphs**	**Mixed**	**Edema**					
A	cath site													
B	cerv star	necrotizing placentitis	2+	3+ cocci	2+			2+			2+	2+		necrotizing placentitis
C	allant UC	necrotizing placentitis	2+	3+	2+			2+			2+			necrotizing placentitis
D	amnio	mild fibrinous funicitis			1+			1+						mild fibrinous funicitis
E	abn CA	allantoic hyperplasia								2+				allantoic hyperplasia
F	lung	necrotizing placentitis	2+		1+									necrotizing placentitis
G	normal CA	bacterial colonies		3+										autolysis; bacterial overgrowth
H	liver	placentitis	1+		1+									placentitis
I	amnion/amn UC	neutrophilic funicitis				2+								neutrophilic funicitis
42 / HS														
B	cerv star	fibrosis, hemorrhage, reactive allantoic epithelium								2+	2+	3+	mineralization	placental fibrosis; degeneration
C	all UC											1+		
D	amnion	reactive												reactive amnion epithelium, amnion nodosum
E	abn CA	necrotizing placentitis		2+	2+	2+					1+	2+	atrophy	necrotizing placentitis
F	lung	bacterial overgrowth		2+										Pulmonary hemorrhage, postmortem bacterial proliferation. Fetal inhalation of keratinaceous debris (fetal in utero stress). Pulmonary hemorrhage, postmortem bacterial proliferation Fetal inhalation of keratinaceous debris (fetal in utero stress)
G	normal CA	reactive allantoic epith											1+	
H	liver													autolysis
I	amn UC	focal hemorrhage												hemorrhage

VITA

The author was born and raised in Gainesville, Florida. She attended Duke University, Durham North Carolina, receiving a Bachelor of Arts in Chemistry, *magna cum laude*, in 1980. She graduated from the University of Florida's College of Veterinary Medicine, Gainesville, Florida, in 1985 with high honors. After practicing for two years in an equine practice in south central Pennsylvania, she returned to the University of Florida to complete a Residency in Theriogenology from 1987 to 1989, and received a Masters of Science in 1991. She attained board certification in the American College of Theriogenologist, by examination, in 1990. Following ten years of private practice in Florida and Kentucky, she became a Clinical Instructor at North Carolina State University's College of Veterinary Medicine. She joined the staff at Louisiana State University's School of Veterinary Medicine as a Clinical Instructor, concurrent to her doctoral candidancy.