PLACENTAL AND FETAL IMMUNITY

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The placenta is essential for development of the fetus and maintenance of pregnancy. Cellular communications between maternal and fetal tissues are critical to create an intrauterine environment acclimated for fetal survival. Successful pregnancy requires modulation of the maternal immune system by placental, among many other needs. Dysfunction of maternal-fetal communication can contribute to detrimental effects and mortality of the fetus during times of reproductive pathology induced by pathogens. In this presentation, an overview of placental physiology and immunity during health and disease conditions.

Non-invasive assessment of the fetal-placental unit, including biomarker characterization, is a growing area of research occurring in both human and veterinary medicine for states of health and disease. This presentation will discuss translating circulating biomarkers and advanced ultrasonography described in recent research to practical approaches to assess reproductive efficiency and placental health. Biomarkers including non-coding RNAs and pregnancy associated glycoproteins (PAGs), will be defined. Current literature review will be presented describing the utilization of different biomarkers to assess placental health during times of disease or determine fetal outcome following conception. The veterinary practitioner and bovine producers will be exposed to cutting edge research in the area of placental health, yet walk away with a practical understanding where these progressive techniques can be used in practice

Learning objectives:

1. Understand the immune system modulation at the maternal-fetal interface during both healthy and complicated pregnancies
2. Describe non-invasive methodologies used in practice and research to determine placental health
3. Understand the mechanism of pregnancy associated glycoproteins production and how production correlates with fetal morbidity and mortality
4. Utilize ultrasonography to assess fetal viability

<u>Placental immunology during healthy pregnancy</u>

Survival of the semi-allograft conceptus is required for successful pregnancy outcome (Clark et al 2007). The variety of placental types may have evolved to avoid immune rejection of the fetus, which is antigenically different from the dam (Hemberger 2013). The less invasive the placentation, the less access of maternal immune cells will have to antigenically different fetal cells. The underlying mechanisms for survival and non-rejection of the fetus is not fully understood, yet several immunologic explanations have been proposed. For starters, the maternal immune system may be unable to

recognize fetal cells. Ruminant and human trophoblast have limited expression of major histocompatibility complex (MHC) on its surface thus restricting the ability of immune cells to recognize fetal antigens (Meeusen et al 1993; Hill et al 2002). Sheep trophoblasts appear to be totally devoid of MHC I expression following gestational day 19 (Gogolin-Ewens et al 1989). MHC molecules can act as antigens themselves, and in the case of tissue graft or organ transplantation, T cells may recognize the tissue antigen and induce rejection. Thus, the lack of MHC expression by trophoblasts prevent maternal recognition by CD8+ T cells, ultimately preventing fetal rejection (Brainbridge 2000). Natural killer cells (NK) are other such innate immune cells that utilize MHC molecules and other cellular receptors including killer inhibitory receptors (KIRs) to recognize antigens. KIRs allow NK cells to recognize the absence of self, allowing them to kill cells that fail to produce MHC. This is a critically important mechanism for elimination of virus-infected cells that down regulated MHC expression that would otherwise have been missed by CD8+ T cells. NK are present in high number in the human and mouse decidua, and recently NK have been described to have increasing numbers during early pregnancy in sheep (Entrican 2002; Oliveira et al 2013). Human trophoblast appear to produce a limited amount of MHC to protect against NK induced apoptosis (King et al 2000). With the known presence of NK in the gravid sheep uterus, it is unclear how fetal trophoblasts that are devoid of MHC expression avoid NK-induced apoptosis; therefore, further research is needed to elucidate these roles.

The population and phenotype of uterine leukocytes is altered with normal pregnancy. In sheep, T cells in the uterine glandular epithelium greatly decrease in number while the number of macrophages increases during pregnancy (Lee et al 1992). Tekin and Hansen demonstrated that the accumulation of macrophages was due to both systemic and local signs by use of unilateral uterine horn ligation studies in sheep (2004). The phenotype of uterine macrophages located in the ruminant endometrium shifts to M2 (alternative) which function in tissue remodeling, healing, and immunomodulation compared to its pro-inflammatory (M1) counter parts during pregnancy (Tekin & Hansen 2004). Concentrations of uterine ɣ/δ T cell increase in ruminant placenta during mid and late pregnancy (Lee et al 1992; Meeusen et al 2001). CD8+ ɣ/δ T cell populations are concentrated in the intercaruncular glandular and luminal epithelium where there is no chorionic attachment, limiting exposure of fetal cells to this subset of maternal immune cells (Lee et al 1992; Majewski et al 2001). T cells expressing a ɣ/δ receptor have less antigen specificity compared to their α/β counterparts (i.e. CD4+ and CD8+ T cells). Yet, CD8+ ɣ/δ T cell presence at the interplacentome tissue suggests they control for excessive trophoblast invasion preventing danger to the dam yet minimizing potentially detrimental immune responses to the placenta (Fox et al 1998). CD4+ T cells are restricted to stromal epithelium of normal pregnancy (Cobb & Watson 1995). B cells are low numbers in the uterus during diestrus and concentrate in the deep stroma surrounding blood vessels (Oliveira et al 2013). IgG is the primary immunoglobulin produced while IgA is in higher concentrations in the vagina (Cobb & Watson 1995; Lander et al 1990; Mestecky et al 2005). Lack of B cell populations and corresponding antibodies is confirmed by population finding of less than 30% of multiparous cattle having anti-fetal antibodies in circulation following parturition. These findings describe minimal maternal leukocytes at the caruncular regions while number of leukocytes increase with the progression of pregnancy in the intercaruncular endometrium ready to recognize and

processing foreign antigens of pathogenic invaders (Leung et al 2000). Mechanisms inducing maternal T cell tolerance to fetal cells have been described in other species. Human and mouse trophoblast expression indoleamine 2,3 dioxygenase is required for successful pregnancy with the concluded action of inhibiting CD8+ T cell function (Kudo & Boyd, 2000). Further, FAS ligand is expressed by human trophoblast and can induce apoptosis in activated T cells that bear Fas/CD95 receptor thus preventing trafficking of activated lymphocytes to the maternal-fetal interface (Mor et al 1998). This supports the hypothesis of an anti-inflammatory giving way to a pro-inflammatory uterine environment during late term pregnancy described in many species (Moffett and Loke 2003).

Cytokines and chemokines, produced by both maternal and fetal cells, are important mechanisms of communication at their interface. During diestrus and early pregnancy, the endometrial immune environment favors a Th2 profile (Oliveira et al 2013). Uterine CD8+ ɣ/δ T cells in pregnant sheep express mRNA for anti-inflammatory cytokines including TGF- β, and IL-10 (Fox et al 1998). Human chorionic villus trophoblasts express mRNA for both pro- and anti-inflammatory proteins including TNF- α, IFN- α, IL-1 β, TGF- β, and IL-10 (king et al 1995; Bennett et al 1999). The concentration of pro-inflammatory cytokines has been confirmed at the fetal-maternal interface in mice using ELISAs (Johki et al 1997). However, the effect of a pro-inflammatory milieu leading to fetal rejection is counteracted by high concentrations of IL-10 (Chaouat et al 1995). Interferons, including IFNT produced by the trophoblast, have inherent antiviral properties and alter the function of target cells (Bazer et al 2012). The function of IFNT produced by the trophoblast as a signal for maternal recognition may have evolved from other type I IFNs (Elay & Wooldridge 2017). At the maternal fetal interface prostaglandins have a pro-inflammatory feature (Hansen 1997). In vitro, ovine INFT reduced PGF2α concentrations confirming its antiluteolytic properties and inferring an immunosuppression role during early pregnancy (Parent el al 2003; Chen et al 2006). Similar to other members of the IFN group, IFNT has been demonstrated to have antiviral activity and inhibitory effect on lymphocytes (Chen et al 2006). Thus, there appears to be a cytokine balance between pro- and anti-inflammatory stimuli that is critical for successful pregnancy.

Another immune alteration during successful pregnancy is maternal immunosuppression. Pregnancy hormones have been demonstrated to have immunomodulatory effects. Beyond the functions of establishment and maintenance of pregnancy, progesterone has many immunologic effects at the maternal-fetal interface. Progesterone exerts this role by influencing immune cell functionality. In humans and mice, progesterone plays a role in inhibiting mature dendritic cells (DCs), favoring immature DCs populations at the decidua. DCs are important APCs that can activate T-cells hence having major influences on the immune response within tissue. Immature DCs exhibit an anti-inflammatory phenotype defined by increased expression of IL-10 (anti-inflammatory cytokine) and induction of T-regulatory cells. Additionally, progesterone can bind to activated T-cells directly, producing progesterone induced blocking factor (PIBF) via signaling through the JAK/STAT pathway (Kozma et al 2006). PIBF influences T cell and NK response, favoring a Th2 response, leading to down regulation of a pro-inflammatory response (Szekeres-Bartho 1996; Kozma et al 2006). Urine concentrations of have been positively correlated with successful pregnancy

outcomes in women (Polgar et al 2003). Progesterone itself can induce TH2-type cytokine production of IL-4 and IL-10 (Monterroso & Hansen 1993; Piccinni et al. 1995). Both IL-4 and IL-10 decrease TH1 and macrophage activity thus preventing allograft type rejection of the fetus. The shift of Th1 to Th2 response seems clinically important for maintenance of pregnancy, where woman who suffer from reoccurring abortion or miscarriage was associated with defective IL-4 production (Piccinni et al 1998). Progesterone appears to have another indirect role for immunosuppression beyond affecting uterine immune cells. Majewski and Hansen demonstrated the infusion of progesterone induced secretory molecule release from the uterine endometrium, thought to increase maternal-fetal communication and prevent fetal rejection (Majewski & Hansen 2002). This action was independent of lymphocyte inhibition (Padua et al 2005). It was further elucidated that progesterone induces the release of bioactive molecules known as serpins from uterine epithelium that have an immunoregulatory role (Lui & Hansen 1993 Monterroso & Hansen 1993). Uterine serpins in sheep have been found to inhibit lymphocyte proliferation and NK cell function (Lui & Hansen 1993; Peltier et al 2000). Uterine milk protein (UTMP) a type of serpin is a progesterone glycoprotein produced by endometrium epithelium (Hansen & Liu 1997). During pregnancy, UTMP is the predominant fluid in sheep uterine secretions (Moffatt et al 1987). In-vitro, UTMP inhibits activated T cells yet in-vivo studies are lacking (Hansen 1998). These roles elude to a progesterone-dependent immunomodulation of the dam during pregnancy (Arck et al 2007).

Another immune theory during pregnancy follows the theory that during normal, healthy pregnancy the semi-allogenic fetus does not propose any threat to the dam (Matzinger 1998). This lack of danger signals is thought to eliminate the necessity of the maternal immune response to reject the fetus (Entrican 2002). Danger associated molecular patterns (DAMPs) are signals produced by cells suffering from damage and non-apoptotic death. DAMPs can bind to cellular receptors such as TLRs that can initiate gene expression of inflammatory products. This and many other theories speak to a multi-mechanistic immune approach for successful pregnancy.

Placental immunology during infectious disease

The most notable infectious reproductive diseases in livestock are those that are vertically transmitted causing placental pathology and abortion (Entrican 2001). Pathogens including Neospora caninum, Chlamydophila abortus, and bovine viral diarrhea virus (BVDV), will create unapparent maternal disease while manifesting severe reproductive clinical disease including abortion. This may be due to the inherently, anti-inflammatory immune milieu at the maternal-fetal interface creating an environment incapable of control or prevention of an infectious pathogen. Conversely, abortion maybe a consequence of the pro-inflammatory shift to Th1 environment resulting in immunopathology. It is important to note that mechanisms of abortion will different between pathogens. For the purpose of this review, select pathogens representing diverse organisms and the mechanism of abortion induction in ruminants will be discussed. Beyond specific pathogens, maternal physiologic stress, with and without disease can induce fatal consequences for the developing fetus. Stress associated with disease processes that activate the hypothalamic-pituitary axis inhibits the female reproductive system thus down regulating progesterone production, inducing spontaneous abortion

(Magiakou et al 1997). Cortisol release associated with inflammation can impair PAG production and activity (Dosogne et al 2000). An abortion outcome may be directly associated with insufficient progesterone to maintain a pregnancy or the loss of progesterone-dependent immune modulation. Consequently, maternal stress alone is of significant consequence to the developing fetus.

Trophoblasts express toll-like receptors (TLRs), allowing binding of specific pathogen associated molecular patterns (PAMPs) leading to immune-related gene expression (Takeda & Akira 2005). The most expression of TLRs and thus strongest ability to recognize pathogens are found on trophoblast located at the chorionic villi which interdigitates with maternal endothelium (Takeda & Akira 2005). Following activation, trophoblasts can produce cytokines associated with a pro-inflammatory immune response (Mineo et al 2010). Maternal uterine endothelium also recognizes PAMPs and can produce a variety of chemokines, cytokines, and antimicrobial compounds in response. Defensins are one such compound that can produced and highly conserved across species. Beta-defensins have been identified at the mucosal surface of the gastrointestinal, respiratory, and reproductive system of sheep (Meyerholz et al 2004). Expression of these proteins is associated with bacterial and viral pathogens at these surfaces (Grubor et al 2004; Meyerholz et al 2004). In humans, the endometrium produces large concentration of defensins following pathogen recognition. Further, these proteins can acts as chemokines for immature DC and memory T cells (Yang et al 1999). Maternal immune cells within uterine tissues will also recognize pathogens. Leukocyte TLR activation is needed to initiate host defenses against pathogens. Once antigens are processed APCs and presented, CD4+ lymphocytes secrete cytokines like IL-2 and B cells produce specific antibodies as plasma cells (Cobb and Watson, 1995). Yet, with maturation of APCs and NK along with pro-inflammatory cytokine production will affect the feto-placental units access to blood flow, nutrient exchange, and exposure to hostile, activated immune cells.

Pregnancy Associated Glycoproteins

PAGs are placental products abundantly expressed by even-toed ungulates, such as ruminants (Telugu et al. 2009). PAGs are subdivided into ‘ancient’ or ‘modern’ groups based on phylogenetic origination (Hughes et al. 2000). Individual PAGs are further named numerically based on series of which they were sequenced or location on gene loci (Wallace et al 2015). Cattle and sheep, belonging to the suborder Ruminantia produce a larger concentration of ‘modern’ PAGs compared to ‘ancient’ PAGs. Two dozen PAG genes, encoding for corresponding proteins, have been identified in the bovine and ovine genome (Telugu et al. 2009). PAGs can be expressed in both mononuclear and giant binucleated trophoblast (Wallace, 2015). PAG antibodies are identified via western blot in day 16 bovine conceptus, prior to binucleated trophoblast formation (Wallace, 2015). Immunolocalization studies have identified that modern PAGs are more abundant in cotyledons compared to the intercotyledonary chorion. Conversely, ancient PAG transcripts are elevated at intercotyledonary areas (Wallace et al. 2015). Numerically annotated, individual PAGs are produced by different trophoblasts; for example, PAG-1 is produced by binucleate trophoblasts, while PAG-2 is produced by both bi- and mono-nuclear trophoblasts (Garbayo et al. 2000; Sousa and Beckers 2007). Their expression undergoes spatial and temporal patterns through

gestation, yet little is known of how PAG gene transcription is regulated. Circulating PAG concentrations in maternal blood can be influenced by maternal factors (i.e. weight, parity, breed), and fetal factors (i.e. birth weight, sex, number) (Mercadante et al 2016). Circulating PAG concentrations are increased in twin pregnancies in cattle likely due to increased production that would parallel increased total binucleate trophoblast numbers (García-Ispierto et al 2016).

PAGs produced by binucleated giant cells and secreted at the maternal uterine stroma are the basis of blood & milk pregnancy tests in ruminants. PAGs have been extensively evaluated in the scientific literature as a reliable tool to diagnose pregnancy in many two-toed ungulates (Wallace, 2015). Commercial assays are available and can detect PAG concentrations in both maternal blood and milk (Pohler et al. 2013). Accuracy of detecting circulating PAGs is reported to range from 93-96% (Pohler et al. 2013). However, spurious results can occur occasionally. For example, pregnant cows with a viable embryo, with evidence of beating heart of rectal ultrasonography, have resulted with no detectable circulating PAGs (Pohler et al. 2013). It is unknown whether this situation results from a fetus not producing PAGs that can enter and accumulate in maternal circulation; or if the immunoreactive assay is not specific to the circulating PAGs subset. Circulating PAG concentrations are highest prior to parturition and remain elevated post-partum due to a relatively long half-life of up to 8-10 days (Wallace et al. 2015). Pregnant sheep demonstrate a temporal; biphasic production of PAGs with an initial peak at gestation day 60 followed by a sharp decline by 90 days then a steady increase culminating with parturition (Roberts et al 2017). This unique profile is speculated to be caused by a shifting in PAG production by the trophoblast and possibly the insensitivity of the diagnostic assays to detect different subsets. It is interesting to note that the initial decrease of PAGs in sheep corresponds when the trophoblast overtake the corpus luteum producing the majority of progesterone for the remainder of pregnancy.

There are many potential functional roles of PAGs throughout pregnancy. Speculation of PAG function is based on expression pattern and amount of proteolytic activity. Proteolytic PAGs produced at the microvillar space could process growth factors or act to disrupt the connections between trophoblast and uterine epithelium (Wooding et al. 2005). Proteolytic activity is pH dependent, a decline of pH is known to occur at the fetal-maternal microenvironment around parturition (Telugu & Green, 2008). PAGs also may act as a bridging molecule assisting in adhesion at the maternal-fetal interface microenvironment (Wallace et al 2015). PAGs produced by binucleated giant trophoblast can be sequestered in the maternal uterine stroma or enter maternal blood circulation. In cattle with retained placenta, cotyledonary PAG expression is altered compared to control cows, supporting a local regulatory role of PAGs in fetal membrane release (Hooshmandabbasi et al. 2017). Effects of PAGs influence maternal physiology and immunity at the uterus and once distributed via blood circulation could elicit systemic effects. In peri-parturient cattle, polymononuclear neutrophil activity is lowest at late gestation, which corresponds with a marked increase of circulating PAGs (Dosogne et al. 1999). However, correlation between occurrence of peri-parturient infectious disease and PAG concentrations in cattle is speculative. Yet, direct immunomodulatory effects of PAGs are not clear. Administration of PAGs can decrease hematopoietic cell proliferation and directly bind to uterine serpins thus augmenting immune responses

(Mathialagan & Hansen 1996). The immune role of uterine serpins will be discussed later in this chapter. Many PAGs display increased glycosylation, which can provide some protection to the fetal semi-allograft by decreasing susceptibility to NK induced cell apoptosis (Perry et al 2005). Experimentally, PAGs have a luteotrophic role by increasing measurable prostaglandin E2 (PGE2), which has direct luteotrophic and anti-luteolytic effects in ruminants thus increasing measurable progesterone concentration (Weems et al. 2003). Progesterone, itself has immunomodulatory activity, which is discussed later in this review. Furthermore, PGE2 has a direct immunosuppressive effect by inhibiting T-lymphocyte proliferation (Low & Hansen 1988). Thus, PAGs can indirectly modulate immunity through the activation of other pathways that are experimentally defined.

Assessment of the Feto-Placental Unit

Protein Analysis

PAGs have been evaluated for their ability to serve as a marker for embryonic/fetal viability and placental health. Circulating PAG concentrations are lower in cattle likely to undergo embryonic/fetal loss. Beef cattle that maintain a pregnancy beyond 72 days of gestation have statistically higher circulating PAG concentration at day 28 compared to cohorts that experienced embryonic/fetal loss by day 72 of gestation (Pohler et al. 2013). All cows underwent transrectal ultrasonography, which demonstrated a viable embryo at day 28 based on fetal heartbeat. Similarly, circulating PAGs were decreased in beef cattle 41 days post insemination that had embryonic loss compared to cows that maintained their pregnancy (Perry et al. 2005). However ability to detect pregnancy loss or embryonic survival is assay antibody dependent (Gatea et al 2018). Although these studies demonstrate an association between low PAG concentrations and fetal loss, the opposite occurs with somatic cell nuclear transfer (SCNT) derived embryos. Elevation in PAG levels is observed in recipient cows receiving SCNT-derived embryos compared with control at days 35 and 50 of gestation followed by subsequent fetal loss (Hashizume et al. 2002). This increase in circulating PAGs may reflect structural alteration of the placenta allowing increased exposure of maternal epithelia to trophoblast products such as PAGs (Pereira et al. 2013). PAGs have further been evaluated as a marker of placental health during reproductive infections. Several studies have investigated shifting PAG concentration following Neospora caninum infection in cattle. Lopez-Gatius et al. determined that PAG-1 concentrations decreased in aborting animals yet provided no indication of feto-placental health in chronically infected pregnant cattle that did not abort (Lopez-Gatius et al 2007). In another study, circulating PAG-2 concentrations of < 4.5 ng/mL at day 120 of gestation served as an indicator of abortion risk in chronically infected cattle (Garcıa-Ispierto et al 2012). Early PAG concentrations along with seroconversion to Neospora demonstrated an odd ratio of seven for abortion by 5-7 months of gestation. A negative correlation with other placental products such as plasma prolactin follows a similar relationship in the face of Neospora induced abortion (Garcıa-Ispierto et al. 2009). These data demonstrate that measurement of trophoblastic products such as PAG, can serve as a non-invasive marker of placental health, possibly indicating the occurrence of fetal mortality.