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Characterization of circulating extracellular vesicle content and PAG concentrations in pregnant sheep infected with BVDV

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To the Graduate Council:

I am submitting herewith a dissertation written by Andrea Sketch Lear entitled "Characterization of circulating extracellular vesicle content and PAG concentrations in pregnant sheep infected with BVDV." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Characterization of circulating extracellular vesicle content and PAG concentrations in pregnant sheep infected with BVDV

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Abstract

The placenta is an active immunologic organ, modulating the maternal environment to support the conceptus. Fetal trophoblasts produce a variety of products including pregnancy-associated glycoproteins (PAGs) and extracellular vesicles called exosomes. PAGs directly interact with the uterus and maternal immune cells with low concentrations correlating with adverse pregnancy outcomes in cattle. Exosomes contain nucleic acids and proteins that influence intercellular communication during pregnancy. The specific objectives of this body of work were to 1) quantify circulating PAG concentrations, 2) characterize the profiles of circulating exosomal proteins and miRNAs in pregnant and non-pregnant sheep, and 2) determine the difference in exosomal content profiles between non-infected and BVDV-infected pregnant and non-pregnant sheep. Twenty-four yearling ewes, found to be negative to BVDV on serum neutralizing antibody serology, were enrolled in this study. Ewes were inoculated with BVDV NY-1 or sham media. Fifteen days post inoculation, all animals underwent hysterectomy and peripheral blood collection. PAG1 concentrations were quantified using commercially available ELISA. Exosomes were isolated using ultracentrifugation and gradient density separation. Small RNA high through-put sequencing and mass spectroscopy proteomics were performed. BVDV infection status was determined with BVDV PCR of fetal tissue, IHC of placentomes, and VN of dams. Statistical differences between circulating concentrations of PAG1, miRNAs, and proteins were analyzed by analysis of variance for repeated measures. PAG1 concentrations in maternal blood differed between treatment groups, with mean concentrations significantly lower ($P = 0.04$) in BVDV inoculated dams compared to controls. Sixty million reads, identifying 1634 miRNAs were identified following high-throughput sequencing. Several miRNAs were identified to be unique to BVDV exposure and pregnancy status. With a confidence rate of 0.01, 539 proteins were identified with differential relative abundance between viral exposure. In conclusion, infection with BVDV decreases PAG1 concentration in maternal circulation in pregnant sheep compared to healthy controls. Exosomes containing select miRNAs and proteins are present in peripheral circulation likely have a biological role in fetal-maternal interactions

important in times of both disease and health. Maternal PAG concentrations and differential exosome content may be a viable biomarker to determine placental health following reproductive viral infections.

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Chapter 1

Introduction

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 and endometrial production, 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 review, an overview of placental physiology and immunity during health and disease conditions will be presented. Biogenesis and function of exosomes and corresponding miRNA and protein content and their significance associated with pathologic pregnancies is summarized.

1. Placenta form and function in sheep

The placenta is a transient, yet critical organ responsible for maintenance of pregnancy and preparation for lactation following parturition. Additionally, the placenta supports the developing fetus by allowing nutrient exchange and immunologic protection. Both fetal and maternal components make up the formed placenta. Description of the placenta is based on two criteria: 1) histologic separation between maternal and fetal blood and 2) anatomical distribution of villus attachment. The ruminant placenta is classified as synepitheiochorial with cotyledonary attachment that develops during embryogenesis (Spencer & Bazer, 2002). Following fertilization, the sheep embryo enters the uterus by day 3 of gestation and through mitotic development of the zygote a blastocyst forms, which is comprised of cells required for extraembryonic membrane formation (Bazer, 2012; Sherer & Abulafia, 2001). Prior to implantation, the extraembryonic membranes consist of the yolk sac, chorion, amnion, and allantois (Clemente et al., 2009; Senger, 2004). The yolk sac derived from the primitive endoderm, is transient and regresses with conceptus development, yet its lasting contribution are the germ cells lining the genital ridge in the embryo (Senger, 2004). The trophectoderm, comprised of mononuclear trophoblast cells, along with the mesoderm give rise to the chorion. Combination of the trophoblasts and endoderm forms the fluid filled amnion immediately surrounding the developing fetus. The allantois, an invagination of the primitive gut, forms to collect liquid waste originating from the embryo. Eventually the chorion and

allantois will fuse, forming the fetal portion of the attaching placenta (Senger, 2004). Interdigitation of the fetal chorionic cotyledon with the maternal caruncular regions begins at 16 days following fertilization with attachment of the embryo occurring around 18 days (Stewart et al., 1992). The trophoblast of the developing conceptus produces interferon-tau (IFNT), which serves as the signal for maternal recognition of pregnancy (Reynolds et al., 2015; Spencer & Bazer, 2002). INFT achieves this function by inhibiting endometrial oxytocin receptors which in-turn inhibits estrogen receptors and prostaglandin F2-alpha (PGF2α) synthesis (Martal et al., 1997; Spencer & Bazer, 2002). Without the luteolytic activity of PGF2α, the ovarian corpus luteum continues to produce progesterone, the hormone required for maintenance of pregnancy.

Epitheliochorial placenta, the least intimate of placenta types, has three intact maternal tissue layers and three fetal layers, 6 in total separating maternal and fetal blood. However, ruminants' epitheliochorial placenta erodes into the maternal endometrium allowing access to maternal circulation by the invading fetal chorion (R. M. Wallace, Pohler, Smith, & Green, 2015). This placental feature of endometrial erosion defines the synepitheiochorial placenta type found in ruminants. Two trophoblast populations histologically comprise the developing synepitheiochorial ruminant placenta: binucleated giant and mononuclear trophoblasts. Giant binucleated cells arise from mononuclear trophoblasts at 2 weeks of gestation in sheep (Grazul-Bilska et al., 2011). The binucleated trophoblast migrate through the chorionic microvillar junction eventually fusing with maternal uterine epithelium forming multinucleated fetal-maternal syncytial cells (R. M. Wallace et al., 2015). In cattle, these syncytia are transient and disappear by day 40 of pregnancy. Yet in small ruminants (sheep and goats), these syncytia persist throughout gestation (R. M. Wallace et al., 2015). Villous chorionic cotyledons interdigitate with aglandular maternal uterine caruncles creating regions of fetal-maternal interaction in the form of placentomes (R. M. Wallace et al., 2015). Direct engagement with maternal tissues is unique to the ruminant placenta compared to other epitheliochorial placenta. By gestational day 30, placental attachment is well established (Senger, 2004). In ewes, the placentomes form a concave structure that

will increase in surface area throughout fetal development thus increasing nutrient exchange to keep up with fetal growth demands.

Cotyledons are discrete button-like structures that contain abundant amounts of blood supply. As mentioned above, the surface are of these structure increase through pregnancy, increasing the possibility for maternal-fetal interactions. The endocrine activity of the placenta increases with fetal development as well and placentally produced hormones gain access to both maternal and fetal compartments (Reynolds et al., 2015). The gestation length in the ewe is approximately 150 days; binucleate trophoblasts begin producing progesterone following 2 weeks of gestation. By gestational day 50-75 in sheep, the placenta overtakes the ovarian corpus luteum, making the majority of progesterone needed to maintain pregnancy until parturition (Reynolds et al., 2015; Wathes et al., 1996). After this time, luteolytic agents (i.e. PGF2α), whether endogenous or exogenously derived, are unlikely to induce abortion alone. The high concentration of placentally produced progesterone also blocks myometrium contractility preventing fetal expulsion (Spencer & Bazer, 1995). Furthermore, high progesterone concentration aids in increased blood supply to the uterus needed for the developing (Spencer & Bazer, 2002). Binucleated trophoblast also create many other products including pregnancy-associated glycoproteins (PAGs), placental lactogens, and prelatic-related proteins. These trophoblast-expressed proteins are delivered to the maternal uterine connective tissue through secretory granules. All of these products can reach maternal blood circulation as verified by positive measurements in maternal blood (R. M. Wallace et al., 2015). The exchange of these proteins does not necessarily go both ways. In epitheliochorial, endometrium proteins and lipids do not cross the placental barrier into the fetal compartment (Bairagi et al., 2016). Instead, the maternal tissues utilize other forms of cross talk with the fetal placenta in the forms of extracellular vesicles (described below).

2. Pregnancy Associated Glycoproteins (PAGs)

PAGs are placental products abundantly expressed by even-toed ungulates, such as ruminants (Telugu, Walker, & Green, 2009). PAGs are subdivided into ‘ancient’ or ‘modern’ groups based on phylogenetic origination (Hughes, Green, Garbayo, & Roberts, 2000). Individual PAGs are further named

numerically based on series of which they were sequenced or location on gene loci (R. M. 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 (R. M. Wallace et al., 2015). PAG antibodies are identified via western blot in day 16 bovine conceptus, prior to binucleated trophoblast formation. 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 (R. M. 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 (de Sousa et al., 2003; Garbayo et al., 2000). 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, Ribeiro, Risco, & Ealy, 2016). Circulating PAG concentrations are increased in twin pregnancies in cattle likely due to increased production that would parallel increased total binucleate trophoblast numbers (Garcia-Ispierto et al., 2013).

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 (R. M. Wallace et al., 2015). Commercial assays are available and can detect PAG concentrations in both maternal blood and milk (Reese, Pereira, Edwards, Vasconcelos, & Pohler, 2018). Accuracy of detecting circulating PAGs is reported to range from 93-96% (Pohler et al., 2016). 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., 2015). 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 (R. M. 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, May, & Veiga-Lopez, 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, Roberts, & Green, 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 (R. M. 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., 2018). 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, Kim, Humphreys, Tsuda, & Weems, 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 (P. J. Hansen & Liu, 1997). Thus, PAGs can indirectly modulate immunity through the activation of other pathways that are experimentally defined.

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, Almeria, 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 (Garcia-Ispierto et al., 2013). 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 (Lopez-Gatius, Almeria, et al., 2007). 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.

3. Placental immunology during healthy pregnancy

Survival of the semi-allograft conceptus is required for successful pregnancy outcome (M. B. 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 (Hill, Schlafer, Fisher, & Davies, 2002; E. Meeusen, Fox, Brandon, & Lee, 1993). Sheep trophoblasts appear to be totally devoid of MHC I expression following gestational day 19 (Gogolin-Ewens, Lee, Mercer, & Brandon, 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 (Bainbridge, Ellis, & Sargent, 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). Human trophoblast appear to produce a limited amount of MHC to protect against NK induced apoptosis (King, Allan, 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, Meeusen, Gogolin-Ewens, & Brandon, 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 γ/δ T cell increase in ruminant placenta during mid and late pregnancy (Lee et al., 1992; E. N. Meeusen, Bischof, & Lee, 2001). CD8+ γ/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 (Majewski, Tekin, & Hansen, 2001). T cells expressing a γ/δ receptor have less antigen specificity compared to their α/β counterparts (i.e. CD4+ and CD8+ T cells). Yet, CD8+ γ/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, Lee, Brandon, & Meeusen, 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 (Pereira et al., 2013). IgG is the primary immunoglobulin produced while IgA is in higher concentrations

in the vagina (Cobb & Watson, 1995; Leung, Derecka, Mann, Flint, & Wathes, 2000; Mestecky, Moldoveanu, & Russell, 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, Gutierrez, Eliza, Kahyaoglu, & Arici, 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 (Gardner & Moffett, 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 (Mineo, Oliveira, Gutierrez, & Silva, 2010). Uterine CD8+ γ/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 (Bennett et al., 1999; King, Hiby, et al., 2000). The concentration of pro-inflammatory cytokines has been confirmed at the fetal-maternal interface in mice using ELISAs (Jokhi, King, & Loke, 1994; Jokhi, King, Sharkey, Smith, & Loke, 1994). 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, Song, & Thatcher, 2012). The function of IFNT produced by the trophoblast as a signal for maternal recognition may have evolved

from other type I IFNs (Ealy & Wooldridge, 2017). At the maternal fetal interface prostaglandins have a pro-inflammatory feature (P. J. Hansen & Liu, 1997). In vitro, ovine INFT reduced PGF2α concentrations confirming its antiluteolytic properties and inferring an immunosuppression role during early pregnancy (Chen et al., 2006; Parent, Villeneuve, Alexenko, Ealy, & Fortier, 2003). 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, Halasz, Polgar, et al., 2006). PIBF influences T cell and NK response, favoring a Th2 response, leading to down regulation of a pro-inflammatory response (Kozma, Halasz, Palkovics, & Szekeres-Bartho, 2006; Szekeres-Bartho, Faust, Varga, Szereday, & Kelemen, 1996). 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., 1995). Progesterone appears to have another indirect role for immunosuppression beyond affecting

uterine immune cells. Majewski demonstrated the infusion of progesterone induced secretory molecule release from the uterine endometrium, thought to increase maternal-fetal communication and prevent fetal rejection (Majewski et al., 2001). This action was independent of lymphocyte inhibition (Padua, Tekin, Spencer, & Hansen, 2005). It was further elucidated that progesterone induces the release of bioactive molecules known as serpins from uterine epithelium that have an immunoregulatory role (Liu & Hansen, 1993; Monterroso & Hansen, 1993). Uterine serpins in sheep have been found to inhibit lymphocyte proliferation and NK cell function (Liu & Hansen, 1993; Peltier, Liu, & Hansen, 2000). Uterine milk protein (UTMP) a type of serpin is a progesterone glycoprotein produced by endometrium epithelium (P. J. Hansen & Liu, 1997). During pregnancy, UTMP is the predominant fluid in sheep uterine secretions (Moffatt, Bazer, Hansen, Chun, & Roberts, 1987). In-vitro, UTMP inhibits activated T cells yet in-vivo studies are lacking (P. J. Hansen et al., 1987). These roles elude to a progesterone-dependent immunomodulation of the dam during pregnancy (Arck, Hansen, Mulac Jericevic, Piccinni, & Szekeres-Bartho, 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.

4. 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, Buxton, & Longbottom, 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, Mastorakos, Webster, & Chrousos, 1997). Cortisol release associated with inflammation can impair PAG production and activity (Dosogne et al., 1999). 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, Ramirez-Romero, Gallup, Bailey, & Ackermann, 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 & 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.

C. abortus, a gram-negative bacterium, is the causative agent of ovine enzootic abortion. In non-pregnant sheep, infection is unapparent. Infection during pregnancy results in a delayed abortion, 6-7 weeks following the organism reaching the fetal compartment (Entrican et al., 2001). C. abortus has a strong placental tropism in many species. In sheep, the organism replicates in the chorionic villus trophoblasts. As discussed above, the binucleate trophoblasts at the placentome create and release significant products required for successful pregnancy. Infected trophoblast have insufficient production of necessary products like progesterone. A crucial consequence of decreasing progesterone leads to increasing estrogen concentration, resembling a temporal hormone pattern associated induction of parturition. Increasing estrogen concentrations along with decreasing progesterone increases oxytocin activity and myometrium contraction, initiating labor. This reduction of progesterone can also result in the loss of the immunomodulatory benefits of this pregnancy hormone at the maternal-fetal interface. Resultant immunopathology of the placenta also plays a role with infectious abortion with *C. abortus*. Innate immune cells like neutrophils, not usually found in the gravid uterus, infiltrate the caruncular regions (Buendia et al., 1998). Neutrophils are beneficial in phagocytizing and killing the bacterium, yet the pro-inflammatory environment defined by the presence of cytokines such as TNF- α can have detrimental effects on pregnancy. A link between IL-8 and neutrophil recruitment has been examined between placental endothelium damage and consequently pregnancy termination from lack of blood supply to the fetal compartment (D. E. Clark, Salvig, Smith, & Charnock-Jones, 1998). Consequently, the combined costs of *C. abortus* placental infection leads to fetal rejection and expulsion.

Neospora caninum, a protozoon, is the most common cause of infectious abortion in cattle, worldwide (Reichel, Alejandra Ayanegui-Alcerreca, Gondim, & Ellis, 2013). *Neospora* associated

disease and pregnancy outcomes depends on maternal immunity and stage of gestation at time of infection. Infection during the first trimester results in severe congenital defects in the offspring, yet risk of transmission to the fetus increases with gestational progression (Innes, 2007). Similar mechanisms of placental pathogenesis and resultant abortion are shared between *N. caninum* and *Toxoplasma gondii*, a cause of protozoan abortion in small ruminants (Menzies, 2011). Reproductive pathogenesis and fetal loss is associated with placental insufficiency (Lopez-Perez, Collantes-Fernandez, Aguado-Martinez, Rodriguez-Bertos, & Ortega-Mora, 2008). In cattle, placental invasion by tachzoites results in a pro-inflammatory shift of Th1 based cell population resulting in a cytokine storm. Although the Th1 influenced immune response is needed to control the infection, an excess of INF-ɣ, IL-4, and IL-12 is detrimental toward the fetus (Arranz-Solis et al., 2016). Following mid-gestation inoculation of pregnant ewes, *N. caninum* induced CD8+ T cell infiltrates and upregulation of INF- ɣ, IL-4, TNF- α, and IL-10 thus depicting a mixed Th1-Th2 response. In cattle, uterine serpin expression was negatively correlated with IL-4 and INF- ɣ cytokine concentrations at the maternal fetal interface following *N. caninum* infection (Serrano-Perez et al., 2018). This microenvironment contributes to the observed necrotizing inflammation associated with placental and fetal infection (Lopez-Perez et al., 2008). Consequently, the large inflammatory response leading to hemodynamic changes to local tissue can further lead to fetal hypoxia. Not surprisingly, the fetal trophoblast expression also changes following parasitic invasion of placental tissues. Bovine trophoblasts have an altered transcriptome demonstrating impaired cholesterol biosynthesis and extracellular matric disorganization with upregulated TLR-2 expression and cytokine production (Horcajo et al., 2017). Also, decreased concentration of circulating prolactin and PAG-2 have been associated with abortion at 5-7 months following *N. caninum* infection (Garcia-Ispierto et al., 2013). All of these factors lead to fetal morbidity and mortality following *N. caninum* placental infection.

Placental pathogenesis associated with viral infections can induce fetal loss via several mechanisms. In the mouse, viral infection induces an upregulation of INF- ɣ, which in turn can increase trophoblast MHC expression (Vassiliadis & Athanassakis, 1994). This aberrant expression of MHC may contribute to viral pathogen associated abortion, as increased maternal INF- ɣ expression is associated

with spontaneous abortion (Vassiliadis & Athanassakis, 1994). Many viruses interrupt the type I IFN pathway following cellular infection allowing evasion of the immune cells (Baigent et al., 2002). Type I IFN family proteins consist of IFNT, IFN- α, IFN- β. The benefits and roles of type I IFNs in pregnancy successes have already been discussed in this review.

5. BVDV fetal infections

BVDV is a pathogen of cattle worldwide. The virus is differentiated by genotype, one and 2, now defined as pesitvirus A and B, and biotype, cytopathic (cp) and non-cytopathic (ncp). Both genotypes present with similar clinical manifestation in livestock. The biotype, immune status of the dam, and the stage of gestation of infection determines the outcome of reproductive BVDV induced disease. Subclinical disease in the dam can result in failure of conception, embryonic death, abortion, fetal persistent infection, congenital defects, and congenital-transient infections. Maternal peak viremia occurs around 7 days post inoculation (dpi) with elimination of the virus by 14 dpi. Both phenotypes can cross the placental to cause fetal infection, yet only ncp BVDV viruses are capable of producing persistently infected (PI) offspring. In PI neonates, the viral antigen is recognized as self, limiting the type I IFN response needed for viral control. These animals continually shed the virus, maintaining the pathogen within a population. Disease associated with reproductive BVDV infection in sheep parallels what is observed in cattle.

During pregnancy, acutely infected dams transmit BVDV vertically to their fetus. Although the dam has effectively cleared the virus, she demonstrates a compromised immune response. Viral RNA is a recognized PAMP triggering the innate immune response characterized by IFN response. An antiviral state is set into motion with IFN pathway activation, continuing with an adaptive immune response. Following infection spreading to the fetal compartment, dams demonstrate sustained down regulation of chemokine receptor 4 (CXCR4) and TCR pathways (T. R. Hansen et al., 2010; Shoemaker et al., 2009). CXCR4 is expressed on platelets, immune, and neuronal cells and participate in many biological processes most notably immune cell trafficking, and embryonic development in vasculo- and neurogenesis (Nagasawa, Tachibana, & Kishimoto, 1998). Down regulation of TCR pathways interferes

with cellular defense. BVDV antigen is not found again in maternal circulation after viral elimination even when carrying an infected fetus (T. R. Hansen et al., 2010). However, pregnant heifers carrying a PI fetus will have low, positive virus neutralization (VN) antibody titers 15 dpi which continually increase through 115 dpi. These data support the continual exposure of the maternal immune response to fetally derived virus yet local immunity prevents repeating maternal viremia. This gestational titer profile is not observed in heifers carrying congenital, transiently infected (CI) fetuses (T. R. Hansen et al., 2010). Smironova et al. described differential gene expression of circulating leukocytes of heifers carrying these two different populations of BVDV infected fetuses. Both groups had increased expression of interferon stimulated genes (ISG) including ISG15, 44, and 28; oligoadenylate synthetase 1 (OAS-1) and myxovirus resistance factor 2 (MX2) (Smirnova et al., 2008). Upregulated expression of these mRNA are prominent in innate immune responses to viral pathogen. An upregulated type I IFN response has also been described in developing calves during gestation following BVDV infection (Shoemaker et al., 2009). Type I IFN can act as a growth-suppressive with long-term increased expression thus contributing to overall fetal morbidity associated with viral infection (Iwase et al., 1997).

Following reproductive infection BVDV antigen is localized to uterine luminal and glandular epithelium, placentomes, and intercotyledonary fetal membranes (Fredriksen, Press, Sandvik, Odegaard, & Loken, 1999). BVDV antigen can also be detected in uterine macrophages and T cells (Baszler, Evermann, Kaylor, Byington, & Dilbeck, 1995; Fredriksen et al., 1999; Lopez, Osorio, Kelling, & Donis, 1993). In ewes inoculated with ncp BVDV-2 isolate at 55-60 days of gestation, viral replication at the placentome from seven to 21 dpi was observed, with severe fibrinous ulcerative placentitis developing over time (Scherer et al., 2001). Binucleate trophoblasts demonstrated a larger portion of BVDV antigen compared to mononuclear cells (Swasdipan, Bielefeldt-Ohmann, Phillips, Kirkland, & McGowan, 2001). Consequently, trophoblasts suffering from BVDV infection have altered creation of need products for successful pregnancy including INFT and progesterone. As a type I IFN, INFT may have inherent anti-viral properties yet the role of IFNT in BVDV infected placenta has not been elucidated (Swasdipan et al., 2001). In ovaries from PI cattle, luteal cells are positive for BVDV antigen and resulting in altered

hormone production (Booth, Stevens, Collins, & Brownlie, 1995; Fredriksen et al., 1999). Luteal cell production of progesterone is required for maintenance of pregnancy in early gestational sheep. Disruption of this production can lead to pregnancy failure. We can infer that the immunomodulatory effects of progesterone leading to a pro-inflammatory shift at the maternal-fetal interface can also contribute to viral pathogenesis. These described immune modifications following transplacental BVDV infection leads to intra-uterine growth restriction (IUGR) associated with placental insufficiency. Evidence of IUGR has been demonstrated in several studies evaluating birth weight and bone structure of PI versus TI and control calves (T. R. Hansen et al., 2010; Nuss et al., 2005; Webb, McGilvray, Smirnova, Hansen, & Norrdin, 2013). As with other infectious pathogens, placental and uterine pathology caused by alteration of multiple pathways results in detrimental side effects to the fetus.

6. Exosomes – biogenesis and function

Extracellular vesicles (EV) are produced by almost all mammalian cells. Currently EVs are classified based on size, content, and mechanism of production. Exosomes are one type of EV and are distinct in their biogenesis. Exosomes are formed with in intraluminal vesicles inside multivesicular bodies (MVBs). These MVBs are created from budding from the endoplasmic reticulum. Cargo installation into exosomes requires several steps involving endosomal proteins. Eventually, MVB fuse with the plasma membrane effectively dumping the exosomes via exocytosis into the extracellular space. Exosomes can be identified based on the presence surface marker proteins, size, and density. Once in the extracellular environment, exosomes can interact with cells locally in a paracrine manner or distributed in circulation to act in an endocrine mode. Recipient cells can uptake exosomes via catharadin-induced endocytosis, phagocytosis, or pinocytosis (Tian, Wang, Wang, Zhu, & Xiao, 2010). EVs such as exosomes play a role in cell-to-cell communication. Exosomes have been shown to effect a wide arrange of regulatory functions including immunity (Raposo & Stoorvogel, 2013). EV contain protein, nucleic acid, and lipid contents that can be shared with recipient cells thus imparting effects on cellular processes. EV can be isolated from a variety of biological fluids including serum, whole blood, milk and amniotic fluid in livestock and humans (G. Burns et al., 2014). Studies in humans have investigated EV content as

a non-invasive biomarker during disease conditions including complicated pregnancies (Hausler et al., 2010).

EV are believed to be a mode of maternal-fetal communication during pregnancy via transfer of EV contents between the conceptus and endometrium. Further, it is hypothesized that embryonic implantation is depended on uterine exosomes, critical for pregnancy recognition in ewes (Brooks, Burns, & Spencer, 2015; Ng et al., 2013; Ruiz-Gonzalez et al., 2015). Labeled maternal EV from the luminal and glandular epithelium traffic and interact directly with the trophoblast of the conceptus (G. Burns et al., 2014). It is proposed that lipid content of EV help with embryonic cell growth and other cargo may effect function, proliferation, adhesion and attachment. In vitro, EV stimulate trophoblast proliferation and IFNT production (Ruiz-Gonzalez et al., 2015). In sheep, the total number of endometrial derived EV in uterine lumen are positively correlated with progesterone concentration therefore helping to establish an embryotrophic environment (G. W. Burns et al., 2018). This concept in confirmed in women, who have 50 fold greater exosomes in circulation during pregnancy (Salomon et al., 2016). Exosome concentration also increases considerable with gestational age when fetal-maternal contact surface area and blood flow also increase (Sarker et al., 2014).

Several roles of EV have been proposed during healthy pregnancy. Exosome exchange between the endometrium and cytotrophoblasts is associated with structural remodeling, allowing increased uterine blood blow during gestation (Desrochers, Antonyak, & Cerione, 2016). EV demonstrate an immunomodulatory role during pregnancy. Vesicles are thought to suppress maternal immunity by inhibiting T cell and NK activation, which are prominent in uterine endometrium during pregnancy (Mincheva-Nilsson & Baranov, 2014). Placental exosomes modulate cytokine release attenuating NK and CD8+ and ɣ/δ T cells in a dose dependent manner (Hedlund et al., 2009). Placental EV can content and transfer immunosuppressive messengers like syncytin-1, which decreases pro-inflammatory cytokines, TNF-α, and IFN-ɣ concentrations (Than et al., 2008). It is important to remember that all activity of EVs is associated with their content and bioactivity.

Exosomes are released under both normal and pathologic conditions. Secretion of EV is altered in pathologic pregnancies conditions such as in pre-eclampsia (Adam et al., 2017). Furthermore, oxygen tensions and glucose concentrations, both indicators of placental metabolism and blood flow, influence EV release (Rice et al., 2015). Circulating exosome concentration is greater in pregnant woman compared to controls, yet pre-eclampic woman have 40% greater exosomes than women with healthy pregnancies (Goswami et al., 2006). This eludes to a role in maternal-fetal communication during times of placental disease. Utilization of EV as a form of cell-cell communication has been well described in human pregnancy, yet warrants further research in livestock.

Exosomal content can be altered with viral infection. When infected with Dengue Virus, exosomes contain a larger amount of type I IFN based proteins needed for cellular antiviral responses (Zhu et al., 2015). Many RNA viruses, including HIV and PRRSV, utilize exosomes from virally infected cells to spread infectious viral RNA and proteins to susceptible cells (Narayanan et al., 2013; L. Wang et al., 2018). With the help of exosomes, HIV+ exosomes target CD4+ T cell, inducing activation, and making them susceptible to HIV replication (Arenaccio et al., 2014). Hepatitis A virus mimics exosome formation by encapsulating fully infectious viral particles into vesicles derived from endosomal membranes (White, Llinas-Brunet, & Bos, 2006). Within the membrane, hiding viral antigens from antibodies capable of neutralization. The use of exosomes by important livestock viruses during reproductive pathology has not been explored.

7. MicroRNAs during pregnancy & disease

MircoRNAs (miRNAs) are less than 25 nucleotide base pairs in length and pay a role in gene expression (Reid, Kirschner, & van Zandwijk, 2011). These non-coding RNA products regulate gene expression by repression of translation or inducing transcript decay ultimately leading to intracellular silencing (Ouyang, Mouillet, Coyne, & Sadovsky, 2014). Pre-miRNA genes are transcribed and processed in the nucleus, then exported to the cytoplasm as an immature, stem-loop miRNA (Guo & Lu, 2010). In the cytoplasm, they are further processed by endonucleases to form mature, short sequences (Hutvagner et al., 2001). There are over 1500 miRNAs identified in humans (Ouyang et al., 2014).

Protein or EV bound MiRNA can be isolated from blood, urine, and milk in many species including ruminants (Pohler et al., 2015; Reid et al., 2011). Packaging of miRNA within the lipid bilayer of EV prevent breakdown by endogenous RNA-ases, perhaps increasing their effectiveness by increasing the likelihood of reaching recipient cells (Reid et al., 2011). Human based studies have determined differential abundance of specific miRNAs associated with neoplasia, sepsis, and pregnancy status (Chen et al., 2006; Hausler et al., 2010; J. Wang et al., 2010). Thus, miRNAs are thought to have important regulatory function in many tissues including the reproductive system. In immunity, miRNA profiles are associated with decreasing cortisol production and DAMPs signaling associated with inflammation eluding to an anti-inflammatory role (Fleshner & Crane, 2017). Certain miRNAs are upregulated by pro-inflammatory pathway activation of NFκ-b in macrophages, aiding in a robust immune response following LPS stimulation (Taganov, Boldin, Chang, & Baltimore, 2006). Furthermore, miRNAs have been shown to increase granulocyte and NK activity (Mehta and Baltimore, 2016).

Roles of miRNAs within the reproductive system are well studied in humans and more recently, livestock. MiRNAs have been demonstrated in ovarian function including follicular and ganulosa cell development (Andreas et al., 2016; Gebremedhn et al., 2015). During diestrus, progesterone appears to play a role in miRNA expression. In sheep, progesterone administration altered miRNA cargo of EV found in the uterine lumen (G. W. Burns et al., 2018). Progesterone-up-regulated miRNAs have been explored in other species as well (Cochrane et al., 2012). Burns et al. were able to characterize unique miRNAs within in EV associated with progesterone treatment in sheep (G. W. Burns et al., 2018). Pregnancy specific miRNAs have been extensively investigated in pregnant human and now more research is focused on this population of miRNAs in horses, cattle, and sheep (G. Burns et al., 2014; Klohonatz et al., 2016; Pohler et al., 2017). Burns et al. characterized EVs and miRNA profile in pregnant sheep in the uterine lumen and hypothesized their role in maternal-fetal communication (Brooks et al., 2015). Several studies have identified circulating miRNAs in cattle as potential biomarkers for early pregnancy detection (Ioannidis & Donadeu, 2016; Pohler et al., 2017). In humans a placenta-cluster of miRNAs, chromosome 19 miRNA cluster (C19MC) has been distinguished as trophoblast specific that

are eliminated following (Ouyang et al., 2014). Furthermore, this group of miRNAs has been characterized within exosomes. Expression of C19MC in trophoblast appears to be dependent on location and behavior, with invasive villous trophoblasts expressing more of this miRNA cluster (Xie, Han, Liu, Han, & Liu, 2014; Y. F. Xie et al., 2014). Greater C19MC expression correlates with maternal uterine stroma interaction, increasing the likelihood that miRNAs will gain access to maternal circulation. Chromosome 14 miRNA cluster (C14MC) are also associated with pregnancy in humans, however, unlike C19MC, their concentration decreases as pregnancy progresses (Ouyang et al., 2014). Recently, several miRNAs were identified in serum of pregnant cattle at 24 days of gestation (Gebremedhn et al., 2018). These miRNAs identified shared homologous structure to C14MC miRNAs, and thought to be pregnancy specific. Further research is needed to determine trophoblast specific miRNAs and quantify their presence in maternal circulation in livestock.

Once in circulation, miRNAs have the ability to affect maternal physiology through gene silencing. The full understanding of miRNAs biological actions during pregnancy remain to be elucidated, however, their presence has been associated with a variety of pregnancy related pathophysiological conditions. In humans, circulating miRNAs are biomarker candidates for pregnancy related diseases including ectopic pregnancy, intrauterine growth restriction, and eclampsia (P. Li et al., 2013; Miura et al., 2015; Mouillet et al., 2010). Differential expression and abundance of miRNA has been investigated as a marker for pregnancy outcome in livestock. In cattle, differential miRNA are defined as markers of embryonic viability, with evidence supporting differential expression prior to embryonic mortality (Pohler et al., 2017). In SNCT pregnancy, overall low abundance of exosomal miRNAs at 21 days of gestation was associated with unsuccessful pregnancy outcome (De Bem et al., 2017). Twenty-seven miRNAs were more abundant in SCNT pregnancy that reached term. Using gene ontology, the miRNAs were strongly associated with cell proliferation, angiogenesis, and embryonic development (De Bem et al., 2017). Based on these recent studies and the body of literature in human pregnancy, miRNAs have a potential to serve as a biomarker to assess pregnancy status and placental health yet standardization of methodology is needed.

MiRNAs activity in the face of viral infection has been investigated. Trophoblastic C19MC confer resistance to viral infection by promoting autophagy in cell culture yet concentration of miRNA was not influenced by viral infection in in-vivo modeling (Dumont et al., 2017)). This resistance effect was not observed with bacterial or protozoan challenges (Bayer et al., 2018). However, this finding has not been reproduced in vivo. In cell culture, bta-miR-2411 decreased BVDV replication in infected cells (Shi et al., 2018). MiRNAs have also been used as a mechanism for viruses to avoid host immunity. A portion of the human herpes virus genome encodes for miRNAs that can be transferred to other host cells (Pegtel, van de Garde, & Middeldorp, 2011). Once inside a recipient cell, viral origin miRNA act as host miRNAs and silence gene expression, thus down regulating IFN responses needed to control viral infection. With the current understanding of the role of exosomes and miRNAs in intercellular communication, it is not surprising that viruses would have a means to exploit this host mechanism.

8. EV Proteomics of pregnancy

Proteomic content in EV during pregnancy is a new field of research and thus not well described in published literature; however, more has been described in humans. A proteome analysis of all EV during the first trimester in humans revealed a protein profile involved with vesicle transport and inflammation, including annexin V and complement proteins (Tong et al., 2016). Jia et al performed the only EV proteome profile performed in abnormal pregnancy. They profiled the exosomal proteome in umbilical cord blood comparing healthy and pre-eclampic pregnancy (Jia et al., 2015). Twenty-nine proteins were differential abundant based on disease status, demonstrating an altered expression profile of exosomal proteins in pre-eclampsia pregnancy possibly contributing to the pathogenesis of this condition. In bovine uterine exosomes during early pregnancy, 172 differentially abundant proteins were identified including IFNT (Kusama et al., 2018). The exosomal proteome shifted with a 1.5 fold change with conceptus attachment. Upregulated proteins were associated with regulation of adhesion molecule expression and apoptosis. Kusama et al. concluded that these exosome and their cargo are required for conceptus attachment (Kusama et al., 2018). Studies have evaluated change in endometrial proteome of pregnant sheep prior to attachment of the conceptus. Using a unilateral pregnant sheep model,

Arianmanesh et al. determined expression of endometrial proteins in early gestation (Arianmanesh, Fowler, & Al-Gubory, 2016). Several proteins associated with metabolism and cellular adhesion, including apolipoptroin A and heat shock proteins were differential abundant. With what is known of the production and interaction of endometrial EV and corresponding nucleic acid content with the conceptus, investigation of EV proteome content is a worthy area of exploration.

9. Conclusions

Communication between the maternal and fetal tissues is critical for allow successful pregnancy. This is supported by the exchange of proteins, nucleic acids, biologically active lipids at this interface at the time of attachment and progression through a healthy gestation. The alteration of these compounds are notable during times of pregnancy complications associated with infectious placental pathogenesis. Further exploration of these maternal and fetal products is critical to gain a full understanding of the milieu environment during healthy and disease pregnancy. After identification of markers associated with feto-placental health and pathology, mechanisms of biologic activity can be elucidating using in-vitro and in-vivo methodology. Furthermore, biomarkers associated with pathologic conditions may be identified and used to assess placental health during gestation.

Chapter 2

Alteration of pregnancy associated glycoprotein concentration in pregnant sheep infected with bovine viral diarrhea virus

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Abstract

Pregnancy-associated glycoproteins (PAGs) are proteins produced in large quantities from the placenta of even-toed ungulates. PAGs are used as a marker of pregnancy status and exhibit temporal expression patterns. The purpose of this study was to evaluate alterations of PAG1 concentrations in pregnant sheep following exposure to Bovine Viral Diarrhea Virus. Fifteen pregnant, yearling sheep were randomly assigned to two treatments groups. At 65 days of gestation, ewes underwent intranasal inoculation with 10^7 TCID50/mL of non-cytopathic BVDV New York-1 clinical isolate or equivalent volume of media for sham inoculation. Venous blood was collected at gestational day 60 and 85. At gestational day 85, uterine venous blood, amniotic fluid, and fetal tissue were collected via laparotomy. Successful fetal transmission of BVDV was confirmed with RT-PCR of fetal tissue. Blood PAG1 concentrations were determined using a commercially available ELISA. PAG1 concentrations were compared between treatment group and site of collection using ANOVA analysis. PAG1 concentrations in jugular and uterine vein blood differed between treatment groups, with mean concentrations significantly lower ($P = 0.04$ and 0.01, respectively) in BVDV inoculated dams compared to controls at gestational day 80. No significant concentration difference was found in amniotic fluid PAG1 concentrations ($P > 0.05$). In conclusion, infection with BVDV decreases PAG1 concentration in maternal circulation in pregnant sheep compared to healthy controls. Maternal PAG concentrations may be a viable biomarker to determine placental health following reproductive viral infections.

Keywords

Pregnancy-associated glycoproteins

Sheep

Bovine Viral Diarrhea Virus

Placenta

1. Introduction

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of livestock, worldwide. The reproductive outcome of BVDV fetal infection is largely dependent on the immune status of the dam, stage of gestation at the time of infection, biotype and virulence of the infecting virus (Kelling & Topliff, 2013). Data collected from a recent meta-analysis demonstrated that BVDV infection of naive dams during gestation results in fetal infection in greater than 95% of exposures (Newcomer, Walz, Givens, & Wilson, 2015). Potential reproductive sequelae include reduced conception, embryonic death, abortion, development of persistent infection, congenital malformations, and transient fetal infection (McGowan, Kirkland, Rodwell, Kerr, & Carroll, 1993). Many ungulate species are susceptible to BVDV infection (Passler, Ditchkoff, & Walz, 2016; Passler et al., 2014). Reproductive effects of pregnant ewes exposed to BVDV demonstrate similar disease progression and outcome as cattle, therefore insights into maternal-fetal health in the face of BVDV infection can be directly correlated (Scherer et al., 2001).

Pregnancy associated glycoproteins (PAG) are produced by the ruminant trophoblast and accumulate at the microvillar junction of the placenta (R. M. Wallace et al., 2015; Wooding et al., 2005). The measurement of circulating PAGs in maternal blood and milk is commonly used to determine pregnancy status in ruminants (Commun et al., 2016; Reese et al., 2018). Although the function of PAGs are not fully understood, circulating concentrations have been evaluated as a biomarker of placental health (R. M. Wallace et al., 2015). Decreases in circulating PAG concentrations have been associated with embryonic loss and *Neospora caninium* induced abortion (Gatea et al., 2018; Pohler et al., 2013; J. M. Wallace, Aitken, Cheyne, & Humblot, 1997). Additionally, PAG concentrations are associated with abnormal placental development in dams carrying somatic cell nuclear transfer fetuses (Constant et al.,

2011). Variations in PAG concentrations have never been evaluated in regard to reproductive viral infections.

The purpose of this study was to determine the effect of BVDV fetal infection at gestational age 85 on maternal systemic blood, uterine outflow blood, and amniotic fluid concentrations of PAG1. Our hypothesis is that PAG1 concentrations in BVDV infected sheep would decrease in each compartment compared to sham-inoculated, pregnant controls and the ensuing decline in concentration can be used as a marker of fetal health.

2. Materials and Methods

2.1 Animals

All animal work was approved under the guidance of the University of Tennessee Institutional Animal Care and Use committee (protocol 2479). Fifteen, yearling primiparous Kahtadin ewes were acquired from local sources and enrolled in the study. The ewes underwent estrous synchronization as described: an intravaginal insert containing 0.9 g progesterone (EAZI-BREED CIDR sheep insert, Zoetis Inc., Parsippany, New Jersey) was placed then removed after seven days, 5 mg dinoprost tromethamine (Lutalyse Injection, Zoetis Inc.) was intramuscularly administered at the time of removal. Ewes were housed with a fertile ram for natural service. Pregnancy diagnosis and confirmation using transabdominal ultrasound occurred at gestational day (GD) 30 and 60, respectively. At confirmation, 11 ewes exposed to the ram had similar pregnancy lengths at GD 60 while the remaining 4 ewes carried younger fetuses and were excluded from the study. After confirmation of pregnancy, sheep were then randomly allocated between two treatment groups: BVDV (n = 6) or CTRL (n = 5). A larger number of experimental units were allotted to the BVDV group to account for expected fetal loss following inoculation. At GD 65, ewes received intranasal inoculation of non-cytopathic BVDV type 2 isolate NY- 1 (10^7 $TCID^{50}$/mL) or equivalent volume of media to serve as sham. Blood was collected at GD 65 and 80 via jugular venipuncture. Whole blood was allowed to clot for 30 minutes then centrifuged at 3000 x *g* for 10 minutes, then the serum removed, aliquotted and stored at – 80 ^{0}C until analysis. At GD 80, 15 days post inoculation (dpi), sheep were placed under general anesthesia and hysterectomy performed. A 12 to 15

cm long incision was made on midline, starting immediately cranial to the udder. The linea alba was identified and incised to enter the abdomen. The uterine horns were carefully exteriorized and the uterine vein ipsilateral to the gravid horn identified to collect 3 mL of blood via catheterization with a 21 g butterfly catheter, afterward a fine needle aspiration of 3 mL of amniotic fluid was collected. *En bloc* resection of the complete uterus containing all fetal tissue was obtained by creating window in the broad ligaments to double ligate 1 cm apart the ovarian pedicle with #1 or #2 absorbable suture material (either polydioxanone or polyglactin sutures) before transection. The uterine body was ligated at the cervix using one encircling ligature and one transfixing ligature using #1 or #2 absorbable suture material before section of the uterine body and *en bloc* resection of the complete uterus. The body wall was closed in 3 layers: peritoneum with the linea alba and subcutaneous tissues in simple continuous patterns using #1 absorbable suture material, and the skin in a Ford interlocking pattern using #1 non-absorbable braided suture material (polyamide) as described by Noordsy (Noordsey, 1995). Fetal and maternal tissue where placed on ice and processed within 30 minutes of removal. Representative tissues of each fetus and placentomes were flash frozen in liquid nitrogen. All samples were then stored at -80^{o}F until analysis.

2.2 PAG analysis

PAG concentrations were determined in serum originating from the jugular and uterine vein as well as amniotic fluid using a commercially available pregnancy specific protein B (PSPB) enzyme-linked immunoassay (ELISA) (BioPRYN, BioTracking LLC, Moscow, Idaho). PSPB is a placental protein consisting of an unknown number of PAG variants (Roberts et al., 2017). For the purpose of this paper, the authors will refer to PSPB as PAG1. The ELISA assay was performed following manufacturer's instructions. All samples were performed in duplicate including protein standard, non-pregnant sheep serum and late gestation pregnant sheep serum as negative and positive controls respectively. Plates were read within 30 minutes of completion of the ELISA on a plate reader at 650 nm and concentrations estimated based on derivation from a standard curve run in duplicate with each plate. Standard best fit line was accepted with a fit $R^2 > 0.989$. Mean intraassay CVs based on two quality

control pools were less than 3 and 5%. The interassay CVs for the same quality control pools were 2 and 15%, respectively.

2.3 Progesterone measurement

Progesterone concentrations were determined on serum collected from the jugular vein at GD 85 using commercially available radioimmunoassay (ImmuneChem Progesterone kit, MP Biomedicals, LLC, Orangeburg, NY). The assay was performed following manufacturer's guidelines with an additional 15-minute incubation period prior to centrifugation of the precipitated solution. Intra- and interassay coefficients of variation were both <10%.

2.4 BVDV inoculum preparation

Viral isolate BVDV non-cytopathic genotype 1b (pestivirus A), strain NY-1 was propagated in Madin-Darby bovine kidney cells in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum, L-glutamine, and penicillin G and streptomycin. Infection was induced as described by Walz et al. (Walz, Givens, Cochran, & Navarre, 2008). All sheep where inoculated intranasally at GD 60, to mimic naturally occurring maternal exposure (Swasdipan et al., 2001). Briefly, sheep were physically restrained, haltered and the head elevated and stabilized. Using a disposable intranasal cannula, sheep received 10^7 median tissue culture infective dose (TCID50) of the NY-1 isolate in EMEM (2mL aliquot/nostril).

2.5 BVDV Polymerase chain reaction

Real time polymerase chain reaction (RT-PCR) was performed as described by La Rocca and Sandvik on fetal tissue collected at GD 80 (15 dpi) to identify the presence of BVDV NY-1 (La Rocca & Sandvik, 2009). Briefly, tissue was homogenized and RNA was isolated from samples using QIAamp viral RNA mini kit according to manufacturer's instructions (Qiagen, Hilden Germany). The reverse and forward PCR primers for this reaction were GYGTCGAACCAYTGACGACT and CCATRCCCDTAGTAGGACTAGC, respectively. A TaqMan probe (TaqMan, Applied Biosystems, Foster City, CA) (TGGATGGCYRAABCCCTGAGT) with dye label 6-FAM on the 5' end and Black

Hole Quencher (BHQ, Integrated DNA Technologies, Coralville IA) on the 3' end. Cycle threshold (Ct) values over 20 were considered positive for BVDV genetic material.

2.6 Immunohistochemistry

Placentome tissues were fixed by immersion in 10% formalin. Tissues were routinely processed and, paraffin embedded. Staining for BVDV was carried out using standard immunohistochemical procedures developed for use in formalin fixed tissues. Five micrometer thick tissue sections were cut on charged slides. Immunohistochemistry for BVD antigen was performed using the Discovery Ultra Automated Staining System (Roche, Ventana Medical Systems, Tucson, AZ). Briefly, following automated deparaffinization and antigen retrieval for 8 minutes using protease 2 retrieval solution (Roche, Ventana Medical Systems, Tucson, AZ) at 37 degrees, sections were incubated with a monoclonal anti-BVD antibody that specifically targets the BVDV glycoprotein E (clone 15.C.5., Syracuse Bioanalytical, East Syracuse, NY, USA) at a concentration of 1:400 for 1 hour. Labeling was detected using the multimer based ultra-map anti-mouse detection system with a red chromogen (Roche, Ventana Medical Systems, Tucson, AZ). Positive controls included ear notch tissues from BVD positive cows as confirmed by PCR. For negative controls, the primary antibody was replaced with homologous nonimmune serum.

2.7 Statistical analysis

Normal distribution of data was determined using PROC UNIVARIATE. Statistical analysis was conducted using a one-way ANOVA using PROC GLM to test for differences in jugular vein PAG concentration at GD 65 and 80, taking into account time and time x treatment interactions. PROC GLM was used to determine difference in progesterone, amniotic fluid and uterine vein PAG concentrations. Pearson and ranked-Spearman correlations were determined using PROC CORR. Power of test to determine observations required to find equivalence between two treatments was determined using POWER with TWOSAMPLEMEANS and TEST=EQUIV_DIFF. Significant difference between the groups was considered significant when $P < 0.05$. All results are represented as mean with standard error of the mean. All analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC).

3. Results

3.1 Confirmation of BVDV fetal-infection using PCR and IHC

Successful maternal infection and passage of BVDV into the fetal compartment was determined by RT-PCR. Fetal tissue collected from a single sheep assigned to the BVDV inoculated group was found to be negative on RT-PCR and excluded from the study leaving 5 experimental units in each treatment group used for final analysis. Fetal tissues from dams inoculated with virus (n = 5) were found to be positive on BVDV RT-PCR (mean Ct = 23.29, standard error of the mean 4.44). All fetuses of control sheep (n= 5) were confirmed negative for the virus on RT-PCR (Ct = 0).

Microscopic evaluation of placentomes with hematoxylin and eosin stained slides did not identify any significant light microscopic differences between inoculated and control animals. In control animals, low numbers of mononuclear cells, predominantly lymphocytes were present in all levels of the endometrial stroma. In inoculated animals, the intercaruncular luminal endometrial epithelium had mildly increased numbers of intraepithelial mononuclear cells; however, the increase was not statistically significant. BVDV antigen was detected by IHC within the trophoblastic epithelium lining chorioallantoic villi in all pregnant sheep inoculated with BVDV. Figure A-1 is a representative image from a single pregnant sheep inoculated with BVDV.

3.2 Changes in progesterone and PAG concentrations in sheep infected with BVDV

Mean progesterone concentrations are depicted in figure A-2. Mean progesterone concentration at GD 80 trended downward in BVDV infected sheep compared to CTRL (P = 0.08). No significant correlation was observed between progesterone concentrations and jugular PAG concentrations at GD 80 (see figure A-3; $R^2 < 0.1$, P = 0.13). Mean jugular PAG concentrations at GD 65 and GD 80 are depicted in figure A-4. Mean jugular PAG1 concentrations did not differ between treatment groups at GD 65 (P = 0.08), but significantly diverged at GD 80, 15 dpi (P = 0.04). Serum jugular PAG1 concentration differed over time regardless of treatment group (P = 0.04). Concentrations at GD 65 significantly affected mean PAG1 concentration at GD 80 (P = 0.03). Mean amniotic PAG1 concentrations for BVDV (45.59 ± 25.88 ng/mL) and CTRL (59.91 ± 22.05 ng/mL) sheep were not significantly different (P = 0.86). Mean

uterine vein PAG concentrations are depicted in figure A-5. Mean uterine vein PAG1 concentrations were significantly affected by treatment ($P = 0.01$) and significantly correlated using Pearson's correlation with jugular PAG1 concentration at GD 80 ($P < 0.01$). Figure A-6 is a scatter plot displaying significant correlation between mean jugular and uterine vein PAG concentrations ($R^2 = 0.8$, $P < 0.01$).

4. Discussion

The results of this study demonstrate successful experimental BVDV infection of pregnant ewes at GD 65 and extension of the infection to the fetus by GD 80. The effect of this infection was demonstrated by BVDV nucleic material found in fetal tissues and histopathologic evidence of BVDV co-located with placental inflammation. In addition, BVDV infection resulted in a significant decrease in PAG concentrations associated between inoculation at GD 65 and experimental termination of the pregnancy at GD 80 in both maternal systemic circulation and uterine vein outflow. To the authors' knowledge, this is the first description of the effects of viral pathogenesis on PAG concentrations and their correlation with fetal health.

4.1 BVDV induces maternal-fetal pathology

BVDV passage into the fetal compartment was confirmed with positive RT-PCR results in viral inoculated sheep. The successful inoculation method performed in this study mimics natural maternal exposure and resulting fetal infection in susceptible animals (Swasdipan et al., 2001). BVDV non-cytopathic genotype 1b (pestivirus A), strain NY-1 was used for the infectious inoculation in this study. As a non-cytopathic biotype, this BVDV strain is capable of producing an array of reproductive sequelae following inoculation, including the creation of persistently infected offspring (Kelling & Topliff, 2013). Efficient creation of a persistently infected offspring requires passage into the fetal compartments and continued maintenance of pregnancy by the dam. The placental pathology observed on histopathology was minimal in infected dams compared to controls, yet the presence of BVDV antigen within the trophoblast was confirmed using IHC. Future studies, utilizing a more virulent strain of BVDV may create significant placental pathology, thus altering maternal and fetal PAG concentrations even more dramatically. It is important to note that no abortion associated with BVDV inoculation was observed in

this study. This is consistent with other studies inducing fetal disease in small ruminants with BVDV. In does infected with BVDV at GD 65, abortion occurred 7-65 dpi in less than 50% of inoculated animals (Desouky, Younis, Ahmed, & Hegazy, 2011).

4.2 Changes in P4 and PAG concentrations with maternal-fetal BVDV infection

Although mean serum progesterone concentrations trended lower (P = 0.08) in BVDV infected ewes, no significant difference was determined between infected and control sheep in this study. BVDV infection can result in both oophritis and fetal death with subsequent release of $PGF2_a$ from the endometrium, therefore it is often not clear which process has the greatest effect on serum progesterone (Fray, Mann, Clarke, & Charleston, 1999, 2000). Several cell types in the bovine ovary are permissive to BVDV, including cumulus cells, ovarian stroma, and the oocyte throughout its development, but luteal cells appear to be inconsistently affected (Brownlie, Booth, Stevens, & Collins, 1997; Fray, Prentice, Clarke, & Charleston, 1998). In non-pregnant naïve heifers, the serum progesterone and luteal tissues were unaffected by BVDV infection in naturally cycling heifers, however synchronized heifers demonstrated reduced serum progesterone during the early diestral phase and premature luteolysis (Fray et al., 1999; Fray, Mann, et al., 2000). Yet, any cause of embryonic loss is associated with decreased serum progesterone concentrations as result of indirect luteolysis (Gabor et al., 2016). Desouky et al described evidence of luteolysis and oophiritis as well as falling progesterone concentrations in pregnant does from GD 72 – 135 following inoculation at GD 65 (Desouky et al., 2011). In this report, luteloysis was also either induced by fetal mortality and subsequent release of $PGF2_a$ or direct pathology to the luteal tissue as 4 of 9 does aborted from 7 – 65 days post-inoculation. Progesterone, produced by the corpus luteum and eventually the placenta, is important for the establishment and maintenance of pregnancy (Spencer & Bazer, 2002). Moreover, the ovine placenta is the primary source of progesterone after 50 days of gestation (Senger, 2004). Histopathology in this study suggested minimal placentitis at 15 dpi (GD 80), inferring that progesterone production was most likely normal in infected ewes at that time, but the termination of gestations makes it impossible to know if ongoing pathology would have impaired progesterone production at a later time point.

Egen et al. described a biphasic profile of PAG concentration in maternal circulation among 31 tested ewes, where PAG concentration initially peaked at 185 ng/ml at GD 63 then decreased below 100 ng/ml by GD 125 (Egen, Ealy, Landon, Roberts, & Green, 2009). A similar temporal decrease was observed in the current study between GD 65 and 80 in both treatment groups. However, the decline among the BVDV infected ewes statistically exceeded that of the control ewes suggesting that by GD 80 the infected ewes were experiencing trophoblastic injury. This observation is supported by a similar decrease in progesterone concentration and histopathologic evidence of placental inflammation co-located with BVDV antigen. At GD 65, prior to inoculation, there was no significant difference in PAG1 concentrations between treatment groups, supporting that the expected temporal decline was not the sole reason for decreasing PAG1 concentrations by GD 80. Wallace et al. also described a pattern of decreasing PAG concentration among pregnant ewes fed two rations during this gestational period and noted a sharper decrease in circulating PAG concentration among ewes experiencing non-infectious abortion than ewes carrying fetuses to term (J. M. Wallace et al., 1997). Likewise, other authors have correlated decreased PAG concentrations with embryonic loss in cattle in association with ovulatory follicle size, *Neospora caninum* infection, heat stress, high milk production, and other unidentified etiologies of fetal death (Garcia-Ispierto et al., 2013; Lopez-Gatius, Garbayo, et al., 2007; Mur-Novales et al., 2016; Perry et al., 2005; Pohler et al., 2013). In the present study, because all gestations were experimentally terminated at GD 80, it is not clear if the affected ewes would have demonstrated a continued drop in PAG concentration, ultimately indicative of ensuing abortion, or once the fetus and placenta had cleared the infection, PAG concentrations would have rebounded.

Jugular venous PAG concentrations were strongly correlated with uterine vein PAG concentrations. As expected, the mean uterine venous PAG concentration was higher compared to jugular samples, indicative of dilution and or decay of PAGs upon entry into the periphery. The fate of peripherally circulating PAGs is unknown. Bovine PAGs have a variable half-life depending on stage of pregnancy and range from 21.9 hours to 10 days. Nor is it known if the half-life changes in relation to maternal physiology or in times of fetal stress or death. In experimentally induced, late embryonic

abortion, the estimated half-life of PAGs ranged 21.9 hours to 3.9 days (Pohler et al., 2013; Szenci et al., 2003)while immediately prior to parturition half-life increased to 8-10 days (de Sousa et al., 2003). As a secretory product of fetal trophoblasts, several functions have been proposed. Experimental evidence supports immunomodulatory actions of PAGs allowing some protection to the semi-allograft placenta (R. M. Wallace et al., 2015). Treatment with bovine PAG1 alters hematopoietic cell proliferation and endometrial chemokine production (Austin, King, Vierk, Sasser, & Hansen, 1999; Hoeben et al., 1999). Futhermore, uterine serpins, which directly inhibit lymphocyte proliferation, can be bound by PAGs in vitro thus augmenting immunomodulatory actions (Peltier et al., 2000). PAGs that accumulate in the maternal stroma would allow these trophoblastic products to directly influence maternal immune cells. In addition to immunomodulatory roles, evidence suggests a luteotrophic action by PAGs. When treated with PAGs, luteal cell increase production of prostaglandin E2, which is inherently luteotrophic and anti-luteolytic in ruminants (Weems, Lammoglia, Vera-Avila, Randel, King, et al., 1998). It is interesting to note that PGE2 is directly immunosuppressive, working by inhibiting lymphocyte proliferation, possibly an indirect mechanism of PAG immunomodulary capabilities (Low & Hansen, 1988). Luteal cells exposed to PAG resulted in increased measurable progesterone, thus supporting luteal tissue function and thus maintenance of pregnancy (Weems, Lammoglia, Vera-Avila, Randel, Sasser, et al., 1998). The effect of PAGs at the maternal-fetal interface and elsewhere in the maternal body requires further investigation to define their function during pregnancy.

No significant differences in amniotic PAG1 concentrations were observed between infected and non-infected control groups in the current study. This finding was in contrast that of Mur-Novales et al. where PAG concentrations was elevated in the fetal compartments of dams infected with *N. caninum* compared to non-infected controls (Mur-Novales et al., 2016). In that study, the authors proposed that the increase in PAGs within fetal fluid was associated with placental damage and leakage into the fetal compartment. In the current study, there was minimal placentitis most likely due to the non-cytopathic nature and mild severity of the inoculating strain, both suggesting there was insufficient trophoblastic injury to induce release of PAGs in significant concentrations into fetal fluid.

5. Conclusions

This is the first study investigating alterations of maternal and fetal fluid PAG concentrations following reproductive viral infection in ruminants. Data presented here demonstrate that BVDV infection results in trophoblastic injury and a significant decrease in PAG concentrations in maternal circulation and uterine vein blood. This indicates that circulating PAG concentrations may be used as a marker of placental function in the face of reproductive infection with BVDV and adds to the growing evidence that low PAG concentrations are correlated with placental dysfunction and fetal loss. Further refinement into the temporal relationship of PAG production and function with a model of fetal infection such as the viral model introduced here will yield future insights in evaluating these proteins as biomarkers of the fetal-maternal unit.

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7. Competing interests

The authors have no competing interests to disclose.

Chapter 3

Characterization of circulating exosomal microRNA transcriptome in pregnant and non-pregnant sheep infected with Bovine Viral Diarrhea Virus

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Abstract

The placenta is an active immunologic organ, modulating the maternal immune response during pregnancy. Trophoblasts from the placenta release small membrane-bound vesicles called exosomes which play an important role in intercellular communication by transferring nucleic acids and proteins between cells. Exosomes have been found in the uterine luminal fluid within pregnant sheep, yet little is known of about their role in intercellular communication during pregnancy in times of health and disease. The hypothesis for this study states that exosomal cargo detected in maternal circulation will vary depending on reproductive health status and viral exposure. The specific objectives were to 1) characterize the profiles of circulating miRNAs in pregnant and non-pregnant sheep, and 2) determine the difference in exosome miRNA profiles between non-infected and BVDV-infected pregnant and non-pregnant sheep. Twenty-three yearling ewes, found to be seronegative to BVDV on serum neutralizing antibody serology, were enrolled in this study. Ewes were inoculated with BVDV NY-1 or non-infective sham media. Fifteen days post inoculation, all animals underwent hysterectomy and peripheral blood collection. Exosomes were isolated using ultracentrifugation and gradient density separation. Small RNA were extracted and high throughput sequencing was performed. BVDV infection status was determined

with BVDV RT-PCR, immunohistochemistry of fetal tissue, and virus neutralization. Statistical differences between circulating concentrations of miRNAs were analyzed by analysis of variance for repeated measures. Sixty million reads, identifying 1634 miRNAs were identified following high-throughput sequencing. Several unique miRNAs were isolated from BVDV exposed and/or pregnant ewes. The identification of unique miRNAs supports the hypothesis that exosomes have a biological role in fetal-maternal interactions that can be modulated by infection or pregnancy status.

Keywords

Micro RNA

Exosome

Sheep

Bovine Viral Diarrhea Virus

Placenta

1. Introduction

The placenta is the bridge between the maternal and fetal tissues. The fetal trophoblasts are immunologically active, producing products that interact with maternal cells. Some products include native molecules such as pregnancy associated glycoproteins (PAGs) and membrane protected extracellular vesicles containing multiple forms of bioactive molecules (Pohler et al., 2017; Pohler et al., 2016). Exosomes are small microvesicles, 30-20 nm in size, comprised of a lipid bilayer. The production of exosomes form as a membrane extrusions of the endoplasmic reticulum known as a microvesiclar bodies (MVB) which are ultimately released into the extracellular milieu via fusion with the interior of the parent cell's plasma membrane (Barile & Vassalli, 2017). Exosomes have received more attention recently due to the diverse type of cells that produce them and their presence in bodily fluids (G. Burns et al., 2014; Vlassov, Magdaleno, Setterquist, & Conrad, 2012). Exosomal cargo generally contains proteins, nucleic acids, and bioactive lipids such as prostaglandins (Subra et al., 2010). The exosomal content activity is determined by cell or tissue of origin and suggested to be involved with intercellular communication thus effecting physiologic processes in recipient cells (Schorey & Bhatnagar, 2008;

Vlassov et al., 2012). Once released, exosomes have paracrine effects on the local cell population or more distant endocrine effects via peripheral circulation. The lipid bilayer surrounding exosomes serve as a protective shield preventing non-specific degradation of the bioactive content of small non-coding RNA and protein. Moreover, membrane bound proteins studding the lipid bilayer likely mediate interactions with target cell receptors which may drive specific or more ubiquitous messaging. Using cantharadin induced endocytosis, the exosomal membrane merges with the cell's plasma membrane thereby exuding its contents into the cytoplasm (Barile & Vassalli, 2017).

Exosomes have been found in venous circulation in humans, cattle, and sheep (Adam et al., 2017; Gebremedhn et al., 2018; Ruiz-Gonzalez et al., 2015). Exosomal roles have been described in reproductive setting including conceptus attachment in cattle, and placental complications in humans (Jin & Menon, 2018; Kusama et al., 2018). In humans, exosomes released from trophoblasts have been demonstrated to carry out an important role in intercellular communication by transferring nucleic acids and proteins between fetal and maternal cells. Exosomes have been discovered in uterine luminal and fetal fluid in sheep in times of healthy pregnancy (G. Burns et al., 2014). However, description of circulating exosomes in livestock species during pregnancy associated infectious disease in absent.

MicroRNAs (miRNAs), less than 25 bp non-coding RNA, are one of the bioactive molecules isolated from exosomes (Ouyang et al., 2014). These small RNA regulate gene expression by repressing translation or inducing transcript decay, with an overall effect of silencing gene expression and largely down regulating protein synthesis (Glazov et al., 2009). Pregnancy specific miRNAs have been in pregnant women and recently investigated in miRNAs in horses, cattle, and sheep (G. Burns et al., 2014; Klohonatz et al., 2016; Pohler et al., 2017). Attempts to infer the function of miRNAs associated with trophoblastic exosomes has been through isolation and nucleotide sequencing which has yielded profiles correlating with placental health and risk of fetal morbidity and mortality in humans (J. Y. Li, Yong, Michael, & Gleadle, 2014; Mouillet, Ouyang, Bayer, Coyne, & Sadovsky, 2014; Ouyang et al., 2014). In humans, circulating miRNAs are biomarker candidates for pregnancy related diseases including ectopic pregnancy, intrauterine growth restriction, and eclampsia (P. Li et al., 2013; Miura et al., 2015; Mouillet

et al., 2010). Bovine Viral Diarrhea Virus (BVDV) is an economically important virus of artiodactyls worldwide (Passler & Walz, 2010). Susceptible animals infected with BVDV can suffer from a variety of clinical manifestations including immunosuppression, diarrhea, and an array of reproductive disease. The reproductive consequences seen in livestock are dependent on the stage of estrous or gestation the animals is exposure (Newcomer et al., 2015). Fetal infections, when coinciding with specific developmental timeframes, in cattle and sheep suffer from embryonic mortality, persistent fetal infection, congenital developmental defects, and abortion (Walz et al., 2018). Previous studies have profiled the maternal cytokine production and circulating mRNA transcriptome signature at differing stages of in-utero BVDV infection from uterine vein blood revealing a prominent interferon response typical of viral infections (T. R. Hansen et al., 2010; Smirnova et al., 2014).

In the face of viral infections, circulating exosome content and miRNAs are altered. RNA viruses, including human-immunodeficiency virus and porcine respiratory reproductive syndrome virus, utilize host cell exome production to aid in spread of viral proteins or infectious RNA to susceptible cell (Narayanan et al., 2013; L. Wang et al., 2018). Chromosome 19 miRNA cluster, considered the trophoblast specific miRNA in humans, confer viral resistance to non-placental cell in vitro (Dumont et al., 2017). Recently, bovine miRNA 2411 was found to decreased BVDV replication in cell culture (Shi et al., 2018)

MiRNA profiles have not been characterized during times of pathogen-associated placental pathology in livestock. Describing this exosomal content may help characterize reproductive viral pathogenesis and aid in potential biomarkers associated with placental pathology. The purpose of this study was to identify and describe the small RNA transcriptome from exosomes found in circulation in sheep. Furthermore, this study characterizes the differential miRNA expression found in relation to pregnancy status and BVDV exposure in sheep.

2. Materials and Methods

2.1 Animals

All animal work was approved under the guidance of the University of Tennessee Institutional Animal Care and Use committee (protocol 2479). Twenty-three, yearling primiparous Kahtadin ewes were acquired from local sources and enrolled in the study. Fifteen ewes underwent estrous synchronization as described: an intravaginal insert containing 0.9 g progesterone (EAZI-BREED CIDR sheep insert, Zoetis Inc., Parsippany, New Jersey) was placed then removed after seven days, 5 mg dinoprost tromethamine (Lutalyse Injection, Zoetis Inc.) was intramuscularly administered at the time of removal. Ewes were then housed with a fertile ram for natural service. Pregnancy diagnosis and confirmation using transabdominal ultrasound occurred at gestational day (GD) 30 and 65, respectively. At confirmation, 11 ewes exposed to the ram had similar pregnancy lengths at GD 65 while the remaining 4 ewes carried younger fetuses and were excluded from the study. After confirmation of pregnancy, sheep were then randomly allocated between two treatment groups: PREGNANT-BVDV (n = 6) or PREGNANT-CTRL (n = 5). A larger number of experimental units were allotted to the BVDV group to account for expected fetal loss following inoculation. The remaining 8 ewes not exposed to the ram were randomly allocated between two additional treatment groups: NON PREGNANT-BVDV (n =4) or NON PRENANT-CTRL (n= 4). All Ewes received intranasal inoculation of non-cytopathic BVDV type 1 isolate NY- 1 (10^7 TCID50/mL) or equivalent volume of media to serve as sham at GD 65 based on pregnancy confirmation. All non-pregnant ewes received intranasal viral inoculation or sham on the same day as pregnant ewes. Blood was collected at GD 80 or 15 days post inoculation (dpi) via jugular venipuncture. Whole blood was allowed to clot for 30 minutes then centrifuged at 3000 x g for 10 minutes, then the serum removed, aliquotted and stored at – 80 0 C until analysis. At 15 dpi, all sheep were placed under general anesthesia and hysterectomy performed. A 12 to 15 cm long incision was made on midline, starting immediately cranial to the udder. The linea alba was identified and incised to enter the abdomen. The uterine horns were carefully exteriorized. En bloc resection of the complete uterus, both gravid and non gravid were obtained by creating window in the broad ligaments to double ligate 1 cm apart the ovarian pedicle with #1 or #2 absorbable suture material (either polydioxanone or polyglactin sutures) before transection. The uterine body was ligated at the cervix using one encircling

ligature and one transfixing ligature using #1 or #2 absorbable suture material before section of the uterine body and en bloc resection of the complete uterus. The body wall was closed in 3 layers: peritoneum with the linea alba and subcutaneous tissues in simple continuous patterns using #1 absorbable suture material, and the skin in a Ford interlocking pattern using #1 non-absorbable braided suture material (polyamide) as described by Noordsy (Noordsy, 1976). Fetal and uterine tissue where placed on ice and processed within 30 minutes of removal. Representative tissues of each fetus and placentomes in pregnant sheep were flash frozen in liquid nitrogen. Representative tissues of caruncular tissue in non-pregnant sheep were also handled as described. All samples were then stored at -80^{o}F until analysis.

2.2 BVDV inoculum preparation

Viral isolate BVDV non-cytopathic genotype 1b (pestivirus A), strain NY-1 was propagated in Madin-Darby bovine kidney cells in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum, L-glutamine, and penicillin G and streptomycin. In-vivo infection was induced as described by Walz et al. (Walz et al., 2008). All sheep where inoculated intranasally at GD 65, to mimic naturally occurring maternal exposure (Swasdipan et al., 2001). Briefly, sheep were physically restrained, haltered and the head elevated and stabilized. Using a disposable intranasal cannula, sheep received 10^7 median tissue culture infective dose (TCID50) of the NY-1 isolate in EMEM (2mL aliquot/nostril). Sheep in the control groups were handled similarly, but received a equivalent volume of media to serve as a sham inoculation.

2.3 Virus neutralizing assay for BVDV

Virus neutralization (VN) was used to detect serum antibodies against BVDV-1 and BVDV-2. Serum was collected for VN assay prior to inoculation and 30 dpi. The BVDV-1 cytopathic stain NADL and BVDV cytopathic strain 125c was used for this assay. VN was performed as described by Walz et al. (Passler et al., 2014). Serum titer <1:4 was described as negative. A rising titer (4x) in paired serum samples collected 3 to 4 weeks apart constitutes a good basis for the diagnosis of acute BVDV infection (Lanyon, Hill, Reichel, & Brownlie, 2014)

2.3 BVDV Polymerase chain reaction

Real time polymerase chain reaction (RT-PCR) was performed as described by La Rocca and Sandvik on fetal tissue collected at GD 80 (15 dpi) to identify the presence of BVDV NY-1 (La Rocca & Sandvik, 2009). Briefly, tissue was homogenized and RNA was isolated from samples using QIAamp viral RNA mini kit according to manufacturer's instructions (Qiagen, Hilden Germany). The reverse and forward PCR primers for this reaction were GYGTCGAACCAYTGACGACT and CCATRCCCDTAGTAGGACTAGC, respectively. A TaqMan probe (TaqMan, Applied Biosystems, Foster City, CA) (TGGATGGCYRAABCCCTGAGT) with dye label 6-FAM on the 5' end and Black Hole Quencher (BHQ, Integrated DNA Technologies, Coralville IA) on the 3' end. Cycle threshold (Ct) values over 20 were considered positive for BVDV genetic material.

2.4 Immunohistochemistry

Uterine and placental tissues were fixed by immersion in 10% formalin. Tissues were routinely processed and, paraffin embedded. Staining for BVDV was carried out using standard immunohistochemical procedures developed for use in formalin fixed tissues. Five micrometer thick tissue sections were cut on charged slides. Immunohistochemistry for BVD antigen was performed using the Discovery Ultra Automated Staining System (Roche, Ventana Medical Systems, Tucson, AZ). Briefly, following automated deparaffinization and antigen retrieval for 8 mins using protease 2 retrieval solution (Roche, Ventana Medical Systems, Tucson, AZ) at 37 degrees, sections were incubated with a monoclonal anti-BVD antibody that specifically targets the BVDV glycoprotein E (clone 15.C.5., Syracuse Bioanalytical, East Syracuse, NY, USA) at a concentration of 1:400 for 1 hour. Labeling was detected using the multimer based ultra-map anti-mouse detection system with a red chromogen (Roche, Ventana Medical Systems, Tucson, AZ). Positive controls included ear notch tissues from BVD positive cows as confirmed by PCR. For negative controls, the primary antibody was replaced with homologous nonimmune serum.

2.5 Progesterone measurement

Progesterone concentrations were determined on serum collected from the jugular vein at GD 80 or 15 dpi using commercially available radioimmunoassay (ImmuneChem Progesterone kit, MP Biomedicals, LLC, Orangeburg, NY). The assay was performed following manufacturer's guidelines with an additional 15-minute incubation period prior to centrifugation of the precipitated solution. Intra- and interassay coefficients of variation were both <10%.

2.6 Exosome isolation

Extracellular vesicles were isolated from 2 mL of serum 15 dpi using a combination of centrifugation gradient isolation. Each sample was centrifuged for 15 minutes at 3000 x g and cleared supernatant transferred to sterile tube. Exosome precipitation was performed using a commercial kit according to manufacturer's instructions (ExoQuick, System Biosciences, Palo Alto CA). Cleaned exosome pellets were suspended in 250 uL of PBS; 20 uL was saved for western blot analysis while the remainder was used for miRNA extraction described below.

2.6.1 Western blot

Western blots were performed as described by Pohler et al (Pohler et al., 2017). Exosomes were identified for immunopositivity to surface markers Cluster of differentiation 81 (CD 81) and Heat-shock-protein 70 (HSP 70). Both of these markers well characterized as identifiers of exosomes(Vlassov et al., 2012).

2.7 MiRNA extraction and validation

Total miRNA was extracted from each biological replicate by using the miRNeasy mini-isolation kit (Qiagen, Germantown, MD). The quantity and quality of miRNAs were evaluated on a small RNA Labchip kit (Agilent Technologies, Santa Clara CA) using an Agilent 2100 Bioanalyzer according to manufacturer's instructions.

2.8 Illumina RNA library preparation and sequencing

Three experimental units per treatment group that were positive on serum exosome immunoreactivity for CD81 and HSP70 collected at 15 dpi were submitted for deep sequencing. All miRNA sequencing was performed on the Illumina Hi Seq2500 system at University of Kansas Medical Center Genomics Core (Kansas City, KS). All methods below have been validated and published by author Pohler (Pohler et al., 2017). The final volume of extracted small RNAs were converted to cDNA with the reverse transcriptase SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions for oligo dT priming. Second-strand cDNA was then synthesized by following a protocol provided by Illumina; the protocol uses the Klenow fragment of DNA polymerase I (Illumina, San Diego, CA). The double-stranded samples were purified with a QIAquick PCR spin column (Qiagen). Libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq miRNA stranded sample preparation kit. Briefly, the polyadenylated miRNA was purified from total RNA and fragmented. Double-stranded cDNA will be generated from fragmented RNA, and the index containing adapters ligated. The final construct of each purified library was evaluated using the Fragment Analyzer instrument, quantified with the Qubit fluorimeter using the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol for sequencing on an Illumina HiSeq 2500 sequencer. Sequence runs were presented as 100 bp paired-end reads.

2.9 Statistical analysis

Normal distribution of data was determined using PROC UNIVARIATE. Statistical analysis was conducted using a one-way ANOVA using PROC GLM to test for differences in mean progesterone concentrations. Power of test to determine observations required to find equivalence between two treatments was determined using POWER with TWOSAMPLEMEANS and TEST=EQUIV_DIFF. Significant difference between the groups was considered significant when $P < 0.05$. All results are represented as mean with standard error of the mean. All analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC).

2.9.1 Bioinformatics

Read quality was verified using FastQC, a quality control program that determines base content, sequence quality, and adaptor content [1]. Next, the sequences were trimmed of their adaptor content using Skewer, a program that takes user-defined transcripts and adapter sequences and trims them based on the user's parameters [2]. FastQC analysis indicated the presence of a single adaptor: the Illumina small RNA 3' adaptor; using this as the sole adapter sequence, Skewer was run using mostly default parameters for single end trimming. Due to the smaller size of miRNAs, a minimum read length of 10 was chosen instead of Skewer's default of 18. One of the 12 samples was split into two files due to the total file size exceeding Illumina's maximum file size; additionally, all samples were run on two lanes, meaning that each sample had two files corresponding to each lane. These files were concatenated into a single file for each sample after trimming, and FastQC was run again to confirm the removal of all adapter content. Based on the methods of another small RNA study in Ovis aries, the sequences were filtered to remove any reads with more than 30 base pairs [3].

The microRNA database, miRBase, contains the sequences for 106 known miRNAs annotated in the O. aries genome [4-8]. To determine the percentage of reads that align with these known miRNAs, UCSC's BLAST-like alignment tool (BLAT) was used with special parameters to account for the smaller size of small RNAs [9]. In particular, the step size, or spacing between tiles, was set to 1, minimum sequence identity and score were set to 0, a high number of repetitions was used to avoid sequences being marked as "overused," and the "fine" option was selected. Accounting for the relative small number of known miRNAs in O. aries, 5 additional species were used: fellow ruminants Bos Taurus (cow) and Capra hircus (goat), and well-annotated mammalians Mus musculus, Rattus norvegicus, and Homo sapiens. The design for this procedure was as follows; run BLAT to align sequences with one of the miRBase hairpin libraries, filter out multi-mapping reads, and filter out any reads that match with a miRNA. This process is repeated until reads matching any miRNA across the 6 miRNA libraries were identified. After filtering, each sample had six files – one for each set of miRNAs – containing a list of reads that aligned to known miRNAs from one of the six libraries. The filtering process ensured that no

read was repeated between any of the six files, and any redundant miRNAs were collapsed into a single instance. These files were concatenated into single files containing every known miRNA aligning to reads from a sample, and a custom python script was used to count every instance of a miRNA in the combined file and save these results to a new file.

Two factors were used in analysis to determine differential expression: the status of the uterus (Non-Pregnant vs. Pregnant) and treatment with bovine viral diarrhea virus (CTRL vs. BVDV). The count files for the 12 samples were exported to RStudio, and analyzed using the DESeq2 and EdgeR packages [10-12]. DESeq2 was used to combine the individual counts files into a single data set, while EdgeR was used to perform a quasi-likelihood F-test to identify genes with significant differential expression. This analysis produced a list of miRNAs, based on EdgeR's default adjusted p-value of 0.05 for QLF tests, that were deemed differentially expressed. To visualize these results through heatmaps, the reads were converted into log counts per million with an added count of 2 to avoid taking the log of zero. Additionally, the top 50 genes – ranked on their adjusted p-value with a false discovery rate of 0.1– were used to create Venn diagrams to identify genes unique to each treatment type. To be considered biologically relevant, at least 2 out of 3 sheep sequenced per group were required to have abundant miRNA expression defined by a count per million reads.

To identify if samples obtained from ewes infected with BVDV contained genetic data from the virus, all remaining reads from the blat alignment and filtering step were aligned to the Ovis aries genome (assembly Oar_v4.0, ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/298/735/GCF_000298735.2_Oar_v4.0/GCF_000298735.2_Oar_v4.0_genomic.fna.gz) using sensitive Bowtie2 alignment [14]. Bowtie2 produces a sequence alignment/map (SAM) format file that marks the number of times a read aligns to the reference genome. SAM Tools was used to create a separate binary SAM (BAM) file containing only reads that did not align to the O. aries genome, and to generate a fasta file containing the sequences of these unaligned reads [15]. These reads were aligned to the completed BVDV genome (GenBank: KJ689448.1) with BLAT, using the same parameters for short miRNA reads described above, to identify any overlap between the virus'

genome and the unknown reads. In theory, the reads collected from infected ewes would contain more reads that overlap with the BVDV genome than those collected from healthy ewes.

3. Results

3.1 Confirmation of BVDV infection using VN, PCR, and IHC

Prior to enrollment of the study, all sheep were negative (<1:4) for neutralizing antibodies to BVDV, confirming immunologic naivety of the animals. All animals sham inoculated with sterile media remained negative (<1:4) on VN throughout the study period. All animals enrolled in the BVDV group who received intranasal viral inoculation had a positive titer on VN assay 30 days following viral exposure. The mean titer for PREGNANT-BVDV was 1:32 (standard error of the mean (SEM) ± 6) and Non-Pregnant-BVDV was 1:24 (SEM ± 6). Successful maternal infection and passage of BVDV into the fetal compartment was determined by RT-PCR. Fetal tissues from dams inoculated with virus (n = 5) were found to be positive on BVDV RT-PCR (mean Ct = 23.29 ± 4.44). All fetuses of PREGNANT-CTRL sheep (n= 5) were negative for the virus on RT-PCR (Ct = 0).

Microscopic evaluation of placentomes with hematoxylin and eosin stained slides did not identify any significant light microscopic differences between inoculated and control animals. In control animals, low numbers of mononuclear cells, predominantly lymphocytes were present in all levels of the endometrial stroma. In inoculated animals, the intercaruncular luminal endometrial epithelium had mildly increased numbers of intraepithelial mononuclear cells. BVDV antigen was detected by IHC within the trophoblastic epithelium lining chorioallantoic villi in all PREGNANT-BVDV sheep inoculated with BVDV. Figure A-1 is a representative image from a single pregnant sheep inoculated with BVDV. Histopathology of NON PREGNANT-BVDV sheep subjectively demonstrated mild cellular infiltration of mononuclear cells with a mean of 17 cells compared to mean of 11 cells observed in NON PREGNANT CTRL sheep. BVDV antigen was not visualized in uterine tissue of any sheep in the NON PREGNANT-BVDV group.

3.2 Progesterone concentrations between treatment groups

Mean progesterone concentrations measured 15 dpi for all treatment groups are depicted in figure A-7. Mean concentrations for PREGNANT-CTRL sheep (2.77 ng/ml ± 0.86) was not significantly different compared to PREGNANT-BVDV (1.65 ng/mL ± 0.52) and NON PREGNANT-CTRL (1.65 ng/mL ± 0.63) ($P > 0.1$). Mean progesterone concentrations for NON PREGNANT-BVDV was significantly lower at 0.77 ng/mL (SEM ± 0.57) compared to PREGNANT-CTRL ($P = 0.01$) but did not significantly differ compared to the remaining treatment groups ($P = 0.1$).

3.3 Evaluation of isolated exosomes and miRNA content

Exosomes were successfully isolated from circulating blood using chemical gradient methodology. Confirmation of exosomes isolated from serum was performed using western blot, which demonstrated immunostaining for exosome surface markers CD 81 and HSP 70. Figure A-8A displays a representative image from a sheep assigned to the PREGNANT-CTRL group demonstrating a positive western blot result for both CD 81 and HSP 70 along with positive and negative controls. All biologic replicates for each treatment group were positive for these surface markers. Quantification of miRNAs were performed for each biological replicate prior to sequencing. Mean miRNA concentration for each treatment group are as follows: PREGNANT-CTRL 706.96 pg/ µL (SEM ± 177.87), PREGNANT-BVDV 525.7 pg/µL (SEM ± 46.34), NON PREGNANT-CTRL 446.53 pg/µL (SEM ± 50.96), and NON PREGNANT-BVDV 565.9 pg/µL (SEM ± 175.37). No significant difference was observed in miRNA concentration isolated for each treatment group ($P > 0.5$). Figure A-8B portrays Agilent electropherogram profiles of small RNAs extracted from circulating exosomes representative for each treatment group. Clustering of small RNA species below 60 base pair in all samples is indicative of the presence of miRNAs (Pohler et al. 2017).

3.4 Differential abundance of miRNAs between treatment groups

To determine the representative relative abundance of miRNAs between treatment groups, miRNAs were sequenced from exosomes isolated from a subset of three sheep per treatment. Deep sequencing of miRNA yielded 60 million reads in total with 1634 miRNAs identified. On average, 6.7

million raw reads were obtained per sample with 2.8 million reads containing less than 30 base pairs. The majority of reads mapped to bovine genome, yet miRNAs that were annotated to human, murine, ovine and caprine genomes were also present. BVDV RNA was not identified in the sequenced products from exosomes of any treatment group. Principal coordinate analysis of the total the 1634 miRNAs sequenced produced overlapping populations of miRNA observed within each treatment group (figure A-9). Unique miRNAs were detected in each treatment group and displayed in figure A-10. No miRNAs were consistently present across all treatment groups. Ninety miRNAs were differentially expressed between treatment groups. The 30 most abundant miRNAs identified that and were differentially expressed based on viral exposure and pregnancy status are displayed in table A-1. From these 30 miRNAs, 14 were differentially expressed based on exposure to BVDV and 16 miRNAs were differentially expressed by pregnancy status.

4. Discussion

This study represents the first examination of exosomal miRNA content in pregnant and non-pregnant ruminants infected with BVDV. Exosomes were successful isolated from maternal circulation using gradient methodology and confirmed with western blot. Following successful small RNA extraction, sequencing, and transcriptomic analysis a total of 90 miRNAs were differentially expressed between treatment groups defined by pregnancy status and exposure to BVDV. To the authors' knowledge, this is the first study to characterize the miRNA content of exosomes produced during viral induced reproductive pathogenesis.

4.1 BVDV induces maternal-fetal pathology

Confirmation that BVDV infection was achieved following inoculation used a combination of RT-PCR, IHC, and VN assay results. Seroconversion with following BVDV exposure was observed 30 dpi. A rising titer (4x) in paired serum samples constitutes a good basis for the diagnosis of BVDV (Lanyon et al., 2014). Observed histologic pathology was comprised of mild infiltration of white blood cells to the caruncular and intercaruncular tissue. However, no major histologic lesions were observed in either pregnant and non-pregnant sheep infected with BVDV. BVDV NY-1 is a relatively low virulence

non-cytopathic strain of BVDV which accounts for the underwhelming amount of histologic inflammation observed (Swasdipan et al., 2001). Perhaps the use of a more virulent strain of BVDV would produce a more marked appearance of local tissue inflammation. BVDV antigen was identified within the trophoblast in all pregnant sheep inoculated with the virus, confirming viral spread to the fetal compartment by 15 dpi. Viral antigen was not identified at the caruncular tissue in non-pregnant sheep infected with BVDV. BVDV is known to have a proclivity for reproductive tissue including the fetal maternal interface and ovary (Fray, Paton, & Alenius, 2000; Swasdipan et al., 2001). In non-pregnant sheep blood supply to the caruncular tissue and local tissue immunity is vastly different compared to pregnant sheep (Swasdipan et al., 2001). This inherent difference is tissue activity may have limited the presence of viral replication at endometrium observed 15 dpi. Progesterone in all sheep infected with BVDV trended lower than non-infected counterparts with similar reproductive status, yet no significant difference was observed. These data may have suffered from type II error, where a statistically significant difference may have been observed with a larger sample size. BVDV infection can result in both oophritis and fetal death with subsequent release of $PGF2_a$ from the endometrium, inferring that both processes will have an effect on serum progesterone (Fray et al., 1999; Fray, Mann, et al., 2000).

4.2 Exosomal miRNAs are differentially expressed based on pregnancy and viral status

Exosomes are small microvesicles comprised of a lipid bilayer. In this study, exosomes were isolated using a chemical gradient and were positive on western blot for exosomal surface markers CD81 and HSP70, which are well-described exosome markers (Conde-Vancells et al., 2008). Exosomal cargo generally contains proteins, nucleic acids, and bioactive lipids such as prostaglandins (Subra et al., 2010). MiRNAs impose their biological function by modulating gene expression by silencing mRNA leading to suppression of translation and thus protein synthesis (Glazov et al., 2009). Profiles of miRNAs are being investigated as markers of placental health during human pregnancy (Adam et al., 2017). This study compared miRNA concentrations between sheep based on pregnancy status and exposure to BVDV. A total of 90 miRNAs were differentially expressed between treatment groups using a FDR 0.1. Of those, 30 of the most abundant miRNAs were identified with expression difference based on pregnancy and

BVDV exposure in 16 and 14 miRNAs, respectively. Several abundant miRNAs identified in this study are novel for differential expression for both pregnancy status and viral associated infection. Spencer et al. described oar-mir-19b in healthy pregnant sheep (G. W. Burns, Brooks, & Spencer, 2016). That finding holds true in this study, as this miRNA is upregulated in only pregnant animals. Several miRNAs whose differential expression found to be determined by pregnancy status did not hold true in this study (Cleys et al., 2014; Ioannidis & Donadeu, 2016). Differences between other published results may stem from our small number of experimental units per treatment group, thus lower the power and leading to a type 2 error. Further, this study evaluated a different population of miRNAs by only sequencing exosomal miRNAs compared to free miRNAs in circulation. The difference in methodology may account for inconsistent results when trying to define miRNA profiles based on pregnancy status.

Several miRNAs that were upregulated in the BVDV exposed group were also described in the literature as associated with other infectious conditions of livestock and humans. Bta-mir-2330 and bta-mir-10225b have been associated with BHV-1 infection in bovine derived cells in vitro (Glazov et al., 2009). In small ruminants exposed to peste des petits ruminants, bta-mir-144 was found to be upregulated in the clinically ill (Pandey et al., 2017). The miRNA has-mir-7-1 which was upregulated in the BVDV exposed sheep was found to be associated with reproductive viral infections in humans. Other differentially expressed miRNAs discovered in this study are were identified in fetal disease. For example, hsa-mir-674 associated with fetal alcohol syndrome is upregulated in the BVDV-infected group (Balaraman et al., 2014). Fetal alcohol syndrome results in dysregulation of nutrient and oxygen exchange at the placental level (Balaraman et al., 2014; Balaraman, Tingling, Tsai, & Miranda, 2013). A similar disequilibrium can be expected in placentitis associated with infectious condition.

Annotation of sequenced miRNA sequences was performed by aligning to several genomes including ovine, bovine, caprine, human, and murine. The majority of the sequences were annotated to the bovine miRNA genome, yet alignment to the other aforementioned species was observed in differentially expressed miRNA. Homologous structures are described between bovine and both human and murine miRNAs (Gebremedhn et al., 2018). However, it is unknown if the function of well-studied

human or murine miRNA is the same in ruminant species. A pathway analysis using mirPATH v. 3 Diana Tool was attempted yet the majority of bovine and ovine derived miRNA are relatively novel without unknown mechanisms in biologic pathways (Vlachos et al., 2015). Prediction of miRNA targets in sheep published in currently literature is indirect and based on conserved sequences across multiple species (G. W. Burns et al., 2016).

Viruses including PRRSV and HIV utilize exosome-mediated transport of viral RNA allowing cell-to-cell transmission (Arenaccio et al., 2014; L. Wang et al., 2018). Nucleic acids from BVDV genome were not identified in the sequenced product isolated from exosomes. During the sequencing process, researchers selected for nucleic acid segments of less than 100 bp, with the hope of identifying miRNAs which are less than 50 bp. Narrowing the sequencing product of this size could have eliminated the identification of viral nucleic acids, which could be closer to 250 bp in length (Ridpath & Bolin, 1995). Further research is needed to determine if BVDV may utilize the use of exosomes to mediate viral transmission.

5. Conclusions

MiRNAs are known to play a role in a wide range of biological processes and thus their expression may be disease specific making them excellent biomarkers. Many miRNAs identified in this study are novel and not previously described in livestock with reproductive viral infection. The miRNAs described in this study may provide a useful resource for future investigators to further characterize and select candidate miRNAs for further analysis for biological relevance.

6. Acknowledgements

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7. Competing interests

The authors declare no conflicts of interest.

Chapter 4

Characterization of the exosomal proteome in pregnant and non-pregnant sheep infected with Bovine Viral Diarrhea Virus

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Abstract

The placenta is a transient organ, critical for growth and protection of the developing fetus. Trophoblasts from the fetal placenta produce a variety of products including extracellular vesicles, such as exosome. Exosomes have been found in pregnant sheep, yet little is known of about their role in maternal-fetal communication during pregnancy during times of health and disease. The hypothesis of this study states that exosomal proteome detected in maternal circulation will vary depending on reproductive health status and viral exposure. The specific objectives were to 1) characterize the profiles of circulating exosomal proteins in pregnant and non-pregnant sheep, and 2) determine the differential abundance in proteome between non-infected and BVDV-infected pregnant and non-pregnant sheep. Twenty-four yearling ewes, found to be negative to BVDV on serum neutralizing antibody serology, were enrolled in this study. Ewes were inoculated with BVDV NY-1 or sham media. Fifteen days post inoculation, all animals underwent hysterectomy and peripheral blood collection. Exosomes were isolated using ultracentrifugation and gradient density separation. Mass spectroscopy proteomics were performed and predicted protein forms identified using Proteome Discoverer Percolator. BVDV infection status was determined with BVDV PCR of fetal tissue. Statistical differences between abundance of proteins were analyzed of variance for repeated measures. With a confidence rate of 0.01, 539 proteins were identified with differential relative abundance between viral exposure. This studies supports the idea that exosomes containing select proteins are present in peripheral circulation likely have a biological role in fetal-maternal interactions important in times of both disease and health.

Keywords

Proteome

Exosome

Sheep

Bovine Viral Diarrhea Virus

Placenta

1. Introduction

The placenta is an essential organ needed for growth and survival of the fetus. Maternal-fetal cellular communication is critical to maintain a healthy pregnancy, and any dysfunction can result in fetal morbidity or mortality (Adam et al., 2017). Exosomes (30-150 nm particles) are released from various types of cells and can serve as communication channels between the dam and fetus (Jin & Menon, 2018). In sheep production of exosomes is associated with progesterone production, with higher concentration of vesicles isolated during pregnancy compared to non-pregnant sheep (G. W. Burns et al., 2018). This concept in confirmed in women, who have 50 fold greater exosomes in circulation during pregnancy (Salomon et al., 2016). Exosomal cargo comprised of proteins, lipids, and nucleic acids. Once released from the parent cell, exosomes cause a function change in recipient cells dependent on their contents (Barile & Vassalli, 2017). Studies in human pregnancy have linked exosomal content with placental dysfunction, leading health complications for the fetus and dam(Jin & Menon, 2018). Examination of exosomal content in livestock is limited, with a narrow scope evaluating only normal, uncomplicated pregnancy (G. W. Burns et al., 2016). Proteomic evaluation of exosomes in pregnancy is relatively novel with only a few publish reports in humans. Jia et al described differentially abundant proteins in cord blood isolated from pre-eclampic woman compared to healthy controls (Jia et al., 2015). Characterization of the protein contents of exosomes may help elucidate pathophysiologic mechanism during complicated pregnancies.

Bovine viral diarrhea virus (BVDV) is a positive sense, RNA virus that affects all even-toed ungulates (Passler & Walz, 2010). It is an economically important virus of livestock, worldwide, and can

yield dramatic reproductive pathology resulting in failure to conceive, embryonic death, persistent fetal infection, congenital fetal infection, and abortion (Newcomer et al., 2015). The virally induced pathology at the maternal fetal interface tissue has been investigated using histopathology, protein arrays, and mRNA transcription (T. R. Hansen et al., 2010; Smirnova et al., 2012; Smirnova et al., 2014). Other RNA viruses, including HIV and PRSSV, have been demonstrated to alter exosome production and content in infected cells (Narayanan et al., 2013; L. Wang et al., 2018). However, this viral mechanism has not been evaluated in relation to BVDV infection. Examination of exosomal content may further elucidate viral pathogenic mechanism during pregnancy in livestock.

Proteome profiling of circulating exosomes in pregnant animals is needed to better understand placental immunology in times of health and disease. With the hypothesis that the exosomal proteome would vary based on pregnancy and viral exposure status, we set out with the specific objectives to 1) characterize the profiles of circulating exosomal proteins in pregnant and non-pregnant sheep, and 2) determine the difference in exosome profiles between non-infected and BVDV-infected pregnant and non-pregnant sheep.

2. Materials and Methods

2.1 Animals

All animal work was approved under the guidance of the University of Tennessee Institutional Animal Care and Use committee (protocol 2479). Twenty-four, yearling primiparous Kahtadin ewes were acquired from local sources and enrolled in the study. The ewes underwent estrous synchronization as described: an intravaginal insert containing 0.9 g progesterone (EAZI-BREED CIDR sheep insert, Zoetis Inc., Parsippany, New Jersey) was placed then removed after seven days, 5 mg dinoprost tromethamine (Lutalyse Injection, Zoetis Inc.) was intramuscularly administered at the time of removal. Ewes were housed with a fertile ram for natural service. Pregnancy diagnosis and confirmation using transabdominal ultrasound occurred at gestational day (GD) 30 and 60, respectively. At confirmation, 11 ewes exposed to the ram had similar pregnancy lengths at GD 65 while the remaining four ewes carried younger fetuses and were excluded from the study. After confirmation of pregnancy, sheep were then randomly allocated

between two treatment groups: BVDV (n = 6) or CTRL (n = 5). A larger number of experimental units were allotted to the BVDV group to account for expected fetal loss following inoculation. At GD 65, ewes received intranasal inoculation of non-cytopathic BVDV type 2 isolate NY- 1 (10^7 TCID50/mL) or equivalent volume of media to serve as sham. Blood was collected at GD 80 via jugular venipuncture. Whole blood was allowed to clot for 30 minutes then centrifuged at 3000 x g for 10 minutes, then the serum removed, aliquotted and stored at – 80 ^{0} F until analysis.

2.2 BVDV inoculum preparation

Viral isolate BVDV non-cytopathic genotype 1b (pestivirus A), strain NY-1 was propagated in Madin-Darby bovine kidney cells in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum, L-glutamine, and penicillin G and streptomycin. Infection was induced as described by Walz et al. (Passler et al., 2007). All sheep where inoculated intranasally at GD 65, to mimic naturally occurring maternal exposure (Swasdipan et al., 2001). Briefly, sheep were physically restrained, haltered and the head elevated and stabilized. Using a disposable intranasal cannula, sheep received 10^7 median tissue culture infective dose (TCID50) of the NY-1 isolate in EMEM (2mL aliquot/nostril).

2.3 BVDV Polymerase chain reaction

Real time polymerase chain reaction (RT-PCR) was performed as described by La Rocca and Sandvik on fetal tissue collected at GD 80 (15 dpi) to identify the presence of BVDV NY-1 (La Rocca & Sandvik, 2009). Briefly, tissue was homogenized and RNA was isolated from samples using QIAamp viral RNA mini kit according to manufacturer's instructions (Qiagen, Hilden Germany). The reverse and forward PCR primers for this reaction were GYGTCGAACCAYTGACGACT and CCATRCCCDTAGTAGGACTAGC, respectively. A TaqMan probe (TaqMan, Applied Biosystems, Foster City, CA) (TGGATGGCYRAABCCCTGAGT) with dye label 6-FAM on the 5' end and Black Hole Quencher (BHQ, Integrated DNA Technologies, Coralville IA) on the 3' end. Cycle threshold (Ct) values over 20 were considered positive for BVDV genetic material.

2.4 Immunohistochemistry

Placentome tissues were fixed by immersion in 10% formalin. Tissues were routinely processed and, paraffin embedded. Staining for BVDV was carried out using standard immunohistochemical procedures developed for use in formalin fixed tissues. Five micrometer thick tissue sections were cut on charged slides. Immunohistochemistry for BVD antigen was performed using the Discovery Ultra Automated Staining System (Roche, Ventana Medical Systems, Tucson, AZ). Briefly, following automated deparaffinization and antigen retrieval for 8 minutes using protease 2 retrieval solution (Roche, Ventana Medical Systems, Tucson, AZ) at 37 degrees, sections were incubated with a monoclonal anti-BVD antibody that specifically targets the BVDV glycoprotein E (clone 15.C.5., Syracuse Bioanalytical, East Syracuse, NY, USA) at a concentration of 1:400 for 1 hour. Labeling was detected using the multimer based ultra-map anti-mouse detection system with a red chromogen (Roche, Ventana Medical Systems, Tucson, AZ). Positive controls included ear notch tissues from BVD positive cows as confirmed by PCR. For negative controls, the primary antibody was replaced with homologous nonimmune serum.

2.5 Exosome isolation

Extracellular vesicles were isolated from 2 mL of serum 15 dpi using a combination of centrifugation gradient isolation. Each sample was centrifuged for 15 minutes at 3000 x g and cleared supernatant transferred to sterile tube. Exosome precipitation was performed using a commercial kit according to manufacturer's instructions (ExoQuick, System Biosciences, Palo Alto CA). Cleaned exosome pellets were suspended in 250 uL of PBS; 20 uL was saved for western blot analysis while the remainder was used for proteomic analysis described below.

2.5.1 Western blot

Western blots were performed as described by Pohler et al (Pohler et al., 2017). Exosomes were identified for immunopositivity to surface markers Cluster of differentiation 81 (CD 81) and Heat-shock-protein 70 (HSP 70). Both of these markers well characterized as identifiers of exosomes (Conde-Vancells et al., 2008).

2.6 Mass spectroscopy

Each multidimensional protein identification technology (MudPIT) fraction was subjected to in-line Reverse Phase (RP) chromatography and tandem mass spectrometry to identify proteins in each fraction. Briefly, each sample was loaded onto a C18 nano column using a Thermo Scientific RSLCnano system. The flow rate was 200nl/min. Peptides were eluted from the column using a linear acetonitrile gradient from 2 to 80% acetonitrile over 180 minutes followed by high and low organic washes into an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) via a nanospray source. The spray voltage was set to 1.8kV and the ion transfer capillary was set at 275°C. A data-dependent Top 10 method was used where a full MS scan from m/z 350-1600 was followed by MS/MS scans on the 10 most abundant ions. Data dependent MS/MS data was collected using a collision induced dissociation(CID) energy of 35%, at a scan range of 400-1200m/z, and using higher energy collision dissociation (HCD) energy of 65% with a scan range of 100-500 m/z. A dynamic exclusion of 60s was set.

2.7 Statistical analysis

Raw data files were searched against the most recent database for Ovis Aries downloaded from NCBInr using the MudPIT option in Proteome Discoverer 2.2 (Thermo Scientific) and the Sequest HT search algorithm. For protein identification results, only peptides identified with high confidence were used. Trypsin was the selected enzyme allowing for up to two missed cleavages per peptide. Carbamidomethylation of cysteine, N-terminal TMT6-plex, and Lysine TMT6-plex were used as a static modifications whereas oxidation of Methionine and TMT6-plex of Threonine were used as variable modifications. For confidence, the Percolator algorithm was used for peptide-spectrum match (PSM) validation in database searches. The False Discovery Rate (FDR) threshold calculated in Proteome Discoverer Percolator (Thermo Fischer Scientific, Waltham, MA) when high confidence peptides was used for protein identification is 0.01. The relative abundance of identified proteins in each sample was determined by calculating the ratios in pairwise comparisons using SAS version 9.4 (SAS Institute Inc., Cary, NC). Graphical representation of proteome distribution via scatter plot and up and down regulated biological processes were created using XLSTAT 2018 (Addinsoft, New York, NY)

3. Results

3.1 Confirmation of BVDV infection using VN, PCR, and IHC

Prior to enrollment of the study, all sheep were negative (<1:4) for neutralizing antibodies to BVDV, confirming immunologic naivety of the animals. All animals undergoing sham inoculation with media remained negative (<1:4) on VN 1 month following intranasal inoculation. All animals enrolled in the BVDV group who received intranasal viral inoculation had a positive titer on VN assay 1 month following viral exposure. The mean titer for PREGNANT-BVDV was 1:36 (standard error of the mean (SEM) ± 6) and Non-Pregnant-BVDV was 1:24 (SEM ± 6). A rising titer (4x) in paired serum samples constitutes a good basis for the diagnosis of BVDV (Lanyon et al. 2014). Successful maternal infection and passage of BVDV into the fetal compartment was determined by RT-PCR. Fetal tissues from dams inoculated with virus (n = 5) were found to be positive on BVDV RT-PCR (mean Ct = 23.29 ± 4.44). All fetuses of PREGNANT-CTRL sheep (n= 5) were negative for the virus on RT-PCR (Ct = 0).

Microscopic evaluation of placentomes with hematoxylin and eosin stained slides did not identify any significant microscopic differences between inoculated and control animals. In control animals, low numbers of mononuclear cells, predominantly lymphocytes, were present in all levels of the endometrial stroma. In inoculated animals, the intercaruncular luminal endometrial epithelium had mildly increased numbers of intraepithelial mononuclear cells. BVDV antigen was detected by IHC within the trophoblastic epithelium lining chorioallantoic villi in all PREGNANT-BVDV sheep inoculated with BVDV. Figure A-1 is a representative image from a single pregnant sheep inoculated with BVDV. Uterine tissue from NON PREGNANT-BVDV was not positive for BVDV antigen on IHC.

3.2 Evaluation of isolated exosomes

Exosomes were successfully isolated from circulating blood using chemical gradient methodology. Confirmation of exosomes isolated from serum was performed using western blot, which demonstrated immunostaining for exosome surface markers CD 81 and HSP 70. Figure A-11 displays a representative image from a sheep assigned to the PREGNANT-CTRL group demonstrating a positive

western blot result for both CD 81 and HSP 70 along with positive and negative controls. All biologic replicates for each treatment group were positive for these surface markers.

3.4 Differential proteome content between treatment groups

Briefly, 539 proteins were identified as high confidence peptides. When high, medium and low confidence peptides are considered the total number of identified proteins increases to 694. Figure A-12 depicts a volcano scatter plot representing the distribution of proteome data discovered with high confidence between treatment groups. Twenty proteins identified were significantly abundant ($P < 0.05$) between treatment groups. Table A-2 displays these significant proteins and the TMT ratios used in estimating the relative concentration of each identified protein in samples. A fold-change threshold of 1.5 is used for up/down regulation, a TMT Ratio >1.5 is classified as up-regulated, and a ratio <0.67 is classified as down- regulated. Ratios from 0.67 – 1.5 are considered moderate to no changes. Ratios above 100 exceed the maximum allowed threshold that is reasonably expected. Pathway analysis determined biological and cellular processes of proteins found to be differentially abundant based on BVDV exposure; these are depicted in figure A-13 and A-14. Pathways associated with immunologic processes, enzymatic regulation, protein binding and metabolic processes were upregulated in animals exposed to BVDV. Notably, antioxidant activity, and catalytic activity, and defense responses were down regulated with BVDV exposure.

4. Discussion

This study was successful in describing the proteome of circulating exosomes isolated from sheep exposed to BVDV. Further, this is study is novel in describing the circulating exosomal proteome in apparently healthy pregnant and non-pregnant sheep. Following successful isolation of circulating exosomes, 539 proteins were identified with high confidence. To the authors' knowledge, this is the first description of the effects of viral reproductive pathogenesis on exosomal proteome profiles in livestock.

4.1 BVDV induces maternal-fetal pathology

Confirmation that BVDV infection was achieved following inoculation used a combination of RT-PCR, IHC, and VN assay results. Observed histologic pathology was comprised of mild infiltration

of white blood cells to the caruncular and intercaruncular tissue. However, no major histologic lesions were observed in either pregnant and non-pregnant sheep infected with BVDV. BVDV NY-1 is a relatively low virulence non-cytopathic strain of BVDV which accounts for the underwhelming amount of histologic inflammation observed (Swasdipan et al., 2001). Perhaps the use of a more virulent strain of BVDV would produce a more marked appearance of local tissue inflammation. BVDV antigen was identified within the trophoblast in all pregnant sheep inoculated with the virus, confirming viral spread to the fetal compartment by 15 dpi. Viral antigen was not identified at the caruncular tissue in non-pregnant sheep infected with BVDV. BVDV is known to have a proclivity for reproductive tissue including the fetal maternal interface and ovary (Fray, Mann, et al., 2000; Swasdipan et al., 2001). In non-pregnant sheep blood supply to the caruncular tissue and local tissue immunity is vastly different compared to pregnant sheep (Swasdipan et al., 2001). This inherent difference is tissue activity may have limited the presence of viral replication at endometrium observed 15 dpi.

4.2 Exosomal proteins are differentially abundant based on viral exposure status

Many of the exosomal proteins identified are known to be associated with innate immunity and inflammation, including fibrinogen and complement. Other than its role in coagulation, fibrinogen is an acute phase protein produced by the liver during acute inflammation (Davalos & Akassoglou, 2012). Several other acute phase proteins including haptoglobin, C-reactive protein, and serum amyloid A were found to be abundant within BVDV exposure sheep yet significantly (data not shown). Complement proteins are produced constitutively, yet production can be increased during inflammation (Daha & van Kooten, 2016). These proteins aid in opsoninzation of antigens and direct killing of microbial pathogens via complement complex formation. Other abundant proteins included proteins associated with the innate immune arm such as immunoglobulin segments. Variable portions of immunoglobulins are upregulated in pregnant BVDV inoculated animals compared to non-pregnant. B-lymphocytes are the producers of circulating immunoglobulins. Further, this cell type are avid producers of exosomes during inflammation (Saunderson & McLellan, 2017). The fact that the variable light chain was abundant without the presence of the constant heavy chain indicated the smaller, antigen-binding portion of the immunoglobulins was

selectively being released. The presence of this profile of proteins supports a humoral immune response following BVDV infection. The production of neutralizing antibodies is also supported by the VN titer increase 30 days following experimental infection with BVDV. Concurrent peak viremia and viral shedding of BVDV is expected to occur at 7 dpi, with resolution of viral shedding by 14 dpi (Grissett, White, & Larson, 2015). This study looked at samples at 15 dpi, yet evidence of acute inflammation was still present in the exosomal proteome of BVDV infected animals. In pregnant animals, the fetus and trophoblast had confirmed presence of BVDV antigen, perhaps serving as a nidus of ongoing acute inflammation.

Retinol binding protein 4 was down regulated in animals exposed to BVDV compared to controls. Recent literature describes the role of retinol binding proteins inducing pro-inflammatory response by T lymphocytes (Zabetian-Targhi, Mahmoudi, Rezaei, & Mahmoudi, 2015). It is interesting that this protein would be down regulated in the face of induced inflammation associated with experimental infection. Following acute BVDV, transient lymphopenia is observed 3-9 dpi, with peripheral numbers recovering by 14 dpi (Falkenberg, Dassanayake, Neill, & Ridpath, 2017). BVDV has a cellular tropism for lymphocytes and that cellular proclivity may thus interfere with lymphocyte inflammatory response inducing immunosupression (Chase, Elmowalid, & Yousif, 2004; Howard, 1990). The down regulation of this effector protein may represent a novel mechanism of immunosuppression induced by BVDV infection.

Different forms of serpins were present and had a variable abundance pattern in BVDV infected animals. Serpins are produced by the endometrium and appear to be progesterone dependent. Uterine serpins' mechanism of immune modulation during pregnancy was recently reviewed (Padua et al., 2005). Serpin acts to down regulate immune cells, by inhibiting lymphocyte responses against the fetus, thus inducing an anti-inflammatory environment at the maternal fetal interface during pregnancy (Liu, Gottshall, & Hansen, 1997). The role of serpins during infectious placental pathology are less described. One recent study described a negative association between *Neospora caninum* antibody titers and serpin expression within the bovine placenta (Serrano-Perez et al., 2016). Furthermore, bovine uterine serpin

concentrations are negatively correlated with interferon expression following *N. caninum* infection (Serrano-Perez et al., 2018). In reproductive infection of naive cattle carrying fetuses transiently or persistently infected with BVDV, interferon associated genes and interferon cytokines were elevated at the maternal fetal interface and in maternal circulation compared to controls (Smirnova et al., 2014). With the complex abundance pattern of serpins observed in this study, placental viral infection may disrupt the immunosuppressive function of uterine serpin.

Several alpha-glycoproteins were up regulated in BVDV infected sheep. The binucleate trophoblasts of pregnancy ruminants produce a variety of products including pregnancy-associated glycoproteins (PAGs) (R. M. Wallace et al., 2015). Trophoblast are known to produce exosomes in many species, indicating that PAGs may be present within these exosomes found in maternal circulation (Adam et al., 2017; G. Burns et al., 2014). However, the predicted molecular structure of PAGs would need to compared with the other glycoproteins discovered in this study to confirm this postulate. Histidine rich glycoprotein was observed to be down regulated in pregnant sheep exposed to BVDV. Lower circulating concentrations of this protein is associated with pre-eclampsia in women (Bolin, McClurkin, Cutlip, & Coria, 1985). Exosome concentrations and content has been extensively investigated in human as biomarkers to predict pre-eclampsia in women (Adam et al., 2017). Total numbers of circulating exosomes is greater in pregnant woman compared to controls, yet pre-eclampic woman have 40% greater exosomes than women with healthy pregnancies (Goswami et al., 2006). For this study, the total concentration of exosomes was not determined between treatment groups.

Many forms of apolipoproteins were identified within treatment groups. This finding is expected since the composition of exosomes is a lipid bilayer (Subra et al., 2010). In addition, the method of isolated exosomes may yield other lipoprotein contamination from the lipid fraction of the blood (Karimi et al., 2018). Apolipoprotein C III, which was downregulated in BVDV infected sheep, is active in the peroxisome proliferator-activated receptors signaling pathway (PPAR) (Lui et al 2015). PPAR have been demonstrated to play a significant role in human placentogenesis and a disruption of this signaling pathway is thought to contribute to pathologic pregnancy conditions such as preeclampsia and intra-

uterine growth restriction (Ruebner et al., 2012). The dysregulation of this pathway is a possible novel mechanism of BVDV-induced placental inflammation.

5. Conclusions

This is the first study profiling the proteome of exosomes isolated from pregnant and non-pregnant sheep exposed to BVDV. Differentially abundant proteins described in this study give insights into novel mechanisms of immunosuppression and placental pathology not previously described with BVDV infection. These data will serve future studies in helping to define placental immunity during times of health and disease.

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7. Competing interests

The authors declare no conflicts of interest.

Chapter 5

Conclusions

Communication between the maternal and fetal tissues is critical for allow successful pregnancy. This is supported by the exchange of proteins, nucleic acids, biologically active lipids at this interface at the time of attachment and progression through a healthy gestation. The alteration of these compounds are notable during times of pregnancy complications associated with infectious placental pathogenesis. Further exploration of these maternal and fetal products is critical to gain a full understanding of the milieu environment during healthy and disease pregnancy. After identification of markers associated with feto-placental health and pathology, mechanisms of biologic activity can be elucidating using in-vitro and in-vivo methodology. These data collected in this body of work serve as foundational research to answer mechanistic questions of placental pathology. Furthermore, these works looked to identify biomarkers associated with pathologic conditions that may be used to assess placental health during gestation.

These data presented are the first investigating alterations of maternal and fetal fluid PAG concentrations following reproductive viral infection in ruminants. Our study supports that trophoblastic injury associated with BVDV infection caused a significant decrease in PAG concentrations in maternal circulation. This indicates that circulating PAG concentrations are a useful marker of placental function in the BVDV infection and adds to the growing evidence that low PAG concentrations are correlated with placental dysfunction and fetal loss with reproductive infections. Comparing PAG concentrations between dams carrying PI and CI fetuses may demonstrate a pattern of trophoblastic injury through gestation. Further characterization of the relationship between temporal PAG production using a fetal infection model such as the viral model introduced here will yield future insights in evaluating these proteins as biomarkers of the fetal-maternal unit. This is also the first study to compare PAG concentrations between maternal jugular and venous blood. Further studies evaluating dilution or removal of PAG from systemic circulation may elucidate sites beyond the uterine environment where PAG may exert novel function.

These were the first studies defining circulating exosomal content in pregnant and non-pregnant animals exposed to a viral pathogen.. Many miRNAs identified our study are novel and not previously described in livestock in reproductive viral infection and healthy pregnancy. Using the information gleaned from this and publish work, a list of potential miRNAs that are biologically relevant during both

healthy and pathology pregnancies can be created. Future studies using cell culture and infection models like the one described here can help define the biologic impacts on trophoblasts and maternal cells. The miRNAs described in this study may provide a useful resource for future investigators to further characterize and select candidate miRNAs for further analysis for biological relevance. However, the need for standardized methodology for miRNA isolation and annotation will better the current and future databases allowing accurate available information. Defining the proteome of exosomes during pathologic pregnancies is relatively novel, with few published reports to date. In our study, differentially abundant proteins were discovered between infected and non-infected animals. This data gives insights into novel mechanisms of immunosuppression and placental pathology not previously described with BVDV infection. These data will serve future studies in helping to define placental immunity during times of health and disease.

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Appendix

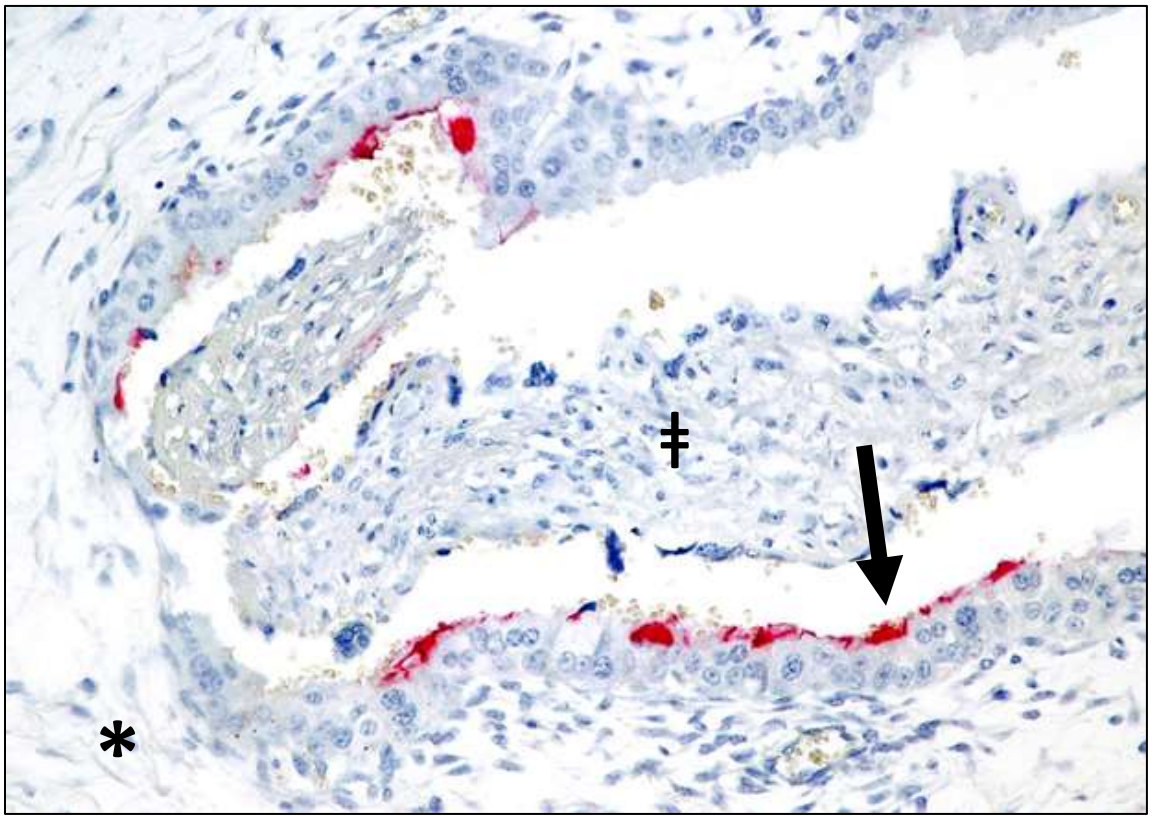

Figure 1:

Representative image of the maternal-fetal interface (placentome), BVDV inoculated animal, 80 days of gestation. The chorioallantois (*) is lined by plump trophoblastic epithelium which is multifocally immunopositive (arrows) for BVDV. The maternal endometrium (ǂ) interdigitates with the chorioallantois and is lined by attenuated epithelium which does not exhibit immunopositivity for BVD.

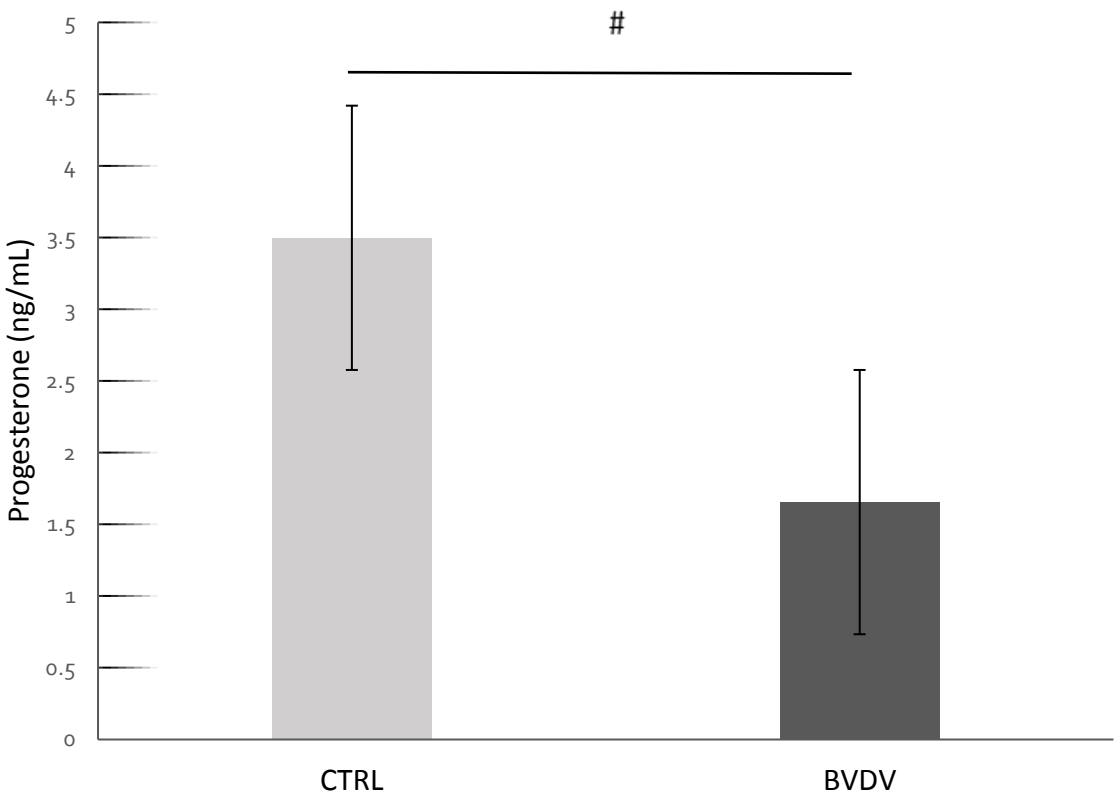

Figure 2:

Mean progesterone concentrations (ng/mL) (mean ± SEM) of control and BVDV inoculated ewes at gestational day (GD) 80. No significant difference was determined, denoted as # (P = 0.08). SEM, standard error of the mean.

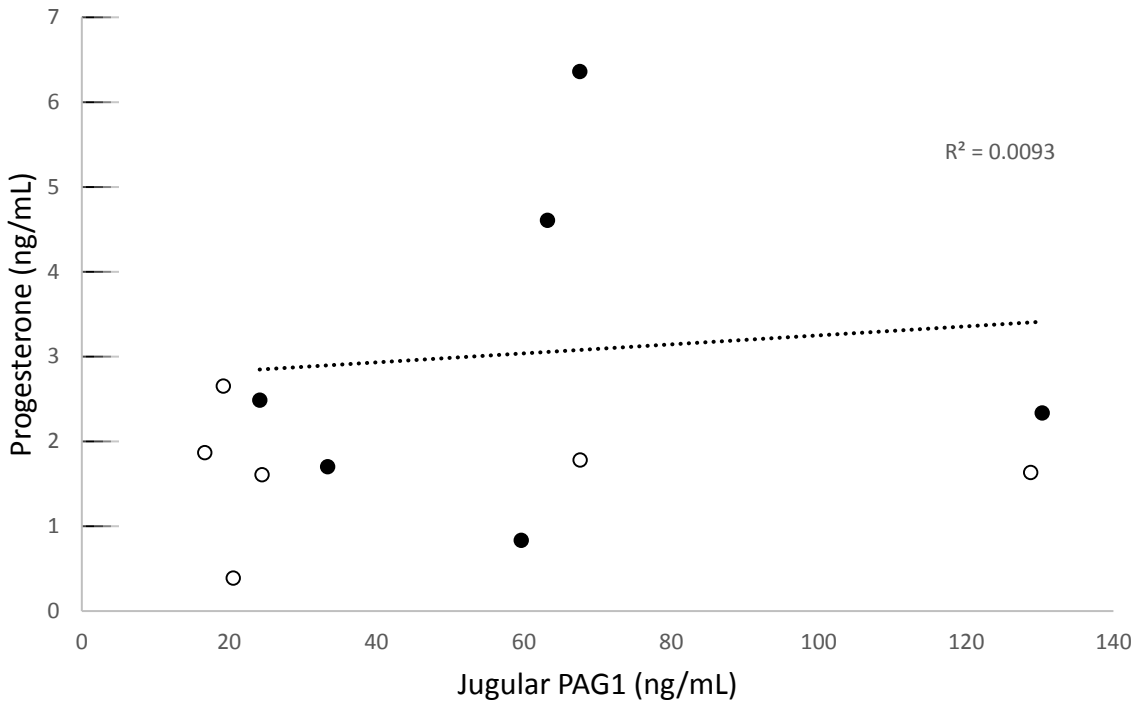

Figure 3:

Pearson's correlation plots between progesterone and PAG1 concentrations between control (open symbols) and BVDV (closed symbols) groups at GD 80. No significant correlation was observed (R^2 = 0.0093).

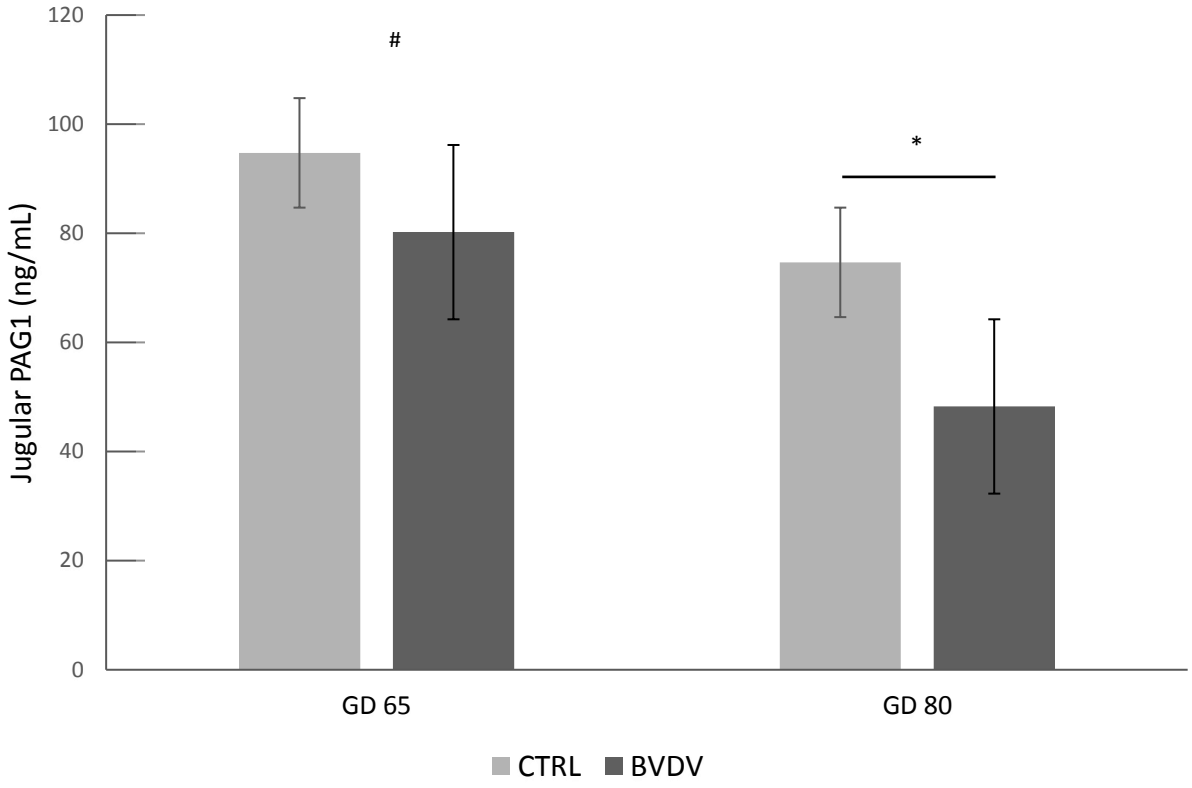

Figure 4:

Mean serum PAG1 concentration (ng/mL, mean ± SEM) of control and BVDV inoculated ewes at GD 65 and 80. Differences between gestational days (GD) denoted as # (P = 0.08) and * (P = 0.04). SEM, standard error of the mean.

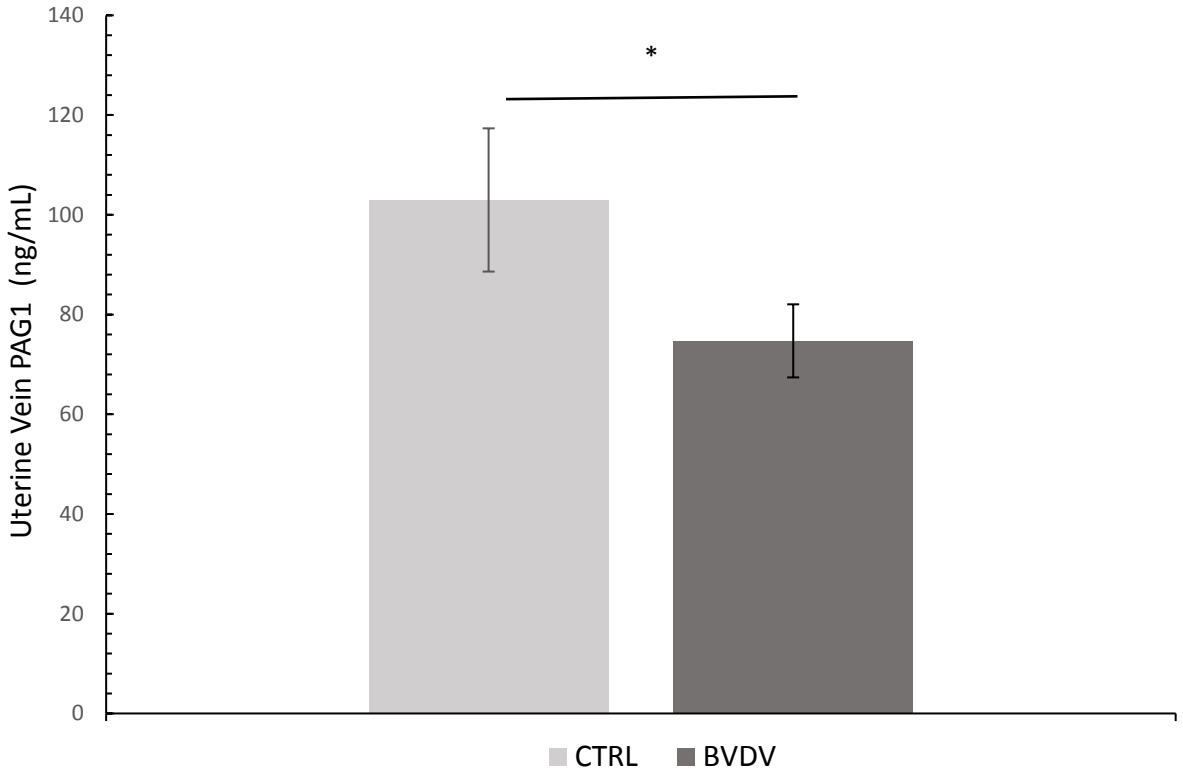

Figure 5:

Mean uterine vein PAG1 concentration (ng/mL) (mean ± SEM) of control and BVDV inoculated ewes at GD 80. Differences between treatment groups denoted as * (P = 0.01). SEM, standard error of the mean

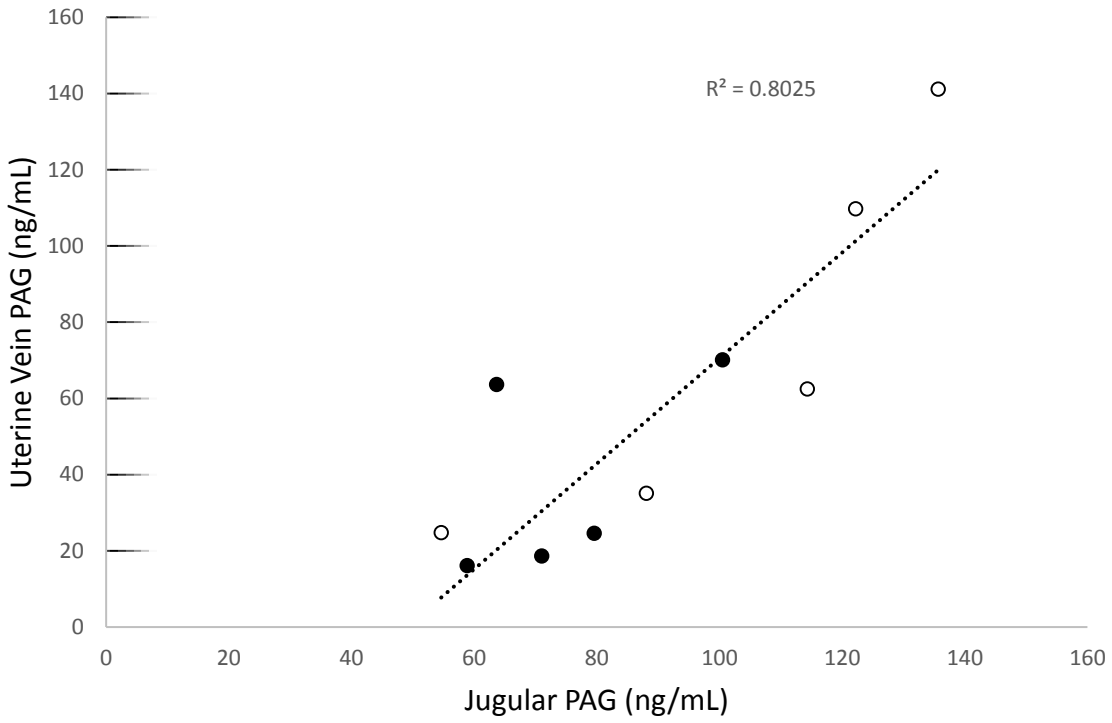

Figure 6:

Pearson's correlation plots between uterine vein and jugular PAG1 concentrations between control (closed symbols) and BVDV (open symbols) groups at GD 80. A significant correlation was observed ($R2 = 0.8$, $P < 0.01$).

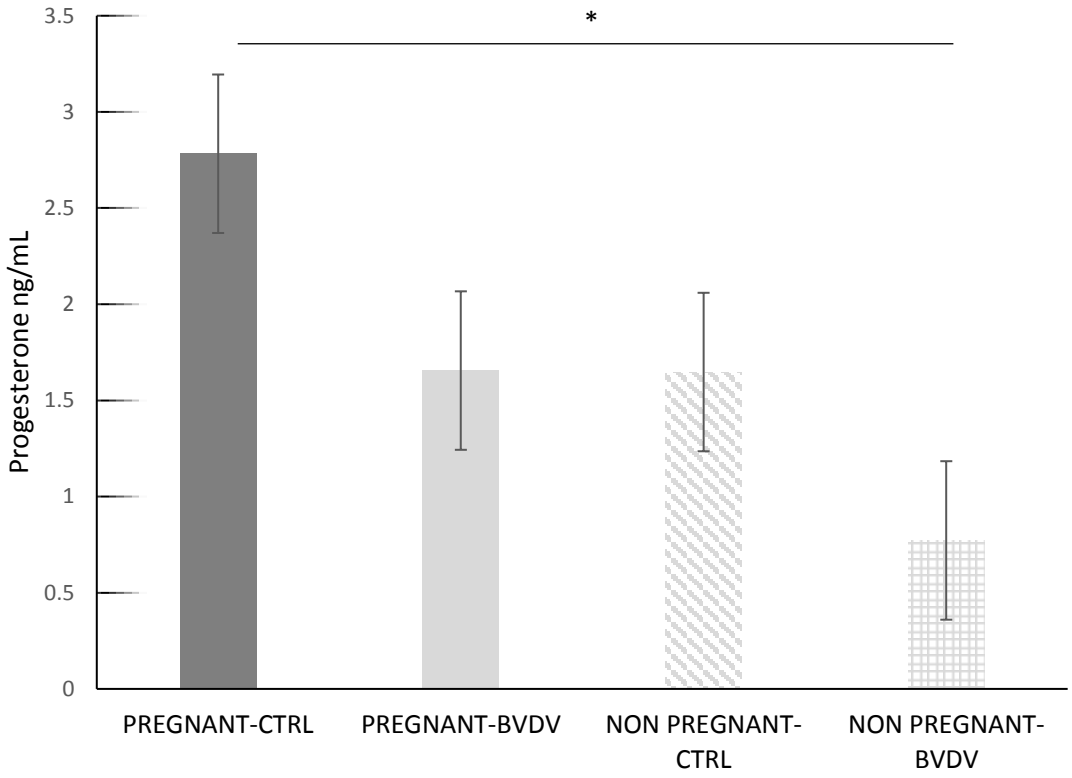

Figure 7:

Mean progesterone concentrations (ng/mL) (mean ± SEM) of ewes from all treatment groups 15 dpi. Mean progesterone concentrations for NON PREGNANT-BVDV group was significantly lower compared to PREGNANT-CTRL (P = 0.01, denoted as *). No significant difference was determined between other treatment groups. SEM, standard error of the mean.

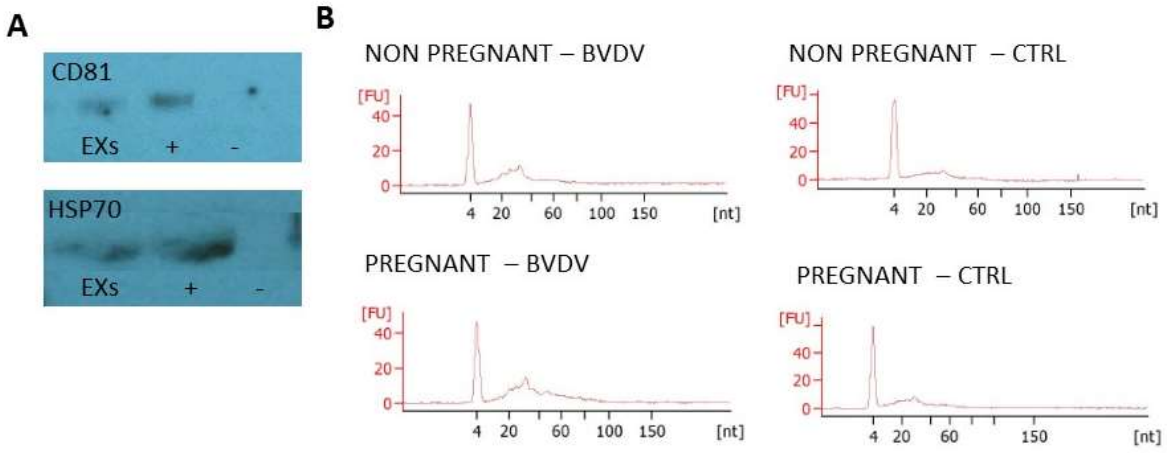

Figure 8:

Evaluation of isolated exosomes and associated miRNA. (A) representative western blot showing the prescence of CD 81 and HSP 70, two characterized markers that define exosomes, isolated from a sheep assigned to the PREGNANT – CTRL group along with positive (+) and negative (-) controls. (B) Agilent electropherogram profiles of small RNAs extracted from circulating exosomes. The base pair length [nt] (x axis) ploted against the fluorescence units [FU] (y axis). Note the clustering of RNA species below 60 base pair in all samples.

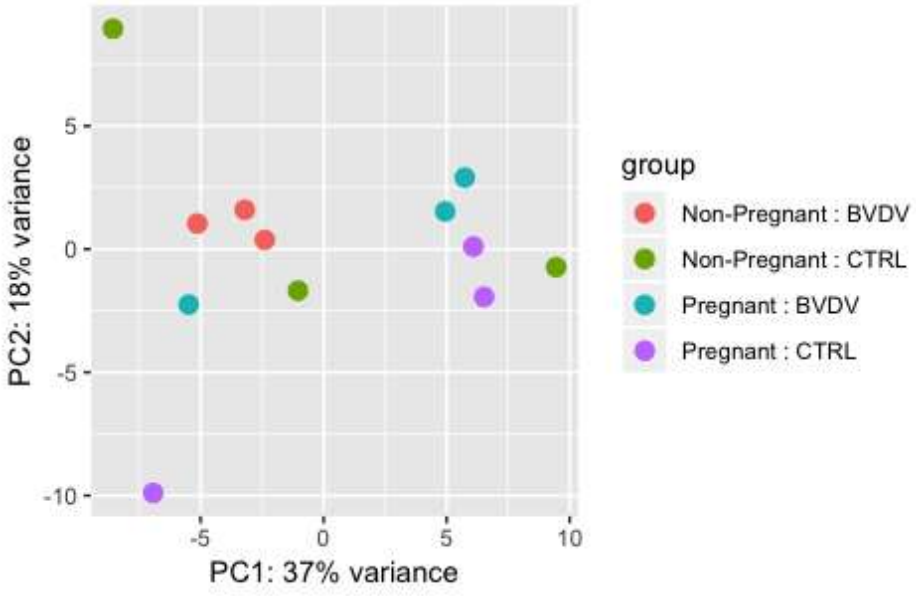

Figure 9:

Principle coordinate analysis illustrating degree of variance for miRNA abundance within each treatment group.

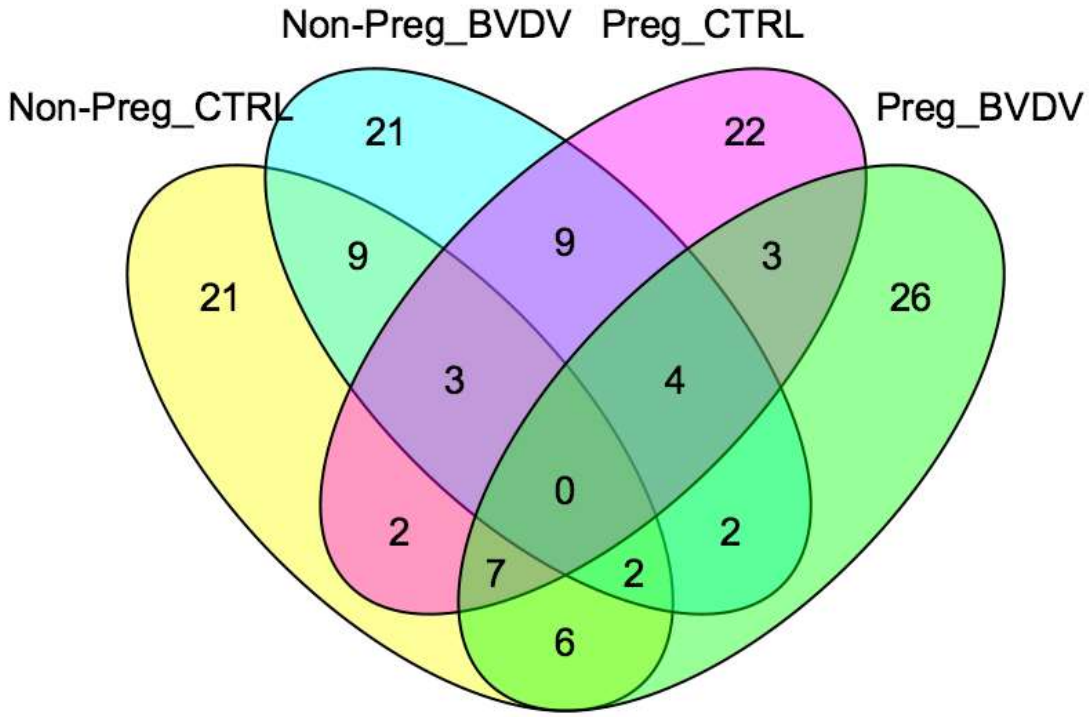

Figure 10:

Venn diagram representing number of differentially abundant miRNAs between treatment groups.

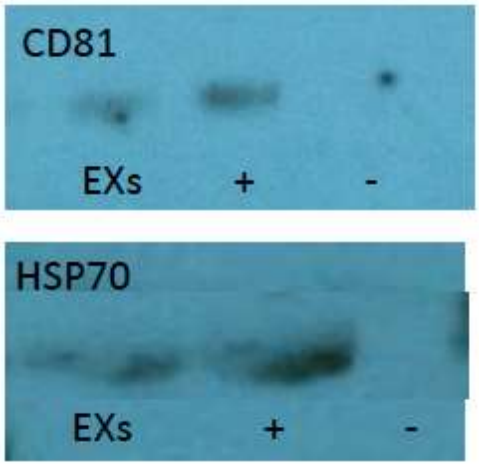

Figure 11:

Representative western blot showing the presence of CD 81 and HSP 70, two characterized markers in serum derived exosomes from a sheep assigned to the PREGNANT – CTRL group along with positive (+) and negative (-) controls.

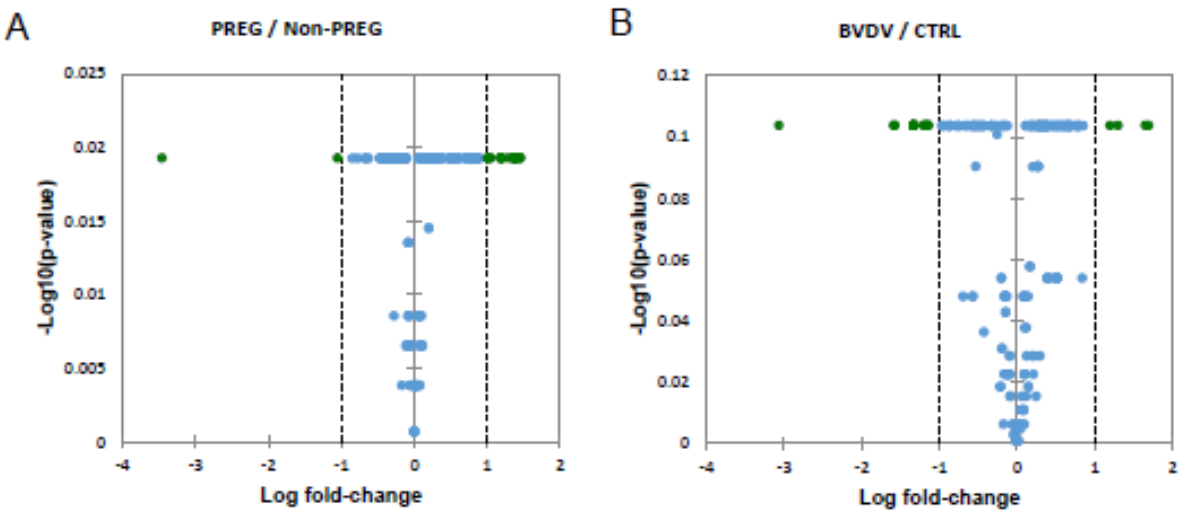

Figure 12:

Volcano Plot showing proteomic data between pregnant versus non-pregnant sheep (A) as well as control versus BVDV exposed sheep (B). The x-axis depicts large magnitude fold changes with statistical significance (-log10 of p value) depicted on the y-axis. The plot is colored such that the points have a fold change less than 2 (log2 =1) are shown in blue.

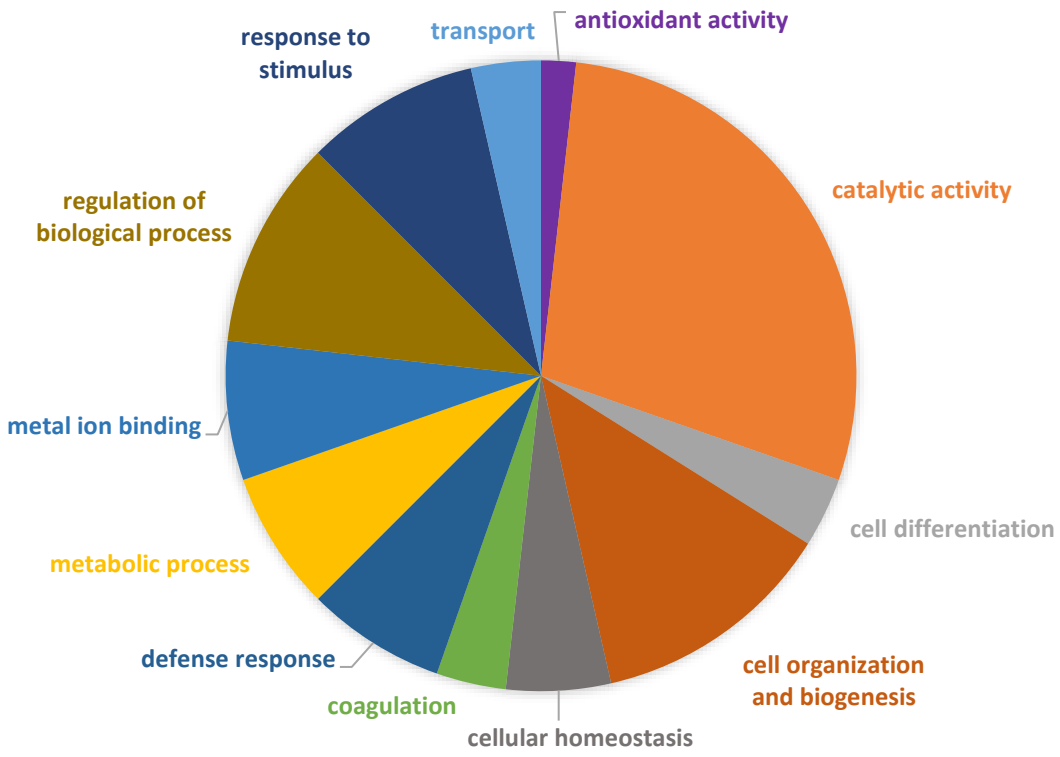

Figure 13:

Pie chart representing down-regulated biologic and cellular processes from pathway analysis based on BVDV exposure.

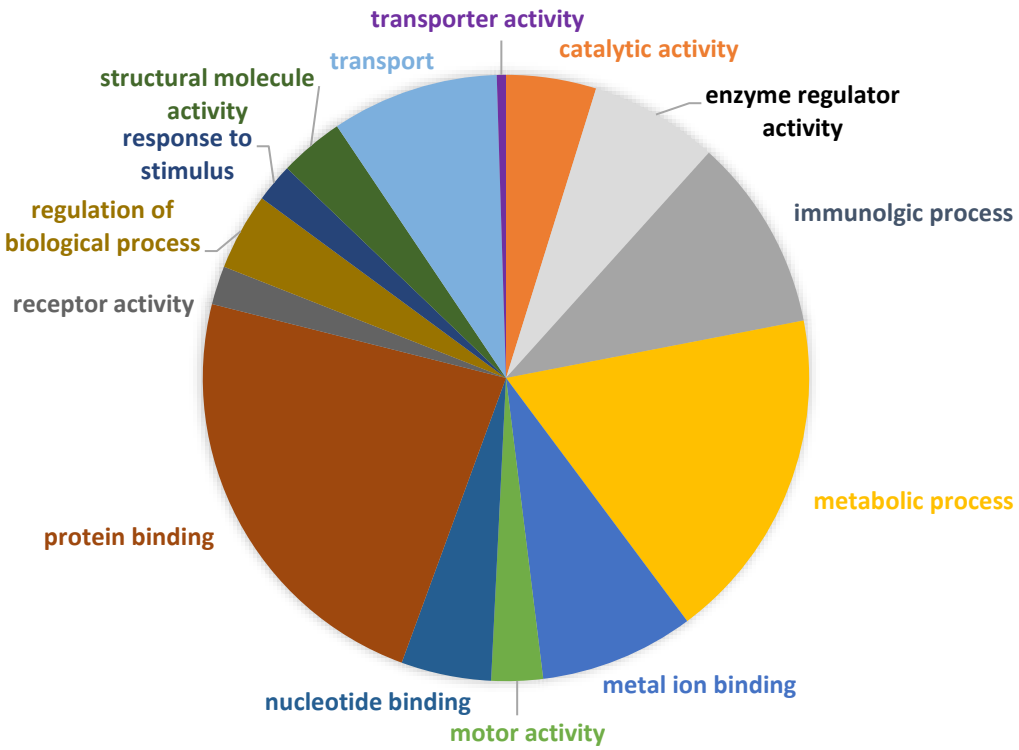

Figure 14:

Pie chart representing upregulated biologic and cellular processes from pathway analysis based on BVDV exposure.

Table 1:

Most abundant circulating-exosome-derived miRNAs with differential abundance in sheep at 15 dpi. Mean abundance for each treatment group is displayed as log count per million reads. Shading of counts represents the quantity of reads (red = increased quantity, blue = decrease quantity).

miRNA	Non-Pregnant, BVDV	Non-Pregnant, CTRL	Pregnant, BVDV	Pregnant, CTRL
bta-mir-10225b	1.719024	2.345936	1.719024	3.035236333
bta-mir-11998	5.078831	4.268056	4.779501	3.382291333
bta-mir-12022	8.028356	7.506199	7.784214	6.674143333
bta-mir-1291	2.213358	2.650805	3.615292	5.544595667
bta-mir-1388	3.969205	5.223099	5.747218	5.272334333
bta-mir-144	6.702682	7.192592	8.381197	7.924628667
bta-mir-187	1.866785	1.857453	3.304917	2.165693
bta-mir-193a	1.719024	2.289512	3.375086	2.788254
bta-mir-19a	2.829216	3.888565	4.998951	4.481507333
bta-mir-2330	2.213358	1.719024	2.861422	1.719024
bta-mir-2895	3.866853	3.124122	3.337584	2.255131667
bta-mir-29c	1.719024	1.964811	1.719024	2.838988333
bta-mir-34c	2.734612	2.942509	5.569437	3.891579
bta-mir-449c	2.734612	2.066833	3.170745	2.056786667
bta-mir-451	10.79098	11.29406	12.3562	11.95457633
bta-mir-767	3.760724	4.237765	3.398481	3.158994667
chi-mir-16a	3.693114	3.7939	5.393907	4.149750667
chi-mir-2284c	4.160002	4.827474	3.810683	3.591750333
chi-mir-29c	1.719024	1.857453	3.083628	2.056786667
hsa-mir-4453	1.866785	1.719024	2.059024	2.395147333
hsa-mir-4508	5.792865	4.48439	4.74379	4.442682
hsa-mir-5193	3.280715	2.638698	3.228549	1.719024
hsa-mir-6084	1.979656	1.719024	2.209571	2.724193
hsa-mir-6128	2.053924	2.54147	1.719024	2.451586667
hsa-mir-624	3.565997	1.914122	2.746937	1.991630333
mmu-mir-5106	8.924946	7.892783	8.743697	7.599069
mmu-mir-5119	7.6162	6.358208	7.318954	5.984148333
mmu-mir-5124a	3.196456	2.461247	2.753091	1.719024
mmu-mir-6368	2.510064	1.997888	3.885062	1.719024

Table 1 continued.

miRNA	Non-Pregnant, BVDV	Non-Pregnant, CTRL	Pregnant, BVDV	Pregnant, CTRL
mmu-mir-6990	3.230875	2.861578	1.984056	2.329991
mmu-mir-719	3.351146	1.719024	3.129163	2.629221667
oar-mir-194	3.19406	3.389861	5.459918	3.490387
oar-mir-19b	5.035718	5.413829	6.307035	6.000914

Table 2:

Select circulating-exosome-derived proteins with differential abundance in sheep at 15 dpi. Mean abundance is displayed as a ratio. Shading of counts represents the quantity of reads (red = increased quantity, blue = decrease quantity).

	Abundance Ratios		
Protein	(BVDV) / (CRTL)	(BVDV, Non-Preg) / (CRTL,Non-Preg)	(BVDV,Preg) / (CRTL,Preg)
Alpha-1-acid glycoprotein 1	1.579	1.735	1.437
Alpha-1-antiproteinase	1.633	1.874	1.423
Alpha-2-HS-glycoprotein	0.283	0.333	0.241
Antithrombin-III	0.701	0.744	0.66
Apolipoprotein A-II	0.589	0.587	0.59
Apolipoprotein C-III	0.621	0.666	0.578
Complement factor H-related protein 2	0.653	0.655	0.651
Complement factor H	0.556	0.649	0.476
Fibrinogen alpha chain	0.639	0.628	0.651
Fibrinogen beta chain	0.66	0.623	0.7
Fibrinogen gamma chain	0.601	0.629	0.575
Histidine-rich glycoprotein	0.711	0.821	0.615
Immunoglobulin kappa-2.1 light chain variable region	1.419	1.288	1.564
Immunoglobulin light chain variable region	1.397	1.535	1.273
Lumican	1.859	1.91	1.81
Retinol-binding protein 4	0.586	0.605	0.567
Serpin A3-5	2.181	2.711	1.755
Serpin A3-7	0.666	0.65	0.682

Table 2 continued.

Protein	Abundance Ratios (BVDV) / (CRTL)	(BVDV, Non-Preg) / (CRTL,Non-Preg)	(BVDV,Preg) / (CRTL,Preg)
Transferrin receptor protein 1	2.007	1.721	2.34
Zona pellucida sperm-binding protein 3 receptor	0.46	0.479	0.442

Vita

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Dr. Andi Lear holds a DVM from Auburn University College of Veterinary Medicine. While performing a large animal internal medicine residency, with an emphasis in livestock medicine and surgery, at Colorado State University College of Veterinary Medicine, she obtained a MS. Afterward, Dr. Lear joined the Farm Animal Field Service team at the University of Tennessee and is concurrently working toward her PhD in immunology and infectious disease. Her clinical interests include livestock medicine and surgery, backyard milk quality, herd disease investigation, and neonatology. Her research interests focus around maternal-fetal interactions, including placental immunology and reproductive physiology.